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Form Revision Date: March 1, 2019

Ascendis EX1002
U.S. Patent No. 12,233,106

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Form Revision Date: 07 January 2022

PTO/AIA/15 (10-17)
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	30610/57038
First Named Inventor	Jonathan Day
Title	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
Priority Mail Express® Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [X] **Specification** [*Total Pages* 74]
   Both the claims and abstract must start on a new page.
   (See MPEP § 608.01(a) for information on the preferred arrangement)
5. [X] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 4]
6. **Inventor's Oath or Declaration** [*Total Pages*]
   (including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d))
7. [X] **Application Data Sheet** ** See note below.*
   See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [X] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s))
    Name of Assignee
11. [ ] **37 CFR 3.73(c) Statement** (*when there is an assignee*) [X] **Power of Attorney**
12. [ ] **English Translation Document** (*if applicable*)
13. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [ ] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [ ] **Other:**

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 90849 **OR** [ ] Correspondence address below

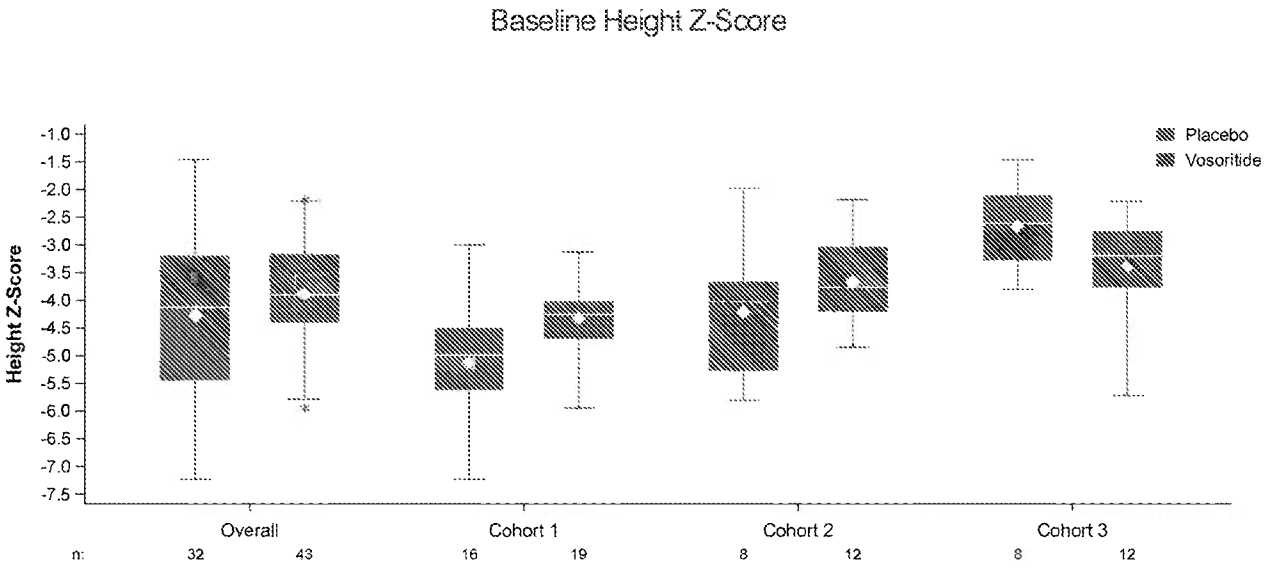
Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Katherine L. Neville/	Date	July 11, 2022
Name (Print/Type)	Katherine L. Neville	Registration No. (Attorney/Agent)	53,379

Figure 1

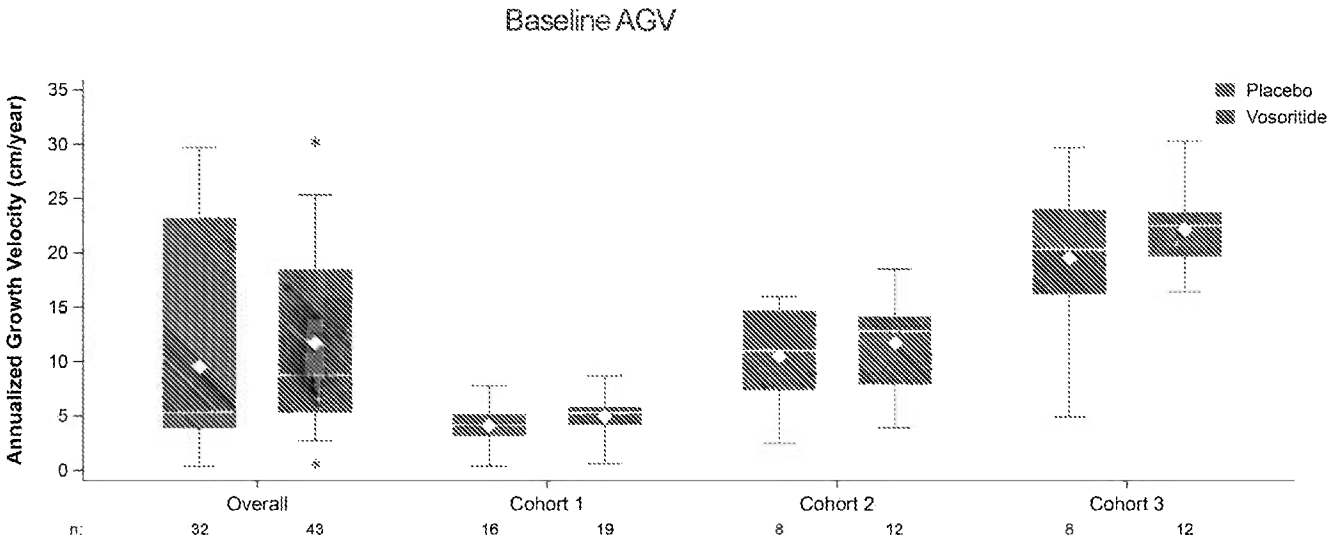
	Overall		Cohort 1		Cohort 2		Cohort 3	
	Vosoritide (N=32)	**Placebo (N=32)**	**Vosoritide (N=15)**	**Placebo (N=16)**	**Vosoritide (N=8)**	**Placebo (N=8)**	**Vosoritide (N=9)**	**Placebo (N=8)**
Age on Day 1, Months								
Mean (SD)	24.39 (16.83)	27.82 (19.25)	39.62 (10.11)	44.33 (11.54)	17.00 (5.79)	16.87 (6.21)	5.56 (0.44)	5.76 (0.59)
Median	23.29	26.43	36.86	40.39	17.92	18.56	5.78	5.91
Q1, Q3	5.93, 36.58	7.67, 40.39	30.19, 49.38	34.55, 56.36	11.50, 22.52	10.46, 22.16	5.32, 5.88	5.72, 5.95
Sex, n(%)								
Male	17 (53.1)	13 (40.6)	7 (46.7)	7 (43.8)	5 (62.5)	5 (62.5)	5 (55.6)	1 (12.5)
Female	15 (46.9)	19 (59.4)	8 (53.3)	9 (56.3)	3 (37.5)	3 (37.5)	4 (44.4)	7 (87.5)
Race*								
White	21 (65.6)	25 (78.1)	8 (53.3)	13 (81.3)	6 (75.0)	6 (75.0)	7 (77.8)	6 (75.0)
Asian	10 (31.3)	6 (18.8)	6 (40.0)	3 (18.8)	2 (25.0)	1 (12.5)	2 (22.2)	2 (25.0)
Japanese	4 (12.5)	4 (12.5)	2 (13.3)	3 (18.8)	1 (12.5)	1 (12.5)	1 (11.1)	0

Figure 2



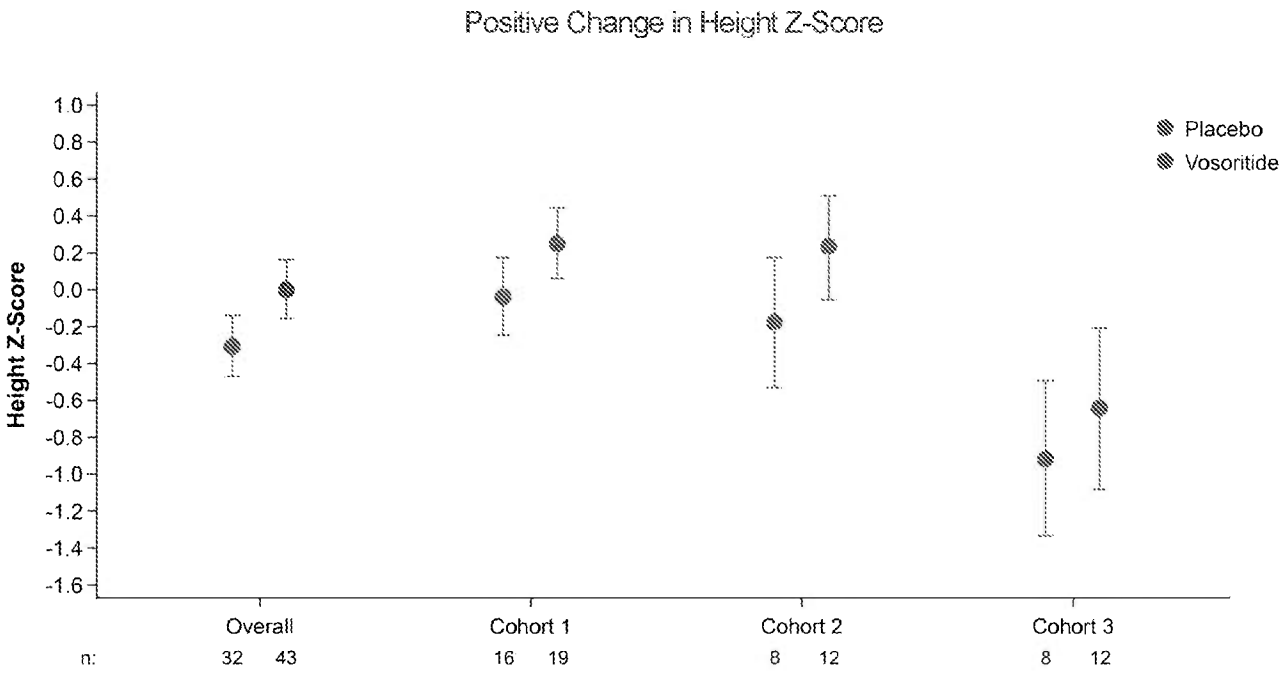
Cohort 1: Ages 24 to <60 months
Cohort 2: Ages 6 to <24 months
Cohort 3: Ages 0 to <6 months

Figure 3



Cohort 1: Ages 24 to <60 months
Cohort 2: Ages 6 to <24 months
Cohort 3: Ages 0 to <6 months

Figure 4



Cohort 1: Ages 24 to <60 months
Cohort 2: Ages 6 to <24 months
Cohort 3: Ages 0 to <6 months

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	07/11/2022 04:49:31 PM ET	30610/57038

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	60772868	AUTHORIZED BY	Katherine Neville
CUSTOMER #	90849	FILING DATE	-
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Jonathan Day

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0428	E20227AG50271385	Valeria Rodriguez

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1051	SURCHARGE- LATE FILING FEE, SEARCH FEE, EXAMINATION FEE, INVENTOR'S OATH OR DECLARATION, OR APPLICATION FILED WITHOUT AT LEAST ONE CLAIM OR BY REFERENCE	160.00	1	160.00
1111	UTILITY PATENT APPL. SEARCH FEE	700.00	1	700.00
1011	BASIC FILING FEE - UTILITY (PAPER FILING ALSO REQUIRES NON-ELECTRONIC FILING FEE UNDER 1.16(T))	320.00	1	320.00

1311	PATENT APPL. EXAMINATION FEE	800.00	1	800.00

TOTAL AMOUNT: **$1,980.00**

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

C-TYPE NATRIURETIC PEPTIDE VARIANTS TO TREAT SKELETAL DYSPLASIA IN CHILDREN

Cross-Reference to Related Applications

[0001] The present application claims the priority benefit of U.S. Provisional Patent Application No. 63/220,275, filed July 9, 2021 and U.S. Provisional Patent Application No. 63/350,196 filed June 8, 2022, hereby incorporated by reference in their entireties.

Incorporation by Reference of Material Submitted Electronically

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification in .XML format. The name of the text file containing the Sequence Listing is " 57038_Seqlisting.xml", which was created on July 7, 2022 and is 65,087 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

Field of the Disclosure

[0003] The disclosure relates, in general, to the use of variants of C-type natriuretic peptide (CNP) to treat skeletal dysplasias in children.

Background

[0004] The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP) (Genbank Accession No. NP_006163, for the ANP precursor protein, NPPA), brain natriuretic peptide (BNP) (GenBank Accession No. NP_002512, for the BNP precursor protein, NPPB), and C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)). These small, single chain peptides (ANP, BNP, CNP) have a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. ANP and BNP bind to and activate the natriuretic peptide receptor A (NPR-A), also termed guanalyl cyclase A (GC-A), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Likewise, CNP interacts with NPR-B (GC-B) to stimulate the generation of cGMP (J. Hypertens., 10: 1111-1114 (1992)). A third type of receptor, NPR-C, binds each of the natriuretic peptides with high affinity and functions primarily to capture the peptides from the extracellular compartment and deposit the peptides into lysosomes, where they are degraded (Science, 238: 675-678 (1987)). ANP and BNP are produced primarily within the muscle cells of the heart, and are believed to have important roles in cardiovascular homeostasis (Science, 252: 120-123 (1991)). CNP is

expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

[0005] In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). The predominant CNP form in cartilage is unknown. Both CNP-53 and CNP-22 bind similarly to NPR-B. Furthermore, they both induce cGMP production in a dose-dependent and similar fashion (VT Yeung, Peptides, 17: 101-106 (1996)).

[0006] Natural CNP genes and polypeptides have been previously described. U.S. Patent No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Patent No. 6,034,231 discloses the human gene and polypeptide of proCNP (126 amino acids) and the human CNP-53 gene and polypeptide.

[0007] Clearance of CNP from the extracellular space occurs through the action of membrane-bound neutral endopeptidase (NEP), which rapidly degrades CNP (Biochem. J., 291 (Pt 1): 83-88 (1993)), and through NPR-C, which binds to and deposits CNP into lysosomes, where CNP is degraded. CNP has been shown to have an in vivo half-life of 2.6 min in the normal human (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). The low plasma concentration of CNP (J. Bone Moner. Res., 19 (Suppl.1)S20 (2004)) and its co-expression with NPR-B in a number of tissues suggests that CNP functions primarily through an autocrine/paracrine mechanism.

[0008] As stated above, CNP binds to and activates natriuretic peptide receptor B (NPR-B), also termed guanylyl cyclase B (GC-B), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. Accordingly, elevated or depressed levels of any of the components in this pathway may lead to aberrant bone or cartilage growth. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae.

Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, et al., J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, et al., Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

[0009] Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. Both chondrocyte proliferation and differentiation appear to be disturbed, leading to remarkably short growth plate cartilage (P. Krejci et al., J. Cell Sci. 118: 5089-5100 (2005)). Endochondral ossification is the process that governs longitudinal long-bone growth. There are four zones of the growth plate – resting, proliferative, hypertrophic and zone of calcification. In the growth plate, NPR-B is expressed by proliferative cells while NPR-C is expressed by hypertrophic cells (Yamashite et al., J. Biochem. 127: 177-179 (2000)). In normal endochondral bone growth, chondrocytes organize in columns and proliferate in the proliferative zone of the growth plate. These columns are disorganized in achondroplasia patients. Additionally, the hypertrophic zone is where the cells become large and eventually apoptose (lyse), leading to osteocyte invasion and mineralization. The hypertrophic chondrocytes and the overall size of the zone are much smaller in achondroplasia patients than in normal patients. CNP is an agonist for NPR-B, a positive regulator of chondrocyte and bone growth. Downstream signaling of CNP/NPR-B inhibits the FGFR-3 pathway at the level of mitogen-activated protein kinase (MAPK). Inhibition at MAPK promotes proliferation and differentiation of the chondrocytes in the proliferative and hypertrophic zones of the growth plate, resulting in bone growth.

[0010] In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

[0011] Therapeutic use of CNP is currently limited by its short plasma half-life, which has been shown to be 2.6 minutes in vivo in humans (Hunt et al., J Clin. Endocrinol. Metab., 78: 1428-35 (1994)). To increase CNP concentration above intrinsic levels (about 5 pM) typically found in human plasma, continuous infusion has been necessary in all human and animal

studies using systemically administered CNP. Two mechanisms by which the half-life of CNP is reduced in human plasma are degradation by neutral endopeptidase (NEP) and clearance by natriuretic peptide receptor C (NPR-C) (Olney, Growth Horm. & IGF Res., 16: S6-S14 (2006)). A CNP variant having a longer in vivo serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a sustainable therapeutic strategy.

[0012] The biological activities of various analogs and derivatives of CNP have been evaluated. See e.g., US Patent 7,276,481, PCT Publication No. WO 94/20534 which discloses a chimera of CNP-22 and the 5-amino acid C-terminus of ANP designated as the vasonatrin peptide (VNP). U.S. Patents 8,198,242, 8,598,121, 9,907,834 and 10,646,550 disclose use of CNP variants to treat skeletal dysplasias, such as achondroplasia. U.S. Patents 7,642,243 and 8,658,373 describe use of variants of CNP-22 or CNP-53 to treat arthritis.

Summary

[0013] The present disclosure relates to use of CNP variant peptides to treat skeletal dysplasias in children, for example, children between 2 and 5 years old, and in particular children less than or about 2 years old, to treat one or more skeletal dysplasia-associated symptom(s), or to improve one or more consequence(s) or physiological symptom(s) of a skeletal dysplasia in a subject suffering therefrom, when the CNP variant peptide is administered above a certain dose amount and/or under a certain drug administration regime as herein described. It is disclosed herein that administration of CNP variant peptides can lead to improved growth velocity in achondroplasia subjects.

[0014] In various embodiments, the disclosure provides a method of treating skeletal dysplasia in a subject from about 2 years old to about 5 years old, comprising administering to the subject a composition comprising a C type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, or improve at least one symptom or physiological consequence of the skeletal dysplasia, wherein the CNP variant is selected from the group consisting of the CNP variants disclosed herein.

[0015] In various embodiments, the disclosure provides a method of treating skeletal dysplasia in a subject less than or about 2 years old, comprising administering to the subject a composition comprising a C type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, or improve at least one symptom or physiological consequence of the skeletal dysplasia, wherein the CNP variant is selected from the group consisting of the CNP variants disclosed herein.

[0016] In various embodiments, the composition is administered at a dose of 30 µg/kg. In various embodiments, the subject is about 6 months to about 2 years old. In various embodiments, the subject is 0 to about 6 months old. In various embodiments, the subject is about 3 to about 6 months old. In various embodiments, the subject is 0 to about 3 months old. In various embodiments, if the subject starts treatment at younger than about 2 years old, the dosage may be decreased to 15 µg/kg when the subject reaches 2 years old.

[0017] In one embodiment, the treatment is an improvement in one or more symptoms of skeletal dysplasia selected from the group consisting of increased absolute growth, improved or increased growth velocity, increased QCT bone mineral density (BMD), improvement in growth plate morphology, increased long-bone growth, improvement in the morphology of the spine, improved or increased elbow joint range of motion and decreased sleep apnea.

[0018] In one embodiment, the skeletal dysplasia is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature. In one embodiment, the skeletal dysplasia is achondroplasia.

[0019] Also provided is a method of increasing long bone growth in a subject less than or about 2 years old, comprising administering a composition comprising a CNP variant peptide to a subject in need thereof, wherein the administering increases long bone growth. Also provided is a method of increasing long bone growth in a subject about 2 years old to about 5 years old, comprising administering a composition comprising a CNP variant peptide to a subject in need thereof, wherein the administering increases long bone growth. In one embodiment, the subject has achondroplasia.

[0020] The disclosure also contemplates a method of enhancing or increasing the velocity of growth (i.e., growth velocity) in a subject less than or about 2 years old, comprising administering a composition comprising a CNP variant peptide to a subject in need thereof, wherein the administering enhances or increases growth velocity in the subject. Also provided is a method of enhancing or increasing the velocity of growth (i.e., growth velocity) in a subject about 2 years old to about 5 years old, comprising administering a composition comprising a CNP variant peptide to a subject in need thereof, wherein the administering enhances or increases growth velocity in the subject. In one embodiment, the subject has achondroplasia. In various embodiments, the enhancement or increase in growth velocity is an increase in annualized growth velocity in the range of 25%-50% change from baseline in the subject. In one embodiment, the enhancement or increase in growth velocity is an increase in annualized growth velocity of at least about 25%, more preferably at least about 40%, change from baseline in the subject.

[0021] It is contemplated that the enhancement in growth velocity may be assessed by measuring standing height, sitting height, weight, head circumference, upper arm length, lower arm length, upper leg length, lower leg length, hand length and/or foot length.

[0022] In the various methods and compositions described herein, the CNP variant peptide is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);

KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

[0023] In various embodiments, the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); and PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24).

[0024] In various embodiments, the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);

[0025] In the various methods described herein, the amount of CNP variant peptide administered ranges from about 15 µg/kg to about 100 µg/kg, preferably about 15 µg/kg to about 60 µg/kg, more preferably about 30 µg/kg to about 60 µg/kg. In various embodiments, the amount of CNP variant peptide administered is at least about 15 µg/kg, or at least about 30 µg/kg.

[0026] In the various methods described herein, the CNP variant peptide or composition or formulation comprising the same is administered either subcutaneously or parenterally, preferably subcutaneously. It is also contemplated that the CNP variant peptide is administered by other routes. Exemplary routes of administration include, but are not limited to subcutaneous, intraarticular, intravenous, intra-arterial, intraperitoneal, intramuscular, intradermal, intrathecal, intraperitoneal, intramuscular, intradermal, intrathecal, topical, transdermal, or transmucosal administration.

[0027] In the various methods described herein, the CNP variant peptide or composition or formulation comprising the same is administered to the subject in a single treatment or in multiple doses. The multiple doses may be administered once daily, or in multiple doses over the course of treatment. In various embodiments, it is contemplated that the CNP variant peptide or composition or formulation comprising the same is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months or other as deemed appropriate by a treating physician. In particularly preferred embodiments, the

CNP variant peptide or composition or formulation comprising the same is administered to the subject once daily for a period of at least one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, twelve months, or longer.

[0028] In certain embodiments of the methods described herein, administration of the CNP variant peptide or composition or formulation comprising the same is adjusted to allow for periods of preventative or therapeutic treatment followed by a recovery period. For example, the CNP variant peptide or composition or formulation comprising the same may be administered intraarticularly, subcutaneously, intravenously, or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant peptide daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant peptide is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

[0029] In the various methods described herein, it is preferred that administration of the CNP variant peptide or composition or formulation comprising the same not cause or result in an adverse event in the subject rated as grade two or higher. In other embodiments of the methods described herein, administration of the CNP variant peptide or composition or formulation comprising the same results in no clinically significant change in blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration and/or blood aspartate aminotransferase concentration in said subject.

[0030] In the various methods described herein, in preferred embodiments, administration of the CNP variant peptide or composition or formulation comprising the same results in a change in (i) the upper body length to lower body length ratio, (ii) the upper arm length to forearm length ratio, or (iii) the upper leg length to lower leg length ratio, of between about - 0.25 and about 0.25, about -0.20 and about 0.20, about -0.15 and about 0.15, about -0.10 and about 0.10, or

about -0.05 and about 0.05, compared to baseline (i.e., prior to administration of the CNP variant peptide or composition or formulation comprising the same).

[0031] In various embodiments, the disclosure provides for compositions or formulations comprising a CNP variant peptide, or use of a composition or formulation comprising a CNP variant peptide in the methods described herein. In one embodiment, the composition or formulation further comprises a pharmaceutically acceptable excipient, carrier or diluent. In certain embodiments, the composition is prepared from a formulation, either liquid or lyophilized, comprising a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6. In various embodiments, the pH is about 5.5.

[0032] Also contemplated is a method of treatment as described herein further comprising administration of a second agent.

[0033] In various embodiments, the CNP variant peptides used in the methods and compositions or formulations described herein can be attached to a hydrophobic acid, and can be attached to one or more hydrophobic acids. Non-limiting examples of hydrophobic acids include straight-chain or branched, saturated or unsaturated C5-C12 carboxylic acids (e.g., pentanoic acid, heptanoic acid, etc.) and natural fatty acids. The hydrophobic acids can be attached to the N-terminus, the C-terminus, and/or the side chain of one or more amino acid residues. In one embodiment, the hydrophobic acids are conjugated to the N-terminus.

[0034] In yet another embodiment, the CNP variant peptides used in the methods and compositions of the present invention are chimera, or fusion proteins, comprising a CNP variant peptide, and a cleavable peptide or protein, or peptide tag. Exemplary cleavable proteins or peptides include, but are not limited to, histidine (e.g., hexa-His) tags; TAF12: human transcription factor TAF12; KSI: ketosteroid isomerase; MBP: maltose-binding protein; 13-Gal: 13-galactosidase; GST: glutathione-S-transferase; Trx: thioredoxin; CBD: chitin binding domain; BMPM: BMP-2 mutation, SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly(Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

[0035] In various embodiments, the CNP variant peptide useful in the methods may be a monomer or a dimer. In a related embodiment the monomers of dimeric CNP variant peptides

can be attached N-terminus to N-terminus via a linker or no linker, N-terminus to C-terminus via a linker or no linker, or C-terminus to C-terminus via a linker or no linker.

[0036] In any of the embodiments disclosed herein, the CNP variant peptides may have substantially the same or better biological activity than wild-type CNP-22. For example, the CNP variant peptides may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP-22, or may have greater activity than CNP-22, e.g., with respect to interaction with NPR-B (GC-B) to stimulate the generation of cGMP. Alternatively, or in addition, the CNP variant peptides may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP-22, or may have greater activity than CNP-22, with respect to regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation, chondrocyte differentiation, inhibition of the mitogen activated protein (MAP) kinase/ MEK (Raf-1) kinase signaling pathway, and promoting endochondral ossification. In any of the embodiments described herein, the CNP variant peptides may comprise an amino acid sequence that is at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or more identical or homologous to amino acids 6-22 or 1-22 of wild-type CNP-22.

[0037] In various embodiments, the CNP variant peptides can optionally have conjugation(s) or extension(s), e.g., at the N- and/or C-terminus to facilitate cartilage targeting, reduce renal clearance, and/or increase resistance to NEP degradation. Such conjugation(s) or extension(s) can comprise molecules or sequences formed or derived from, e.g., polyAsp, polyGlu, cartilage-targeting peptides, sialoprotein, PEGs, carbohydrates, hydrophobic acids, NPPC or non-CNP (poly)peptides, or combinations thereof.

[0038] It is further contemplated that the CNP variant peptides can be conjugated to a hydrophobic polymeric or non-polymeric moiety, such as, e.g., heptanoic acid, pentanoic acid, or fatty acids. The hydrophobic moiety can be conjugated to the side chain of an amino acid residue, including but not limited to a lysine, a serine, a cysteine or a threonine, or can be attached to the N-terminus and/or C-terminus of the CNP variant.

[0039] In various embodiments, the CNP variant comprises a synthetic polymeric group. In various embodiments, the variant comprises a synthetic polymeric group coupled to the variant through a hydrolysable linker. In various embodiments, the synthetic polymeric group comprises a hydrophilic polymer moiety. In various embodiments, the hydrophilic polymer moiety comprises polyethylene glycol (PEG).

[0040] In various embodiments, the disclosure provides for use of a pharmaceutical composition comprising a CNP variant peptide, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In various embodiments, the compositions are sterile pharmaceutical compositions suitable for parenteral injection. In some embodiments, the compositions comprise substantially pure CNP variant peptide, e.g. at least about 90% or 95% pure. In some embodiments, the compositions contain less than about 5%, 4%, 3%, 2%, 1% or 0.5% contaminants, such as other human proteins, porcine proteins, or CNP-53 or fragments thereof (other than the desired CNP variant peptide). In various embodiments, the sterile composition is administered to a subject for treating or preventing a skeletal dysplasia or one or more symptoms or physiological consequences of a skeletal dysplasia disclosed herein.

[0041] CNP variant peptides useful herein advantageously retain CNP activity and exhibit increased serum half-life. Retention of CNP activity can be shown, for example, as retention of desired in vivo biological effect, or retention of at least about 50%, 60%, 70%, 80%, 90%, 95% or at least about 100% of the cGMP stimulating activity of CNP-22, under the same concentration (e.g., 1 μM of CNP peptide or greater than the ED80). In some embodiments, CNP variant peptides exhibit at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or 40-fold increase in serum half-life compared to CNP-22.

[0042] In a related embodiment, the CNP variant peptides described herein have increased NEP resistance and exhibit increased half-life compared to wild-type CNP-22. In one embodiment, the half-life of the CNP variant peptides is increased by about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or about 100% compared to wild-type CNP-22.

[0043] Use of any of the foregoing CNP variant peptides, or composition or formulation comprising the same, described herein in preparation of a medicament for treatment of skeletal dysplasia in children less than or about 2 years old and symptoms or other physiological manifestations described herein associated with skeletal dysplasia is also contemplated. Use of any of the foregoing CNP variant peptides, or composition or formulation comprising the same, described herein in preparation of a medicament for treatment of skeletal dysplasia in children about 2 to about 5 years old and symptoms or other physiological manifestations described herein associated with skeletal dysplasia is also contemplated. Syringes, e.g., single use or pre-filled syringes, sterile sealed containers, e.g. vials, bottle, vessel, and/or kits or packages

comprising any of the foregoing CNP variant peptides, or composition or formulation comprising the same, optionally with suitable instructions for use, are also contemplated.

[0044] It is also contemplated that the CNP variants are in a formulation comprising (a) a CNP variant peptide described herein and (b) one or more components selected from the group consisting of a buffering agent, an isotonicity agent, a stabilizer and an anti-adsorbent agent. In particularly preferred embodiments, buffering agents employed in the formulations may be citric acid monohydrate, sodium citrate dihydrate, or a combination of the two. In yet other preferred embodiments, isotonicity agents employed in the formulations of the present invention may be trehalose dihydrate, D-mannitol, or a combination of the two. In other preferred embodiments, the stabilizer employed in the formulations of the present invention is L-methionine. In yet other preferred embodiments, the anti-adsorbent agent employed in the formulations of the present invention is polysorbate 80.

[0045] In various embodiments, the formulations useful in the methods are lyophilized, in liquid form, or in liquid form that has been reconstituted from a previously lyophilized form. In certain embodiments, the formulations useful in the methods are preservative-free and, optionally, may be contained within a type 1 untreated borosilicate glass vial. Optionally, the formulations have a pH in the range of between about 5.0 and about 6.0, preferably about 5.5.

[0046] In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1 mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 0.5 to 5 mg/ml, or from about 0.5 to 3 mg/ml, or from about 0.5 mg/ml to about 2.0 mg/ml, or from about 0.8 to about 2.0 mg/ml, or from about 1 mg/ml to about 10 mg/ml. In various embodiments, the CNP variant is in a concentration of 0.5 mg/mL to 2 mg/mL. In various embodiments, the CNP variant is in a concentration of 0.8 mg/mL to 2 mg/mL. In various embodiments, the CNP variant is at a concentration of 0.8 mg/mL. In various embodiments, the CNP variant is at a concentration of 2.0 mg/mL. In various embodiments, the CNP variant is reconstituted from a lyophilized powder. In various embodiments, the CNP variant peptide of the formulation is CNP-38 or Pro-Gly-CNP-37 (BMN111).

[0047] In other embodiments, the formulations useful in the methods comprise a CNP variant peptide as described herein, citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80. In certain embodiments, the CNP variant peptide is present at a concentration between about 0.5 mg/ml and about 2.0 mg/ml, the

citric acid monohydrate is present at a concentration between about 0.15 mg/ml and about 0.40 mg/ml, the sodium citrate dihydrate is present at a concentration between about 0.5 mg/ml and about 1.5 mg/ml, the trehalose dihydrate is present at a concentration between about 30 mg/ml and about 70 mg/ml, the D-mannitol is present at a concentration between about 10 mg/ml and about 20.0 mg/ml, the L-methionine is present at a concentration between about 0.5 mg/ml and about 1.5 mg/ml and the polysorbate 80 is present at a concentration between about 0.01 mg/ml and about 0.1 mg/ml. In various embodiments, the CNP variant is present at a concentration of about 2.0 mg/ml, the citric acid monohydrate is present at a concentration of about 0.28 mg/ml, the sodium citrate dihydrate is present at a concentration of about 1.08 mg/ml, the trehalose dihydrate is present at a concentration of about 58.01 mg/ml, the D-mannitol is present at a concentration of about 15.0 mg/ml, the L-methionine is present at a concentration of about 0.73 mg/ml and the polysorbate 80 is present at a concentration of about 0.05 mg/ml. In various embodiments, the CNP variant is present at a concentration of about 0.8 mg/ml, the citric acid monohydrate is present at a concentration of about 0.28 mg/ml, the sodium citrate dihydrate is present at a concentration of about 1.08 mg/ml, the trehalose dihydrate is present at a concentration of about 58.01 mg/ml, the D-mannitol is present at a concentration of about 15.0 mg/ml, the L-methionine is present at a concentration of about 0.73 mg/ml and the polysorbate 80 is present at a concentration of about 0.05 mg/ml.

[0048] It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the disclosure and, as such, is meant to be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as "one embodiment", "some embodiments", "further embodiment", "specific exemplary embodiments", and/or "another embodiment", each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the disclosure. Where examples of values falling within ranges are disclosed, any of these examples are contemplated as possible endpoints of a range, any and all numeric values between such endpoints are contemplated, and any and all combinations of upper and lower endpoints are envisioned.

Brief Description of the Figures

[0049] Figure 1 shows the demographic makeup of the study groups for 52-week vosoritide treatment in Cohorts 1-3.

[0050] Figure 2 represents baseline Z score for study Cohorts 1-3 at 52 weeks of treatment.

[0051] Figure 3 represents baseline AGV and change in baseline AGV for study Cohorts 1-3 over 52 weeks.

[0052] Figure 4 illustrates the positive change in height Z score over time after 52 weeks of treatment.

Detailed Description

[0053] The present disclosure relates to methods of using CNP variant peptides to treat skeletal dysplasias in children less than or about 2 years old, one or more symptoms or physiological consequences of skeletal dysplasias and other disorders having a skeletal dysplasia and/or CNP-associated symptom or component. Treatment with a CNP variant peptide in skeletal dysplasia reduces one or more symptoms or physiological consequence of a skeletal dysplasia, increases long bone growth in a subject in need thereof, or improves or increases growth velocity in a subject in need thereof, for example in a subject suffering from a skeletal dysplasia.

Definitions

[0054] Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below.

[0055] As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

[0056] The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood

that the term "about" or "approximately" applies to each one of the numerical values in that series.

[0057] Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present disclosure may employ, unless otherwise indicated, certain conventional methods of synthetic organic chemistry, mass spectrometry, preparative and analytical chromatography, protein chemistry, biochemistry, recombinant DNA technology and pharmacology, within the skill of the art. See, e.g., T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

[0058] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

[0059] The following amino acid abbreviations are used throughout the text

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

[0060] Conventional notation is used herein to portray polypeptide and peptide sequences: the left-hand end of a polypeptide or peptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

[0061] In one embodiment, the CNP variant peptides described herein useful in the methods are generated via recombinant means, using a polynucleotide encoding a CNP variant peptide.

CNP variant peptides expressed by such polynucleotides may be produced by methods including growing host cells in culture medium under conditions suitable for expression of the polynucleotide encoding a CNP variant, and isolating the expression product from the host cells or culture medium. Actual expression products may vary slightly from the encoded protein product depending on any post-translational processing. Methods for producing the CNP variant peptides of the present invention are disclosed at least in US Patent No. 8,198,242, incorporated herein by reference.

[0062] The terms "identical" and percent "identity", in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

[0063] The phrase "substantially homologous" or "substantially identical", in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In certain embodiments, the substantial homology or identity exists over regions of the sequences that are at least about 25, 50, 100 or 150 residues in length. In another embodiment, the sequences are substantially homologous or identical over the entire length of either or both comparison biopolymers.

[0064] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0065] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math., 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison,

WI), or by visual inspection. One example of a useful algorithm is PILEUP, which uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35: 351-360 (1987) and is similar to the method described by Higgins & Sharp, CABIOS, 5: 151-153 (1989). Another algorithm useful for generating multiple alignments of sequences is Clustal W (Thompson et al., Nucleic Acids Research, 22: 4673¬4680 (1994)). An example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm (Altschul et al., J. Mol. Biol., 215: 403-410 (1990); Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA, 89: 10915 (1989); Karlin & Altschul, Proc. Natl. Acad. Sci. USA, 90: 5873-5787 (1993)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[0066] "Wild-type" (wt) is a term referring to the natural form, including sequence, of a polynucleotide, polypeptide or protein in a species. A wild-type form is distinguished from a mutant form of a polynucleotide, polypeptide or protein arising from genetic mutation(s).

[0067] In one embodiment, a first peptide that is an "analog" or "variant" or "derivative" of a second peptide is a peptide having at least about 50%, 60% or 70% sequence homology, but less than 100% sequence homology, with the second peptide. Such analogs, variants or derivatives may be comprised of non-naturally occurring amino acid residues, including without limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues.

[0068] The natriuretic peptide precursor C (NPPC) polypeptide is a single chain 126-amino acid pre-pro polypeptide, and which upon cleavage ultimately results in wild type CNP-22 (wtCNP-22). Removal of the signal peptide from NPPC yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again to produce the mature 22-amino acid peptide (CNP, or CNP-22). In one embodiment, a "CNP variant peptide" is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that a CNP variant peptide may comprise from about 1 to about 53, or 1 to 38, or 1 to 37, or 1 to 35, or 1 to 34, or 1 to 33, or 1 to 32, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, a CNP variant may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

[0069] The term “effective amount” means a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate “effective” amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

[0070] "Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, “treatment” refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

[0071] A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

[0072] A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

[0073] "Pharmaceutical composition" or "formulation" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a CNP variant peptide, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the

pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

[0074] "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

[0075] A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, etc.) and salts of ammonia or organic amines.

[0076] By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

[0077] "Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

[0078] As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm

animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

[0079] The terms "polyethylene glycol", "PEG", "polyethylene oxide" and "PEO" are used interchangeably herein unless indicated otherwise. A CNP variant conjugated via an amino group to a "PEOn" polymer associated with the number n, in general has the formula: CH3– [–O–CH2CH2–]n–C(=O)–NHR, where n is the number of ethylene oxide units and R denotes the rest of the peptide. The "PEOn" polymer can optionally have an alkylene group, (CH2)m, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. Such a "PEOn" (e.g., PEO12 or PEO24) polymer is monodispersed, i.e., is a single discrete polymer of a particular molecular weight. Similarly, a CNP variant conjugated via an amino group to a "PEGnK" polymer associated with the number nK, in general has the formula: CH3–[–O–CH2CH2–]p–C(=O)–NHR, where p is an integer greater than 1. The "PEGnK" polymer also can optionally have an alkylene group, (CH2)m, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. However, such a "PEGnK" (e.g., PEG1K, PEG2K, PEG5K or PEG20K) polymer is polydispersed, i.e., contains a mixture of polymers having a distribution of molecular weights, where the number nK denotes the polymer number-average molecular weight (Mn) in kilo Daltons. For example, "PEG2K" conjugated to a CNP variant denotes a polydispersed PEG polymer having a polymer number-average molecular weight of around 2 kDa.

[0080] When a range of the mass of a polymer (e.g., PEG) is given (e.g., in units of kDa), the range refers to a range of polymer number-average molecular weights, not to a range of molecular weights of multiple polymers in a polydispersed mixture, unless expressly indicated otherwise.

CNP Variant Peptides

[0081] The use of CNP-22 as a therapeutic is limited by its short half-life in plasma (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). In human plasma, the concentration of CNP-22 typically is less than five picomolar. CNP-22 is degraded and cleared from circulation by NEP and NPR-C in humans (Growth Hormone & IGF Res., 16: S6-S14). In all human and animal studies using systemically administered CNP-22, continuous infusion has been used to increase the CNP-22 concentration in the subjects. A CNP peptide having a longer half-life and at least a similar level of functionality would be beneficial to a CNP-based therapeutic strategy. CNP

variant peptides having improved properties are disclosed in International Application Nos. WO 2009/067639 and WO 2010/135541 and U.S. Patent Nos. 8,198,242, 8,598,121, 8,377,884, 9,907,834 and 10,646,550 all specifically incorporated herein by reference.

[0082] Exemplary CNP variant peptides contemplated for use in the methods include: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6); RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7); VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);

KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO:48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO:54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); and/or PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64).

[0083] In certain embodiments, the CNP variant peptides are derivatives of CNP-37 or CNP-38. The CNP-37 variant peptides can contain amino acid addition(s), deletion(s), and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)) at any one or more of the 37 positions of CNP-37. Non-limiting examples of substitutions that can be made in CNP-37, based on the numbering of CNP-22, include K4R, G5S, G5R, G8S, K10R, G15S, S16Q, M17N, G19R, and combinations thereof.

[0084] In one embodiment, the CNP variant peptides is a CNP-38, CNP-37, CNP-36, CNP-35, CNP-34, CNP-33 or CNP-32 that has been modified to comprise a hydrophobic moiety. In various embodiments the hydrophobic moiety is a polyethylene glycol.

[0085] Additional CNP variants are disclosed in International Application PCT/US2020/045885, which describes hydrophobic peptide salts of CNP variants useful to treat skeletal dysplasias, and incorporated herein by reference. Still other CNP variants contemplated for use are described in PCT/US2020/051100 (herein incorporated by reference), which discloses CNP sequence variants PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 66); PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 1); PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 67); and

PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 68), optionally wherein the variants further comprise an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the peptide further comprises an OH or an NH2 group at the C-terminus. In various embodiments, the variant peptide comprises a conjugate moiety. In various embodiments, the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the conjugate moiety is on a lysine residue. In various embodiments, the conjugate moiety comprises one or more acid moieties. In various embodiments, the acid moiety is a hydrophobic acid. In various embodiments, the conjugate moiety comprises one or more acid moieties linked to a hydrophilic spacer. In various embodiments, the hydrophilic spacer is any amino acid. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu). In various embodiments, the hydrophilic spacer is OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) or OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) linked to one or two or more OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the acid moiety is a fatty acid. Exemplary fatty acids include short chain, medium chain, or long chain fatty acids, or a dicarboxylic fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. Contemplated are C-6 to C-20 fatty acids, including but not limited to, C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acids, saturated or unsaturated. In various embodiments, the fatty acid is decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid, arachidic acid, or diacids of the same.

[0086] In one embodiment, the CNP variant peptides are modified CNP-37 or CNP-38 peptides having mutation(s)/substitution(s) at the furin cleavage site, designed to improve in vivo resistance to the furin protease, and/or containing glycine or proline-glycine at the N-terminus, designed to improve plasma stability and prevent pyroglutamine formation. Exemplary CNP-37 variants include but are not limited to:

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ NO: 1); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID

NO:45); and GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43).

[0087] In certain embodiments, the CNP variants may comprise a synthetic polymeric group. In various embodiments, the variant comprises a synthetic polymeric group coupled to the variant through a hydrolysable linker. In various embodiments, the synthetic polymeric group comprises a hydrophilic polymer moiety. In an embodiment, the hydrophilic polymers are water-soluble so that the CNP peptides conjugated thereto do not precipitate out in an aqueous (e.g., physiological) environment. Further, the hydrophilic polymers are biocompatible, i.e., do not cause injury, toxicity or an immunological reaction in vivo. The hydrophilic polymers can be branched or unbranched. In one embodiment, the hydrophilic polymers are not branched. In various embodiments, the hydrophilic polymer moiety comprises polyethylene glycol (PEG). Various sites of conjugation of CNP variants to a hydrophilic polymer are possible, including but not limited to: (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site (e.g., Lys4 and/or Lys 10); (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site.

[0088] Methods for preparing PEGylated CNP variant peptides generally comprise the steps of (a) reacting CNP variant with a PEGylation reagent under conditions suitable for attaching PEG to the CNP peptide (e.g., at the N-terminus), and (b) obtaining the reaction product(s), using techniques known in the art.

Synthesis and Purification of CNP Variant Peptides

[0089] In some embodiments, the CNP variant peptides useful herein are produced by recombinant expression, using certain techniques known in the art in certain embodiments. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. (1989)); DNA Cloning: A Practical Approach, Volumes I and II, D. N. Glover, Ed. (1985); and Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

[0090] In certain embodiments, the CNP variant peptides are produced by a recombinant process that comprises culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant peptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant peptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker. In some

embodiments, the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant peptide linked to the polynucleotide encoding the cleavable peptide or protein. In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. The expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant peptide.

[0091] Methods of producing CNP variant peptides are described in U.S. Patents 8,198,242, 8,377,884 and 8,598,121, 9,907,834 and 10,646,550 herein incorporated by reference.

[0092] Host cells used to produce CNP variant peptides can be bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells. Bacterial cells include without limitation E. coli cell lines and strains. Non-limiting examples of E. coli cell lines and strains include BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In an embodiment, CNP variant peptides and CNP fusion proteins are produced using BL21(DE3) cells. Mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), chondrocytes and other bone-derived cells, and precursors of these somatic cell types. Host cells containing the CNP variant DNA or RNA are cultured under conditions appropriate for growth of the cells, expression of the DNA or RNA and identification/selection of cells expressing the CNP variant peptide.

[0093] In certain embodiments, the CNP variant peptides are recombinantly expressed as fusion proteins comprising a CNP variant peptide and a cleavable carrier protein or cleavable tag (e.g., peptide tag), wherein the fusion protein comprises the CNP variant peptide directly linked to the cleavable carrier protein or tag or indirectly linked thereto via a linker. Use of a carrier protein or tag facilitates, e.g., detection, isolation and/or purification of the fusion protein. Cleavable carrier proteins and tags include, but are not limited to, histidine (e.g., hexa-His) tags; human transcription factor TAF12 (TAF12), TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A &

6D/6E), TAF12(C/A & 10D/10E); ketosteroid isomerase (KSI); maltose-binding protein (MBP); ß-galactosidase (ß-Gal); glutathione-S-transferase (GST); thioredoxin (Trx); chitin binding domain (CBD); BMP-2, BMP-2 mutants, BMP-2(C/A); SUMO; and mutants and fragments thereof.

[0094] In other embodiments, the CNP variant peptides described herein are synthesized using a peptide synthesizer and purified according to methods known in the art, e.g., according to the methods of Atherton and Sheppard, Solid Phase Peptide Synthesis: a Practical Approach, IRL Press (Oxford, England (1989)).

Methods of Using CNP Variant Peptides

Bone-Related Disorders/Skeletal Dysplasias

[0095] Fibroblast growth factors (FGFs) play important roles in bone formation, and mutations in FGF receptor genes (FGFR 1, 2 and 3) give rise to a variety of inherited skeletal malformations (Curr. Biol., 5: 500-507 (1995)). In particular, activating mutations in FGFR-3 are responsible for disorders of the long bones, including achondroplasia, the most common form of human genetic dwarfism (Nature, 371: 252-254 (1994); Cell, 78: 335-342 (1994)), the milder disorder hypochondroplasia (Ann. N.Y. Acad. Sci., 785: 182-187 (1996)), and the more severe and neonatal lethal thanatophoric dysplasia (TD) types I and II (Hum. Mol. Genet., 5: 509-512 (1996); Nat. Genet., 9: 321-328 (1995)). Mouse models overexpressing FGF-2, and consequentially activating FGFR-3, show shortened long bones and macrocephaly (Mol. Biol. Cell, 6: 1861-73 (1995)). Consistent with this model, mice deficient in FGFR-3 show remarkable skeletal overgrowth with wider growth plates (Nature Genet., 12: 390-397 (1996)).

[0096] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variant peptides of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic

dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature. Further, the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

[0097] In addition, the CNP variant peptides are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

[0098] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome).

[0099] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome), or insulin growth factor 1 receptor (IGF1R).

[0100] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3.

[0101] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0102] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from a RASopathy. In various embodiments, the RASopathy is Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, or LEOPARD syndrome.

[0103] In one embodiment, the RASopathy is hereditary gingival fibromatosis type 1.

[0104] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R).

[0105] In various embodiments, the short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0106] RASopathies are a group of rare genetic conditions caused by mutations in genes of the Ras/mitogen-activated protein kinase (MAPK) pathway. RASopathies are a group of disorders characterized by increased signaling through RAS/MAPK pathway. This pathway leads to downstream activation of the RAF/MEK/ERK pathway. Short stature is a characteristic feature of certain RASopathies. For example, CNP signaling inhibits RAF and leads to decreased MEK and ERK activation.

[0107] Treatment of RASopathies are contemplated herein. RASopathies associated with short stature include Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, and LEOPARD syndrome. Hereditary gingival fibromatosis type 1 is also a RASopathy contemplated herein. RASopathy patients (including Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, LEOPARD syndrome, hereditary gingival fibromatosis type 1) include patients with heterozygous variants in one or more of the following genes: BRAF, CBL, HRAS, KRAS, LZTR1, MAP2K1, MAP2K2, MRAS, NF1, NRAS, PPP1CB, PTPN11, RAF1, RRAS, RIT1, SHOC2, SOS1, or SOS2 (Tajan et al. Endocr. Rev. 2018;39(5):676–700).

[0108] Mutations in multiple genes can cause Noonan syndrome, which is characterized by short stature, heart defects, bleeding problems, and skeletal malformations. Mutations in the PTPN11 gene cause about half of all cases of Noonan's syndrome. SOS1 gene mutations cause an additional 10 to 15 percent, and RAF1 and RIT1 genes each account for about 5 percent of cases. Mutations in other genes each account for a small number of cases. The cause of Noonan syndrome in 15 to 20 percent of people with this disorder is unknown.

[0109] The PTPN11, SOS1, RAF1, and RIT1 genes all encode for proteins that are important in the RAS/MAPK cell signaling pathway, which is needed for cell division and growth (proliferation), differentiation, and cell migration. Many of the mutations in the genes associated

with Noonan syndrome cause the resulting protein to be turned on (active) and this prolonged activation alters normal RAS/MAPK signaling, which disrupts the regulation of cell growth and division, leading to the characteristic features of Noonan syndrome. See, e.g., Chen et al., Proc Natl Acad Sci U S A. 111(31):11473-8, 2014, Romano et al., Pediatrics. 126(4):746-59, 2010, and Milosavljević et al., Am J Med Genet 170(7):1874-80, 2016. It is contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve bone growth and short stature. It is also contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve other comorbidities associated with an overactive MAPK pathway in other cells throughout the body where the NPR2 receptor is expressed on its surface.

[0110] In certain embodiments, the CNP variant peptides and compositions and formulations described herein are useful for improving one or more of the symptom(s) or physiological consequences of a skeletal dysplasia, wherein the improvement may be increased absolute growth, increased growth velocity, increased qualitative computed tomography (QCT) bone mineral density, improvement in growth plate morphology, increased long bone growth, improvement in spinal morphology, improved elbow joint range of motion and/or decreased sleep apnea. In this regard, it is noted that the terms "improved", "improvement", "increase", "decrease" and grammatical equivalents thereof are all relative terms that when used in relation to a symptom or physiological consequence of a disease state, refer to the state of the symptom or physiological consequence of the disease after treatment with a CNP variant peptide (or composition or formulation comprising the same) of the present invention as compared to the same symptom or physiological consequence of the disease before treatment with a CNP variant peptide (or composition or formulation comprising the same) of the present invention (i.e., as compared to "baseline"). As described above, a "baseline" state can be determined either through measurement of the state in the subject prior to treatment (which can subsequently be compared to the state in the same subject after treatment), or through measurement of that state in a population of subjects suffering from the same affliction that share the same or similar characteristics (e.g., age, sex and/or disease state or progression).

Increasing or Enhancing Growth Velocity

[0111] The compositions and formulations of the present disclosure may also be administered for the purpose of enhancing or increasing growth velocity in a subject suffering from skeletal dysplasia. In a preferred embodiment, the subject suffers from achondroplasia.

Efficacy of treatment is measured by various parameters. In various embodiments, efficacy is assessed as the change in annualized growth velocity from the baseline period to the intervention period. Efficacy is also assessed as the change in height standard deviations score (SDS), also referred to as a height Z-score, from baseline to end of treatment as measured using the CDC growth curves, and growth velocity SDS is based on the Bone Mineral Density in Childhood Study (Kelly et al., J. Clin. Endocrinol. Metab. 2014;99(6):2104–2112).

[0112] Measurements of growth velocity in subjects may be made over time using standard techniques well known in the art. In certain embodiments, measurements of parameters such as standing height, sitting height, weight, head circumference, upper arm length, lower arm (forearm) length, upper leg length, lower leg length (knee to foot), hand length (wrist to end of finger) and/or foot length may be made over a specified period of time to determine the specific rate of growth as measured by any particular parameter (i.e., the growth velocity). Measurements of growth velocity over a specified period of time may be "annualized", wherein the rate of growth calculated over a specific period of time is converted to the expected rate of growth over a period of one year. Measurements of growth velocity in a subject prior to treatment with a CNP variant peptide of the present invention (i.e., a "baseline" growth velocity) may be compared to measurements of growth velocity during or after treatment with a CNP variant peptide of the present invention to determine the effect of treatment on changes in growth velocity in the subject. A "baseline" growth velocity may also be determined from a population of subjects of the same general age, sex and disease status as the individual being treated with a CNP variant peptide of the present invention.

[0113] Improvements in growth velocity as measured by assessment of one of more of the parameters described above (e.g., standing height, etc.) induced or caused by treatment of a subject in need thereof with a CNP variant peptide of the present invention (or composition or formulation comprising the same) may be quantitatively measured. In this regard, in certain embodiments, annualized increase or improvement of growth velocity of any particular parameter in a subject treated with a CNP variant of the present invention (or a composition or formulation comprising the same) are at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%, or more above baseline. In certain embodiments, the methods described herein result in an annualized increase in growth velocity as measured by standing height of at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%, or more above baseline. In other embodiments, the methods described herein result in an annualized increase in growth velocity as measured by either sitting height, weight, head circumference, upper arm length, lower arm

(forearm) length, upper leg length, lower leg length (knee to foot), hand length (wrist to end of finger) or foot length of at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%, or more above baseline. As described herein, the baseline comparator may be the annualized growth velocity as measured by the specific parameter prior to treatment of the same subject with a CNP variant peptide of the present invention, or may be the growth velocity determined from a population of subjects of the same general age, sex and disease status as the individual being treated with a CNP variant peptide of the present invention.

[0114] Anthropometry data relating to proportionality of certain body segments to others may also be obtained from subjects both before and after treatment with a CNP variant peptide to determine whether administration of a CNP variant peptide results in changes in such proportionality. For example, ratios of upper body length (waist to top of head) to lower body length (waist to foot) may be calculated both prior to treatment with a CNP variant peptide (i.e., baseline) and after treatment with a CNP variant peptide to determine whether treatment with the CNP variant peptide has an adverse effect on body proportionality. In younger subjects, e.g., patients 0 to 2 years old, measurement of body length and crown to rump length may take precedence over standing height and sitting height for measurement of annualized growth velocity and Z-score.

[0115] Ratios of upper arm to forearm length or upper leg to lower leg (knee to foot) may also be calculated and compared to determine the effect of CNP variant peptide treatment on body proportionality. In a preferred embodiment, administration of a CNP variant peptide to a subject does not result in a significant change in body proportionality as measured by any specific ratio as compared to baseline. In this regard, in certain embodiments, treatment with a CNP variant peptide, or composition or formulation comprising the same, of the present invention results in a change in any of the above described ratios of no more than 0.5, 0.25, 0.20, 0.15, 0.10 or 0.05, preferably no more than 0.05.

[0116] In the various methods described herein, it is preferred that administration of the CNP variant peptide or composition or formulation comprising the same not result in an adverse event related to the study drug of grade two or higher, or grade three or higher, in the treated subject. In this regard, techniques for measuring and grading physiological events associated with the treatment of a subject with a compound, and a listing of graded adverse events, maintained by the US National Institute for Health (NIH) under the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 (Quick Reference Guide, Common Terminology

Criteria for Adverse Events (CTCAE) Version 5.0 Published: November 27, 2017) which is herein incorporated by reference.

[0117] Moreover, in the various methods described herein, it is preferred that administration of the CNP variant peptide or composition or formulation comprising the same not result in a clinically significant change (either increase or decrease as compared to baseline) in one or more of the following physiological parameters; blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration or blood aspartate aminotransferase concentration. By "clinically significant change" is meant a change in any one or more of the above parameters that is observed to result in a detectable/observable, adverse physiological change in the subject's overall health.

Pharmaceutical Compositions and Formulations of CNP Variant Peptides

[0118] In additional embodiments, the disclosure contemplates use of pharmaceutical compositions and formulations comprising a CNP variant peptide, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (e.g., inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

[0119] Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactant, emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (e.g., water, ethanol); diluents of various buffer content (e.g., Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (e.g., Polysorbate 20, Polysorbate 80); anti-oxidants (e.g., methionine, ascorbic acid, sodium metabisulfite); preservatives (e.g., Thimerosol, benzyl alcohol, m-cresol); and bulking substances (e.g., lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, e.g., Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)), which is incorporated herein by reference in its entirety.

[0120] For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (e.g., oil/water

emulsions). A carrier can be a solvent or dispersing medium containing, e.g., ethanol, a polyol (e.g., glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

[0121] In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant peptide in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1 mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 1 mg/ml to about 10 mg/ml, or from about 2 mg/ml to about 10 mg/ml, or about 2 mg/ml or about 10 mg/ml. In other embodiments, the formulation may be a lyophilized formulation or may be a liquid formulation that was previously reconstituted from a lyophilized formulation.

[0122] In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, citric acid monohydrate and sodium citrate dihydrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. For example, acetate is a more efficient pH buffer at pH 5 than pH 6, so less acetate may be used in a solution at pH 5 than at pH 6. In some embodiments, the buffering agent has a concentration of about 10 mM ± 5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0 ± 1.0, or is at about pH 5.5 ± 1.0.

[0123] In other embodiments, the compositions contain an isotonicity agent to render the solution or suspension isotonic and more compatible for injection. Non-limiting examples of isotonicity agents include NaCl, trehalose, mannitol, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is trehalose or mannitol, which can be employed individually or in combination. In certain embodiments, trehalose or mannitol is in a concentration of about 160 ± 20 mM , or about 140 mM ± 20 mM, or about 120 ± 20 mM , or about 100 mM ± 20 mM, or about 80 mM ± 20 mM, or about 60 mM ± 20 mM.

[0124] In various embodiments, the compositions may comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4% ± 0.2%, or about 1% ± 0.5%, or about 1.5% ± 0.5%, or about 2.0% ± 0.5%. In certain embodiments of the invention, the composition or formulation does not contain a preservative.

[0125] In various embodiments, the compositions contain an anti-adsorbent agent (e.g., to mitigate adsorption of a CNP variant to glass or plastic). Anti-adsorbent agents include without limitation benzyl alcohol, polysorbate 20, and polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

[0126] In various embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%.

[0127] In various embodiments, the compositions contain an antioxidant. An exemplary anti-oxidant is, without limitation, ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP variant peptide is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

[0128] Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (e.g., hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (e.g., acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (e.g., isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Company, (Easton, Pennsylvania (1990)).

[0129] Formulations for parenteral administration can be prepared, e.g., as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (e.g., buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile

fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

[0130] Exemplary CNP peptide-containing formulations are described in U.S. Patents 8,198,242, 8,598,121, 9,907,834 and 10,646,550. Use of CNP formulations having a pH in the range from about 4 to about 6 is contemplated.

[0131] In various embodiments, the composition is an extended release composition.

[0132] In various embodiments, CNP variant peptides can be formulated in pharmaceutical carriers for administration to subjects affected by skeletal dysplasia. In some embodiments, liquid formulations of CNP variant peptides are formulated according to any combinations of the ingredients and their amounts or concentrations are described below:

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant peptide	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent	NaCl	140 mM ± 20 mM
Isotonicity-adjusting agent	Trehalose; mannitol	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.1% or 0.2%

Ingredient Class	Ingredient	Concentration Range
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5% - 100% (neat)
Stabilizer	Methionine	0.01% - 0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1% - 1%
Stabilizer	Thioglycerol	0.1% - 1%

Anti-adsorbent	Polysorbate 20 Polysorbate 80 Benzyl alcohol	0.001% - 0.5% 0.001% - 0.5% 0.5% - 1.5%

[0133] Compositions comprising a CNP variant peptide can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dextran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol and/or trehalose is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%.

[0134] In various embodiments, lyophilized formulations of CNP variant peptides are prepared from formulations formulated according to any combinations of the ingredients and their amounts or concentrations described below:

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent/ bulking agent	Sorbitol	5% ± 3%
Isotonicity-adjusting agent/ bulking agent	Mannitol/Trehalose	5% ± 3%
Isotonicity-adjusting agent/ bulking agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%

Ingredient Class	Ingredient	Concentration Range
Stabilizer	Glycerin (glycerol)	5% - 100% (neat)
Stabilizer	Methionine	0.01% - 0.2%

Stabilizer	Ascorbic acid/ ascorbate salt	0.1% - 1%
Stabilizer	Thioglycerol	0.1% - 1%
Anti-adsorbent	Polysorbate 20	0.001% - 0.5%
	Polysorbate 80	0.001% - 0.5%
	Benzyl alcohol	0.5% - 1.5%

[0135] In various embodiments, a formulation comprising a CNP variant peptide has a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5. In some embodiments, for pH 4-5.5 a suitable buffering agent is acetic acid/acetate (e.g., sodium acetate), and for pH 5.5-6 a suitable buffering agent is citric acid/citrate. Citric acid/citrate (e.g., sodium citrate) is also a suitable buffering agent in the range of pH 3-6 or pH 4-6. In certain embodiments, the buffering agent has a concentration in the formulation of about 2-50 mM, or about 2-40 mM, or about 2-30 mM, or about 5-30 mM, or about 2-20 mM, or about 5-20 mM, or about 5-15 mM.

[0136] Also to minimize or avoid deamidation of a CNP variant peptide, water can be removed from the formulation by lyophilization. In some embodiments, lyophilized formulations contain any combinations of the following components: buffer: sodium acetate and acetic acid, or sodium citrate and citric acid; isotonicity/bulking agent: mannitol (e.g., 3-10%, 2-8% or 4-6%); sucrose (e.g., 6-20%, 5-15% or 8-12%); antioxidants: methionine and/or ascorbic acid with molal ratio of each antioxidant to CNP variant peptide from about 0.1:1 to about 1:1, or from about 0.5:1 to about 5:1, or from about 1:1 to about 15:1, or from about 1:1 to about 10:1, or from about 3:1 to about 10:1.

[0137] Deamidation can also be minimized or avoided by storing a CNP composition (e.g., a liquid formulation or a lyophilized formulation) at lower temperature, such as at about 5 °C, 0 °C, -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, or -100 °C.

[0138] To minimize or avoid oxidation of oxidizable residues (e.g., methionine) in a CNP variant peptide, the variant can be formulated with one or more antioxidants. Exemplary antioxidants include, but are not limited to, methionine, ascorbic acid, and thioglycerol. Oxidation of, e.g., methionine residues can also be minimized or prevented by purging oxygen from a liquid medium (if a liquid formulation) with nitrogen or argon, and/or by purging oxygen from a container or packaging with nitrogen or argon.

[0139] In some embodiments, to minimize or prevent adsorption (e.g., adsorption of a CNP variant peptide to plastic or glass), polysorbate 20, polysorbate 80 or benzyl alcohol, or a combination thereof, is added to a CNP formulation. In certain embodiments, each of the anti-adsorbent(s) is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%. Exemplary range(s) of anti-adsorbent(s) in the formulation include without limitation from about 0.001% to about 0.5% of polysorbate 20, from about 0.001% to about 0.5% of polysorbate 80, and/or from about 0.5% to about 1.5% of benzyl alcohol.

[0140] In various embodiments, the formulation comprises citric acid, sodium citrate, trehalose, mannitol, methionine, polysorbate 80, and optionally sterile water for injection (WFI).

[0141] The disclosure also provides kits containing, e.g., bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, e.g., sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices, such as aerosol or injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

Dosages and Frequency of Dosing

[0142] As used herein, the term "therapeutically effective amount" of an active agent (e.g., a CNP variant peptide) refers to an amount that provides therapeutic benefit to a patient. The amount may vary from one individual to another and may depend upon a number of factors, including the overall physical condition of the patient. A therapeutically effective amount of a CNP variant peptide can be readily ascertained by one skilled in the art, using publicly available materials and procedures. For example, the amount of a CNP variant peptide used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (214 females and 189 males), which list height for age, head circumference, and segmental growth (Horton WA et al., Standard growth curves for achondroplasia, J. Pediatr., 93: 435-8 (1978)). CDC charts can be used to assess weight for

age and weight for height or BMI for age. Secondary outcomes with courses that are more chronic in nature can also be measured.

[0143] The dosing frequency for a particular individual may vary depending upon various factors, including the disorder being treated and the condition and response of the individual to the therapy. In certain embodiments, a pharmaceutical composition containing a CNP variant peptide is administered to a subject about one time per day, one time per two days, one time per three days, or one time per week, twice per week, three times per week, once every two weeks, or monthly. In one embodiment, for treatment of bone-related disorders (e.g., skeletal dysplasias, including achondroplasia), a daily or weekly dose of a CNP variant peptide is administered to patients until and/or through adulthood.

[0144] In certain embodiments, the CNP variant compositions described herein are administered at a dose in the range from about 3, 4, 5, 6, 7, 8, 9 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175,180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variant compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variant compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400,450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 mg/kg, or about 0.5, 0.8, 1.0, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of CNP or CNP variant described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc. In various embodiments, the CNP or CNP variant is administered daily subcutaneously. In various embodiments, the CNP or CNP variant is administered weekly subcutaneously. In various embodiments, the CNP variant is administered at a dose of 2.5 µg/kg/day to 60 µg/kg/day, 10 µg/kg/day to 45 µg/kg/day, or 15 µg/kg/day to 30 µg/kg/day. In various

embodiments, the CNP variant is administered at a dose of 15 μg/kg/day. In various embodiments, the CNP variant is administered at a dose of 30 μg/kg/day. Administration of a CNP variant peptide may occur over an extended period of time, in some cases, over one month, three months, six months, 12 months or more. In various embodiments, treatment may start at 3 months or 6 months, or when a subject is determined to have a skeletal dysplasia, e.g., by genetic testing, and may be continued until the growth plates are closed.

[0145] The CNP variants or compositions thereof can also be administered by implantation of a depot at the target site of action (e.g., an abnormal or degenerated joint or cartilage area). Alternatively, the CNP variant can be administered sublingually under the tongue (e.g., sublingual tablet) by transdermal delivery (e.g., by means of a patch on the skin) or orally in the form of micro-spheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

[0146] The CNP variant compositions described herein can be administered to patients in need thereof at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia). The safety and therapeutic efficacy of the CNP variant can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50 /ED50. Active agents exhibiting a large therapeutic index are normally preferred.

[0147] The frequency of dosing/administration of a CNP variant peptide for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The CNP variant peptide can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the CNP variant peptide is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months, or as deemed appropriate by the treating physician.

[0148] In some embodiments, a CNP variant peptide is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant peptide may be administered intravenously, subcutaneously,

intraarticularly or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant peptide daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant peptide is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

Modes of Administration

[0149] The CNP variant peptides, or pharmaceutical compositions or formulations comprising them, can be administered to subjects in various ways such as, e.g., by injection subcutaneously, intraarticularly, intravenously, intra-arterially, intraperitoneally, intramuscularly, intradermally, or intrathecally. In one embodiment, the CNP variant peptides are administered by a single subcutaneous, intraarticular, intravenous, intra-arterial, intraperitoneal, intramuscular, intradermal or intrathecal injection once a day, one every other day, once every three days, or once a week.

[0150] The CNP variant peptides can also be administered by direct injection at or near the site of disease. Further, the CNP variant peptides can be administered by implantation of a depot at the target site of action (e.g., an abnormal or degenerated joint or cartilage area). Alternatively, the CNP variant peptides can be administered sublingually under the tongue (e.g., sublingual tablet) or by inhalation into the lungs (e.g., inhaler or aerosol spray), by delivery into the nasal cavity (e.g., intranasal spray), by delivery into the eye (e.g., eye drop), or by transdermal delivery (e.g., by means of a patch on the skin). The CNP variant peptides may also be administered orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

[0151] A further method of administration is by osmotic pump (e.g., an Alzet pump) or mini-pump (e.g., an Alzet mini-osmotic pump), which allows for controlled, continuous and/or slow-release delivery of the CNP variant peptide or pharmaceutical composition over a pre-

determined period. The osmotic pump or mini-pump can be implanted subcutaneously, or near the target site (e.g., the long bones of limbs, the epiphyses, etc.).

[0152] It will be apparent to one skilled in the art that the CNP variant peptides or compositions thereof can also be administered by other modes. Determination of the most effective mode of administration of the CNP variant peptides or compositions thereof is within the skill of the skilled artisan.

Biomarkers

[0153] For treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements in utero and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, Collagen X, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

[0154] Biomarkers useful for assessing treatment of skeletal dysplasia also include NTproCNP, N terminal fragment of collagen X (CXM), CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I and fragments thereof, collagen type I and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, collagen X, and alkaline phosphatase. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine. In some embodiments, the biomarkers are measured in blood, plasma or serum from animals undergoing efficacy/pharmacodynamic in vivo studies and/or from the conditioned media of ex vivo studies.

[0155] One CNP signaling marker is cGMP (guanosine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (in vitro) after CNP exposure, conditioned media from bone ex-plant studies (ex vivo) after CNP exposure, and in the plasma (in vivo) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

[0156] Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into

circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover pre-cedes new bone formation.

[0157] A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

[0158] Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type II collagen (cartilage-specific marker for cartilage formation), collagen type I C-telopeptide (CTx), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic in vivo studies and from the conditioned media of ex vivo studies, using commercially available kits. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine.

[0159] In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP variant or composition described herein in order to monitor the effects of the CNP composition on bone and cartilage formation and growth in vivo. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP variant or composition has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g., endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, collagen type I C-telopeptide (CTx), osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, collagen X, aggrecan chondroitin sulfate, and alkaline phosphatase. In various embodiments, measured biomarkers include NTproCNP, N terminal fragment of collagen X (CXM), CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I

and fragments thereof, collagen type I and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, collagen X, and alkaline phosphatase.

[0160] In various embodiments, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP variant. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

[0161] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

Example 1 – Phase 2 Trial of CNP Variant in Achondroplasia Patients 0-5 Years Old

[0162] In order to determine the effect of CNP variant peptides on symptoms of skeletal dysplasia, such as achondroplasia, and on long bone growth and/or growth velocity (as measured by the rate of increase in the patient's standing height post-treatment as compared to pre-treatment) in younger achondroplasia patients, dose studies were carried out in achondroplaisa (ACH) children less than 2 years old (inclusive) and 2 to 5 years old who have documented ACH, as documented by clinical grounds and confirmed by genetic testing.

[0163] Prior to administration of a formulation comprising a CNP variant peptide as described herein. More specifically, Pro-Gly-CNP-37 peptide was formulated into a sterile, preservative-free composition comprising either 2.0 mg/ml CNP peptide in combination with 0.28 mg/ml citric acid monohydrate, 1.08 mg/ml sodium citrate dihydrate, 58.01 mg/ml trehalose dihydrate, 15 mg/ml D-mannitol, 0.73 mg/ml L-methionine and 0.05 mg/ml polysorbate 80, pH 5.5. This liquid composition was reconstituted from an originally lyophilized formulation comprising the described components and was supplied in a sterile, single use, type I untreated borosilicate clear glass vial. This reconstituted liquid formulation was employed in all human clinical treatment studies described herein.

[0164] To assess the safety and efficacy of daily SC injections of vosoritide in younger children with ACH a Phase 2, randomized, double-blind, placebo-controlled, global, multicenter from 0 to 5 years old was undertaken (Study 111-206 and extension study 111-208).

[0165] Cohort 1 – children aged ≥ 24 to < 60 months (n ≥ 30 total: three sentinel subjects who receive vosoritide, and at least 27 additional subjects randomized 1:1 to treatment or placebo control), stratified by age (≥ 24 to < 36 months and ≥ 36 months to < 60 months). All subjects Cohort 1: 15 µg/kg/day subcutaneous injection (all subjects).

[0166] Cohort 2 – children aged ≥ 6 to < 24 months (n ≥ 20 total: three sentinel subjects who receive vosoritide, and at least 17 additional subjects randomized 1:1 to treatment or placebo control), stratified by age (≥ 6 months to < 15 months and ≥ 15 months to < 24 months). All subjects in this cohort are treated with 30 µg/kg/day adjusted to 15 µg/kg/day when subjects reach 2 years of age.

[0167] Cohort 3 – children aged 0 to < 6 months (n ≥ 20 total: three sentinel subjects receive vosoritide, and at least 17 additional subjects randomized 1:1 to treatment or placebo control). Treatment begins at ≥ 3 months to < 6 months after 3 months of observation.

[0168] Cohort 1 and 2 subjects must have at least a 6-month period of pre-treatment growth assessment immediately before screening, and have one documented measurement of height/body length a minimum of 6 months prior to the screening visit for 111-206. Cohort 3 subjects must have a minimum of 3 months of observation prior to treatment. This observational period can be obtained either (1) via prior enrolment in another related study or (2) via enrollment in this 111-206 for a minimum of 3 months of non-treatment observation prior to commencement of treatment.

[0169] Primary endpoints include length/height Z-score at Week 52 and evaluation of any adverse events. Secondary endpoints include AGV at Week 52, Bilateral X-rays of lower extremities, Lumbar spine X-rays, DXA of whole body and spine, AUC0-∞, AUC0-t, C_{max}, T_{max}, $t_{1/2}$, CL/F, V_z/F, Hip monitoring clinical assessments, Bayley-III, WeeFIM, and ITQOL scores, CBCL scores, Anti-vosoritide total antibody (TAb), Anti-vosoritide antibody cross-reactivity with endogenous CNP, ANP, and BNP (TAb), Anti-vosoritide neutralizing antibody (NAb), CXM and bone-specific alkaline phosphatase, Collagen type II (CTX-II), Cyclic guanosine monophosphate (cGMP), anthropometric measurements, upper to lower body segment ratio, upper arm length to lower arm (forearm) length ratio, upper leg length (thigh) to knee to heel length ratio, upper leg length (thigh) to tibial length ratio, and arm span to standing height ratio.

[0170] Exploratory imaging assessment data included measurements and morphology of the spine, long bones and extremities, as well as measures of growth plate, bone age, and bone mineral density. Assays include anterior-posterior (AP) x-ray of pelvis for baseline hip

assessment; posterior-anterior (PA) radiographs of the left hand and wrist to assess bone age (Greulich, 1971, Stanford University Press.); (Tanner, 1975, Academic Press.), growth plates, hand length, and cortical thickness; AP lower extremity radiograph to assess growth plates, tibial length, cortical thickness, and bowing, AP radiographs of lumbar spine to assess transverse interpedicular distance. Lateral radiographs of lumbar spine to assess thoracolumbar lordosis angle, vertebral morphology, as well as other potential changes related to spinal stenosis, QCT scan of forearm and tibia to assess bone mineral density, growth plate morphology, and bone length are per-formed. This scan can be acquired with a standard CT scanner, calibration phantom, and designated software, using a predetermined low radiation dose protocol, which avoids direct radiation to the head and torso.

[0171] Flexion-extension measures of elbow joint range of motion are measured with a goniometer.

[0172] Biomarkers are evaluated by change from baseline and include, but are not limited to, assessment for cartilage turnover (CTX-II), chondrocyte and osteoblast activity (bone-specific alkaline phosphatase), bone formation (P1NP), bone turnover (osteocalcin); and markers of CNP bioactivity (cGMP, NT-proCNP, and ANP) as well as additional biomarkers. Samples for blood and urine biochemical markers of collagen and bone turnover, and for markers of CNP activity, are collected at the designated time points.

[0173] Adverse events. All patients in each of the above described cohorts were regularly assessed for the presence of adverse events (as defined by Common Terminology Criteria for Adverse Events (CTCAE) (Version 5.0 Published: November 27, 2017) specifically caused by treatment with the CNP variant peptide. No patient in any of the three study cohorts evidenced an adverse event of grade two or higher that was determined to be related to the study drug, demonstrating that treatment of achondroplasia patients with CNP variant peptides does not result in serious adverse physiological events.

[0174] All patients in each study cohort are regularly monitored for blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration and blood aspartate aminotransferase concentration.

[0175] Each measurement of standing height was converted to age- and sex-appropriate standard deviations score (SDS), also referred to as a height Z-score, by comparison with reference data available for average stature children from the Centre for Disease Control

(CDC). Height Z-score, and its changes from baseline at Week 26 and Week 52 was summarized and presented for sentinel subjects by cohort and overall.

[0176] AGV, upper to lower body segment ratio, and standing and sitting height were summarized similarly to height Z-score. Results were summarized for sentinel subjects by cohort and overall. Other anthropometric measures (sitting height, head circumference, etc.) were summarized at each time point and were evaluated for changes from baseline). For younger subjects, body length was measured and used in the analyses instead of standing height. Similarly, crown-to-rump was measured instead of sitting height.

[0177] 44 subjects had enrolled into Study 111-206 and received treatment, four sentinel subjects and 31 randomized subjects in Cohort 1, and 4 sentinel subjects and 16 randomized subjects in Cohort 2. 3 sentinel subjects and 4 randomized subjects were included in Cohort 3. At the first assessment period, in Cohort 1, four sentinels subjects have completed 52 weeks of treatment, while all 26 randomized subjects have completed the Week 13 visit and nine randomized subjects have completed the Week 39 visit. In Cohort 2, two sentinel subjects have completed the Week 26 visit. No subjects had discontinued from the study drug or the 111-206 study at the time of the data-cutoff date. At a later assessment period, 67 subjects had been enrolled and all 37 treated subjects were included in both the safety analysis population and the full analysis set (FAS), which was considered the primary efficacy analysis set. In addition, study 206 and study 208 are both ongoing, and the randomized subjects were still blinded as of the data cutoff point, so the results that are presented below are the results from the sentinel subjects. Figure 1 provides demographics of the study participants.

[0178] Results: 52 Weeks of Treatment

[0179] A summary of the results for the sentinel subjects is presented in Table 1.

Table 1. Change from Baseline in Annualized Growth Velocity, Height Z-score, and Body Proportion (Sentinel Subjects)

Endpoint	Analysis Visit [a)]	Cohort (n=4)	Cohort 2 (n=4)	Cohort 3 (n=3)
AGV (cm/year) [c)]	Baseline [b)]	6.21±1.73 (n=4)	11.93±1.32 (n=4)	24.78±5.39 (n=3)
	Week 26	6.90±1.64 (n=4)	10.40±2.15 (n=4)	14.43 (n=1)
	Change from baseline to Week 26	0.69±1.70 (n=4)	-1.53±3.03 (n=4)	-5.03 (n=1)
	Week 52	6.78±1.00 (n=4)	9.17±1.06 (n=4)	-
	Change from baseline to Week 52	0.57±0.91 (n=4)	-2.75±1.65 (n=4)	-
	Week 78	6.48±0.90 (n=4)	9.39 (n=1)	-
	Change from baseline to Week 78	0.27±1.13 (n=4)	-3.26 (n=1)	-
	Week 104	6.45±0.70 (n=3)	-	-
	Change from baseline to Week 104	-0.27±1.09 (n=3)	-	-
	Baseline	-4.51±0.33 (n=4)	-4.72±0.53 (n=4)	-4.18±0.77 (n=3)
	Week 26	-4.35±0.23 (n=4)	-4.29±0.65 (n=4)	-4.39 (n=1)

Height Z-score	Change from baseline to Week 26	0.15±0.19 (n=4)	0.43±0.69 (n=4)	0.08 (n=1)
	Week 52	-4.16±0.47 (n=4)	-3.88±0.35 (n=4)	-
	Change from baseline to Week 52	0.34±0.27 (n=4)	0.84±0.25 (n=4)	-
	Week 78	-4.06±0.50 (n=4)	-3.35 (n=1)	-
	Change from baseline to Week 78	0.45±0.29 (n=4)	0.78 (n=1)	-
	Week 104	-3.77±0.41 (n=3)	-	-
	Change from baseline to Week 104	0.62±0.36 (n=3)	-	-
Upper to Lower Body Segment Ratio	Baseline	2.25±0.36 (n=4)	2.56±0.25 (n=4)	3.12±0.37 (n=3)
	Week 26	2.21±0.35 (n=4)	2.49±0.22 (n=4)	3.00 (n=1)
	Change from baseline to Week 26	-0.04±0.06 (n=4)	-0.07±0.05 (n=4)	-0.37 (n=1)
	Week 52	2.23±0.24 (n=4)	2.37±0.21 (n=4)	-
	Change from baseline to Week 52	-0.02±0.12 (n=4)	-0.19±0.28 (n=4)	-
	Week 78	2.15±0.29 (n=4)	2.30 (n=1)	-
	Change from baseline to Week 78	-0.11±0.08 (n=4)	-0.29 (n=1)	-
	Week 104	2.19±0.22 (n=3)	-	-
	Change from baseline to Week 104	-0.19±0.10 (n=3)	-	-

Mean±SD, Individual values for 2 or less subjects, - : Not measured
AGV: annualized growth velocity.
a) Period from the start of study 206
b) For Cohorts 1 and 2, the calculations were based on height data from study 901 over the past 6 months; for Cohort 3, the calculations were based on height data from study 901 over the past 3 months.
c) AGV at each visit was calculated using height data for the previous 12 months.

[0180] Height Z-score Change from Baseline

[0181] For Cohort 1 sentinel subjects the mean (SD) baseline height Z-score was -4.51 (0.33). For Cohort 1 sentinel subjects (N=4), the mean (SD) change from Baseline in height Z score at Week 26 was +0.15 (0.19) and at Week 52 was +0.34 (0.27).

[0182] For Cohort 2 sentinel subjects the mean (SD) baseline height Z-score was -4.72 (0.53). For Cohort 2 sentinel subjects (N=4), mean (SD) change from Baseline at Week 26 was +0.43 (0.69) and at Week 52 was +0.84 (0.25). For cohort 3, sentinel subjects the mean (SD) baseline height Z-score was -4.18 (0.77). For Cohort 3 sentinel subjects (N=3), the mean (SD) change from Baseline in height Z score at Week 26 was +0.08.

[0183] Annualized Growth Velocity Change from Baseline

[0184] Baseline AGV was 6.21 cm/year for Cohort 1 sentinel subjects, 11.93 cm/year for Cohort 2 sentinel subjects, and 24.78 cm/yr for Cohort 3. In Cohort 1 sentinel subjects (N=4), following 26 weeks of treatment, there was a mean (SD) increase in AGV from Baseline of 0.69 (1.70) cm/year and after 52 weeks of treatment there was a mean (SD) increase of 0.57 (0.91) cm/year in AGV from Baseline.

[0185] In four Cohort 2 sentinel subjects, following 26 weeks of treatment, there was a mean (SD) decrease of 1.53 (3.03) cm/year in AGV from Baseline and of 2.75 (1.65) cm/year in AGV from Baseline at Week 52. In Cohort 3 sentinel subjects, following 26 weeks of treatment, there was a mean (SD) decrease of 5.03 cm/year in AGV from Baseline.

[0186] Upper to Lower Body Segment Ratio

[0187] There was no change in upper to lower body ratio over time in both cohorts at the initial checkpoint. In Cohort 1 sentinel subjects (N=4), mean (SD) change in upper to lower body segment ratio from Baseline to Week 26 was -0.04 (0.06) and -0.02 (0.12) to Week 52. For Cohort 2 sentinel subjects (N=2), the mean (SD) change in upper to lower body segment ratio from Baseline to Week 26 was -0.07 (0.05) and -0.19 (0.28) cm at Week 52. At later checkpoints, the same effect was noted for Cohort 3, with the change in ratio of -0.37 at week 26.

[0188] Standing Height and Sitting Height

[0189] In Cohort 1 sentinel subjects (N=4), mean (SD) change in standing height from baseline was 3.38 (0.82) cm at Week 26 and 6.78 (1.12) cm at Week 52. A consistent improvement was noted in standing height over 2 years of treatment in each of the subjects. Mean (SD) change in sitting height from baseline was 1.98 (1.12) cm at Week 26 and 4.69 (0.43) cm at Week 52.

[0190] In the Cohort 2 sentinel subjects (N=2), mean (SD) change in standing height from baseline was 5.15 (1.01) cm at Week 26 and 9.22 (0.98) cm at Week 52. Mean (SD) change in sitting height from baseline in 2 sentinel subjects was 3.70 (0.19) cm at Week 26.

[0191] Body Proportion Ratios of the Extremities Change from Baseline and Growth Measures Change from Baseline

[0192] There were no clinically significant changes in body proportions at Weeks 26 and 52. Across all growth measures (head circumference, arm span, upper arm length, lower arm length, lower body length, upper leg length, knee to heel length, and tibial length) there were consistent positive improvements in growth in both Cohort 1 and 2.

[0193] In Study 111-206, over 52 weeks of treatment at 15 µg/kg daily, the mean Cmax and AUC0-∞ of ACH subjects aged 2 to 5 years were generally consistent with 15 µg/kg in older ACH subjects (aged 5-18 years), ranging from 3810 to 6860 pg/mL and 118000 to 350000 pg-min/mL, respectively. Median Tmax ranged from 14.0 to 15.5 minutes, and mean t1/2 ranged from 15.2 to 29.3 minutes. Mean CL/F and Vz/F ranged from 82.1 to 150 mL/min/kg and 2650 to 3800 mL/kg respectively. evidence suggesting that at 15 rig/kg, exposure from study 111-206 Cohort 1 subjects aged 2 to < 5 years was similar to that from subjects aged 5 to 18 years in study 111-301.

[0194] The same dose of 15 µg/kg was used for Cohort 2 sentinels. Analysis of PK data for Cohort 2 indicated that the appropriate dose to achieve the desired exposure is 30 µg/kg/day, subsequent for 3 sentinels were increased to 30 µg/kg/day until the subjects reach 2 years where they were returned to 15 µg/kg dosing. Cohort 3 will be evaluated the same way.

[0195] Analysis of the full data set (FAS) for the placebo and Cohorts 1-3 at week 52 is presented below.

Table 2. Analysis of Covariance of Height Z-Score at Week 52 (Overall, Cohort 1, 2 and 3)

Analysis Population	Assessment	Overall		Cohort 1		Cohort 2		Cohort 3	
		Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide
FAS (randomized)	N	32	32	16	15	8	8	8	9
	Baseline, mean (SD)	-4.28 (0.26)	-3.79 (0.17)	-5.13 (1.15)	-4.27 (0.81)	-4.21 (1.24)	-3.39 (0.84)	-2.65 (0.28)	-3.34 (0.34)
	LSM change from baseline (95% CI)	-0.31 (-0.48, -0.13)	-0.06 (-0.26, 0.15)	-0.06 (-0.28, 0.16)	0.27 (0.04, 0.50)	-0.19 (-0.58, 0.20)	0.02 (-0.38, 0.41)	-0.91 (-1.36, -0.45)[c]	-0.68 (-1.21, -0.15)[c]
	Difference in LSM (95% CI)[a]	**0.25 (-0.02, 0.53)**		**0.33 (0.00, 0.67)**		**0.21 (-0.37, 0.79)**		**0.23 (-0.45, 0.91)[c]**	
	p-value[b]	0.0712		0.0510		0.4427		0.5083	
FAS	N	32	43	16	19	8	12	8	12
	Baseline, mean (SD)	-4.28 (0.26)	-3.88 (0.14)	-5.13 (1.15)	-4.32 (0.73)	-4.21 (1.24)	-3.67 (0.83)	-2.65 (0.28)	-3.39 (0.28)
	LSM change from baseline (95% CI)	-0.30 (-0.47, -0.13)	0.01 (-0.15, 0.17)	-0.03 (-0.24, 0.18)	0.26 (0.07, 0.45)	-0.17 (-0.53, 0.18)	0.24 (-0.05, 0.52)	-0.91 (-1.33, -0.49)[c]	-0.64 (-1.08, -0.20)[c]
	Difference in LSM (95% CI)[a]	**0.30 (0.07, 0.54)**		**0.29 (-0.01, 0.58)**		**0.41 (-0.06, 0.87)**		**0.27 (-0.34, 0.89)[c]**	
	p-value[b]	0.0110		0.0589		0.0804		0.3876[c]	

CI: confidence interval; FAS: full analysis set; SD: standard deviation
[a] Difference is vosoritide minus placebo. [b] Two-sided p-value. [c] Based on 10 imputed datasets.
LS means and difference in LS means were obtained from an analysis of covariance model. Model terms included treatment, sex, age stratum, baseline age, baseline AGV and baseline height Z-score.
For participants aged < 24 months, body length takes precedence over standing height. Subjects aged < 24 months at baseline and >= 24 months at Week 52, body length takes precedence.
If there was no height assessment available within the Week 52 analysis visit window, height Z-score at Week 52 was derived based on imputed height.

Table 3. Analysis of Covariance of Annualized Growth Velocity at Week 52 (Overall, Cohort 1, 2 and 3)

Analysis Population	Assessment	Overall		Cohort 1		Cohort 2		Cohort 3	
		Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide
FAS	N	32	32	16	15	8	8	8	9

Analysis Population	Assessment	Overall		Cohort 1		Cohort 2		Cohort 3	
		Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide
	Baseline, mean (SD)	9.60 (1.37)	11.06 (1.34)	4.20 (1.78)	4.74 (1.68)	10.55 (4.78)	11.51 (4.66)	19.45 (2.67)	21.19 (0.93)
	LSM change from baseline (95% CI)	-2.95 (-3.45, -2.45)	-2.17 (-2.76, -1.58)	0.89 (0.23, 1.55)	1.99 (1.31, 2.67)	-3.00 (-3.86, -2.13)	-2.36 (-3.22, -1.50)	-10.14 (-11.48, -8.79)[c]	-9.34 (-10.78, -7.91)[c]
	Difference in LSM (95% CI)[a]	**0.78 (0.02, 1.54)**		**1.10 (0.13, 2.07)**		**0.63 (-0.60, 1.87)**		**0.79 (-1.08, 2.67)[c]**	
	p-value[b]	0.0452		0.0276		0.2802		0.4072	
FAS	N	32	43	16	19	8	12	8	12
	Baseline, mean (SD)	9.60 (1.37)	11.66 (1.19)	4.20 (1.78)	5.07 (1.74)	10.55 (4.78)	11.66 (4.61)	19.45 (2.67)	22.09 (1.07)
	LSM change from baseline (95% CI)	-3.32 (-3.81, -2.84)	-2.41 (-2.88, -1.94)	0.77 (0.14, 1.40)	1.78 (1.20, 2.35)	-3.14 (-3.96, -2.31)	-2.23 (-2.90, -1.55)	-10.84 (-12.11, -9.57)[c]	-9.79 (-10.97, -8.62)[c]
	Difference in LSM (95% CI)[a]	**0.92 (0.24, 1.59)**		**1.00 (0.13, 1.88)**		**0.91 (-0.17, 1.99)**		**1.04 (-0.62, 2.71)[c]**	
	p-value[b]	0.0075		0.0256		0.0932		0.2200	

CI: confidence interval; FAS: full analysis set; SD: standard deviation

[a] Difference is vosoritide minus placebo. [b] Two-sided p-value. [c] Based on 10 imputed datasets.

LS means and difference in LS means were obtained from an analysis of covariance model. Model terms included treatment, sex, age stratum, baseline age, and baseline AGV.

For Subjects in Cohorts 1 and 2, AGV at baseline is defined as [(Height at baseline - last height measurement in 111-901 6 months prior to baseline)/(Date of baseline - Date of last height assessment in 111-901 6 months prior to baseline)] x 365.25. For participants in Cohort 3, the height 3 months prior to baseline in 111-901 or 111-206 is used instead. AGV at post-baseline visit was derived over baseline. For example, AGV at Week 52 = [(Height at Week 52 visit - Height at Baseline visit)/(Date of Week 52 visit - Date of Baseline Visit)] x 365.25.

[0196] Figure 2 represents baseline Z score for each cohort at 52 weeks of treatment. Figure 3 represents baseline AGV for each cohort over 52 weeks and Figure 4 illustrates the positive change in height Z score over time after 52 weeks of treatment. Overall results at week 52 showed that vosoritide (n=43), compared to placebo (n=32): increased height Z-score by 0.30 SD (95% CI 0.07, 0.54), increased AGV by 0.92cm/year (95% CI 0.24, 1.59), and did not worsen upper-to-lower body segment ratio, which changed by -0.06 (95% CI -0.15, 0.03). The data at week 52 show that vosoritide treatment improves annualized growth velocity, and also improves overall height Z score in treated patients compared to controls in all cohorts.

[0197] The pharmacokinetics of vosoritide in the different populations of patients was also analyzed for any change in PK (Table 4). PD information (plasma cGMP and serum CXM) in 4 sentinel subjects and 33 randomized subjects (aged ≥ 24 to < 60 months) receiving 30 µg/kg vosoritide was available.

Table 4. Pharmacokinetic Parameters at Multiple Subcutaneous Doses of BMN 111

Cohort (age)	Dose (μg/kg)	Analysis Visit	n	Cmax (ng/mL)	AUC0-t (ng·min/mL)	tmax (min)	t1/2 (min)	CL/F (mL/min/kg)	Vz/F (mL/kg)
Cohort 1 (24 to <60 months)	15	Day 1	3	4.47±3.30	167±110	15.0 [15.0, 15.0]	21.9±3.4	111±58.6	3700±2410
		Week 13	4	3.81±1.67	114±64.3	15.5 [15.0, 16.0]	15.2±2.7	150±58.5	3240±1180
		Week 26	4	6.86±4.59	327±336	15.0 [15.0, 17.0]	29.3±11.0	82.1±57.6	3800±3950
		Week 39	4	4.98±1.33	204±123	15.0 [15.0, 21.0]	27.1±9.0	90.3±51.6	3170±1350
		Week 52	4	5.65±3.01	218±138	14.0 [7.0, 17.0]	21.8±4.2	85.7±44.4	2650±1470
Cohort 2 (6 months to <24 months)	15	Day 1	3	2.84±1.23	65.9±33.6	15.0 [4.0, 15.0]	26.1 a)	147 a)	5550 a)
		Week 26	1	10.1	398	17.0	37.6	35.4	1920
		Week 39	2	1.01, 4.39	29.9, 229	5.0, 35.0	22.1 a)	63.1 a)	2010 a)
		Week 52	2	1.01, 2.87	17.7, 77.1	5.0, 15.0	12.5 a)	182 a)	3290 a)
	30	Week 13	3	12.5±9.19	530±370	13.0 [5.0, 16.0]	25.8±4.67	77.3±54.2	2740±1820
		Week 26	1	8.96	243	6.0	6.41	123	1130
		Week 39	1	6.18	333	15.0	22.3	86.0	2770
		Week 52	1	17.6	798	14.0	22.6	35.9	1170
Cohort 3 (birth to <6 months)	30	Day 1	3	12.3±3.68	372±119	5.0 [5.0, 13.0]	60.4±14.9	82.0±25.9	6800±577
		Week 13	1	16.4	489	5.0	23.3	59.3	1990
		Week 39	1	23.6	638	12.0	-	-	-

Mean±SD, tmax: Median [minimum, maximum], Individual values for 2 or less subjects, -: uncalculated
Cmax: maximum plasma concentration, AUC0-t: area under the plasma concentration-time curve from time 0 to t after administration, tmax: time taken to reach the maximum plasma concentration, t1/2: elimination half-life at terminal phase, CL/F: apparent total body clearance, Vz/F: apparent volume of distribution
a) n=1

[0198] Table 5 shows the changes from baseline at each assessment time point in the urine cGMP/Cr, and Table 6 shows the changes over time from baseline in the serum CXM. The PD parameters were analyzed only in the sentinel subjects in Cohorts 1 and 2.

Table 5. Change from Pre-dose in Urinary cGMP/Cr at Each Evaluation Time Point

Cohort (age)	Dose (μg/kg)	Collection Time	Day 1	Week 13	Week 26	Week 39	Week 52
Cohort 1 (24 to <60 months)	15	pre-dose	4620±1730 (4)	3350±515 (4)	3290±917 (4)	3420±1230 (4)	3290±400 (3)
		2 - 4 hr post dose	4880±6710 (4)	2450±1130 (4)	6290±4790 (4)	7620±4220 (4)	4820±4110 (3)
Cohort 2 (6 months to <24 months)	15	pre-dose	6770±3440 (3)	9020 (1)	4940 (1)	-	-
		2 - 4 hr post dose	-1260±2230 (3)	-1260 (1)	6040 (1)	-	-
	30	pre-dose	-	3340 (1)	5600±1120 (3)	8900 (1)	-
		2 - 4 hr post dose	-	10300 (1)	5090±5140 (3)	4910 (1)	-

Mean±SD(n), Individual values for 2 or less subjects, -: uncalculated, unit: pmol cGMP /mg Cr
a) Evaluated using values approximately 2 to 4 hours after administration.

Table 6. Change from Baseline in Serum CXM a) at Each Evaluation Time Point

Cohort	Dose (μg/kg)	Baseline	Day 8	Week 6	Week 20	Week 39

Cohort 1 (24 to <60 months)	15	8050±1017 (4)	3645±4392 (4)	10325±7962 (4)	5750±2859 (4)	4880±3724 (4)
Cohort 2 (6 months to <24 months)	15	8920±3662 (4)	5180±2963 (4)	2325±1633 (4)	10300 (1)	-
	30	-	-	-	-2790, 6210 (2)	-

Mean±SD (n), Individual values for 2 or less subjects, -: uncalculated、unit: pg/mL
a) Serum CXM was evaluated using values prior to BMN 111 administration.

[0199] Increases in post-dose plasma cGMP concentrations were observed, which remained constant over the time period of 52 weeks and were similar to the changes observed in older age group. CXM (mean and median) on vosoritide was increased in serum throughout the study (over up-to 39 weeks) compared to baseline and placebo. However, values overlapped considerably between vosoritide and placebo. High variability was noted in the measured parameters.

[0200] An updated blinded analysis in Cohort 1 subjects (19 vosoritide-treated subjects and 16 placebo-treated subjects aged ≥ 24 to < 60 months) showed that levels in serum CXM (averaged across all visits and per treatment) were roughly similar to those observed in older children (i.e. study 111-301) for the respective treatment.

[0201] Results: Greater Than 100 Weeks of Treatment

[0202] Updated results for subjects treated over 100 weeks demonstrated similar results as at week 52. Subjects in Cohort 1 (N=4) have received vosoritide for a median of 978 days (range: 921 to 1012 days) and in Cohort 2 (N=4) for a median of 733.5 days (range: 706 to 741 days). All sentinel subjects in Cohort 1 had treatment follow up for at least 130 weeks across the two studies 111-206/208 and in Cohort 2 up to Week 104; of note, one subject in Cohort 2 did not have a height assessment at the Week 104 visit as the visit was scheduled post the data cut-off date.

[0203] Height Z-Score

[0204] In Cohort 1 sentinels, the reduction in height deficit as evaluated by the height Z-score was sustained with vosoritide treatment over 2.5 years. At Week 104, two of the three sentinel subjects showed an improvement in the height Z-scores of +0.77 SDS and +0.86 SDS, while an improvement of +0.27 SDS (at Week 78) and +0.20 SDS was noted in the other two subjects. Cohort 1 exhibited mean (standard deviation [SD]) change from baseline at Week 52 (N=4) of +0.34 (0.27) standard deviation score (SDS), at Week 104 (N=3) of +0.62 (0.36) SDS, and at Week 130 (N=4) of +0.49 (0.34) SDS. For Cohort 2 sentinels, the mean (SD) change from

baseline in height Z-score at Week 52 (N=4) was +0.84 (0.25) SDS and was sustained at Week 104 (N=3) with a mean (SD) change from baseline of +0.69 (0.55) SDS.

[0205] AGV

[0206] In Cohort 1 sentinels the mean (SD) AGV at baseline was 6.21 (1.73) cm/year. The AGV at Week 104 in 2 subjects and Week 78 for 1 subject was higher than the AGV at Baseline; with only a slight decline noted in the fourth subject at Week 104. The subject with the decline in the AGV had a high baseline AGV and was youngest in the Cohort; the decline in the AGV is likely due to the subject being in the steeper curve of the growth decline. The mean (SD) AGV in the first year of treatment in 111-206 remained at 6.78 (1.00) cm/year and in the second year of treatment in 111-208 was 5.85 (0.39) cm/year. In the Cohort 2 sentinels, the mean (SD) AGV at baseline was 11.93 (1.32) cm/year, in the first year of treatment was 9.17 (1.06) and was 6.55 (0.38) in the second year of treatment. This decline in AGV is not as pronounced as would be expected in the untreated children with ACH of this age range, thus illustrating positive effect of vosoritide on growth velocity.

[0207] Standing Height/Body Length

[0208] For Cohort 1 sentinels, at week 104, a consistent reduction was noted in upper to lower body segment ratios in each of the subjects. The mean (SD) increase in standing height from baseline at Week 52 was 6.78 (1.12) cm, at Week 104 was 12.91 (1.61) and at Week 130 was 15.28 (1.39). In Cohort 2 sentinels, mean (SD) change in standing height from baseline was 9.22 (0.98) cm at Week 52 and at Week 104 was 15.76 (1.42).

[0209] Upper to Lower Body Ratio

[0210] In Cohort 1 sentinels, there was a reduction in the upper to lower body segment ratio over time with treatment with mean (SD) change in the ratio from baseline at Week 52 of -0.02 (0.12), at Week 104 (N=3) of -0.19 (0.10), and at Week 130 (N=4) of -0.19 (0.14). In Cohort 2 sentinels, the mean (SD) reduction in the upper to lower body segment ratio at Week 52 (N=4) was -0.19 (0.28) with further decline observed at Week 104 (N=3), showing a mean (SD) change from baseline of -0.33 (0.20).

[0211] Based on biological/mechanistic considerations, vosoritide is expected to exert favorable effects in all patients with ACH as long as their growth plates are open, although the benefit in young patients with a higher growth potential is likely greater than in older children.

[0212] Based upon the human clinical data described above, daily subcutaneously administered doses of 30 µg/kg/day to child and infant achondroplasia patients will also be tested for efficacy and safety as described above. It is fully expected that such administration regimes will provide both efficacious and safe for the treatment of achondroplasia in human patients.

[0213] Numerous modifications and variations to the disclosure, as set forth in the embodiments and illustrative examples described herein, are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the disclosure.

WHAT IS CLAIMED IS:

1. A method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6); RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID

NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48);MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64): and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

2. A method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO:);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7); VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO:40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59);PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

3. A method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in the subject wherein the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7); VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15);WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);

KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC(CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC[CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)]) (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO:

60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

4. The method of claim 3, wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 25% above baseline in said subject or wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 40% above baseline in said subject.

5. The method of claim 3 or 4, wherein the increase in growth velocity is assessed by measuring standing height, sitting height, weight, head circumference, upper arm length, lower arm length, upper leg length, lower leg length, hand length or foot length.

6. The method of any one of claims 3 to 5 that results in no clinically significant change in blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration or blood aspartate aminotransferase concentration in said subject.

7. The method of any one of claims 3 to 6 that results in

(a) a change in the upper body length to lower body length ratio of between -0.05 and 0.05;

(b) a change in the upper arm length to forearm length ratio of between -0.05 to 0.05; and/or

(c) a change in the upper leg length to lower leg length ratio of between -0.05 and 0.05.

8. The method of any one of claims 1 to 7, wherein

(a) said composition is administered once daily;

(b) said composition is administered once daily over a period of at least 6 months; or

(c) said composition is administered once daily over a period of at least 12 months.

9. The method of any one of the preceding claims, wherein the composition is administered to the subject via subcutaneous administration.

10. The method of any one of the preceding claims, wherein the composition is administered daily, every other day, 3 times weekly, 2 times weekly, or once weekly.

11. The method of any one of the preceding claims, wherein the composition is administered at a dose of 30 µg/kg.

12. The method of any one of the preceding claims, wherein the subject is about 6 months to about 2 years old.

13. The method of any one of the preceding claims, wherein the subject is about 0 to about 6 months old.

14. The method of any one of claims 11-13, wherein the dosage may be decreased to 15 µg/kg when the subject is 2 years old.

15. The method of claim any one of the preceding claims wherein the skeletal dysplasia is selected from the group consisting of hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature.

16. The method of claim 9, wherein the skeletal dysplasia is achondroplasia.

17. The method of any one of the preceding claims wherein the composition further comprises one or more pharmaceutical excipients.

18. The method of claim 17 wherein the composition comprises citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80.

19. The method of any one of the preceding claims wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); and PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24).

20. The method of any one of the preceding claims wherein the CNP variant is selected from the group consisting of
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20).

21. A composition comprising a C type natriuretic peptide (CNP) variant for use in treating skeletal dysplasia in a subject less than 2 years old wherein said CNP variant peptide is

administered to the subject in an amount of about 30 μg/kg and is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25)(SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO:35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO:3*);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-

CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

22. Use of a formulation comprising a C-type natriuretic peptide (CNP) variant for the manufacture of a medicament for treating skeletal dysplasia in a subject less than 2 years old wherein said CNP variant peptide is administered to the subject in an amount of about 30 µg/kg and is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

ABSTRACT

The present disclosure provides for use of variants of C-type natriuretic peptide (CNP), for the treatment of skeletal dysplasias in children, and improvement in one or more symptoms of skeletal dysplasias, such as long bone growth or growth velocity, and use in other disorders having a skeletal dysplasia and/or CNP-associated symptom or component.

Application Data Sheet

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Postal or Zip Code of mailing address:: 94949

Correspondence Information

Correspondence Customer Number::	90849

Application Information

Application Type::	Nonprovisional
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	Yes
Computer Readable Form (CRF)?::	Text
Title::	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
Attorney Docket Number::	30610/57038
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	4
Small Entity?::	No
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::	No

Representative Information

Representative Customer Number::	90849

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::	Prior Appl Status::
This Application	Claims benefit of provisional	63/350,196	06/08/22	Pending
This Application	Claims benefit of provisional	63/220,275	07/09/21	Pending

Foreign Priority Information

Applicant Information

Applicant Number::	1
Applicant Type::	Assignee
Organization Name::	BIOMARIN PHARMACEUTICAL INC.
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City of mailing address::	Novato
State or Province of mailing address::	CA
Country of mailing address::	US
Postal or Zip Code of mailing address::	94949

Assignee Information Including Non-Applicant Assignee Information

Authorization or Opt-Out of Authorization to Permit Access

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

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A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

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☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Katherine L. Neville/	Date (YYYY-MM-DD)	2022-07-11
Name	Katherine L. Neville	Registration Number	53,379

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
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NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Jonathan Day
Title	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	30610/57038

SIGNATURE of Applicant or Patent Practitioner

Signature	/Katherine L. Neville/	Date (Optional)	July 11, 2022
Name	Katherine L. Neville	Registration Number	53,379
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of 1 forms are submitted.

Doc Code: PA..
Document Description: Power of Attorney

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I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

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[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 90849

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

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OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

BIOMARIN PHARMACEUTICAL INC.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	Luisa Bigornia	Date (Optional)	
Name	Luisa Bigornia		
Title	Vice President, Intellectual Property, BioMarin Pharmaceutical Inc.		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[X] Total of 1 forms are submitted.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**07/11/2022 04:49:31 PM ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	60772868	FILING DATE	-
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 8

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
Application Data Sheet57038.pdf		6	Application Data Sheet	29 KB
Warning: This is not a USPTO supplied ADS fillable form. Data in the form cannot be automatically loaded to other USPTO systems.				
57038 Application.pdf		74	-	660 KB
57038 Application-SPEC.pdf	(1-58)	58	Specification	577 KB
57038 Application-CLM.pdf	(59-73)	15	Claims	218 KB
57038 Application-ABST.pdf	(74-74)	1	Abstract	88 KB

Transmittal 57038.pdf	1	Transmittal of New Application	129 KB
57038 Figures.pdf	4	Drawings-only black and white line drawings	442 KB
57038 Seqlisting Listing.xml	-	Sequence Listing (ST.26 XML File)	64 KB
Power of Attorney 57038.pdf	2	Power of Attorney	140 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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Transmittal 57038.pdf	9EA4CC4085CBB19248124EAC122875ECF00EBE7E08519EADB7DB5B902B911FAEE7BB2CA9E938B3BB825D8060B4DD44E3C8866D434853220B0DF919EF6CA8A4CE

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Power of Attorney 57038.pdf	D9BB639C4300B1731B93554581EEE62F503536122A452E0519E53A5E11EDAC26F2C49AFF6A985F1DF775DDAFC259846C199BF639CAD4B4E5E4A33619BEBABD7A

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Sequence Listing Report for Application No: 17811748

File Name: 57038 Seqlisting Listing.xml (65087 B), **Created:** July 11, 2022, 05:41 PM
USPTO Receipt Date: July 11, 2022
Validation Date: August 24, 2022, 04:13 PM
Reviewed by: Nieves-Herrera, Joanie

DEFECTIVE: This validation report concerns the sequence listing filed 07-11-2022 and indicates the errors found by the USPTO during validation of the sequence listing. Any resolution of the errors identified in this validation report should not be construed as meaning that the sequence listing will comply with all of the requirements of 37 CFR §§ 1.821- 1.825, WIPO Standard ST.25, or WIPO Standard ST.26.
For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV

Reviewer's Comments

1. E261 ==========
Validation Report for Application: 17811748 cannot be generated. This file is not compliant with the ST.26 DTD. Timestamp: Wed Aug 24 16:13:47 EDT 2022
==========

This sequence listing is not compliant with the current ST.26 DTD which is version 1.3. Also, this sequence listing was generated using the outdated, beta version of WIPO Sequence, version 1.0.0. WIPO Sequence 1.0.0 does NOT produce a sequence listing in XML format that is compliant with WIPO Standard ST.26.

Please download the most recent version of WIPO Sequence, version 2.1.2, and regenerate this sequence listing.

WIPO Sequence version 2.1.2 can be downloaded from WIPO at:

https://www.wipo.int/standards/en/sequence/index.html

The messages shown below do not indicate non-compliance with the sequence rules, and do not require correction:

Validated By CRFValidator v 1.5.0

Application No: 17811748 Version No: 1.0

CRF Copy Request from application : Version :

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Output Set: /slic/CRF_WORKFLOW/17/811/748/1.0/57038 Seqlisting Listing.xml.pdf

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Finished: 2022-08-24 16:13:47.304
Elapsed: 0 hr(s) 0 min(s) 0 sec(s) 0 ms
Total Warnings: 0
Total Errors: 1
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code	Error Description
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Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Jonathan Day et al.

Application No.: 17/811,748 | Confirmation No.: 2988

Filed: July 11, 2022 | Art Unit: Not Yet Assigned

For: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children | Examiner: Not Yet Assigned

SUBMISSION OF OATH OR DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant respectfully submits the executed inventor Oath and Declarations of Jonathan Day and Elena Fisheleva.

No fees are believed due with this submission. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge such fees to our deposit account no. 13-2855.

Dated: October 21, 2022

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

As the below named inventor, I hereby declare that:

This declaration is directed to:

- [ ] The attached application, or
- [x] United States application or PCT international application number 17/811,748 filed on 07/11/2022.
  - [ ] As Amended on _____ (if applicable)

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein. I acknowledge the duty to disclose information that is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information that became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Jonathan Day Date (Optional): 8-9-2022

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

As the below named inventor, I hereby declare that:

This declaration is directed to:

- ☐ The attached application, or
- ☒ United States application or PCT international application number 17/811,748 filed on 07/11/2022.
  - ☐ As Amended on _____ (if applicable)

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein. I acknowledge the duty to disclose information that is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information that became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

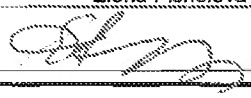
I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Elena Fisheleva Date (Optional): 8-9-2022

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

Electronic Acknowledgement Receipt

EFS ID:	46870024
Application Number:	17811748
International Application Number:	
Confirmation Number:	2988
Title of Invention:	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
First Named Inventor/Applicant Name:	Jonathan Day
Customer Number:	90849
Filer:	Katherine Lynn Neville/Valeria Rodriguez
Filer Authorized By:	Katherine Lynn Neville
Attorney Docket Number:	30610/57038
Receipt Date:	21-OCT-2022
Filing Date:	
Time Stamp:	16:02:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	Submission_of_Declarations_57038.pdf	14040 f46781603e3f1e50f17b55ef2ef881bc6b2b358d	no	1

Warnings:

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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
17/811,748	07/11/2022		1980	30610/57038	22	5

CONFIRMATION NO. 2988

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

FILING RECEIPT

OC00000005103998?

Date Mailed: 11/23/2022

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Jonathan Day, Buckinghamshire, UNITED KINGDOM;
Elena Fisheleva, Novato, CA;

Applicant(s)

BIOMARIN PHARMACEUTICAL INC., Novato, CA;

Power of Attorney: The patent practitioners associated with Customer Number 90849

Domestic Priority data as claimed by applicant

This appln claims benefit of 63/350,196 06/08/2022
and claims benefit of 63/220,275 07/09/2021

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 11/22/2022

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/811,748**

Projected Publication Date: To Be Determined - pending completion of Sequence Disclosures

Non-Publication Request: No

Early Publication Request: No
Title

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
17/811,748	

Applicant(s) Jonathan Day

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
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2	1					
3	1					
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Total Depend	18	↵		↵		↵
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/811,748

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	23 minus 20 =	* 3			OR	x 100 =	300
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 480 =	960
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							860
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	3640

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/811,748	07/11/2022	Jonathan Day	30610/57038

CONFIRMATION NO. 2988

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

FORMALITIES LETTER

OC000000051039990

Date Mailed: 11/23/2022

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Filing Date Granted

Applicant is given **TWO MONTHS FROM THE DATE OF THIS NOTICE** within which to comply with the sequence rules, 37 CFR §§ 1.831-1.835. Failure to comply with these requirements will result in ABANDONMENT of the application under 37 CFR §1.835(d)(1). Extension of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR §1.136.

Summary of Requirements for Patent Applications Filed On Or After July 1, 2022, That Have Sequence Disclosures

37 CFR 1.831(a) requires that patent applications which contain disclosures of nucleotide and/or amino acid sequences that fall within the definitions of 37 CFR 1.831(b) must contain a "Sequence Listing XML", *as a separate part of the disclosure*, which presents the nucleotide and/or amino acid sequences and associated information using the symbols and format in accordance with the requirements of 37 CFR 1.831-1.835. This "Sequence Listing XML" part of the disclosure may be submitted:

1. In accordance with 37 CFR 1.831(a) using the symbols and format requirements of 37 CFR 1.832 through 1.834 via the USPTO's patent electronic filing system (currently Sequence Listing XML files may **only** be properly filed electronically using **Patent Center**) (see Section I.1 of the Legal Framework for Patent Electronic System (https://www.uspto.gov/patents-application-process/filing-online/legal-framework-efs-web), hereinafter "Legal Framework") in XML format, together with an incorporation by reference statement of the material in the XML file in a separate paragraph of the specification as required by 37 CFR 1.835(a)(2) or 1.835(b)(2) identifying:
   a. the name of the XML file
   b. the date of creation; and
   c. the size of the XML file in bytes; or
2. In accordance with 37 CFR 1.831(a) using the symbols and format requirements of 37 CFR 1.832 through 1.834 on read-only optical disc(s) as permitted by 37 CFR 1.52(e)(1)(ii), labeled according to 37 CFR 1.52(e)(5), with an incorporation-by-reference statement of the material in the XML format according to 37 CFR 1.52(e)(8) and 37 CFR 1.835(a)(2) or 1.835(b)(2) in a separate paragraph of the specification identifying:
   a. the name of the XML file;
   b. the date of creation; and
   c. the size of the XML file in bytes.

SPECIFIC DEFICIENCIES AND THE REQUIRED RESPONSE TO THIS NOTICE ARE AS FOLLOWS:

- This application contains a "Sequence Listing" as a PDF file (37 CFR 1.821(c)(2)) or as physical sheets of paper (37 CFR 1.821(c)(3)). A copy of the "Sequence Listing" in computer readable form (CRF) has been submitted. However, the content of the CRF does not comply with one or more of the requirements of 37 CFR 1.822 through 1.824, as indicated in the "Error Report" that indicates the "Sequence Listing" could not be accepted. Refer to attachment or PAIR document "Computer Readable Form (CRF) for Sequence Listing – Defective" dated 08/24/2022.

  Applicant must provide:
  - A replacement "Sequence Listing" part of the disclosure, as described above in item 1); **together with**
  - **An amendment specifically directing its entry into the application in accordance with 37 CFR 1.825(b)(2)**;
  - A statement that identifies the locations of any deletions, replacements, or additions to the "Sequence Listing" as required by 37 CFR 1.825(b)(3);
  - A statement that the "Sequence Listing" includes no new matter as required b y 37 CFR 1.825(b)(5); and
  - A statement that indicates support for the amendment in the application, as filed, as required by 37 CFR 1.825(b)(4).
- If the replacement "Sequence Listing" part of the disclosure is submitted according to item 1) a) or b) above, Applicant must also provide:
  - A substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 inserting the required incorporation-by-reference paragraph, consisting of:
    - A copy of the previously-submitted specification, with deletions shown with strikethrough or brackets and insertions shown with underlining (marked-up version);
    - A copy of the amended specification without markings (clean version); and
    - A statement that the substitute specification contains no new matter; and
    - An amendment to the specification to remove the "Sequence Listing" previously submitted as a PDF filed (37 CFR 1.821(c)(2)) or as physical sheets of paper (37 CFR 1.821(c)(3)).
- If the replacement "Sequence Listing" part of the disclosure is submitted according to item 1) c) or d) above, Applicant must also provide:
  - A CRF in accordance with 1.821(e)(1) or 1.821(e)(2) as required by 37 CFR 1.825(b)(6); and
  - Statement according to item 2) a) or b) above.

Where to obtain assistance:

USPTO Sequence listing Resource Center at: https://www.uspto.gov/patents/apply/sequence-listing-resource-center.

For rules interpretation and filing requirements, call the Sequence Systems Service Center at (571) 272-2510, the Office of Patent Legal Administration at (571) 272-7701, or, for 35 U.S.C. 371 applications, the PCT Help Desk at (571) 272-4300.

For WIPO Standard ST.26 questions, call (571) 272-2510.

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For assistance with submission of a Sequence Listing XML file via Patent Center, call the Electronic Business Center at (866) 217-9197 or email ebc@uspto.gov.

- Additional claim fees of **$2120** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

The fee(s) required (including the fees noted above) are as follows. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- **$960** for 2 independent claims over 3.
- **$300** for 3 total claims over 20.
- **$860** for multiple dependent claim surcharge.
- $(**0**) previous unapplied payment amount.
- **$2120** TOTAL FEE BALANCE DUE.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be addressed to:

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Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

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A reply may be submitted via electronic filing only by registered users of the appropriate USPTO patent electronic filing system and must include:

- A separate document having the document description, "**Applicant response to Pre-Exam Formalities Notice**"; and
- the item(s) for which a reply period is set forth above.

For more information about electronic filing, contact the Patent Electronic Business Center (EBC) 1-866-217-9197 or visit https://www.uspto.gov/ebc

NOTE: If the reply is not being submitted electronically, a copy of this notice must be included.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/hchin/

PTO/SB/08a (01-22)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17811748
	Filing Date	2022-07-11
	First Named Inventor	Jonathan Day
	Art Unit	NA
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	30610/57038

U.S.PATENTS						
Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5352770	A	1994-10-04	MATSUO HISAYUKI	
	2	6034231	A	2000-03-07	TANAKA et al.	
	3	7276481	B2	2007-10-02	GOLEMBO et al.	
	4	7642243	B2	2010-01-05	NAKAO et al.	
	5	8198242	B2	2012-06-12	WENDT et al.	
	6	8377884	B2	2013-02-19	WENDT et al.	
	7	8598121	B2	2013-12-03	WENDT et al.	
	8	8658373	B2	2014-02-25	NAKAO et al.	

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	9	9907834	B2	2018-03-06	BULLENS et al.	
	10	10646550	B2	2020-05-12	BULLENS et al.	

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U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	94/20534	WO	A1	1994-09-15	MAYO FOUNDATION		☐
	2	2009/067639	WO	A2	2009-05-28	BIOMARIN PHARM INC		☐
	3	2010/135541	WO	A2	2010-11-25	BIOMARIN PHARM INC		☐
	4	2021/030411	WO	A1	2021-02-18	BIOMARIN PHARM INC		☐

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	5	2021/055497	WO	A1	2021-03-25	BIOMARIN PHARM INC		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	ALFONZO et al., Characterization of a G protein-coupled guanylyl cyclase-B receptor from bovine tracheal smooth muscle, J. Recept. Signal. Transduct. Res., 26:269-297 (2006).	☐
	2	ALTSCHUL et al., Basic local alignment search tool, J. Mol. Biol., 215(3):403-410 (1990).	☐
	3	BARTELS et al., Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux, Am. J. Hum. Genet., 75:27-34 (2004).	☐
	4	BELLUS et al., Hypochondroplasia: molecular analysis of the fibroblast growth factor receptor 3 gene, Ann. N.Y. Acad. Sci., 785:182-187 (1996).	☐
	5	CHEN et al., Next-generation sequencing identifies rare variants associated with Noonan syndrome, Proc. Natl. Acad. Sci. USA., 111(31):11473-8 (2014).	☐
	6	COFFIN et al., Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice, Mol. Biol. Cell., 6:1861-73 (1995).	☐
	7	COLVIN et al., Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3, Nature Genet., 12:390-397 (1996).	☐
	8	FENG et al., Progressive sequence alignment as a prerequisite to correct phylogenetic trees, J. Mol. Evol., 25:351-360 (1987).	☐

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	9	GARDNER et al., Molecular biology of the natriuretic peptide system: implications for physiology and hypertension, Hypertension, 49:419-426 (2007).	☐
	10	GenBank: Accession No. NP_002512: natriuretic peptides B preproprotein [Homo sapiens], dated November 27, 2022.	☐
	11	GenBank: Accession No. NP_006163: natriuretic peptides A preproprotein [Homo sapiens], dated December 27, 2022.	☐
	12	GenBank: Accession No. NP_077720: C-type natriuretic peptide preproprotein [Homo sapiens], dated December 18, 2022.	☐
	13	HENIKOFF et al., Amino acid substitution matrices from protein blocks, Proc. Natl. Acad. Sci. USA., 89:10915-10919 (1992).	☐
	14	HIGGINS et al., Fast and sensitive multiple sequence alignments on a microcomputer, Comp. Appl. Biosci., 5:151-153 (1989).	☐
	15	HUNT et al., Bioactivity and metabolism of C-type natriuretic peptide in normal man, J. Clin. Endocrinol. Metab., 78:1428-35 (1994).	☐
	16	International Application No. PCT/US2022/073605, International Search Report and Written Opinion, mailed December 15, 2022.	☐
	17	KARLIN et al., Applications and statistics for multiple high-scoring segments in molecular sequences, Proc. Natl. Acad. Sci. U.S.A., 90(12):5873-5877 (1993).	☐
	18	KELLY et al., Age-based reference ranges for annual height velocity in US children, J. Clin. Endocrinol. Metab., 99(6):2104-2112 (2014).	☐
	19	KENNY et al., Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11, Biochem. J., 291(Pt 1):83-88 (1993).	☐

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	20	KOLLER et al., Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP), Science, 252:120-123 (1991).	☐
	21	KREJCI et al., Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis, J. Cell Sci., 118:5089-5100 (2005).	☐
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	25	MILOSAVLJEVIC et al., Two cases of RIT1 associated Noonan syndrome: Further delineation of the clinical phenotype and review of the literature, Am. J. Med Genet., 170(7):1874-80 (2016).	☐
	26	NAKAO et al., Molecular biology and biochemistry of the natriuretic peptide system. I: Natriuretic peptides, J. Hypertens., 10:907-912 (1992).	☐
	27	NAKAO et al., Molecular biology and biochemistry of the natriuretic peptide system. II: Natriuretic peptide receptors, J. Hypertens., 10:1111-1114 (1992).	☐
	28	NEEDLEMAN et al., A general method applicable to the search for similarities in the amino acid sequence of two proteins, J. Mol. Biol., 48:443-53 (1970).	☐
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	30	OLNEY, C-type natriuretic peptide in growth: a new paradigm, Growth Hormone & IGF Res., 16:S6-S14 (2006).	☐

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	31	PEARSON et al., Improved tools for biological sequence comparison, Proc. Natl. Acad. Sci. U.S.A., 85(8):2444-2448 (1988).	☐
	32	REMINGTON'S Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)),	☐
	33	ROMANO et al., Noonan syndrome: clinical features, diagnosis, and management guidelines, Pediatrics, 126(4):746-59 (2010).	☐
	34	ROUSSEAU et al., Missense FGFR3 mutations create cysteine residues in thanatophoric dwarfism type I (TD1), Hum. Mol. Genet., 5:509-512 (1996).	☐
	35	ROUSSEAU et al., Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia, Nature, 371:252-254 (1994).	☐
	36	SAVARIRAYAN et al., C-Type Natriuretic Peptide Analogue Therapy in Children with Achondroplasia, the N. Engl. J. Med., 381(1):25-35 (2019).	☐
	37	SAVARIRAYAN, et al., Once-daily, subcutaneous vosoritide therapy in children with achondroplasia: a randomised, double-blind, phase 3, placebo-controlled, multicentre trial, Lancet., 396(10252):684-692 (2020).	☐
	38	SHIANG et al., Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia, Cell, 78:335-342 (1994).	☐
	39	SMITH et al., Comparison of biosequences, Adv. Appl. Math., 2:482-489 (1981).	☐
	40	SUDOH et al., C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain, Biochem. Biophys. Res. Commun., 168: 863-870 (1990).	☐
	41	TAJAN et al., The RASopathy Family: Consequences of Germline Activation of the RAS/MAPK Pathway, Endocr. Rev., 39(5):676-700 (2018).	☐

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	42	TAVORMINA et al., Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3, Nat. Genet., 9:321-328 (1995).	☐
	43	Thomas et al., "Osteocrin, a Local Mediator of the Natriuretic System", J. Bone Miner. Res., vol. 19(S1), pp. S20, 1075 (January 2004).	☐
	44	THOMPSON et al., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22:4673-4680 (1994).	☐
	45	WILKIE et al., Functions of fibroblast growth factors and their receptors, Curr. Biol., 5:500-507 (1995).	☐
	46	Wu et al., "Furin-mediated Processing of Pro-C-type Natriuretic Peptide", J. Biol. Chem. 278: 25847-852 (2003)	☐
	47	YAMASHITA et al., Concentration of mRNA for the natriuretic peptide receptor-C in hypertrophic chondrocytes of the fetal mouse tibia, J. Biochem., 127:177-179 (2000).	☐
	48	YEUNG et al., Binding of CNP-22 and CNP-53 to cultured mouse astrocytes and effects on cyclic GMP, Peptides, 17:101-106 (1996).	☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	30610/57038

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Katherine L. Neville/	Date (YYYY-MM-DD)	2023-01-18
Name/Print	Katherine L. Neville	Registration Number	53,379

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	47387655
Application Number:	17811748
International Application Number:	
Confirmation Number:	2988
Title of Invention:	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
First Named Inventor/Applicant Name:	Jonathan Day
Customer Number:	90849
Filer:	Katherine Lynn Neville/Peter Adams
Filer Authorized By:	Katherine Lynn Neville
Attorney Docket Number:	30610/57038
Receipt Date:	18-JAN-2023
Filing Date:	11-JUL-2022
Time Stamp:	14:38:36
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	NPL_10_GenBank_Accessi_790716.pdf	2777383 1cde585bb8ea06639ab8686e25aeac28064dbfbb	no	3

Warnings:

Information:					
2	Non Patent Literature	NPL_11_GenBank_Accessi_790715.pdf	3864006 1ecb02c1e5582465316320d6d9a94484499 71522	no	4
Warnings:					
Information:					
3	Non Patent Literature	NPL_12_GenBank_Accessi_790717.pdf	2567162 60c799372829274 28e5e28fe9505e7f95448 008a	no	3
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4	Non Patent Literature	NPL_13_Henikoff_et_a_779936.pdf	1153531 f194824b5abe796738611266cb9ad163194 eb59b	no	5
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Information:					
5	Non Patent Literature	NPL_14_higgins1989_779937.pdf	296606 b756d6c48a5d2c246cddaae6f51fb896dba 40356	no	3
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6	Non Patent Literature	NPL_15_NPL_Hunt1428_779940.pdf	802876 777a1409c9d8680cef15387842891cdba23 4dcca	no	8
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7	Non Patent Literature	NPL_16_PCTUS2022073605_780121.pdf	455180 3d397ded163322b4b175a52078a15645a1 6d39f8	no	12
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8	Non Patent Literature	NPL_17_Applications_an_779948.pdf	1025200 2595e2039f91c4b97db270831b7b399c964 583c8	no	5
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9	Non Patent Literature	NPL_18_Kelly_et_al_779949.pdf	800711 123253cf3171ae985fcfca1aef8844ace76f6b16	no	9
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10	Non Patent Literature	NPL_19_KENNY_et_al_779927.pdf	1162270 0c482953399f5147d3efc2c0143f7119cf52659d	no	6
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11	Non Patent Literature	NPL_1_NPL_Alfonzo269_779942.pdf	20359408 b832f61c2001fc771416ac9e0f3c5b43a2618b23	no	29
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12	Non Patent Literature	NPL_20_NPL_Koller120_779915.pdf	1654229 1230007ee65388abd4c511f689e1ebd1c4b3d7a8	no	4
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13	Non Patent Literature	NPL_21_Krejci_et_al_779909.pdf	518826 8404fabecec579e5ab494f32a9c2ddbf1ccec1c4	no	12
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14	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part0.pdf	23216486 c28e63fe3e1ce23f7a189daebc0c107f586b0ff2	no	31
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15	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part1.pdf	23248920 6274f8885bd3bf9a08debd2ced2618ad3b4713ac	no	31
Warnings:					
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16	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part10.pdf	23415827 efe16055f1f65c1b33c8db6f41cd69810792ec99	no	29
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17	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part11.pdf	23329286 0f5c2ae8e79eef8ea29e492a7ef7be830e41180f	no	27
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18	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part12.pdf	23002931 c72ce7061e247b80ba7323f2ddb15fd6ef0c3ba1	no	29
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19	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part13.pdf	23249477 37b6636f63da216927273c111068f323dd6d6276	no	27
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20	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part14.pdf	23302523 917d0c29c0a53ff94a6038b3f75a81eef3748617	no	29
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21	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part15.pdf	23451708 9b4a9f8ffed4229edc344fba1d4825785949 6e49	no	30
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22	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part16.pdf	23517683 27e129f855dee10d939ef1b9cd8cf37b119c384d	no	27
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23	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part17.pdf	22760032 29db1c5c455c7e5da65bd955b5dc9f9e7e7dd29f1	no	22
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24	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part18.pdf	23365887 056bf04238819907 2ae4d7900f2f11e624ae05fb	no	27
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25	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part19.pdf	22680156 f64ce679ee8554c6962d340ddabe1ad7f97e038c	no	31
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26	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part2.pdf	22985633 8d548352ed9ad9c9a8e3481a2ced9eba1f38554d	no	31
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27	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part20.pdf	23249659 88c4dd692781c7a7871257b0ea172c8733b19f41	no	28
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28	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part21.pdf	22753088 4f7142a64a9621f67c58d64a36f2a10a5d41c0e9	no	30
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29	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part22.pdf	23237948 d972e6c72b5f44b51aee2ab105f921712aa44845	no	23
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30	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part23.pdf	23519403 e5807ff76aa6ad906d4b7d0e1595320b8d9610e4	no	31
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31	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part24.pdf	23419287 b896e876b750adf24210c217d76d09b295e08ea9	no	31
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32	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part25.pdf	23611393 65f498229a83a4963798b58dbeabe10935d70059	no	31
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33	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part26.pdf	22999405 7d06b665c53dd5c461f4ea5e53599929ab59db71	no	30
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34	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part27.pdf	23774470 5aac952b342b9b4bc9389f1ba92a3861144 6cb08	no	31
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35	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part28.pdf	22777101 ce304a8f8872857251 9a8048f604f782fa49106e	no	34
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36	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part29.pdf	23039881 12b7d8694afe58811659ef4abcd475cca4740e1b	no	36
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37	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part3.pdf	23272427 d3a0493d6f31590a266735674b8112966db44f89	no	29
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38	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part30.pdf	23431853 bc62be28e04f954387b5c4d18b7e16396dab64ff	no	31
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39	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part31.pdf	23130893 39c966c06777b3f97c08afaaf31f467e9ff603ea	no	32
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40	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part32.pdf	23087901 9f2bec843cce5a83ce25f9007c9ca2d102607afc	no	31
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41	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part33.pdf	22754899 94223a741465915fe0cbff6e8f56ae0b89a7cc7b	no	31
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42	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part34.pdf	23014835 a8c5b2ff96e49635fd1085b2db2edbe4004e2d9e	no	31
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43	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part35.pdf	23095824 910986d697f8c1b12edf5c1267ebe68579140fd6	no	33
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44	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part36.pdf	23443020 321c59c2f13771a45a84f9e7f3a9c497980afd8c	no	32
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45	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part37.pdf	17869638 3db7fa893e7d9f91f3513c036dbea3d3a95ec8ac	no	22
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46	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part4.pdf	22896650 93fb267267d6d37f482e33caf8486cd8257b32ff	no	30
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47	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part5.pdf	22904387 f6371e27bc79db09cc0b10510b51f8b3946c4ab0	no	29
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48	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part6.pdf	23044684 aa712a03e291af443881da77a4812767178fadd4	no	27
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49	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part7.pdf	23397445 4c44b5e24099b757e7b9aaf86bf9c342a35a50df	no	32
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50	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part8.pdf	23818063 6c7b2f1b5951d0b8f6f4a183b1198ac1c5e7964d	no	27
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51	Non Patent Literature	NPL_22_NPL_Lehninger_779922_Part9.pdf	23418166 a5e0abc46f065828910ce66e9c38c29f11b6815b	no	27
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52	Non Patent Literature	NPL_23_NPL_Levin321_779950.pdf	8183720 6d1943ffd42d82a5120a072a5d8e4a617aff398b	no	8
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53	Non Patent Literature	NPL_24_NPL_Maack675_779914.pdf	1244586 e51131b7ec9712fd2e5bb5d306c7a219f8124038	no	4
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54	Non Patent Literature	NPL_25_NPL_Milosavljev_779952.pdf	9128189 b704572cb9de7a19d928cf3ba219d0a049859ed6	no	8
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55	Non Patent Literature	NPL_26_NPL_Nakao907_779947.pdf	950199 de457c6375edd4d74321d164302f4927ad2834c7	no	6
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56	Non Patent Literature	NPL_27_NPL_Nakao1111_779946.pdf	568736 da56be0eac88cab0729d090a1028383fbc7da9f6	no	4
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57	Non Patent Literature	NPL_28_Needleman_et_al_779907.pdf	785375 798d0d1e4eefa15e7b548085cddfa864e61f95ad	no	11
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58	Non Patent Literature	NPL_2_Altschul_3_779923.pdf	1688590 966f190b4e0dd5a22c221dc79665b36acea b7cc6	no	8
Warnings:					
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59	Information Disclosure Statement (IDS) Form (SB08)	IDS_17811748_1.pdf	3563479 91c9ef78492002a097d7d007a44ee98ae73 8a575	no	9
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
		Total Files Size (in bytes):	940039131		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
Neville, Katherine L.
MARSHALL, GERSTEIN & BORUN LLP
233 South Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
ETATS-UNIS D'AMERIQUE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 30610/57038/PC	Date of mailing *(day/month/year)* 15 December 2022 (15-12-2022) **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US2022/073605	International filing date *(day/month/year)* 11 July 2022 (11-07-2022)

Applicant

BIOMARIN PHARMACEUTICAL INC.

1. [X] The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau preferably through ePCT, or on paper to:
   The International Bureau of WIPO, 34 chemin des Colombettes, 1211 Geneva 20, Switzerland

   For more detailed instructions, see the *PCT Applicant's Guide,* International Phase, paragraphs 9.004 - 9.011.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. [ ] **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   [ ] the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

   [ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months f**rom the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040 Fax: (+31-70) 340-3016	Authorized officer FORAL, Christine Tel: +49 (0)89 2399-5432

Form PCT/ISA/220 (revised January 2020)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
30610/57038/PC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2022/073605	11 July 2022 (11-07-2022)	9 July 2021 (09-07-2021)
Applicant		
BIOMARIN PHARMACEUTICAL INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report. submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

Form PCT/ISA/210 (first sheet) (July 2022)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/073605

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed.

   b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      ☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/073605

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/22 A61K47/52 A61P19/08
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2021/055497 A1 (BIOMARIN PHARM INC [US]) 25 March 2021 (2021-03-25) paragraphs [0008] - [0009] paragraphs [0092] - [0094] paragraphs [0036] - [0041] paragraph [0135]; claims 28-32 paragraph [0192] -----	1-22
X	WO 2021/030411 A1 (BIOMARIN PHARM INC [US]) 18 February 2021 (2021-02-18) paragraphs [0044] - [0045] paragraph [0105] paragraphs [0216] - [0219]; claims 14,38-40 paragraph [0211] ----- -/--	1-3, 6-13, 15-22

[x] Further documents are listed in the continuation of Box C. [x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 2022	15/12/2022

Name and mailing address of the ISA/	Authorized officer
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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/073605

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 9 907 834 B2 (BIOMARIN PHARM INC [US]) 6 March 2018 (2018-03-06) claims; examples	1-5, 8-10, 15-20
X	SAVARIRAYAN RAVI ET AL: "Once-daily, subcutaneous vosoritide therapy in children with achondroplasia: a randomised, double-blind, phase 3, placebo-controlled, multicentre trial", THE LANCET, vol. 396, no. 10252, 1 September 2020 (2020-09-01), pages 684-692, XP055923090, AMSTERDAM, NL ISSN: 0140-6736, DOI: 10.1016/S0140-6736(20)31541-5 abstract page 685, right-hand column, paragraph 2 page 691	1-22
X	RAVI SAVARIRAYAN ET AL: "C-Type Natriuretic Peptide Analogue Therapy in Children with Achondroplasia", THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 381, no. 1, 4 July 2019 (2019-07-04), pages 25-35, XP055750932, US ISSN: 0028-4793, DOI: 10.1056/NEJMoa1813446 abstract page 29, right-hand column page 34	1-22

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/073605

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2021055497 A1	25-03-2021	AU 2020349493 A1	07-04-2022
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		WO 2017020034 A1	02-02-2017

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2022/073605	11.07.2022	09.07.2021

International Patent Classification (IPC) or both national classification and IPC
INV. A61K38/22 A61K47/52 A61P19/08

Applicant
BIOMARIN PHARMACEUTICAL INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Escolar Blasco, P Telephone No. +49 89 2399-0

Form PCT/ISA/237 (Cover Sheet) (July 2022)

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed.

   b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),

      ☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

4. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this opinion has been established to the extent that a meaningful opinion could be formed without a WIPO Standard ST.26 compliant sequence listing.

5. Additional comments:

   see separate sheet

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4, 5, 14
	No:	Claims	1-3, 6-13, 15-22
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-22
Industrial applicability (IA)	Yes:	Claims	1-22
	No:	Claims	

2. Citations and explanations

   see separate sheet

Item I

The sequence listing on the date of filing was not WIPO Standard ST.26 compliant, therefore not used for search purposes.

Item V

1 Reference is made to the following documents, and in particular to the passages cited in the ISR:

D1 WO 2021/055497 A1 (BIOMARIN PHARM INC [US]) 25 March 2021 (2021-03-25)

D2 WO 2021/030411 A1 (BIOMARIN PHARM INC [US]) 18 February 2021 (2021-02-18)

D3 US 9 907 834 B2 (BIOMARIN PHARM INC [US]) 6 March 2018 (2018-03-06)

D4 SAVARIRAYAN RAVI ET AL: "Once-daily, subcutaneous vosoritide therapy in children with achondroplasia: a randomised, double-blind, phase 3, placebo-controlled, multicentre trial", THE LANCET, vol. 396, no. 10252, 1 September 2020, pages 684-692, XP055923090, ISSN: 0140-6736, DOI: 10.1016/S0140-6736(20)31541-5

D5 RAVI SAVARIRAYAN ET AL: "C-Type Natriuretic Peptide Analogue Therapy in Children with Achondroplasia", THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 381, no. 1, 4 July 2019, pages 25-35, XP055750932, ISSN: 0028-4793, DOI: 10.1056/NEJMoa1813446

2 C-type natriuretic peptides (CNP) had been known to be useful for increasing bone growth and treating achondroplasia for years before the present application. It was also well known that they have a very short half life and clinical use required modified peptides to be possible. All of D1 to D5 refer to such modified peptides, and in particular to vosoritide, which is formulation

including the peptide of sequence ID No 1 and the excipients set forth in dependent claim 17. The preferred product of the present application (which is the only one used in the examples) had thus already been disclosed for the same treatment in the prior art. The present claims are however limited to a particular subgroup of patients, namely infants under 2 years of age. While the clinical trials described in D3 to D6 were indeed made with patients 5 years old or older, D1 and D2 disclose the claimed medical use in infants.

2.1 Infants are, by definition, between a child in the earliest period of life, before being able to walk. The reference to infant in the prior art is thus interpreted as meaning about 0 to about 12 months. D1 specifies in any case, that the subject may be older than 1 (as opposed to older than 2 or than 3 years old), or older than 6 months in age. This corresponds to an explicit disclosure of the range 6 to 12 months, and 12 to 24 months of age. D1 and D2 anticipate thus the subject-matter of claims 1-3, 6-13 and 15-22. Besides disclosing the same preferred peptide, group of patients and disease, these documents by the applicant also disclose the dose of claims 11 and 21-22 and the diseases of claims 15 and 16, and the type of administration defined in claims 8 to 10. Finally, they also cite the combination of excipients of the preferred formulation of claim 18.

3 The subject-matter of claims 4 and 5 has not been explicitly disclosed in either D1 or D2. The type of measurement of the increases in growth velocity mentioned in these claims correspond to common features in the art, as can be seen in either D4 or D5. Since there is in any case no indication in the description that these features may be related to any unexpected technical effect, the subject-matter of claims 4 and 5 is considered to lack an inventive step.

3.1 The same applies to the additional feature of claim 14, related to a possible change in dose after the subject turns 2 years. Furthermore, we note that claim 14 could be seen as not related to the same method as the preceding claims, which are restricted by the age group zero to two years of age. It is at present not clear what is the novel and/or inventive contribution of this claim to the subject-matter defined by the previous claims.

4 Claims 1 to 20 refer to a method of treatment by therapy, which is excluded from patentability and may be not searched according to Rule 39(iv) PCT. The EPO in particular does not allow claims directed to methods of treatment of the

human or animal body, but allows claims related to products for use in a medical treatment. Hence, the present claims have been interpreted as relating to the claimed C-type natriuretic peptides for use in treating the disorders indicated in the claims in subjects under 2 years of age.

4.1 Claim 22 is drafted in a Swiss type format, which is also not generally allowable. Depending on the regional procedure, such claims may be interpreted as relating merely to a product *per se*, only suitable for the cited therapeutic indication, but not limited to its use. At the EPO, only the composition for use of claim 21 corresponds to a format accepted for further medical uses.

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
PCT/US2022/073605

TITLE: C-TYPE NATRIURETIC PEPTIDE VARIANTS TO TREAT SKELETAL DYSPLASIA IN CHILDREN

APPLICANT: BIOMARIN PHARMACEUTICAL INC.

IPC CLASSIFICATION: A61K38/22, A61K47/52, A61P19/08

EXAMINER: Escolar Blasco, P

CONSULTED DATABASES: NPL, BIOSIS

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: A61K38/2242, A61K47/52, A61P19/08

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
(skeletal 2d dysplasia?) or (bone w growth) or achondroplasia or hypochondroplasia or dwarfism or osteochondrodysplasias or osteogenesis or achondrogenesis or chondrodysplasia or dysplasia or (short femur) or micromelia
((natriuretic w peptide?) 3d C_type) or cnp_37 or cnp_51 or cnp53 or cnp_48 or cnp_46 or cnp_45 or cnp_44 or cnp_42 or cnp_41 or cnp_38 or cnp_37 or m32n or m22n or (cnp 2d peptide?) or PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC or LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
vosoritide or BMN_111 or uosoritide
child??? or year? or infant? or baby or babies or toddler?

EPO FORM P04A42

Publication Number: WO 94/20534 A1
Publication Date: Sep 15, 1994

Abstract

Compounds and compositions comprising synthetic analogs of C-type natriuretic peptide are provided, together with methods for their production and use as natriuretics, diuretics, and/or vasodilators, or as intermediates for or modulators of such useful compounds or of native natriuretic peptides.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/10, A61K 37/24 — A1

(11) International Publication Number: **WO 94/20534**

(43) International Publication Date: 15 September 1994 (15.09.94)

(21) International Application Number: PCT/US94/02391

(22) International Filing Date: 2 March 1994 (02.03.94)

(30) Priority Data:
08/025,935 3 March 1993 (03.03.93) US

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(81) Designated States: AU, CA, CN, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: VASONATRIN PEPTIDE AND ANALOGS THEREOF

(57) Abstract

Compounds and compositions comprising synthetic analogs of C-type natriuretic peptide are provided, together with methods for their production and use as natriuretics, diuretics, and/or vasodilators, or as intermediates for or modulators of such useful compounds or of native natriuretic peptides.

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VASONATRIN PEPTIDE AND ANALOGS THEREOF

Background of the Invention

This invention was made with the support of the U.S. Government under National Heart, Lung and Blood Institute (NHLBI) Grants HL-36634 and HL07111. The U.S. Government has certain rights in the invention.

Atrial natriuretic peptide (ANP) is the first described peptide in a family of hormones known to have important roles in the regulation of body fluid homeostasis. See, B.M. Brenner et al., Physiol. Rev., 70, 665 (1990). The description of the potent diuretic and natriuretic properties of atrial extracts by A.J. de Bola et al., Life Sci., 28, 89 (1981) was the first evidence that the heart could be an endocrine organ. The subsequent isolation and characterization of this activity by T.G. Flynn et al., Biochem. Biophys. Res. Commun., 117, 859 (1981) and other groups characterized ANP as the first secreted cardiac hormone. It is secreted by atrial myocytes in response to increased intravascular volume. Once it is in the circulation, its effects are primarily on the kidney, vascular tissue, and adrenal gland, in which its actions lead to the excretion of sodium (natriuresis) and water (diuresis) by the kidneys and a decrease in intravascular volume and blood pressure. (S.A. Atlas et al., in Atrial Hormones and Other Natriuretic Factors, P.J. Mulrow et al, ed., Am. Physiol. Soc., Bethesday, MD (1987) at pages 53-76.)

More recently, Matsuo and his coworkers have isolated two new natriuretic peptides. Brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were both isolated from porcine brain extracts on the basis of their potent relaxant effects on chick rectum. (See T. Sudeh et al., Nature, 332, 78 (1988); Biochem. Biophys. Res. Commun., 168, 863 (1990)). Like ANP, these hormones are synthesized from large precursor proteins, and the mature, active peptides have a 17-amino-acid loop formed by an intramolecular disulfide linkage. In the human peptides (Figure 1), eleven of these amino acids are identical in ANP, BNP, and CNP, whereas the N- and C-terminal tails vary in both length and composition. See, Y. Kambayashi et al., FEBS Lett., 259, 341 (1990); Y. Tawaragi et al., Biochem. Biophys. Res. Commun., 175, 645 (1991). CNP has no C-terminal tail, and studies of the structure of the gene for CNP demonstrated that translation is terminated by a stop codon immediately after the final cysteine codon in the mRNA.

Among species, the amino acid sequence of both ANP and CNP are highly conserved, whereas the structure of BNP varies greatly. For example, the mature

28-amino-acid human and porcine ANPs are identical, and there is only one substitution in the rat peptide. This existence of this structural variation, coupled with the presence of at least three types of receptors specific for the natriuretic peptides, suggests that the physiological control of body fluid homeostasis is complex. ANP and CNP both decrease cardiac preload. However, unlike ANP, CNP is not natriuretic. See, A.J. Stingo et al., Am. J. Physiol., 262, H308 (1992). The diverse actions of ANP, BNP and CNP on both the cardiovascular system and the kidney as well as their roles in pathophysiological states such as heart failure, hypertension, and renal disease have made the native peptides and their analog molecules of great interest to both clinical and basic scientists. See, for example, J.A. Lewicki et al. (U.S. Patent Nos. 5,114,923, 4,804,650 and 4,757,048), L.K. Johnson et al. (U.S. Patent No. 5,047,397) and L.K. Johnson et al. (U.S. Patent No. 4,935,492). Therefore, a continuing need exists to identify and characterize bioactive peptides belonging, or related to, this class of materials. Such peptides may be useful both to elucidate the mechanism of action of these compounds, as well as to provide new pharmaceutical agents with useful profiles of bioactivity.

Summary of the Invention

The present invention provides a peptide compound having natriuretic, diuretic and/or vasodilator activity in mammals, which has the formula (I):

$$X_1\text{-}A_y\text{-}X_2\text{-}A_1\text{-}A_2\text{-}A_3\text{-}A_4\text{-}A_5\text{-}X_3\text{-}A_z\text{-}X_y \qquad (I)$$

wherein A_1 is Lys, Arg, Orn, Ala, Thr, Asn or Gln; A_2 is Ile, Met, Val or Leu; A_3 is Glu or Asp; A_4 is Lys, Arg, Orn, Ala, Thr, Asn or Gln; A_5 is Val, Leu or Ileu; X_1 is a peptide of from 1-125 amino acid residues, preferably 1-5 amino acid residues comprising Gly at the carboxyl terminus, and preferably Gly at the N-terminus, with the remainder being individually selected from the group consisting of Leu, Ser and Lys. Most preferably, X_1 is (H)Gly-Leu-Ser-Lys-Gly (SEQ ID NO:5), or an N-terminal deleted form such as (H)Leu-Ser-Lys-Gly (SEQ ID NO:6), (H)Ser-Lys-Gly, (H)Lys-Gly or (H)Gly; A_y and A_z are amino acid residues which together form a bridging bond, wherein said bond is selected from the group consisting of disulfide, methylene, sulfide/methylene, amide and ester; preferably, disulfide; X_2 is a peptide of 1-10 amino acid residues, preferably of about 1-3 residues, individually selected from the group consisting of Phe, Trp, Val, Leu, Ile, Gly, Ser and Cys; X_3 is a peptide of 1-10 amino acid residues, preferably about 1-7

residues and X_y is a peptide of about 1-5 residues selected from the group consisting of R-Asn-, R-Ser-Asn, R-Phe-Ser-Asn, R-Arg-Phe-Ser-Asn- (SEQ ID NO:7) and R-Tyr-Arg-Phe-Ser-Asn- (SEQ ID NO:8) wherein R is OH, NH_2, NHR^3 or NR^3R^4, wherein R^3 and R^4 are independently (C_1-C_4)alkyl, (C_3-C_5)cycloalkyl, phenyl or benzyl. Preferably, X_3 is a heptapeptide comprising peptidyl residues individually selected from the group consisting of Gly, Ser, Met and Leu. Preferably, when A_2 is Ile, A_1 is not Arg, and when A_2 is Arg, A_2 is not Ile.

Preferably, the total ring size is equivalent to that obtained by disulfide bridge formation between cysteine residues separated by 7-15 amino acid (peptidyl) residues. Optionally, one or more of the amide linkages between adjacent amino acid residues can be replaced by a linkage such as $-CH_2-NH-$, $-CH_2-S-$, $-CH_2CH_2-$, $-CH=CH-$, $-COCH_2-$, $-CH(OH)CH_2-$ or $-CH_2SO-$. X_1 can also be an amino acid protecting group such as fluorenylmethyloxycarbonyl, benzyloxycarbonyl, (2-(2',6'-methoxynaphthyl)propionyl, diphenylpropionyl, cyclohexylacetyl, 3-indolepropionyl, 2-naphthoxy and the like. Other such groups are disclosed in U.S. Patent No. 4,935,492.

In the peptides of the invention, the L-form of any amino acid residue having an optical isomer is intended unless otherwise indicated. In some cases, A_2 and/or A_5 can be replaced by the D-forms. Bioactive peptides of formula (I) which do not comprise the disulfide bond are also within the scope of the invention.

In a preferred embodiment, the present invention provides a peptide having natriuretic, diuretic and/or vasodilator activity in mammals, of the formula (II):

H-Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-A'-A"-Asp-Arg-
Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-R^2 (II)

(SEQ ID NO:9) wherein A' is Lys or Arg, A" is Leu, Ile or Met and R^2 is R-Asn-, R-Ser-Asn-, R-Phe-Ser-Asn-, R-Arg-Phe-Ser-Asn- (SEQ ID NO:7) and R-Tyr-Arg-Phe-Ser-Asn- (SEQ ID NO:8); wherein R is OH, NH_2, NHR^3 or NR^3R^4 wherein R^3 and R^4 are independently phenyl or (C_1-C_4)alkyl; and wherein the bracket connecting the two cysteinyl moieties of formula (I) indicates that they are connected by a disulfide bond as depicted on Figure 2. Preferably, R^2 is R-Tyr-Arg-Phe-Ser-Asn-, and most preferably, R is OH, i.e., H in formula (I) represents the amino terminus of the peptide and (OH) represents the carboxyl terminus of the peptide. Preferably, A' is Lys (K) and A" is Leu (L). Thus, using single letter amino acid code, this preferred peptide, or "VNP", can be represented as:

G-L-S-K-G-C-F-G-L-K-L-D-R-I-G-S-M-S-G-L-G-C-N-S-F-R-Y

(SEQ ID NO:4) wherein the sequence is read conventionally, from the left, amino terminus to the right, carboxy terminus. The bracket represents a disulfide bond between the cysteinyl residues.

VNP is a more potent, endothelium independent, vasorelaxing peptide in both arteries and veins than ANP or CNP. VNP also has potent natriuretic effects *in vivo*. Thus, the present invention also provides a composition useful as a natriuretic, diuretic and/or vasodilator comprising a therapeutically effective amount of the present peptide in combination with a pharmaceutically acceptable carrier, and a method for inducing natriuresis, diuresis or vasodilation in a mammal, preferably a human, comprising administering to said mammal a pharmaceutically effective amount of said composition. The present compositions can also be employed to modify the renin-angiotensis-aldosterone system.

Thus, the present peptides may be useful, either singly or in combination, to treat (ameliorate or prevent) a number of pathological conditions, including acute or chronic kidney failure, hypertension, congestive heart failure, cirrhosis of the liver, nephrotic syndrome, and other "edematous states".

Brief Description of the Figures

Figure 1 is a diagrammatic comparison of the mature human atrial (ANP) (SEQ ID NO:1), brain (BNP) (SEQ ID NO:2) and C-type (CNP) (SEQ ID NO:3) natriuretic peptides. Conserved residues of ANP, BNP, and CNP are represented by darkened boxes. The line between the two cysteines in each peptide indicates a disulfide bridge. From Circulation, 86, 1082 (1992)). Single-letter and three-letter abbreviations for the amino acid residues are A, alanine (Ala); R, arginine (Arg); N, asparagine (Asn); D, aspartic acid (Asp); C, cysteine (Cys); Q, glutamine (Gln); E, glutamic acid (Glu); G, glycine (Gly); H, histidine (His); I, isoleucine (Ile); L, leucine (Leu); K, lysine (Lys); F, phenylalanine (Phe); M, methionine (Met); P, proline (Pro); S, serine (Ser); T, threonine (Thr); W, tryptophan (Trp); Y, tyrosine (Tyr); V, valine (Val).

Figure 2 depicts the structure of vasonatrin peptide (VNP) (SEQ ID NO:4). The amino terminus of the peptide is at the left-hand end of the peptide, while the carboxyl terminus is at the right-hand end.

Detailed Description of the Invention

1. Synthesis

Peptides of this invention can be synthesized by the solid phase peptide synthesis (or Merrifield) method. This established and widely used method, including the experimental procedures, is described in the following references: Stewart et al., Solid Phase Peptide Synthesis, W.H. Freeman Co., San Francisco (1969); Merrfield, J. Am. Chem. Soc., 85, 2149 (1963); Meienhofer in "Hormonal Proteins and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; and Barany and Merrifield in "The Peptides," eds. E. Gross and F. Meinenhofer, Vol. 2 (Academic Press, 1980), pp. 3-285. The synthesis is commenced from the carboxy-terminal end of the peptide using an alpha-amino protected amino acid. Fluorenylmethyloxy-carbonyl (Fmoc) or t-butyloxycarbonyl (Boc) protective groups can be used for all amino groups even through other protective groups are suitable. For example, Boc-Asn-OH, Boc-Ser-OH, Boc-Phe-OH, Boc-Arg-OH or Boc-Tyr-OH (i.e., selected ANP analog carboxy-terminal amino acids) can be esterified to chloromethylated polystyrene resin supports. The polystyrene resin support is preferably a copolymer of styrene with about 0.5 to 2% divinyl benzene as a cross-linking agent which causes the polystyrene polymer to be insoluble in certain organic solvents. See Carpino et al., J. Org. Chem., 37, 3404 (1972); Meinhofer, Int. J. Pept. Pro. Res., 11, 246 (1978); and Merrifield, J. Am. Chem. Soc., 85, 2149 (1963). These and other methods of peptide synthesis are also exemplified by U.S. Patent Nos. 3,862,925; 3,842,067; 3,972,859, 4,105,602 and 4,757,048.

The immobilized peptide is then N-deprotected and other amino acids having protected amino groups are added in a stepwise manner to the immobilized peptide. At the end of the procedure, the final peptide is cleaved from the resin, and any remaining protecting groups are removed, by treatment under acidic conditions such as, for example, with a mixture of hydrobromic acid and trifluoroacetic acid or with hydrofluoric acid, or the cleavage from the resin may be effected under basic conditions, for example, with triethylamine, the protecting groups then being removed under acid conditions.

The cleaved peptides are isolated and purified by means well known in the art such as, for example, lyophilization followed by either exclusion or partition chromatography on polysaccharide gel media such as Sephadex G-25, or countercurrent distribution. The composition of the final peptide may be confirmed by amino acid analysis after degradation of the peptide by standard means.

Salts of carboxyl groups of the peptide may be prepared in the usual manner by contacting the peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

Acid addition salts of the polypeptides may be prepared by contacting the polypeptide with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid.

Esters of carboxyl groups of the polypeptides may be prepared by any of the usual means known in the art for converting a carboxylic acid or precursor to an ester. One preferred method for preparing esters of the present polypeptides, when using the Merrifield synthesis technique described above, is to cleave the completed polypeptide from the resin in the presence of the desired alcohol either under basic or acidic conditions, depending upon the resin. Thus, the C-terminal end of the peptide when freed from the resin is directly esterified without isolation of the free acid.

Amides of the polypeptides of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the polypeptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

N-acyl derivatives of an amino group of the present polypeptides may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired.

The synthesis may use manual techniques or be completely automated, employing, for example, an Applied BioSystems 431A Peptide Synthesizer (Foster City, Calif.) or a Biosearch SAM II automatic peptide synthesizer (Biosearch, Inc., San Rafael, Calif.), following the instructions provided in the instruction manual and reagents supplied by the manufacturer. Disulfide bonds between Cys residues can be introduced

by mild oxidation of the linear peptide by KCN as taught in U.S. Patent No. 4,757,048 at Col. 20.

Alternatively, selected compounds of the present invention can be produced by expression of recombinant DNA constructs prepared in accordance with well-known methods such as those described in Seihamer et al. (U.S. Patent No. 5,114,923), entitled "Recombinant Techniques for Production of Novel Natriuretic and Vasodilator Peptides." Such production can be desirable to provide large quantities or alternative embodiments of such compounds.

2. Structural Features

The compounds of the invention contain the pentapeptide core sequence A_3-A_4-A_5, which, in preferred embodiments, corresponds to the conserved region DRI present in ANP, CNP and BNP. Preferably, the sequence A_1-A_2-A_3-A_4-A_5 is RMDRI (SEQ ID NO:10), KMDRI (SEQ ID NO:11), KLDRI (SEQ ID NO:12), KIDRI (SEQ ID NO:13) or RLDRI (SEQ ID NO:14), most preferably, is the KLDRI sequence of rat, porcine or human CNP.

Preferably, X_1 is (H)GLSKG- (SEQ ID NO:15) wherein (H) indicates the amino terminus of the peptide; and X_4 is -NSFRY(R) (SEQ ID NO:16), wherein (R) is preferably (OH), indicating the carboxy terminus of the peptide. Preferably, X_2 is a tripeptide, most preferably -FGL-, or a dipeptide such as -FG- or -GL-, and X_3 is a heptapeptide, most preferably -GSMSGLG- (SEQ ID NO:17). X_3 can also be a truncated form of -GSMSGLG- (SEQ ID NO:17) wherein one or more of the Gly or Ser residues may be replaced by another of Gly (G), Ser (S) or Cys (C) or by Ala, and Leu may be replaced by Val or Ile.

The cyclic disulfides included within the invention are directly analogous to the naturally occurring CNPs, which contain 17 amino acid residue-membered disulfide rings, inclusive of the two cysteine residues which provide the sulfhydryl groups for the formation of the disulfide bond. However, those embodiments of the compounds of the invention which contain the cyclic disulfide may contain either more, or less, than 17 amino acid residues in the cyclic structure.

As indicated, the cyclic compounds of the present invention can be provided by bonding cysteine residues, or alternate amino acid residues A_y and A_z with an equivalent bond or linking group such as, for example, -CH_2-CH_2-. The replacement of a sulfhydryl group on the cysteine residue with an alternative group will effectively

replace the cysteine residue with an alternative amino acid. For example, to replace one sulfhydryl group with a -CH_2- group, the cysteine residues will be replaced by the analogous alpha-aminobutyric acid. These cyclic analog peptides can be formed, for example, in accordance with the methodology of M. Lebl and V.J. Hruby, Tetrahedron Lett., 25, 2067 (1984), or by employing the procedure disclosed in U.S. Patent No. 4,161,521.

Ester or amide bridges may also be formed by reacting the OH or serine or threonine and the carboxyl of aspartic acid or glutamic acid, to yield a bridge of the structure -CH_2-CO_2CH_2-. Similarly, an amide can be obtained by reacting the side-chain of lysine and aspartic or glutamic acid to yield a bridge of the structure -$CH_2C(O)NH$-$(CH_2)_4$-. Methods for synthesis of these bridges are found in P.W. Schiller et al., Biochem. Biophy. Res. Comm., 127 558 (1985); Int. J. Peptide and Protein Res., 25, 171 (1985). Other bridge-forming amino acid residues and reactions are provided in U.S. Patent No. 4,935,492.

The following references describe preparation of peptide analogs which include non-peptidyl bonds to link amino acid residues. A.F. Spatola, Vega Data, Vol. 1, Issue 3 (March 1983), "Peptide Backbone Modifications" (general review); A.F. Spatola in "Chemistry and Biochemistry of Amino Acids Peptides and Proteins", B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); J.S. Morley, Trends Pharm. Sci. (1980) at pp. 463-468 (general review); D. Hudson et al., Int. J. Pept. Prot. Res., 14, 177 (1979); (-CH_2NH-, -CH_2C-H_2-); A.F. Spatola et al., Life Sci., 38, 1243 (1986) (-CH_2-S); M.M. Hann, J. Chem. Soc. Perkin Trans. I, 307 (1982) (-CH-CH-), cis and trans); R.G. Almquist et al., J. Med. Chem., 23, 1392 (1980) (-$COCH_2$-); C. Jennings-White et al., Tetrahedron Lett., 23, 2533 (1982) (-$COCH_2$-); M. Szelke et al., European Patent Appln EP 45665 (1982) (-$C(OH)CH_2$-); M.W. Holladay et al., Tetrahedron Lett., 24, 4401 (1983) (-$C(OH)CH_2$-); and V.J. Hruby, Life Sci., 31, 189 (1982) (-CH_2-S-).

3. Doses and Dosage Forms

The compounds of the present invention have natriuretic, diuretic and hypotensive activity in the intact mammal, and may possess vasorelaxant activity and/or inhibit the release of aldosterone and renin.

Thus, these compounds, and compositions containing them, can find use as therapeutic agents in the treatment of various edematous states such as, for example,

congestive heart failure, nephrotic syndrome and hepatic cirrhosis, in addition to hypertension and renal failure due to ineffective renal perfusion or reduced glomerular filtration rate.

The present invention also provides compositions containing an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, together with physiologically acceptable liquid, gel or solid diluents, adjuvants and excipients.

These compounds and compositions can be administered to mammals for veterinary use, such as with domestic animals, and for clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will range from about 0.01 to 1000 mcg/kg, more usually 0.1 to 1000 mcg/kg of the host body weight. Dosages within these ranges can be administered via bolus doses, via a plurality of unit dosage forms, or by constant infusion over an extended period of time, usually exceeding 24 hours, until the desired therapeutic benefits have been obtained.

Typically, such compositions are prepared for injection or infusion, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection or infusion may also be prepared. The preparation may also be emulsified. The active ingredient can be mixed with diluents or excipients which are physiologically acceptable and compatible with the active ingredient(s). Suitable diluents and excipients are, for example, water, saline, PBS, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

Such compositions are conventionally administered parenterally, by injection, for example, either subcutaneously or intravenously. Formulations which are suitable for other modes of administration include suppositories, insufflated powders or solutions, eyedrops, nosedrops, intranasal aerosols, and, in some cases, oral formulations. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of alkylcelluloses, mannitol, dextrose, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. Thus, these compositions can take the form of solutions, suspensions, tablets, pills, hard or soft gelatin capsules, sustained-release formulations such as liposomes, gels or hydrogels; or powders, and can contain 10%-95% of active ingredient, preferably 25%-70%.

The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups) and which are formed by reaction with inorganic acids such as, for example, hydrochloric, sulfuric or phosphoric acids, or organic acids such as, for example, acetic, oxalic, tartaric, mandelic, citric, malic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases such as amines, i.e., isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

4. Other Utility

In addition to the compounds of the present invention which display natriuretic, diuretic or vasorelaxant activity, compounds of the present invention can also be employed as intermediates in the synthesis of such useful compounds. Alternatively, by appropriate selection, compounds of the present invention whose activity levels are reduced or eliminated entirely can serve to modulate the activity of other diuretic, natriuretic or vasorelaxant compounds, including compounds outside the scope of the present invention by, for example, binding to alternate receptors, stimulating receptor turnover, or providing alternate substrates for degradative enzyme or receptor activity and thus inhibiting these enzymes or receptors. When employed in this manner as antagonists, such compounds can be delivered as admixtures with other active compounds or can be delivered separately, for example, in their own carriers.

Compounds of the present invention can also be used to prepare antisera for use in immunoassays employing labeled reagents, usually antibodies. Conveniently, the peptides can be conjugated to an antigenicity-conferring carrier, if necessary, by means of dialdehydes, carbodiimide or by using commercially available linkers. These compounds and immunologic reagents may be labeled with a variety of labels such as chromophores, fluorophores such as, e.g., fluorescein or rhodamine, radioisotopes such as ^{125}I, ^{35}S, ^{14}C, or ^{3}H, or magnetized particles, by means known to the art.

These labeled compounds and reagents, or labeled reagents capable of recognizing and specifically binding to them, can find use as, e.g., diagnostic reagents. Samples derived from biological specimens can be assayed for the presence or amount of substances having a common antigenic determinant with compounds of the present invention. In addition, monoclonal antibodies can be prepared by methods known in the

art, which antibodies can find therapeutic use, e.g., to neutralize overproduction of immunologically related compounds *in vivo*.

The invention will be further described by reference to the following detailed examples wherein VNP was synthesized in the Mayo Protein Core Facility using fluorenylmethoxy-carbonyl (FMOC) chemistry on an ABI 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) with the protocols and reagents supplied by the manufacturer. The peptide was purified by reverse phase high performance liquid chromatography (HPLC) using a Vydac C8 column (The Separations Group, Hesperia, CA). The synthesis was confirmed by amino acid analysis and plasma absorption mass spectrometry.

The samples from the plasma and urine were analyzed using reverse phase HPLC with a Vydac C18 column (4.6 mm x 250 mm) (The Separations Groups, Hesperia, CA). The components of the HPLC system were two Beckman 114 pumps (Beckman Instruments, San Ramon, CA), ABI 759A absorbance detector (Applied Biosystems, Inc., Foster City, CA), and an IBM PS2 50Z computer with Beckman System Gold Chromatography software. The A buffer was 0.1% trifluoracetic acid and the B buffer was 80% acetonitrile/20% water/0.1% trifluoroacetic acid. The separation was performed with a gradient of 5% to 70% B buffer in 60 minutes.

The results obtained are expressed as the means ± SEM. In organ chamber studies, *n* equals the number of dogs from which rings were taken. Rings with and without endothelium were studied in parallel, and Student's t-test for unpaired observations was used to determine statistical significance among the responses of rings with and without endothelium and between responses of arteries and veins. In rat studies, the data were analyzed using ANOVA for repeated measures followed by Fisher's least significant difference test when appropriate within the group. Data between groups were analyzed by Student's unpaired t-test. Statistical significance was determined at $p<0.05$.

Example 1. In Vitro Studies.

Rings cut from femoral, saphenous, renal and pulmonary arteries and veins obtained from random source mongrel dogs (anesthetized with 30 mg/kg pentobarbital sodium intravenously) were suspended for the measurement of isometric force in organ chambers filled with aerated (95% O_2 and 5% CO_2) modified Krebs-Ringer bicarbonate solution (composition in mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium sodium EDTA, and 11.1 dextrose; control

solution) at 37°C. In one-half rings, the endothelium was removed by gently rubbing the intimal surface with a cotton swab wetted with control solution. Each ring was stretched to the optimal point on its length-tension curve as determined by the tension developed to norepinephrine (3×10^{-7} M) at each level of stretch. The presence of endothelium was determined at the beginning of the experiment by a relaxation to acetylcholine (10^{-6} M) during a contraction to norepinephrine at optimal length. The maximal tension of each ring was determined by norepinephrine (10^{-4} M). To study responses to ANP, CNP and VNP, the rings were contracted with phenylephrine (10^{-6} M). These contractions averaged 30-40% of the maximal contraction to norepinephrine. The peptides were added cumulatively once the contraction had stabilized.

The following commercially available drugs were used: acetylcholine chloride (Sigma Chemical, St. Louis, MO), human ANP and human CNP (Peninsula Laboratories, Belmont, CA), L-norepinephrine bitartrate (Sigma), phenylephrine bitartrate (Sigma). All drugs were dissolved in distilled water immediately prior to study, and the concentrations are reported as the final molar concentration (M) in the organ chamber.

At maximal dose (3×10^{-6} log M), ANP and CNP had no effects on femoral and saphenous arteries and veins, but VNP exposure resulted in a potent relaxation (90-100%) in femoral and saphenous arteries. In femoral and saphenous veins, ANP had no effect. CNP relaxes femoral veins by 90% and saphenous veins by 40%. VNP caused a 90% relaxation in both femoral and saphenous veins.

Table 1 summarizes the maximal relaxations (3×10^{-6} M) of ANP, CNP and VNP in isolated normal canine blood vessels.

Table 1.
The maximal responses of ANP, CNP and VNP (3×10^{-6} M) in isolated normal canine blood vessels.

		ANP (n=6-9)	CNP (n=6-9)	VNP (n=4-6)
Systemic blood vessels				
Arteries	Endothelium			
Femoral	with	10 ± 5§	4 ± 3	91 ± 4*†
	without	19 ± 12	10 ± 6	89 ± 5*†
Saphenous	with	0 ± 0	11 ± 7	73 ± 3*†
	without	3 ± 3	14 ± 8	81 ± 5*†
Renal	with	27 ± 6†	8 ± 2	52 ± 4*†
	without	30 ± 6†	8 ± 2	62 ± 12*†
Veins				
Femoral	with	6 ± 3	37 ± 8*	81 ± 6*†
	without	4 ± 4	73 ± 8*¶	86 ± 6*
Saphenous	with	0 ± 0	16 ± 1*	60 ± 11*†
	without	0 ± 0	43 ± 10*	89 ± 6*†
Renal	with	12 ± 3	43 ± 4*	65 ± 6*†
	without	11 ± 4	71 ± 5*¶	90 ± 4*†¶
Pulmonary blood vessels				
Artery	with	60 ± 7†	12 ± 3	57 ± 5†
	without	75 ± 8†¶	33 ± 5¶	63 ± 7†
Vein	with	49 ± 5	31 ± 6*	71 ± 2*†
	without	16 ± 3¶	66 ± 4*¶	84 ± 3*†¶

§ Data are shown as Mean ± SEM of a percent change in tension from a contraction to phenylephrine (10^{-6} M).
* $p < .05$ vs ANP
† $p < .05$ vs CNP
¶ $p < .05$ vs with endothelium
ANP atrial natriuretic peptide
CNP C-type natriuretic peptide
VNP vasonatrin peptide

In systemic arteries (femoral, saphenous and renal), ANP and CNP produced modest relaxations with the maximum being less than 30% of the contraction to phenylephrine. VNP, on the other hand, produced relaxations which ranged between 50-90% of the contraction; these relaxations were not modified significantly by the endothelium. In the comparable systemic veins, relaxations to CNP were significantly greater than relaxations evoked by ANP. Relaxations to CNP were increased significantly by removal of the endothelium. Relaxations induced by VNP were significantly greater than those induced by CNP in all systemic veins tested. Only in the renal veins were the relaxations enhanced by removed of the endothelium. The potency for relaxation in the systemic blood vessels for the peptides can be summarized as: VNP > ANP ≥ CNP in arteries; VNP > CNP > ANP in veins.

In the pulmonary blood vessels, all three peptides produced significant relaxation. The order of potency in pulmonary arteries is: ANP = VNP > CNP. Relaxations induced by ANP and CNP were increased by removal of the endothelium. The order of potency in the pulmonary veins without endothelium is: VNP > CNP > ANP. Relaxations induced by ANP were greater in the presence of endothelium while those to CNP and VNP were greater in the absence of the endothelium.

Example 2. In Vivo Studies.

Experiments were conducted in accordance with the Animal Welfare Act. Wistar rats and spontaneously hypertensive rats (SHR) (400 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with Inactin (100 mg/kg; intraperitoneal; BYK Gulden, Konstanz, Germany). The body temperature was maintained between 36 and 38°C by a heating pad. Tracheostomy was performed; however, the animals were not artificially ventilated. Polyethylene catheters (PE-50; Becton Dickinson Co., Parsippany, NJ) were placed in the left jugular vein for infusions of saline and drugs, in the right jugular vein to right atrium to monitor right atrial pressure, and in the carotid artery for the collection of blood samples and to monitor mean arterial pressure. A PE-90 catheter was placed in the bladder for urine collection.

Experiments were conducted in three groups in normal rats: ANP group (n=4), CNP group (n=4), and VNP group (n=4). VNP also was studied in SHR rats (n=4). Intravenous infusions of saline solution (0.9% NaCl) were performed (1 ml/100 gm body weight/hr) through the left jugular vein catheter. After completion of surgery, rats were allowed to stabilize for 30 minutes. In each group, a 15-min baseline period

followed. After the baseline period, saline solution (0.9% NaCl) was administered in a bolus fashion (0.1 ml) and was followed by a 15-min period. After the saline period, the peptide (ANP, CNP or VNP) was administered in a bolus fashion (0.1 ml) at 5 μg/kg which was followed by a 15-min period. This was followed by a second bolus (0.1 ml) at 50 μg/kg and a 15-min period. After a 30-min washout, a 15-min recovery period followed. During each experimental period, mean arterial pressure (MAP), heart rate (HR), and right atrial pressure (RAP) were measured. At the midpoint of baseline, second bolus fashion (50 μg/kg) and recovery periods, blood was sampled for plasma cGMP. At the end of each period, urine was measured for volume (UV), and samples were stored for electrolytes and cGMP analysis.

Blood for plasma cGMP analysis was collected into EDTA tubes, immediately placed on ice, and centrifuged at 2,5000 rpm at 4°C. Plasma was separated and stored at -20°C until assay. Urine for cGMP determination was heated to > 90°C before storage. Plasma and urine cGMP were determined by a specific RIA as previously described by A.L. Steiner et al., J. Hypertension, 5 (Suppl. 5) 551-553 (1987).

Table 2 summarizes the cardiovascular and renal actions of ANP, CNP and VNP administration in normal rats.

Table 2.
The cardiorenal actions of ANP, CNP and VNP in normal rats

	Baseline	Saline	5 µg/kg	50 µg/kg	Recovery
ANP group (n=4)					
MAP (mmHg)	119±14	120±14	97±12*	69±7*†	92±11*¶
HR (beats/min)	375±23	365±17	353±14	355±19	368±23
RAP (mmHg)	0.8±1.6	0.5±1.8	-2.3±0.8*	-3.8±0.8*†	-0.8±0.9¶
UV (µl/min)	6.7±1.2	7.2±1.8	79±10*	341±41*†	48±14¶
UNaV(µmol/min)	0.4±0.1	0.4±0.1	13.2±3.5*	68.2±15.7*†	8.9±3.5¶
UKV (µmol/min)	1.8±0.1	0.7±0.1	10.3±3.5	24.4±8.7	2.4±0.1
PcGMP(pmol/ml)	4.1±0.7	12±2	-------	25.7±7.1*	3.2±0.3¶
UcGMPV (pmol/min)	11±2	12±2	605±151*	2580±505*†	105±63¶
CNP group (n=4)					
MAP (mmHg)	116±11	115±12	100±12*	87±9*†	104±11*¶
HR (beats/min)	350±9	360±14	363±19	365±19	363±16
RAP (mmHg)	1.2±0.9	0.2±1.4	0±1.5	-2.3±0.6*	-0.1±0.9*
UV (µl/min)	5.7±0.5	5.7±0.6	27.6±2.0*§	66.4±7.4*†§	21.2±6.3¶
UNaV(µmol/min)	0.6±0.2	0.6±0.2	4.1±1.4§	12.1±3.2*†§	2.9±0.3
UKV (µmol/min)	1.2±0.3	1.2±0.3	5.1±1.6	7.6±2.5	2.4±1.1
PcGMP(pmol/ml)	2.2±0.1	-----	-- —	19.4±2.7*	3.6±0.3¶
UcGMPV(pmol/min)	23±7	22±6	97±32§	323±81*§	70±39¶
VNP group (n=4)					
MAP (mmHg)	112±14	109±12	93±15*	79±15*†	93±15*¶
HR (beats/min)	390±24	398±22	393±20	408±25	390±20
RAP (mmHg)	-2.5±0.6	-2.1±0.7	-3.5±0.9	-4.3±0.8*	-2.9±0.9¶
UV (µl/min)	6.5±1.1	6.4±0.3	32.2±7.5*§	136±5.5*†§#	8.4±2.7¶
UNaV(µmol/min)	0.9±0.3	0.7±0.2	5.3±1.9	22.5±2.2*†§#	0.9±0.3¶
UKV (µmol/min)	0.9±0.1	1.0±0.2	3.3±1.4	11.9±4.7	1.3±0.6
PcGMP(pmol/ml)	3.7±1.2	-----	-----	33.3±8.7*	4.4±1.4¶
UcGMPV(pmol/ml)	13±7	12±6	113±27*§	961±82*†§#	88±48¶

	Mean ± SEM		*	p < .05 vs Baseline
5	†	p < .05 vs 5 μg/kg	¶	p < .05 vs 50 μg/kg
	§	p < .05 vs ANP group	#	p < .05 vs CNP group
	ANP	atrial natriuretic peptide	CNP	C-type natriuretic peptide
10	VNP	vasonatrin peptide		
	MAP	mean arterial pressure	HR	heart rate
15	RAP	right atrial pressure	UV	urine volume
	UNaV	urine sodium excretion	UKV	urine potassium excretion
	PcGMP	plasma cGMP	UcGMPV	urine cGMP volume
20				

As demonstrated by the data in Table 2, bolus administration (0.1 ml) of saline solution had no cardiovascular or renal actions. Bolus administration (0.1 ml) of high dose (50 μg/kg) ANP, CNP and VNP resulted in a significant decrease in MAP and RAP, and increased urine flow, sodium excretion, plasma cGMP and urinary cGMP
5 volume. The increase in urine flow, sodium excretion and urinary cGMP volume were significantly higher with VNP than those of CNP, but were less than those of ANP.

Table 3 reports the cardiovascular and renal effects of VNP in normal and SHR rats.

Table 3.
The cardiorenal actions of VNP in normal and SHR rats

	Baseline	Saline	5 µg/kg	50 µg/kg	Recovery
Normal rats (n=4)					
MAP (mmHg)	112±14	109±12	93±15*	79±15*†	93±15*¶
HR (beats/min)	390±24	398±22	393±20	408±25	390±20
RAP (mmHg)	-2.5±0.6	-2.1±0.7	-3.5±0.9	-4.3±0.8*	-2.9±0.9¶
UV (µl/min)	6.5±1.1	6.4±0.3	32.2±7.5*	136±5.5*†	8.4±2.7¶
UNaV(µmol/min)	0.9±0.3	0.7±0.2	5.3±1.9	22.5±2.2*†	0.9±0.3¶
UKV (µmol/min)	0.9±0.1	1.0±0.2	3.3±1.4	11.9±4.7	1.3±0.6
PcGMP (pmol/ml)	3.7±1.2	------	------	33.3±8.7*	4.4±1.4¶
UcGMPV(pmol/min)	13±7	12±6	113±27*	961±82*†	88±48¶
SHR rats (n=4)					
MAP (mmHg)	169±17§	168±17§	132±23*	119±20*†	138±19*¶
HR (beats/min)	388±13	390±11	393±13	405±10	405±17
RAP (mmHG)	0.5±1.2	0.5±1.3	-1.1±0.8	-2.5±0.6*†	-0.9±1.0¶
UV (µl/min)	3.7±0.3§	3.8±0.7§	26.8±3.5*	48.2±7.4*†§	9.1±3.7¶
UNaV(µmol/min)	0.5±0.2	0.6±0.2	5.2±1.6*	9.2±2.1*†§	1.7±0.7¶
UKV (µmol/min)	0.6±0.2	0.6±0.2	3.9±1.5	6.6±2.6	1.1±0.4
PcGMP(pmol/ml)	3.1±0.7	------	------	17.7±3.6*	3.1±0.5¶
UcGMPV(pmol/min)	9±1	8±2	115±27*	234±42*†§	26±12¶

Mean ± SEM
* p < .05 vs Baseline
† p < .05 vs 5 µg/kg
¶ p < .05 vs 50 µg/kg
§ p < .05 vs normal rats
SHR spontaneous hypertensive rat
VNP vasonatrin peptide
MAP mean arterial pressure
HR heart rate
RAP right arterial pressure
UV urine volume
UNaV urine sodium excretion
UKV urine potassium excretion
PcGMP plasma cGMP
UcGMPV urine cGMP volume

As shown by the data in Table 3, the baseline MAP of SHR was significantly higher than that of normal rats, and the baseline urine volume of SHR was markedly lower than that of normal rats. During high dose bolus infusion of VNP, MAP and RAP significantly decreased, urine flow, sodium excretion, plasma cGMP and urinary cGMP volume were significantly increased in both normal and SHR groups. While the lowering of MAP to VNP was similar in both groups, the renal actions and urinary cGMP effects of VNP were attenuated in SHR rats as compared to normal rats. VNP is a more potent, endothelium independent vasorelaxing peptide in both arteries and veins as compared to ANP and CNP. VNP also has a potent natriuretic effect *in vivo*.

Examples 1 and 2 confirm that ANP is a potent natriuretic and vasoactive peptide, which has vasorelaxing actions in renal and pulmonary arteries. As shown by K.J. Koller et al., Science, 251, 120 (1991), these biological actions of ANP are mediated through the ANPR-A receptor. CNP has potent vasodilator actions in veins with less actions in arteries, while not exhibiting natriuretic effects. See C.M. Wei et al., Am. J. Physiol., 264, H71 (1993); A.J. Stingo et al., Am. J. Physiol., 262, H308 (1992). The biological actions of CNP are mediated through ANPR-B receptor. (See K.J. Koller, cited above.) In the present study, CNP was also confirmed to exhibit hemodynamic actions similar to those previously reported. However, CNP in the rat, unlike the dog, has modest but significant natriuretic actions.

The structural difference between ANP and CNP is a difference in amino acid sequences and CNP lacks a COOH-terminus. Thus, preferred peptides of the invention share properties of both ANP and CNP, suggesting that COOH-terminus may play an important role for biological activity, possibly by leading to binding to both the ANPR-A and ANPR-B receptors. Furthermore, in femoral vein and pulmonary artery, CNP has more potent effects in vessels without endothelium, but the actions of VNP are similar with and without the presence of endothelium. This may be due to endothelial mechanism(s) which inhibit the actions of CNP but not VNP. This may also be related to the presence of the VNP COOH-terminus.

Because VNP has potent arterial and venous vasodilating actions and natriuretic effects, the peptides of the invention may be useful in the treatment of cardiovascular and renal diseases, such as hypertension, congestive heart failure, and eclampsia. In preliminary studies, VNP also exhibits potent vasorelaxing actions in isolated vessels in congestive heart failure dogs. Moreover, this peptide is also able to

relax vessels pre-contracted with endothelin, which is a potent endothelium-derived vasoconstrictor.

All publications and patents cited herein are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

It will be apparent to one of ordinary skill in the art that many changes and modifications can be made in the invention without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Mayo Foundation for Medical
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200 First Street S.W.
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(ii) TITLE OF INVENTION: Vasonatrin Peptide and Analogs Thereof

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 3500 IDS Center
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/025,935
(B) FILING DATE: 03 MARCH 1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Woessner, Warren D.
(B) REGISTRATION NUMBER: 30,440
(C) REFERENCE/DOCKET NUMBER: 150.99WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-339-0331
(B) TELEFAX: 612-339-3061

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly
1 5 10 15

Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Pro Lys Met Val Gln Gly Ser Gly Cys Phe Gly Arg Lys Met Asp
1 5 10 15

Arg Ile Ser Ser Ser Ser Gly Leu Gly Cys Lys Val Leu Arg Arg His
20 25 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
20 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Leu Ser Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Ser Lys Gly
1

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Arg Phe Ser Asn
1

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr Arg Phe Ser Asn
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Leu Ser Lys Gly Cys Phe Gly Leu Xaa Xaa Asp Arg Ile Gly Ser
1 5 10 15

Met Ser Gly Leu Gly Cys
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Met Asp Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Met Asp Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys Leu Asp Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Ile Asp Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Leu Asp Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gly Leu Ser Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asn Ser Phe Arg Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Ser Met Ser Gly Leu Cys
1 5

WHAT IS CLAIMED IS:

1. A peptide compound having natriuretic, diuretic and/or vasodilator activity in mammals, of the formula (I):

H-Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-A'-A"-Asp-Arg-
Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-R^2

(SEQ ID NO: 9) wherein A' is Lys or Arg, A" is Leu, Ile or Met and R^2 is R-Asn, R-Ser-Asn, R-Phe-Ser-Asn, R-Arg-Phe-Ser-Asn- (SEQ ID NO: 7) and R-Tyr-Arg-Phe-Ser-Asn- (SEQ ID NO:8); wherein R is OH, NH_2, NHR^3 or $N(R^3)(R^4)$, wherein R^3 and R^4 are independently phenyl or (C_1-C_4)alkyl; and wherein the bracket connecting the two Cys residues indicates that they are connected by a disulfide bond; and the pharmaceutically acceptable salts thereof.

2. The peptide of claim 1 wherein R^2 is R-Tyr-Arg-Phe-Ser-Asn (SEQ ID NO:8).

3. The peptide of claim 2 wherein R is OH or NH_2.

4. The peptide of claim 1 wherein A' is Lys.

5. The peptide of claim 1 wherein A" is Leu.

6. The peptide of claim 1 wherein A'-A" is Lys-Leu.

7. A peptide compound of the formula:

(H)-Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-
Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-(R)

(SEQ ID NO:4) wherein R is OH, NH_2, NHR^3 or $N(R^3)(R^4)$, wherein R^3 and R^4 are independently phenyl or (C_1-C_4)alkyl; wherein the bracket connecting the two Cys residues indicates that they are connected by a double bond; and the pharmaceutically acceptable salts thereof.

8. A composition useful as a natriuretic, diuretic or vasodilator comprising a therapeutically effective amount of the compound of claims 1 or 7, in combination with a pharmaceutically acceptable carrier.

9. A therapeutic method for inducing natriuresis, diuresis or vasodilation in a mammalian host in need of such treatment, which comprises administering to said host a pharmaceutically effective amount of the composition of claim 8.

10. The method of claim 9 wherein the host is a human.

11. The method of claim 9 wherein the composition is administered parenterally, in combination with a liquid vehicle.

12. The method of claim 11 wherein the composition is administered by injection or infusion.

Fig. 1

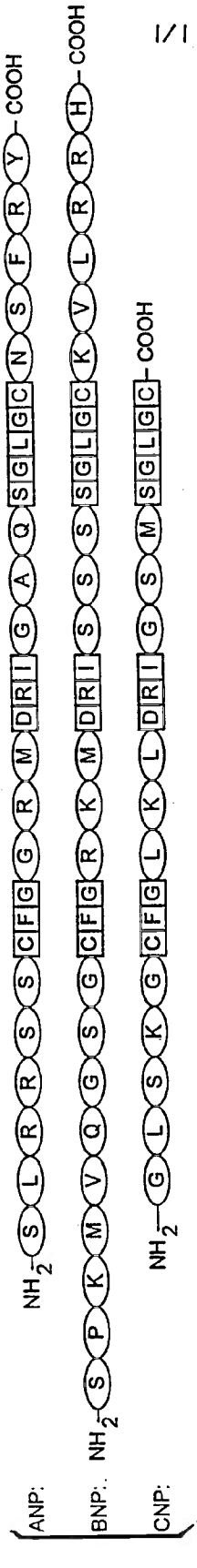
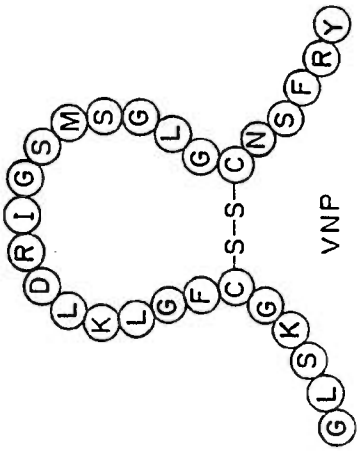


Fig. 2



INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 94/02391

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K7/10 A61K37/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 497 368 (SUNTORY LIMITED) 5 August 1992 *compound 3* see page 7 - page 8; table 2 ---	1-12
X	FEBS vol. 309, no. 2 , September 1992 pages 185 - 189 Y.SHIMEKAKE ET AL. 'The role of the C-terminal region of rat brain natriuretic peptide in receptor selectivity' *compound 10* see table 1 --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1994	27 -06- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Deffner, C-A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 94/02391

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEM.BIOPHS.RES.COMMUN. vol. 183, no. 3 , 31 March 1992 , NEW YORK pages 964 - 969 M.FURUYA ET AL. 'Structural requirements of C-type natriuretic peptide for elevation of cyclic GMP in cultured vascular smooth muscle cells' *compound 2* see tables 1,2 -----	1-12

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/02391

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0497368	05-08-92	JP-A- 6009688	18-01-94

Form PCT/ISA/210 (patent family annex) (July 1992)

Publication Number: WO 2009/067639 A2
Publication Date: May 28, 2009

Abstract

The present invention provides variants of C-type natriuretic peptide (CNP) comprising one or more deletions; additions of and/or substitutions with natural amino acids, unnatural amino acids and/or peptidomimetics (including peptide bond isosteres); amino acid extensions; and/or other chemical moieties such as, e.g., poly(ethylene glycol) and hydrophobic acids. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders such as, e.g., skeletal dysplasias and achondroplasia, and vascular smooth muscle disorders such as, e.g., restenosis and arteriosclerosis.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 May 2009 (28.05.2009)

PCT

(10) International Publication Number
WO 2009/067639 A2

(51) International Patent Classification:
C07K 14/58 (2006.01) *A61K 38/22* (2006.01)

(21) International Application Number:
PCT/US2008/084270

(22) International Filing Date:
21 November 2008 (21.11.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/989,497 21 November 2007 (21.11.2007) US
61/061,488 13 June 2008 (13.06.2008) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) Title: VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

(57) Abstract: The present invention provides variants of C-type natriuretic peptide (CNP) comprising one or more deletions; additions of and/or substitutions with natural amino acids, unnatural amino acids and/or peptidomimetics (including peptide bond isosteres); amino acid extensions; and/or other chemical moieties such as, e.g., poly(ethylene glycol) and hydrophobic acids. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders such as, e.g., skeletal dysplasias and achondroplasia, and vascular smooth muscle disorders such as, e.g., restenosis and arteriosclerosis.

WO 2009/067639 A2

VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

Cross Reference to Related Applications

The present application claims priority to U.S. provisional application no. 60/989,497, filed on November 21, 2007, and U.S. provisional application no. 61/061,488, filed on June 13, 2008. The disclosure of each prior application is incorporated by reference herein in its entirety.

Field of the Invention

The field of the invention relates, in general, to variants of C-type natriuretic peptide (CNP), compositions comprising CNP variants, and methods of using CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as skeletal dysplasias (e.g., achondroplasia) and vascular smooth muscle disorders.

Background of the Invention

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP) (Genbank Accession No. NP_006163, for the ANP precursor protein, NPPA), brain natriuretic peptide (BNP) (GenBank Accession No. NP_002512, for the BNP precursor protein, NPPB), and C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)). These small, single chain peptides (ANP, BNP, CNP) have a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. ANP and BNP bind to and activate the natriuretic peptide receptor A (NPR-A), also termed guanalyl cyclase A (GC-A), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Likewise, CNP interacts with NPR-B (GC-B) to stimulate the generation of cGMP (J. Hypertens., 10: 1111-1114 (1992)). A third type of receptor, NPR-C, binds each of the natriuretic peptides with high affinity and functions primarily to capture the peptides from the extracellular compartment and deposit the peptides into lysosomes, where they are degraded (Science, 238: 675-678 (1987)). ANP and BNP are produced primarily within the muscle cells of the heart, and are believed to have important roles in cardiovascular homeostasis (Science, 252: 120-123 (1991)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). The predominant CNP form in cartilage is unknown. Both CNP-53 and CNP-22 bind similarly to NPR-B. Furthermore, they both induce cGMP production in a dose-dependent and similar fashion (VT Yeung, Peptides, 17: 101-106 (1996)).

Natural CNP gene and polypeptide have been previously described. U.S. Patent No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Patent No. 6,034,231 discloses the human gene and polypeptide of proCNP (126 amino acids) and the human CNP-53 gene and polypeptide.

Clearance of CNP from the extracellular space occurs through the action of membrane-bound neutral endopeptidase (NEP), which rapidly degrades CNP (Biochem. J., 291 (Pt 1): 83-88 (1993)), and through NPR-C, which binds to and deposits CNP into lysosomes, where CNP is degraded. CNP has been shown to have an *in vivo* half-life of 2.6 min in the normal human (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). The low plasma concentration of CNP (J. Bone Moner. Res., 19 (Suppl.1)S20 (2004)) and its co-expression with NPR-B in a number of tissues suggests that CNP functions primarily through an autocrine/paracrine mechanism.

As stated above, CNP binds to and activates natriuretic peptide receptor B (NPR-B), also termed guanylyl cyclase B (GC-B), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. Accordingly, elevated or depressed levels of any of the components in this pathway may lead to aberrant bone growth. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, *et al.*, J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, *et*

al., Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. Both chondrocyte proliferation and differentiation appear to be disturbed, leading to remarkably short growth plate cartilage (P. Krejci et al., J. Cell Sci. 118: 5089-5100 (2005)). Endochondral ossification is the process that governs longitudinal long-bone growth. There are four zones of the growth plate – resting, proliferative, hypertrophic and zone of calcification. In the growth plate, NPR-B is expressed by proliferative cells while NPR-C is expressed by hypertrophic cells (Yamashite et al., J. Biochem. 127: 177-179 (2000)). In normal endochondral bone growth, chondrocytes organize in columns and proliferate in the proliferative zone of the growth plate. These columns are disorganized in achondroplasia patients. Additionally, the hypertrophic zone is where the cells become large and eventually apoptose (lyse), leading to osteocyte invasion and mineralization. The hypertrophic chondrocytes and the overall size of the zone are much smaller in achondroplasia patients than in normal patients. CNP is an agonist for NPR-B, a positive regulator of chondrocyte and bone growth. Downstream signaling of CNP/NPR-B inhibits the FGFR-3 pathway at the level of mitogen-activated protein kinase (MAP K). Inhibition at MAP K promotes proliferation and differentiation of the chondrocytes in the proliferative and hypertrophic zones of the growth plate, resulting in bone growth.

In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

Therapeutic use of CNP is currently limited by its short plasma half-life, which has been shown to be 2.6 minutes *in vivo* in humans (J Clin. Endocrinol. Metab., 78: 1428-35 (1994)). To increase CNP concentration above intrinsic levels (about 5 pM) typically found in human plasma, continuous infusion has been necessary in all human and animal studies using systemically administered CNP. A CNP variant having a longer *in vivo* serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a

sustainable therapeutic strategy. Two mechanisms by which the half-life of CNP is reduced in human plasma are degradation by neutral endopeptidase (NEP) and clearance by natriuretic peptide receptor C (NPR-C) (Growth Horm. & IGF Res., 16: S6-S14 (2006)). Modifications of peptides reportedly can improve resistance to endopeptidase and exopeptidase cleavage (Amino Acids, 30: 351-367 (2006); Curr. Opin. Biotech., 17: 638-642 (2006)).

The biological activities of various analogs and derivatives of CNP have been evaluated. By substituting S-methyl Cys in place of both Cys_6 and Cys_{22}, cyclization of the peptide via a Cys6-Cys22 disulfide linkage was reportedly shown to be important for the activity of CNP in stimulating cGMP formation (Biochem. Biophys. Res. Comm., 183: 964-969 (1992), also using alanine scanning to identify amino acids important for CNP functionality). A significant additional enhancement of activity reportedly results from the combined presence of the amino acids Leu_9, Lys_{10}, and Leu_{11}. U.S. Patent No. 5,434,133 describes CNP analogs comprising CNP-22 with substitutions at amino acid position 6, 7, 9, 11, or 22, wherein the amino acid is selected from Cys or Pmp (pentacyclomercaptopropionic acid) at position 6, Phe, 4-chloro-Phe, 4-fluoro-Phe, 4-nitro-Phe, or Cha (3-cyclohexyl-Ala) at position 7, Gly, Val, Aib, or tLeu at position 9, Leu or Ile at position 11, and Cys or Pmp at position 22.

U.S. Patent Publication No. 2004/0138134 (now US Patent 7,276,481) describes CNP variants comprising amino acids Cys_6 to Cys_{22} of CNP-22 ("CNP-17") which include at least one substitution for another natural amino acid at position 9, 10, 11, 16, 17, 19, or 20, CNP variants with insertions and deletions, such as addition of a His residue at the reported primary site of NEP cleavage, between Cys_6 and Phe_7, and methods of using such variants for increasing the size of a bone growth plate in abnormal bone and elongation of an abnormal bone. However, no significant gains in activity as measured by cGMP production were obtained for these variants, and activity was diminished for nearly all of the variants, as observed in an *in vitro* cell-based method (Example 6). Further no supportive data, such as for example *in vitro* stability or *in vivo* determination of improved pharmacokinetics (PK) were provided to substantiate the asserted NEP resistance and NPR-C resistance of the CNP analogs. U.S. Patent No. 6,743,425 discloses substances for treating achondroplasia which activate NPR-B/GC-B and are peptides or low molecular weight compounds, including the C-type natriuretic peptides CNP-22 and CNP-53. PCT Publication No. WO 94/20534 discloses a chimera of CNP-22 and the 5-amino acid C-terminus of ANP designated as the

vasonatrin peptide (VNP), a limited number of amino acid substitutions and cyclic chimeric peptides that result from formation of a disulfide or double bond.

Approaches for improving the half-life of other natriuretic peptide family members include decreasing the affinity of ANP for NPR-C (U.S. Patent No. 5,846,932), utilizing pentapeptide antagonists of NPR-C (WO 00/61631), and co-administering NEP inhibitors such as thiorphan and candoxatril (Clin. Exp. Pharma. Physiol., 25: 986-991 (1997), Hyperten., 30: 184-190 (1997)). WO 2004/047871 describes conjugates of BNP and BNP variants to polyalkylene glycol moieties, sugar moieties, polysorbate moieties, polycationic moieties, and other hydrophilic polymer moieties that reportedly exhibit improved half-life in circulation and reportedly are useful for the treatment of acute congestive heart failure.

There have been no published reports, however, on a successful strategy for making CNP resistant to NEP while retaining its functionality.

Summary of the Invention

The present invention relates to variants of C-type natriuretic peptide (CNP) which are useful in the treatment of bone-related disorders (e.g., achondroplasia) and vascular smooth muscle disorders. The invention encompasses CNP variants having increased serum half-life, e.g. as a result of reduced ability to bind to neutral endopeptidase (NEP), greater resistance to proteolysis by NEP and/or reduced affinity to the clearance natriuretic peptide receptor C (NPR-C), while retaining the functionality of CNP.

The wild-type sequence of CNP-22 (referred to herein as "wtCNP22" or "CNP22") is set forth below:

(N-terminus) Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 1).

Positions 6 to 22 of CNP22 form a cyclic domain by means of a disulfide bond between Cys6 and Cys22. The 17-amino acid cyclic structure has been shown to be important for binding of CNP to NPR-B (Schiller, Biochem. Biophys. Res. Commun., 138: 880-886 (1986)). The amino acid sequence of positions 6 to 22 of CNP22 is referred to herein as "CNP17" (SEQ ID NO: 2).

CNP is susceptible to NEP cleavage at a number of sites: Cys6-Phe7, Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. In one embodiment, the invention encompasses a CNP variant that is (1) modified to increase its overall size or

molecular weight, e.g., to a range from about 2.6 kDa or 2.8 kDa to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 6.4 kDa, or to about 7 kDa, 7.2 kDa or about 8.2 kDa, and/or (2) modified at certain amino acid positions to reduce its susceptibility to NEP cleavage at 1, 2, 3, 4, 5 or all 6 of the sites listed above. The size or molecular weight of the CNP variant can be increased by various means, e.g., by conjugating additional amino acids and/or other kinds of chemical (e.g., natural or synthetic polymeric) groups to the peptide sequence at, e.g., the N-terminus, the C-terminus and/or side chain(s), and/or by using natural amino acids, unnatural amino acids, and/or peptidomimetics with bulkier side chains. The CNP variant is optionally further conjugated to other functional or structural moieties. Optionally in combination with any of the embodiments described herein, mutation(s) (e.g., substitution(s), addition(s), and/or deletion(s)) may be introduced to certain position(s) of CNP22 to reduce the CNP variants' affinity to NPR-C. Further modifications may be made without affecting NEP resistance or CNP activity, e.g., conservative substitutions, or other modifications known in the art.

In one embodiment, the CNP variant is represented by the general formula: (x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 5), wherein:

the CNP variant comprises one or more modified amino acids, which may result in modified peptide bonds (e.g., through use of peptide bond isosteres), at a position corresponding to one or more of the following CNP residues: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21; and

(x) and (z) independently may be absent or may be an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, ANP, BNP) or a non-natriuretic polypeptide (e.g., human serum albumin (HSA), IgG, etc.).

In an embodiment, the CNP variant includes: (1) a modification at an amino acid position corresponding to one of positions 6, 7 or 8 (Cys6, Phe7 or Gly8) of CNP22, (2) optionally deletion, addition and/or substitution of any or all of the amino acids at positions 1-5 (Gly1, Leu2, Ser3, Lys4, and Gly5) and (3) optionally up to 1, 2, 3, 4, 5, 6 ,7, 8, 9 or 10 further modifications (deletions, additions and/or substitutions) at positions corresponding to positions 6-22, of which 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 may be conservative substitutions or other substitutions described herein or known in the art.

It is understood that a reference to a particular amino acid position by number (e.g. position 7 of CNP22) refers to the corresponding amino acid position in any CNP variant, even if the number of the position in that CNP variant has changed due to preceding insertions or deletions. For example, a reference to "position 7" or "Phe7" would mean the corresponding position 2 for a CNP variant in which the first five amino acids had been deleted. Similarly, a reference to "position 7" would mean the corresponding position 8 for a CNP variant in which one amino acid had been added to the N-terminus.

In any of the embodiments described herein, the CNP variant may be cyclized through a covalent bond between positions corresponding to 6 and 22 of CNP22. It is contemplated that the covalent bond is formed using any methods known in the art. In another embodiment, the CNP variant may be cyclized through a covalent bond formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus (referred to as "terminal" amino acids for this purpose) of the peptide. In one embodiment, the covalent bond is formed between the side chains of the two terminal amino acids or the amino acids at positions corresponding to 6 and 22 of CNP22. In another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of each terminal amino acid. For example, head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail bonds are possible for the covalent bond formed between the terminal amino acids or between the amino acids at positions corresponding to 6 and 22 of CNP22.

In one embodiment, the invention provides a CNP variant having reduced affinity to NEP, and/or greater resistance to cleavage by NEP and/or increased *in vivo* serum half-life, while retaining functionality of CNP (e.g., stimulation of cGMP production). NEP preferably recognizes substrates smaller than about 3 kDa, due to the limited size of its active site cavity (Oefner, J. Mol. Biol., 296: 341-349 (2000)). In an embodiment, the CNP variants are modified to increase their overall molecular weight to a range from about 2.6 or 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa, e.g., by adding about 0.6 to about 5 kDa of amino acids, hydrophilic or water-soluble polymers, hydrophobic acids (including fatty acids), and/or carbohydrates. In specific exemplary embodiments, the CNP variants have a molecular weight between about 2.6 kDa and about 7 kDa, or between about 2.8 kDa and 6 kDa, or between about 2.8 kDa and about 5.8 kDa. In certain embodiments, at least about 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa are added, to increase the total molecular weight of the CNP variant,

e.g., to a range from about 2.6 or 2.8 kDa up to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 7.2 kDa, 8.2 kDa or higher. In some embodiments, such CNP variants comprise an amino acid sequence at least about 70%, 75%. 80%, 85%, 90%, or 95% identical or homologous to amino acids 6-22 of CNP22. In other embodiments, such CNP variants comprise a substitution, insertion or deletion of 1, 2, 3, 4, 5, 6 or 7 amino acids with another natural or unnatural amino acid or peptidomimetic. While both conservative and non-conservative substitutions or insertions are envisioned at any position, introduction of modifications may commence, e.g., by conservative substitutions in regions that have been identified in the art as involved in CNP activity or NPR-B binding, while non-conservative substitutions may be made in those regions that have been previously shown to be tolerant of modification.

In another embodiment, the CNP variants comprise a CNP having an intact cyclized portion between Cys6 and Cys22, and N-terminal and/or C-terminal tails that contain about 1-40, 1-20, 5-40, 5-35, 10-35, 15-35, 5-31, 10-31, or 15-31 amino acids and are fragments derived from a CNP polypeptide and/or a non-CNP polypeptide. In an embodiment, such CNP variants have a molecular weight in a range from about 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa. Non-limiting examples of such CNP variants include wild-type CNP22 or CNP22 with one or more amino acid substitutions (e.g., a K4R substitution), having an N-terminal and/or C-terminal extension derived from natriuretic peptide precursor sequences (e.g., ANP, BNP or CNP) from human or other species, a natriuretic peptide precursor C (NPPC) variant with amino acid substitutions, additions and/or deletions (e.g., the CNP variants may be truncations of CNP-53 which result in peptides with a molecular weight between about 2.8 kDa and 5.8 kDa), or other non-CNP polypeptides such as, e.g., serum albumin or IgG protein (e.g., the CNP variants may be CNP chimeras containing fragments of serum albumin or IgG from human or other species).

In one embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG, also called

polyethylene oxide (PEO)), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may each be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

Gly-Ala-Asn-Lys-Lys (SEQ ID NO:7),

Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 8),

Gly-Ala-Asn-Pro-Arg (SEQ ID NO: 9),

Gly-Ala-Asn-Gln-Gln (SEQ ID NO: 10),

Gly-Ala-Asn-Ser-Ser (SEQ ID NO: 11),

Gly-Ala-Asn-Arg-Gln (SEQ ID NO: 12),

Gly-Ala-Asn-Arg-Met (SEQ ID NO: 13),

Gly-Ala-Asn-Arg-Thr (SEQ ID NO: 14),

Gly-Ala-Asn-Arg-Ser (SEQ ID NO: 15),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala (SEQ ID NO: 16),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg (SEQ ID NO: 17),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg (SEQ ID NO: 18),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 19),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg, (SEQ ID NO: 20)

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 21), and

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 22).

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

Ser-Leu-Arg-Arg-Ser-Ser (SEQ ID NO: 23);

Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 24);

Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 25);

Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 26);

Lys-Val-Leu-Arg-Arg-Tyr (SEQ ID NO: 27);

Lys-Val-Leu-Arg-Arg-His (SEQ ID NO: 28);

Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg (SEQ ID NO: 29);

Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr (SEQ ID NO: 30);

Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln (SEQ ID NO: 31); and

Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser (SEQ ID NO: 32).

In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently may be conjugated to an amino acid extension containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acids. In one embodiment, the amino acid extension is derived from NPPC, CNP53, ANP or BNP. In a specific embodiment, the amino acid extension is Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 33). In a related embodiment, this 15-amino acid extension is added to the N-terminus to provide a CNP variant of the formula Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 34).

In one embodiment, the CNP variants comprise wtCNP22 or a variant thereof (e.g., one having addition(s), deletion(s), and/or substitution(s) such as, e.g., a K4R substitution)

(SEQ ID NO: 35) conjugated at the N-terminus and/or C-terminus to a hydrophilic polymer (e.g., PEG) to increase their overall molecular size to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. Such CNP variants are optionally further conjugated at the N-terminus and/or C-terminus to a polymeric group comprising, e.g., amino acids, carbohydrates, hydrophobic acids and/or phospholipids, a non-limiting example of which is an N-terminal amino acid extension containing 1 to 35, or 5 to 31, amino acids. In an embodiment, a hydrophilic polymeric (e.g., PEG) moiety of at least about 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa, is added to the N-terminus and/or C-terminus of wtCNP22 or a variant thereof.

As shown herein, conjugation of a hydrophilic or water-soluble PEG (or PEO) polymer of about 0.6 kDa or more to CNP22 or variants thereof generally increases resistance to NEP cleavage markedly. However, addition of PEG, even as small as 0.6 kDa, to wtCNP22 may reduce CNP functionality (e.g., stimulation of cGMP signaling), and addition of greater than about 2 or 3 kDa of PEG to CNP22 or variants thereof may reduce CNP functional activity in a size-dependent manner. But CNP functionality (at least comparable to that of wtCNP22) is retained when a PEG (or PEO) polymer of about 0.6 kDa to about 1.2 kDa, or potentially to about 2 kDa, is conjugated to a CNP variant having an N-terminal amino acid extension in which at least one relatively large amino acid that may potentially be positively charged under physiological conditions (e.g., arginine) immediately precedes the position corresponding to Gly1 of CNP22, such as, e.g., GANRR-CNP22(K4R) (**CNP27(Arg4)**) (SEQ ID NO: 36), GANPR-CNP22(K4R) (**CNP27(Pro4)**) (SEQ ID NO: 37), ER-CNP22 (SEQ ID NO: 38), ER-CNP22(K4R) (SEQ ID NO: 39), R-CNP22 (SEQ ID NO: 40) and R-CNP22(K4R) (SEQ ID NO: 41).

Accordingly, in one embodiment, PEGylated CNP variants comprise at the N-terminus of CNP22 or a variant thereof (e.g., one having a K4R substitution) an amino acid extension containing at least 1, 2, 3, 4 or 5 amino acids, wherein the PEG polymer is conjugated to the N-terminus of the amino acid-extended CNP variant to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP cleavage. In an embodiment, for enhanced CNP functionality, such pegylated, amino acid-extended CNP variants contain at least one relatively large natural or unnatural amino acid that may potentially be positively charged under physiological conditions, immediately preceding the position corresponding to Gly1 of CNP22. In a specific embodiment, the pegylated, amino acid-extended CNP variants contain

at least one arginine residue immediately preceding the position corresponding to Gly1 of CNP22.

In addition to CNP variants conjugated at the N-terminus and/or C-terminus to a hydrophilic or water-soluble polymer such as, e.g., PEG (or PEO), the invention encompasses CNP variants conjugated to such a polymer at an internal site. For purposes of brevity here, PEG (or PEO) will be used as a representative example of a hydrophilic or water-soluble polymer. Various sites of PEGylation of a CNP variant are possible, including but not limited to: (1) PEGylation only at the N-terminus; (2) PEGylation only at the C-terminus; (3) PEGylation only at an internal site (e.g., Lys4); (4) PEGylation at both the N-terminus and the C-terminus; (5) PEGylation at the N-terminus and an internal site; and (6) PEGylation at the C-terminus and an internal site. For increased resistance to NEP degradation and retention of CNP functionality, in certain embodiments the total mass of PEGylatedCNP variants is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. In a particular embodiment, CNP17, CNP22, CNP37 (defined below) or variants thereof (including those having amino acid additions, substitutions and/or deletions) are PEGylatedonly at the N-terminus. In another embodiment, the CNP variants are PEGylated only at an internal site (e.g., Lys4). In yet another embodiment, the CNP variants are PEGylated at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP variants are not PEGylated at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). To prevent PEGylation at an internal site, Lys4 and/or Lys10 can be substituted with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

The invention contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy

succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP-22 (2.2kDa), CNP-17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22, or variants with amino acid substitutions, additions and/or deletions, for example, CNP27(Arg4), CNP27(Pro4), R-CNP22, R-CNP22(K4R), ER-CNP22 and ER-CNP22(K4R). In an embodiment, the PEG-CNP variants having a total mass characterized the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) group conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG group conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The invention further contemplates negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds.

In a related embodiment, the invention contemplates PEG-CNP conjugates comprising NHS- or aldehyde-based PEG of the formula $(CH_2CH_2O)_n$, wherein n is an integer from 12 to 50, and the PEG polymer is up to about 2.5 kDa in molecular weight. In a specific embodiment, n is 12 or 24. In an embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In an additional embodiment, the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.4 kDa to about 2.5 kDa or from about 0.6 kDa to about 1.5 kDa.

In a further embodiment, the wtCNP or CNP variant peptide is conjugated to a moiety including, e.g., bisphosphonates, carbohydrates, hydrophobic acids (including fatty acids) or amino acid sequences. Such amino acid sequences include for example polyAsp or polyGlu useful in bone/cartilage targeting, or can be derived from bone proteins with elucidated bone-targeting domains or derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc. In embodiments described herein where CNP22 or a variant thereof is attached to a bone- or cartilage-targeting moiety, such a moiety is designed to promote getting the modified CNP peptide to chondrocytes of bone growth plates, where the peptide can bind and activate NPR-B on the chondrocytes.

In another embodiment, the invention provides CNP variants with a peptide bond that is less susceptible to cleavage by peptidases including NEP. The invention encompasses a CNP variant comprising at least one modified residue at a site of endopeptidase cleavage. In one embodiment, the Cys6-Phe7 peptide bond (-C(=O)-NH-) at an NEP cleavage site in CNP can be replaced with anyone of the following peptide-bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, and

-NHC(=O)NH-.

In another embodiment, Phe7 is substituted with its enantiomer D-Phe. In yet another embodiment, D-enantiomers are introduced to one or more, up to all 22, positions within wtCNP-22. In a further embodiment, a beta amino acid such as 3-amino-2-phenylpropionic acid is substituted for Phe7, which increases the length of the backbone while reducing the length of the side chain. In yet another embodiment, the invention contemplates Cys analogs at the Cys6 position including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid.

Even in the presence of a NEP-resistant bond between Cys6 and Phe7, other peptide bonds can be hydrolyzed by NEP, including Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. Accordingly, the invention encompasses CNP analogs containing peptide bond isosteres at multiple locations in the backbone of the CNP analogs. In one embodiment, CNP analogs or variants comprise modifications at more than one peptidase cleavage site. In a further embodiment, such variant comprises a CNP with substitutions at amino acid residues important in binding to the NEP active site, thereby increasing resistance

to NEP degradation. One or more NEP-binding residues, including but not limited to Gly8, Gly15, Ser18, Gly19 and/or Gly21, are replaced with larger-size natural or unnatural amino acid residues to reduce affinity to the NEP active site. In yet another embodiment, one or more hydrophobic residues essential in NEP recognition, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, are substituted with natural or unnatural amino acids and/or peptidomimetics that decrease NEP binding. In yet another embodiment, one to five of the first five amino acids of CNP can be deleted or substituted with any other natural amino acids or unnatural amino acids or peptidomimetics, or one or more natural or unnatural amino acids or peptidomimetics can be added to any one or to all of the first five positions of CNP.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone proteins with elucidated bone-targeting domains, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; polymeric or non-polymeric molecules that reduce renal clearance such as, e.g., charged PEG molecules; and extensions comprising, e.g., polymers (e.g., PEGs), carbohydrates, hydrophobic acids (including fatty acids), and/or amino acids, and wherein such amino acid extensions can contain, e.g., from 1 to 31, or 1 to 35, or 5 to 35, or 10 to 35, or 15 to 35 amino acid residues, and can be derived from NPPC, ANP, BNP, other non-CNP (poly)peptides such as, e.g., serum albumin or IgG, or variants of the aforementioned polypeptides having substitutions, additions and/or deletions, or combinations thereof;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone or cartilage targeting such as for example polyAsp and polyGlu, amino acid sequences from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein, and amino acid sequences derived from non-CNP (poly)peptides such as, e.g., ANP or BNP;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In one embodiment, the CNP variants comprise a modification at one or more of positions 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and/or 20, and may optionally have modifications at any of the other positions disclosed herein.

In embodiments described herein where CNP22 or variants thereof can be attached to a hydrophobic acid, the CNP peptides can be attached to one or hydrophobic acids. Non-limiting examples of hydrophobic acids include straight-chain or branched, saturated or unsaturated C_5-C_{12} carboxylic acids (e.g., pentanoic acid, heptanoic acid, etc.) and natural fatty acids. The hydrophobic acids can be attached to the N-terminus, the C-terminus, and/or the side chain of one or more amino acid residues. In one embodiment, the hydrophobic acids are conjugated to the N-terminus. In an embodiment, conjugation of CNP22 or a variant thereof to a hydrophobic acid is designed, *inter alia*, to promote non-specific interaction between the modified CNP peptide and serum albumin, thereby increasing the size of the CNP peptide and protecting it from cleavage by proteases such as, e.g., NEP. The interaction between the hydrophobic acid-conjugated CNP peptide and albumin is designed to be not too strong, so that the modified CNP peptide can diffuse through cartilage, get to chondrocytes of bone growth plates, and bind and activate NPR-B.

In a further embodiment, the invention provides CNP variants that *in vitro* stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), comprise at least one modified amino acid at position $(b)_6$, $(c)_7$ and/or $(d)_8$, and are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 47), wherein:

(x) may be absent or may be a peptide sequence containing one to five amino acids which is derived from a natriuretic polypeptide (e.g. NPPC, CNP, ANP or BNP) or a non-natriuretic polypeptide as described herein (e.g., HSA, IgG, a bone-targeting protein, etc.);

(z) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in

bone/cartilage targeting such as, e.g., polyAsp and polyGlu; bone proteins with bone-targeting domains and derivatives thereof, such as fusion proteins or peptides sequences of osteopontin, osteocalcin, and sialoprotein; molecules that reduce renal clearance, such as, e.g., charged PEGs; and molecules that increase resistance of CNP to NEP-mediated degradation, as described herein;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) may be Cys or descarboxy cysteine, or the $(b)_6$-$(c)_7$ peptide bond (-C(=O)-NH-) may be replaced with any one of the following peptide bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, or

-NHC(=O)NH-;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; peptide bond isosteres of Phe such as N-alkylated derivatives of Phe wherein the N-alkyl group is selected from the group consisting of methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not

limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) is any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amino group on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the invention encompasses CNP variants that *in vitro* stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP degradation, and are represented by the general formula:

(x)-(y)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 48), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG), and a non-

limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or FGF2;

(y) may be absent or may be one or more amino acids from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (corresponding to positions 1 to 5 of CNP22) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution);

(h) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (h) is not Arg; and

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG).

In an embodiment, (x), (y) and (z) together contain from about 10 to about 40, or from about 15 to about 35 amino acids. In another embodiment, (x) is an amino acid sequence comprising from 1 to 40 amino acids, or from 1 to 20 amino acids.

Further contemplated are CNP variants that *in vitro* stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), and comprise the sequence:

(y)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 138), wherein:

(y) comprises one or more amino acids selected from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution), and further comprises a hydrophilic or water soluble polymer of molecular weight from about 0.6 kDa to about 5 kDa. In an embodiment, the hydrophilic or water-soluble polymer is conjugated to the N-terminus of such amino acid-extended CNP

variant. In a particular embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In yet another embodiment, the invention provides CNP variants that *in vitro* stimulate the production of at least about 50% of the cGMP level produced under produced under the same concentration of wtCNP22 (e.g. 1 uM), wherein the CNP variants comprise an N-terminal and/or C-terminal peptide extension containing from 1 to 15 amino acids, and are conjugated to a hydrophilic or water soluble polymer. In an embodiment, the peptide extension contains from 5 to 10 amino acids. In a specific embodiment, the peptide extension contains 5 amino acids. In another specific embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In a still further embodiment, the CNP variants of the invention *in vitro* stimulate the production of at least about 50% of the cGMP level produced underthe same concentration of wtCNP22 (e.g. 1 uM), and comprise at least a 15 amino acid fragment derived from natriuretic peptide precursor C (NPPC), wherein the fragment is at least 70% homologous to a sequence from wild type NPPC containing the same number of amino acid residues.

In still another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

$(x)\text{-}(b)_6\text{-}(c)_7\text{-}(d)_8\text{-}(e)_9\text{-}(f)_{10}\text{-}(g)_{11}\text{-}Asp_{12}\text{-}Arg_{13}\text{-}(h)_{14}\text{-}Gly_{15}\text{-}Ser_{16}\text{-}(i)_{17}\text{-}Ser_{18}\text{-}Gly_{19}\text{-}(j)_{20}\text{-}Gly_{21}\text{-}Cys_{22}\text{-}(z)$ (SEQ ID NO: 49), wherein:

(x) may be absent (i.e., the N-terminus ends with an $-NH_2$ group) or may be selected from the group consisting of a sequence of 1, 2, 3, 4 or 5 amino acids from the peptide $Gly_1\text{-}Leu_2\text{-}Ser_3\text{-}Lys_4\text{-}Gly_5$; amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; bone-targeting domains from bone proteins such as for example osteopontin, osteocalcin or sialoprotein; molecules that reduce renal clearance such as hydrophilic or water-soluble polymers, including but not limited to charged PEG molecules; and moieties comprising PEG, carbohydrates, hydrophobic acids, amino acids, or combinations thereof, wherein such moieties can be amino acid extensions including but not limited to amino acid sequences derived from NPPC or non-CNP (poly)peptides such as, e.g., BNP, ANP, serum albumin or IgG;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino

acid sequences derived from bone-targeting proteins, such as for example osteopontin, osteocalcin or sialoprotein; and amino acid sequences derived from NPPC or non-CNP (poly)peptides, as described herein;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-(i)$_{15}$-Ser_{16}-(j)$_{17}$-Ser_{18}-Gly_{19}-(k)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 50), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieties comprising, e.g., hydrophilic polymers (e.g., PEG), carbohydrates, hydrophobic acids, and/or amino acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples

include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants can have amino acid substitution(s) at one or more of any of positions 1 to 22 of CNP22. In one embodiment, Gly1 is substituted with Arg or Glu. In another embodiment, Lys4 is replaced with Arg. In still another embodiment, Gly5 is substituted with Arg, Gln or Ser. In yet another embodiment, Gly15 is substituted with Ser, Asn, Arg or Cit. In a further embodiment, Gly19 is substituted with Ser, Arg or Asn. In yet another embodiment, Gly21 is substituted with Ser, Thr, or Arg.

In one embodiment, the CNP variant is selected from the group consisting of GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (formed using descarboxy-Cys) (SEQ ID NO:

56), GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO: 57), GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC(SEQ ID NO:136), GLSKGCF-(tBuG)-LKLDRIGSMSGLGC (SEQ ID NO: 58), GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO:137), and GLSKGC-[$NHCH_2CH(Ph)CO$]-GLKLDRIGSMSGLGC (formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59). In a further embodiment, a disulfide bond exists between Cys6, descarboxy-Cys or another sulfhydryl-containing cysteine analog at the Cys6 position, and Cys22 of any CNP variant described herein.

In another embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-53**) (SEQ ID NO: 4);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-37, Analog BL**) (SEQ ID NO: 60);

AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (**Analog CA**) (SEQ ID NO: 61);

AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (**Analog CB**) (SEQ ID NO: 62);

DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (**Analog CC**) (SEQ ID NO: 63);

RGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 40);

ERGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 38);

GANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 64);

GANRRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 65);

GANPRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 66);

GANSSGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 67);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144); and

SPKMVQGSG-CNP17-KVLRRH (**Analog CD**) (SEQ ID NO: 68) (CNP17 having N-terminal and C-terminal tails derived from BNP).

In a further embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

GANRRGLSRGCFGLKLDRIGSMSGLGC (**CNP27(Arg4)**) (Analog AY) ((SEQ ID NO: 36);

GANPRGLSRGCFGLKLDRIGSMSGLGC (**CNP27(Pro4)**) (Analog CI) (SEQ ID NO: 37);

RGLSRGCFGLKLDRIGSMSGLGC (**Analog AZ**) (SEQ ID NO: 41);

ERGLSRGCFGLKLDRIGSMSGLGC (**Analog BA**) (SEQ ID NO: 39);

GANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CH**) (SEQ ID NO: 69); and

GANSSGLSRGCFGLKLDRIGSMSGLGC (**Analog CG**) (SEQ ID NO: 70).

In one embodiment, CNP variants having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of such PEGylated CNP variants include PEO24-CNP27(Arg4), PEO12-CNP27(Arg4), PEO24-GANRR-CNP22, PEO12-GANRR-CNP22, PEO24-CNP27(Pro4), PEO12-CNP27(Pro4), PEO24-GANPR-CNP22, PEO12-GANPR-CNP22, PEO24-GANQQ-CNP22, PEO12-GANQQ-CNP22, PEO24-ER-CNP22(K4R), PEO12-ER-CNP22(K4R), PEO24-ER-CNP22, PEO12-ER-CNP22, PEO24-R-CNP22(K4R), PEO12-R-CNP22(K4R), PEO24-R-CNP22, and PEO12-R-CNP22, wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In an embodiment, the PEG (or PEO) polymer is attached to the N-terminus of the CNP variants.

Additional CNP variants include, but are not limited to, derivatives of CNP37 having mutations at the furin cleavage site (underlined) to improve *in vivo* resistance to the furin protease and/or having glycine (underlined) preceding glutamine to prevent pyroglutamate formation, including but not limited to:
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (**Analog CS**) (SEQ ID NO: 71); GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (**Analog CT**) (SEQ ID NO: 72); GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CU**) (SEQ ID NO: 73); GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (**Analog CW**) (SEQ ID NO: 74); and
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Analog DB) (SEQ ID NO: 75).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CQ**) (histidine-rich glycoprotein (HRGP) fragment-CNP22 chimera) (SEQ ID NO: 76);
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CR**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 77);
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CX**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 78);

GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (**Analog CF**) ($IgG_1(F_c)$ fragment-CNP22 chimera) (SEQ ID NO: 79);

GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CY**) (human serum albumin (HSA) fragment-CNP22 chimera) (SEQ ID NO: 80);

GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (**Analog CE**) (HSA fragment-CNP22 chimera) (SEQ ID NO: 81);

FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CZ**) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera) (SEQ ID NO: 82); and

GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (**Analog DA**) (FGF2 "heparin-binding domain" fragment-CNP22 chimera) (SEQ ID NO: 83).

In a further embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22(K4R)"), including but not limited to:

GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CK**) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera) (SEQ ID NO: 84);

GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CL**) (HSA fragment-CNP22(K4R) chimera) (SEQ ID NO: 85

GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CM**) (fibronectin fragment-CNP22(K4R) chimera) (SEQ ID NO: 86);

GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CN**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 87);

GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CO**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 88); and

GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CP**) (zinc finger fragment-CNP22(K4R) chimera) (SEQ ID NO: 89).

In yet another embodiment, the CNP variant may be a monomer or a dimer. In a related embodiment the monomers of dimeric CNP variants can be attached N-terminus to N-terminus via a linker or no linker, N-terminus to C-terminus via a linker or no linker, or C-terminus to C-terminus via a linker or no linker.

Chimera comprising IgG and CNP22 or a variant thereof are designed for, *inter alia*, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, *inter alia*, reduced

immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, *inter alia*, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other advantageous features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In any of the embodiments disclosed herein, the CNP variants may have substantially the same or better biological activity than wild-type CNP22. For example, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, e.g., with respect to interaction with NPR-

B (GC-B) to stimulate the generation of cGMP. Alternatively, or in addition, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, with respect to regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation, chondrocyte differentiation, inhibition of the mitogen activated protein (MAP) kinase/ MEK (Raf-1) kinase signaling pathway, and promoting endochondral ossification. In any of the embodiments described herein, the CNP variants may comprise an amino acid sequence that is at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or more identical or homologous to amino acids 6-22 or 1-22 of wild-type CNP22.

In a further embodiment, the invention provides variants of CNP22 having less affinity to the NPR-C clearance receptor while retaining the ability to bind and activate NPR-B. The present invention encompasses variants that were, or can be, generated from a homology-based structural model of the NPR-B/CNP complex as described in the Detailed Description. In another embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility, which may increase their specificity for binding to NPR-B over NPR-C. Variants of CNP having potentially reduced affinity to the NPR-C include but are not limited to those having one or more of the following substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R.

In yet another embodiment, the CNP variants have a modification and/or substitution at one or more of positions 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and 21, and may optionally have modifications and/or substitutions at any of the other positions disclosed herein. In a further embodiment, the CNP variants can optionally have conjugation(s) or extension(s), e.g., at the N- and/or C-terminus to facilitate bone/cartilage targeting, reduce renal clearance, and/or increase resistance to NEP degradation. Such conjugation(s) or extension(s) can comprise molecules or sequences formed or derived from, e.g., polyAsp, polyGlu, bone- or cartilage-targeting peptides, osteopontin, osteocalcin, sialoprotein, PEGs, carbohydrates, hydrophobic acids, NPPC or non-CNP (poly)peptides, or combinations thereof.

In still another embodiment, the CNP variants are prepared by standard solid-phase peptide synthesis methods with natural or unnatural amino acid(s) or peptidomimetic(s) being substituted and/or added where appropriate. In a further embodiment, PEGylation of the CNP variants occurs following, or part of, peptide synthesis with the conjugation reaction

being performed by NHS- or aldehyde-based chemistry or other chemistry known in the art. In another embodiment, the CNP variants comprise a disulfide bond. In a related embodiment, the disulfide bond forms a cyclic peptide. In a particular embodiment, the disulfide bond is formed between cysteine residues at positions corresponding to positions 6 and 22 of CNP22.

It is further contemplated that the CNP variants can be conjugated to a hydrophobic polymeric or non-polymeric moiety, such as, e.g., heptanoic acid, pentanoic acid, or fatty acids. The hydrophobic moiety can be conjugated to the side chain of an amino acid residue, including but not limited to a lysine, a serine, a cysteine or a threonine, or can be attached to the N-terminus and/or C-terminus of the CNP variant.

In an embodiment, the CNP variants as described herein have a pI in the range from about 8 to about 10.5 or from about 8.5 to about 10.

In a further embodiment, the invention provides a pharmaceutical composition comprising a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the compositions are sterile pharmaceutical compositions suitable for parenteral injection. In some embodiments, the compositions comprise substantially pure CNP variant, e.g. at least about 90% or 95% pure. In some embodiments, the compositions contain less than about 5%, 4%, 3%, 2%, 1% or 0.5% contaminants, such as other human proteins, porcine proteins, or CNP53 or fragments thereof (other than the desired CNP variant).

CNP variants of the invention advantageously retain CNP activity and exhibit increased serum half-life. Retention of CNP activity can be shown, for example, as retention of desired *in vivo* biological effect, or retention of at least about 50% of the cGMP stimulating activity of CNP22, under the same concentration (e.g., 1 uM of CNP peptide or greater than the ED80). In some embodiments, CNP variants exhibit at least about 1.5-fold, 2-fold, 3-fold, 4-fold , 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or 40-fold increase in serum half-life compared to CNP22.

In yet another embodiment, the invention provides methods of treating conditions or disorders responsive to CNP, comprising administering a therapeutically effective amount of a CNP variant or a composition comprising the same to a subject in need thereof. In one embodiment, disorders responsive to CNP are disorders of bone growth, including but not limited to skeletal dysplasias and inherited skeletal malformations such as disorders

associated with fibroblast growth factor receptor 3 (FGFR-3) mutations. In a specific embodiment, the disorder associated with FGFR-3 mutation(s) is achondroplasia. In another embodiment, the disorders responsive to CNP are disorders associated with vascular smooth muscle cells and tissues. In a further embodiment, the CNP variants are useful for increasing the size of the growth plate of a bone (e.g., a limb bone). In another embodiment, the CNP variants are useful for elongating a bone or increasing long bone growth. In still another embodiment, the CNP variants are useful for enhancing matrix production, proliferation and differentiation of chondrocytes.

Brief Description of the Figures

Figure 1 shows the degree of resistance of N-terminal PEGylated CNP22 conjugates to neutral endopeptidase (NEP) *in vitro*.

Figure 2 depicts the degree of NEP resistance of CNP variants having an amino acid extension at the N-terminus. CNP27 in Figure 2 is CNP27(Arg4), i.e., GANRR-CNP22(K4R).

Figure 3 illustrates the degree of NEP resistance of N-terminal PEGylated CNP17 and CNP27(Arg4).

Figure 4 shows the ability of CNP variants having an N-terminal amino acid extension to stimulate cGMP production in NIH3T3 cells *in vitro*. The results are relative to the level of cGMP produced in the presence of 1 uM CNP22. CNP27 in Figure 4 is CNP27(Arg4).

Figure 5 displays the ability of N-terminal PEGylated CNP17 and CNP27(Arg4) to stimulate cGMP production in NIH3T3 cells.

Figure 6 illustrates the effects of N-terminal PEGylation of CNP22 on cGMP production.

Figure 7 demonstrates that exposure of rat chondrosarcoma cells to CNP22 1 hour once daily or 2 hours once daily has substantially similar effectiveness in reversing FGF2-induced arrest of chondrocyte growth as continuous exposure to CNP22.

Figure 8 shows the efficacy of CNP37 and PEO24-CNP27(Arg4) in stimulating longitudinal growth of wild-type femur in an *ex vivo* mouse organ model.

Figure 9 shows that CNP37 and PEO24-CNP27(Arg4) intravenously (i.v.) administered to rats have a much longer half-life and a much greater bioavailability in the plasma than CNP22.

Figure 10 illustrates that PEO24-CNP27(Arg4) subcutaneously (s.c.) administered to rats also has a much longer half-life and a much greater bioavailability in the plasma than CNP22.

Figure 11 demonstrates that i.v. administered CNP37 and PEO24-CNP27(Arg4) stimulate a much greater level of cGMP production in rats than CNP22.

Figure 12 shows that s.c. administered PEO24-CNP27(Arg4) and, to a lesser extent, CNP37 are substantially more effective in stimulating cGMP production in rats than CNP22.

Detailed Description of the Invention

The present invention relates to novel variants of CNP having reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, pharmaceutical compositions comprising such CNP variants, and methods of using such CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as achondroplasia and disorders associated with vascular smooth muscle cells and tissues.

A. Definitions

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below.

As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range.

Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present invention employs, unless otherwise indicated, conventional methods of synthetic organic chemistry, mass spectrometry, preparative and analytical chromatography, protein chemistry, biochemistry, recombinant DNA technology and pharmacology, within the skill of the art. See, *e.g.*, T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

"Polypeptide" and "protein" refer to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof, linked *via* peptide bonds or peptide bond isosteres. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The terms "polypeptide" and "protein" are not limited to a minimum length of the product. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms "polypeptide" and "protein" also include postexpression modifications

of the polypeptide or protein, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" can include "modifications," such as deletions, additions, substitutions (which may be conservative in nature or may include substitutions with any of the 20 amino acids that are commonly present in human proteins, or any other naturally or non-naturally-occurring or atypical amino acids), and chemical modifications (e.g., addition of or substitution with peptidomimetics), to the native sequence. These modifications may be deliberate, as through site-directed mutagenesis, or through chemical modification of amino acids to remove or attach chemical moieties, or may be accidental, such as through mutations arising with hosts that produce the proteins or through errors due to PCR amplification.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Conservative substitution" refers to substitution of an amino acid in a polypeptide with a functionally, structurally or chemically similar natural or unnatural amino acid. In one embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1), Alanine (A) Serine (S), Threonine (T);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

In another embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1) Glycine (G), Alanine (A);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Alanine (A);

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); and

(7) Serine (S), Threonine (T), Cysteine (C).

In a further embodiment, amino acids may be grouped as set out below.

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence backbone orientation: Gly, Pro; and

(6) aromatic: Trp,Tyr, Phe, His.

In one embodiment, the peptides or polypeptide described herein are generated via recombinant means, using a polynucleotide encoding a CNP variant. The invention thus encompasses polynucleotides encoding any of the CNP variants described herein, host cells or vectors comprising such polynucleotides, optionally linked to expression control sequences, and methods of using such polynucleotides, vectors or host cells to produce CNP variants of the invention. CNP variants expressed by such polynucleotides may be produced by methods including growing host cells in culture medium under conditions suitable for expression of the polynucleotide encoding a CNP variant, and isolating the expression product from the host cells or culture medium. Actual expression products may vary slightly from the encoded protein product depending on any post-translational processing.

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), the nucleotide sequence also encompasses an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T." "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Expression control sequence" refers to a nucleotide sequence that regulates the expression of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive), enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, *e.g.*, a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (*e.g.*, promoter, origin of replication, ribosome-binding site, *etc.*) as well.

"Chimera" as used herein refers to a polynucleotide or polypeptide comprising at least two heterologous polynucleotide or polypeptide sequences (i.e. derived from different sources or not associated with each other as a naturally-occurring sequence) which are attached or linked together using techniques commonly known in the art, e.g., recombinant expression or chemical crosslinking.

The terms "identical" and percent "identity," in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially homologous" or "substantially identical" in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In certain embodiments, the substantial identity exists over regions of the sequences that are at least about 25, 50, 100 or 150 residues in length. In another embodiment, the sequences are substantially identical over the entire length of either or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math., 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection. One example of a useful algorithm is PILEUP, which uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35: 351-360 (1987) and is similar to the method described by Higgins & Sharp, CABIOS, 5: 151-153 (1989). Another algorithm useful for generating multiple alignments of sequences is Clustal W (Thompson *et al.*, Nucleic Acids Research, 22: 4673-4680 (1994)). An example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm (Altschul *et al.*, J. Mol. Biol., 215: 403-410 (1990); Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA, 89: 10915 (1989); Karlin & Altschul, Proc. Natl. Acad. Sci. USA, 90: 5873-5787 (1993)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

"Substantially pure" or "isolated" means an object species is the predominant species present (*i.e.*, on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. In one embodiment, a substantially pure composition means that the species of interest comprises at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or more of the macromolecular species present in the composition on a molar or weight basis. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a

single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (*e.g.*, BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In an embodiment, the compounds of the invention are substantially pure or isolated. In another embodiment, the compounds of the invention are substantially pure or isolated with respect to the macromolecular starting materials used in their synthesis. In yet another embodiment, the pharmaceutical compositions of the invention comprise a substantially pure or isolated CNP variant admixed with one or more pharmaceutically acceptable excipients, carriers or diluents, and optionally with another biologically active agent.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) and which has not been intentionally modified by man in the laboratory is naturally occurring. In one embodiment, a "naturally occurring" substance is of human origin.

"Wild-type" (wt) is a term referring to the natural form, including sequence, of a polynucleotide, polypeptide or protein in a species. A wild-type form is distinguished from a mutant form of a polynucleotide, polypeptide or protein arising from genetic mutation(s).

In one embodiment, a first polypeptide that is an "analog" or "variant" or "derivative" of a second polypeptide is a polypeptide having at least about 50%, 60% or 70% sequence homology, but less than 100% sequence homology, with the second polypeptide. Such analogs, variants or derivatives may be comprised of non-naturally occurring amino acid residues, including without limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues. Such analogs, variants or derivatives may also be composed of one or a plurality of D-amino acid residues, and may also contain peptidomimetics or peptide bond isosteres such as non-peptide linkages between two or more amino acid or peptidomimetic residues. In another embodiment, a first polypeptide is an "analog", "variant" or "derivative" of a second polypeptide if the first polypeptide is not a known cleavage product of the second polypeptide or is not a known precursor of the second polypeptide, even if the first polypeptide has 100% sequence homology to the second polypeptide or has a wild-type sequence.

In an embodiment, the term "derived from" as used herein refers to a polypeptide or peptide sequence that is based on a wild type or naturally occurring polypeptide or peptide

sequence and can have one or more deletions, additions, and/or substitutions with natural amino acids, unnatural amino acids or peptidomimetics. In one embodiment, the derivative sequence shares at least about 40%, 50%, 60% or 70%, but less than 100%, sequence similarity to the wild-type or naturally occurring sequence. In another embodiment, the derivative may be a fragment of a polypeptide, wherein the fragment is substantially homologous (e.g., at least about 70%, 75%, 80%, 85%, 90%, or 95% homologous) to the wild-type polypeptide over a length of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids. In still another embodiment, a polypeptide is "derived from" a wild-type polypeptide if it has a moiety (e.g., a polymer such as, e.g., PEG) attached to it which is not present on the wild-type polypeptide, even if both polypeptides share 100% homology in their amino acid sequence.

As used herein, an "NPPC-derived" polypeptide refers to a polypeptide derived from the natriuretic peptide precursor C (NPPC) polypeptide, which is a single chain 126-amino acid pre-pro polypeptide, and which upon cleavage ultimately results in wtCNP22. Removal of the signal peptide from NPPC yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP, or CNP-22). Therefore, CNP22 itself is an "NPPC-derived" polypeptide by virtue of being derived from NPPC. In one embodiment, an NPPC-derived polypeptide is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that an NPPC-derived peptide may comprise from about 1 to about 53, or 1 to 37, or 1 to 35, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, an NPPC-derived peptide may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experi-

mentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the invention may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional, subjective or objective. The compounds of the invention may also be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients.

Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a compound of the invention and a pharmaceutically acceptable excipient, carrier or diluent.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a compound of the invention calculated in an amount sufficient to produce the desired effect, optionally in association with a pharmaceutically acceptable excipient, diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

"Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological

conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

The terms "polyethylene glycol", "PEG", "polyethylene oxide" and "PEO" are used interchangeably herein unless indicated otherwise. A CNP peptide (CNP22 or a variant thereof) conjugated via an amino group to a "PEOn" polymer associated with the number n, in general has the formula: $CH_3–[–O–CH_2CH_2–]_n–C(=O)–NHR$, where n is the number of ethylene oxide units and R denotes the rest of the peptide. The "PEOn" polymer can optionally have an alkylene group, $(CH_2)_n$, where n is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. Such a "PEOn" (e.g., PEO12 or PEO24) polymer is monodispersed, i.e., is a single discrete polymer of a particular molecular weight. Similarly, a CNP peptide conjugated via an amino group to a "PEGnK" polymer associated with the number nK, in general has the formula: $CH_3–[–O–CH_2CH_2–]_p–C(=O)–NHR$, where p is an integer greater than 1. The "PEGnK" polymer also can optionally have an alkylene group, $(CH_2)_n$, where n is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. However, such a "PEGnK" (e.g., PEG1K, PEG2K, PEG5K or PEG20K) polymer is polydispersed, i.e., contains a mixture of polymers having a distribution of molecular weights, where the number nK denotes the polymer number-average molecular weight (M_n) in kilo Daltons. For example, "PEG2K" conjugated to a CNP peptide denotes a polydispersed PEG polymer having a polymer number-average molecular weight of around 2 kDa.

When a range of the mass of a polymer (e.g., PEG) is given (e.g., in units of kDa), the range refers to a range of polymer number-average molecular weights, not to a range of molecular weights of multiple polymers in a polydispersed mixture, unless expressly indicated otherwise.

The term "halogen", "halide" or "halo" refers to fluorine, chlorine, bromine, and/or iodine.

The term "alkyl" refers to a linear or branched saturated monovalent hydrocarbon radical, wherein the alkyl may optionally be substituted with one or more substituents Q as described herein. In certain embodiments, the alkyl is a linear saturated monovalent hydrocarbon radical that has 1 to 20 (C_{1-20}), 1 to 15 (C_{1-15}), 1 to 12 (C_{1-12}), 1 to 10 (C_{1-10}), or 1 to 6 (C_{1-6}) carbon atoms, or a branched saturated monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. As used herein, linear C_{1-6} and branched C_{3-6} alkyl groups are also referred as "lower alkyl." Examples of alkyl groups include, but are not limited to, methyl, ethyl, propyl (including all isomeric forms, including n-propyl and isopropyl), butyl (including all isomeric forms, including n-butyl, isobutyl, sec-butyl and tert-butyl), pentyl (including all isomeric forms), and hexyl (including all isomeric forms). For example, C_{1-6} alkyl refers to a linear saturated monovalent hydrocarbon radical of 1 to 6 carbon atoms or a branched saturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

The term "alkoxy" refers to an –O-alkyl group. In certain embodiments, an alkoxy group may optionally be substituted with one or more substituents Q as described herein.

The term "haloalkyl" refers to an alkyl group that is substituted with one or more halide atoms. In certain embodiments, a haloalkyl group is substituted with one, two, three, four, five or six halide atoms. In certain embodiments, a haloalkyl group may optionally be substituted with one or more additional substituents Q as described herein.

The term "cycloalkyl" refers to a cyclic saturated bridged and/or non-bridged monovalent hydrocarbon radical, which may be optionally substituted with one or more substituents Q as described herein. In certain embodiments, the cycloalkyl has from 3 to 20 (C_{3-20}), from 3 to 15 (C_{3-15}), from 3 to 12 (C_{3-12}), from 3 to 10 (C_{3-10}), or from 3 to 7 (C_{3-7}) carbon atoms. Examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, decalinyl, and adamantyl.

The term "heterocyclyl" or "heterocyclic" refers to a monocyclic non-aromatic ring system or a multicyclic ring system that contains at least one non-aromatic ring, wherein one or more of the non-aromatic ring atoms are heteroatoms independently selected from O, S, or N, and the remaining non-aromatic ring atoms are carbon atoms. In certain embodiments, the heterocyclyl or heterocyclic group has from 3 to 20, from 3 to 15, from 3 to 10, from 3 to 8, from 4 to 7, or from 5 to 6 ring atoms. In certain embodiments, the heterocyclyl is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include a fused or bridged ring system, and in which the nitrogen or sulfur atoms may be optionally oxidized, the nitrogen atoms may be optionally quaternized, and some rings may be partially or fully saturated, or aromatic. The heterocyclyl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. Examples of heterocyclic groups include, but are not limited to, acridinyl, azepinyl, benzimidazolyl, benzindolyl, benzoisoxazolyl, benzisoxazinyl, benzodioxanyl, benzodioxolyl, benzofuranonyl, benzofuranyl, benzonaphthofuranyl, benzopyranonyl, benzopyranyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, benzothiadiazolyl, benzothiazolyl, benzothiophenyl, benzotriazolyl, benzothiopyranyl, benzoxazinyl, benzoxazolyl, benzothiazolyl, β-carbolinyl, carbazolyl, chromanyl, chromonyl, cinnolinyl, coumarinyl, decahydroisoquinolinyl, dibenzofuranyl, dihydrobenzisothiazinyl, dihydrobenzisoxazinyl, dihydrofuryl, dihydropyranyl, dioxolanyl, dihydropyrazinyl, dihydropyridinyl, dihydropyrazolyl, dihydropyrimidinyl, dihydropyrrolyl, dioxolanyl, 1,4-dithianyl, furanonyl, furanyl, imidazolidinyl, imidazolinyl, imidazolyl, imidazopyridinyl, imidazothiazolyl, indazolyl, indolinyl, indolizinyl, indolyl, isobenzotetrahydrofuranyl, isobenzotetrahydrothienyl, isobenzothienyl, isochromanyl, isocoumarinyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolidinyl, isothiazolyl, isoxazolidinyl, isoxazolyl, morpholinyl, naphthyridinyl, octahydroindolyl, octahydroisoindolyl, oxadiazolyl, oxazolidinonyl, oxazolidinyl, oxazolopyridinyl, oxazolyl, oxiranyl, perimidinyl, phenanthridinyl, phenathrolinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, 4-piperidonyl, pteridinyl, purinyl, pyrazinyl, pyrazolidinyl, pyrazolyl, pyridazinyl, pyridinyl, pyridopyridinyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, pyrrolyl, quinazolinyl, quinolinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuryl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydrothienyl, tetrazolyl, thiadiazolopyrimidinyl, thiadiazolyl, thiamorpholinyl, thiazolidinyl, thiazolyl, thienyl,

triazinyl, triazolyl, and 1,3,5-trithianyl. In certain embodiments, a heterocyclic group may optionally be substituted with one or more substituents Q as described herein.

The term "aryl" refers to a monocyclic aromatic group or a multicyclic monovalent aromatic group that contain at least one aromatic hydrocarbon ring. In certain embodiments, the aryl has from 6 to 20 (C_{6-20}), from 6 to 15 (C_{6-15}), or from 6 to 10 (C_{6-10}) ring atoms. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, fluorenyl, azulenyl, anthryl, phenanthryl, pyrenyl, biphenyl, and terphenyl. Aryl also refers to bicyclic or tricyclic carbon rings, where at least one of the rings is aromatic and the others may be saturated, partially unsaturated, or aromatic, for example, dihydronaphthyl, indenyl, indanyl, and tetrahydronaphthyl (tetralinyl). In certain embodiments, an aryl group may optionally be substituted with one or more substituents Q as described herein.

The term "heteroaryl" refers to a monocyclic aromatic group or a multicyclic aromatic group that contain at least one aromatic ring, wherein at least one aromatic ring contains one or more heteroatoms independently selected from O, S, and N. Each ring of a heteroaryl group can contain one or two O atoms, one or two S atoms, and/or one to four N atoms, provided that the total number of heteroatoms in each ring is four or less and each ring contains at least one carbon atom. The heteroaryl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. In certain embodiments, the heteroaryl has from 5 to 20, from 5 to 15, or from 5 to 10 ring atoms. Examples of monocyclic heteroaryl groups include, but are not limited to, pyrrolyl, pyrazolyl, pyrazolinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, isothiazolyl, furanyl, thienyl, oxadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, and triazinyl. Examples of bicyclic heteroaryl groups include, but are not limited to, indolyl, benzothiazolyl, benzoxazolyl, benzothienyl, quinolinyl, tetrahydroisoquinolinyl, isoquinolinyl, benzimidazolyl, benzopyranyl, indolizinyl, benzofuranyl, isobenzofuranyl, chromonyl, coumarinyl, cinnolinyl, quinoxalinyl, indazolyl, purinyl, pyrrolopyridinyl, furopyridinyl, thienopyridinyl, dihydroisoindolyl, and tetrahydroquinolinyl. Examples of tricyclic heteroaryl groups include, but are not limited to, carbazolyl, benzindolyl, phenanthrollinyl, acridinyl, phenanthridinyl, and xanthenyl. In certain embodiments, a heteroaryl group may optionally be substituted with one or more substituents Q as described herein.

The term "optionally substituted" is intended to mean that a group, including alkyl, alkoxy, haloalkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, may be substituted with one or more substituents Q (in one embodiment, one, two, three or four substituents Q), where

each Q is independently selected from the group consisting of cyano, halo, oxo, nitro, C_{1-6} alkyl, C_{1-6} alkoxy, halo-C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, heteroaryl, $-C(O)R^e$, $-C(O)OR^e$, $-C(O)NR^fR^g$, $-C(NR^e)NR^fR^g$, $-OR^e$, $-OC(O)R^e$, $-OC(O)OR^e$, $-OC(O)NR^fR^g$, $-OC(=NR^e)NR^fR^g$, $-OS(O)R^e$, $-OS(O)_2R^e$, $-OS(O)NR^fR^g$, $-OS(O)_2NR^fR^g$, $-NR^fR^g$, $-NR^eC(O)R^f$, $-NR^eC(O)OR^f$, $-NR^eC(O)NR^fR^g$, $-NR^eC(=NR^h)NR^fR^g$, $-NR^eS(O)R^f$, $-NR^eS(O)_2R^f$, $-NR^eS(O)NR^fR^g$, $-NR^eS(O)_2NR^fR^g$, $-SR^e$, $-S(O)R^e$, $-S(O)_2R^e$, and $-S(O)_2NR^fR^g$, wherein each R^e, R^f, R^g, and R^h is independently hydrogen, C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, or heteroaryl; or R^f and R^g, together with the N atom to which they are attached, form heterocyclyl.

B. CNP Variants

The use of CNP22 as a therapeutic is limited by its short half-life in plasma (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). In human plasma, the concentration of CNP22 typically is less than five picomolar. CNP22 is degraded and cleared from circulation by NEP and NPR-C in humans (Growth Hormone & IGF Res., 16: S6-S14). In all human and animal studies using systemically administered CNP22, continuous infusion has been used to increase the CNP22 concentration in the subjects. A CNP peptide having a longer half-life and at least a similar level of functionality would be beneficial to a CNP-based therapeutic strategy.

The present invention provides CNP variants which have reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, but which have substantially the same or better functionality than wild-type CNP22.

Natural substrates of NEP are small and natriuretic peptides (about 2.2 to about 3.2kDa) are the largest of the natural substrates. According to X-ray crystallographic analyses, the NEP active-site is buried deep inside a central cavity, effectively restricting the size of substrate molecules to no more than about 3 kDa (Oefner et al., J. Mol. Biol., 296: 341-349 (2000)). Based on NPR-B signaling studies, variants of CNP-22, such as CNP-17 (retaining only the cyclic domain, Cys6 – Cys22, of CNP22) and CNP-53 (CNP-22 with a 31-amino acid extension at the N-terminus), can still bind and activate NPR-B similarly to the 2.2 kDa wtCNP-22. Accordingly, the invention encompasses CNP variants conjugated to a natural (e.g., peptide) and/or synthetic (e.g., PEG) polymer at the N-terminus and/or C-terminus of CNP22 or variants thereof, which exhibit increased NEP resistance but retain the ability to bind and activate the NPR-B signaling receptor.

In one embodiment, the invention encompasses CNP variants represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:139), or

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:140), wherein:

(x) and (z) each independently are a natural polymer (e.g., a peptide sequence containing at least one amino acid) and/or a synthetic polymer (e.g., PEG) as described herein, such that the total mass of the CNP variant is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the residues from Cys6 to Cys22 form a cyclic portion. In an embodiment, (x) and/or (z) comprise an amino acid extension derived from NPPC or a non-CNP polypeptide (e.g., ANP, BNP, IgG, etc.), wherein the extension contains 1 to 40, 1 to 35, 1 to 31, 5 to 35, 5 to 31 or 5 to 15 amino acids. In another embodiment, the CNP variants comprise one or more modifications and/or substitutions with another natural amino acid, an unnatural amino acid, a peptidomimetic and/or a peptide bond isostere at one or more of the following positions of CNP22: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21.

In another embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z)(SEQ ID NO: 141), or

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z)(SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG (or PEO), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

Gly-Ala-Asn-Lys-Lys(SEQ ID NO: 7),

Gly-Ala-Asn-Arg-Arg(SEQ ID NO: 8),

Gly-Ala-Asn-Pro-Arg(SEQ ID NO: 9),

Gly-Ala-Asn-Gln-Gln(SEQ ID NO: 10),

Gly-Ala-Asn-Ser-Ser(SEQ ID NO: 11),

Gly-Ala-Asn-Arg-Gln, (SEQ ID NO: 12)

Gly-Ala-Asn-Arg-Met, (SEQ ID NO: 13)

Gly-Ala-Asn-Arg-Thr(SEQ ID NO: 14),

Gly-Ala-Asn-Arg-Ser(SEQ ID NO: 15),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala(SEQ ID NO: 16),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg(SEQ ID NO: 17),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg(SEQ ID NO: 18),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys(SEQ ID NO: 19),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg(SEQ ID NO: 20),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 21), and

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 22).

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

Ser-Leu-Arg-Arg-Ser-Ser (SEQ ID NO: 23);

Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 24);

Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly(SEQ ID NO: 25);

Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 26);

Lys-Val-Leu-Arg-Arg-Tyr (SEQ ID NO: 27);

Lys-Val-Leu-Arg-Arg-His (SEQ ID NO: 28);

Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg (SEQ ID NO: 29);

Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr (SEQ ID NO: 30);

Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln (SEQ ID NO: 31); and

Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser(SEQ ID NO: 32).

In an embodiment, the N-terminal (x) group and/or the C-terminal (z) group of any of the CNP variants having an (x) and/or (z) group, as described herein, independently comprise an amino acid sequence that contains a small number of, if any, acidic natural or unnatural amino acids (e.g., Asp or Glu). In another embodiment, (x) and/or (z) are enriched in basic natural or unnatural amino acids (e.g., Lys, Arg or His) to maintain an alkaline pI similar to the pI of CNP22 (pI 8.9). In one embodiment, the pI of the CNP variants is in the range from about 8 to about 10.5, designed so that the CNP variants can diffuse more readily through the extracellular matrix surrounding chondrocytes of bone growth plates. In narrower embodiments, the pI of the CNP variants is from about 8.5 to about 10.5, or from about 8.5 to about 10, or from about 9 to about 10.

In yet another embodiment, (x) and/or (z) are enriched in polar natural or unnatural amino acids, designed for increased aqueous solubility. In still another embodiment, (x) and/or (z) contain a small number of, if any, hydrophobic natural or unnatural amino acids (e.g., Ala, Val, Leu, Ile or Met).

In a further embodiment, the N-terminus of the CNP variants terminates in at least one glycine residue, designed for increased serum half-life. In a related embodiment, to prevent pyroglutamate formation, the N-terminus of CNP variants terminates in a glycine residue if it would otherwise terminate in glutamine. In one embodiment, the (x) group contains an amino acid extension whose N-terminus terminates in at least one glycine residue. In another embodiment, (x) and/or (z) do not contain two adjacent basic natural or

unnatural amino acids (e.g., Lys-Lys or Arg-Arg), designed to reduce susceptibility to cleavage by the protease furin. In an embodiment, (x) does not contain two adjacent basic amino acids immediately preceding the position corresponding to Gly1 of CNP22.

In still another embodiment, the (x) group and/or the (z) group of the CNP variants comprise an amino acid sequence derived from NPPC (e.g., derived from CNP53). In an embodiment, (x) comprises an amino acid sequence derived from the N-terminal tail of ANP or BNP. In another embodiment, (z) comprises an amino acid sequence derived from the C-terminal tail of ANP or BNP. In a further embodiment, (x) and/or (z) comprise an amino acid sequence derived from a non-natriuretic polypeptide such as, e.g., IgG, human serum albumin (HSA), histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, and bone-targeting proteins (e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein).

In any embodiment described herein in which CNP22 or a variant thereof can have an N-terminal (x) group and/or a C-terminal (z) group, (x) and/or (z) independently can contain an amino acid sequence derived from the functional domain of a bone morphogenetic protein (BMP). An N-terminal and/or C-terminal amino acid extension derived from the functional domain of a BMP can increase the NEP resistance, and hence the serum half-life of the CNP variant, by increasing the total mass of the CNP variant to characterized by the ranges described generally herein, e.g., a range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In addition, since certain BMPs are growth factors and cytokines that induce the formation of bone and cartilage, a fragment derived from the functional domain of a BMP can promote chondrocyte, cartilage or bone growth by a mechanism distinct from activation of the guanylyl cyclase function of NPR-B by the cyclic domain of CNP22 or a variant thereof. Non-limiting examples of BMPs that promote bone formation and development, cartilage formation and development, and/or osteoblast differentiation include BMP1, BMP2, BMP3, BMP5, BMP7 and BMP8a. In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently are conjugated to an amino acid sequence derived from the last 140 amino acids in the C-terminal portion of BMP1, BMP2, BMP3, BMP5, BMP7 or BMP8a.

In one embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-53**) (SEQ ID NO: 4);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-37, Analog BL**(SEQ ID NO: 60));

AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (**Analog CA**) (SEQ ID NO: 61);

AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (**Analog CB**) (SEQ ID NO: 62);

DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (**Analog CC**) (SEQ ID NO: 63);

RGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 40);

ERGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 38);

GANQQGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 64);

GANRRGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 65);

GANPRGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 66);

GANSSGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 67);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144); and

SPKMVQGSG-CNP17-KVLRRH (**Analog CD**) (CNP17 having N-terminal and C-terminal tails derived from BNP) (SEQ ID NO: 68).

In another embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

GANRRGLSRGCFGLKLDRIGSMSGLGC (**CNP27(Arg4)**) (Analog AY) (SEQ ID NO: 36);

GANPRGLSRGCFGLKLDRIGSMSGLGC (**CNP27(Pro4)**) (Analog CI) (SEQ ID NO: 37);

RGLSRGCFGLKLDRIGSMSGLGC (**Analog AZ**) (SEQ ID NO: 41);

ERGLSRGCFGLKLDRIGSMSGLGC (**Analog BA**) (SEQ ID NO: 39);

GANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CH**) (SEQ ID NO: 69); and

GANSSGLSRGCFGLKLDRIGSMSGLGC (**Analog CG**) (SEQ ID NO: 70).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:

GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CQ**) (histidine-rich glycoprotein (HRGP) fragment-CNP22 chimera) (SEQ ID NO: 76);

GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CR**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 77);

GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CX**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 78);

GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (**Analog CF**) ($IgG_1(F_c)$ fragment-CNP22 chimera) (SEQ ID NO: 79);

GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CY**) (human serum albumin (HSA) fragment-CNP22 chimera) (SEQ ID NO: 80);

GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (**Analog CE**) (HSA fragment-CNP22 chimera) (SEQ ID NO: 81);

FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CZ**) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera) (SEQ ID NO: 82); and

GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (**Analog DA**) (FGF2 "heparin-binding domain" fragment-CNP22 chimera) (SEQ ID NO: 83).

In yet another embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22(K4R)"), including but not limited to:

GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CK**) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera) (SEQ ID NO: 84);

GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CL**) (HSA fragment-CNP22(K4R) chimera) (SEQ ID NO: 85);

GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CM**) (fibronectin fragment-CNP22(K4R) chimera) (SEQ ID NO: 86);

GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CN**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 87);

GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CO**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 88); and

GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CP**) (zinc finger fragment-CNP22(K4R) chimera) (SEQ ID NO: 89).

Chimera comprising IgG and CNP22 or a variant thereof are designed for, *inter alia*, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, *inter alia*, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, *inter alia*, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from

degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In a further embodiment, the CNP variants are derivatives of CNP37, which is QEHPNARKYKGANKK-CNP22 (SEQ ID NO: 60). The CNP37 variants can contain amino acid addition(s), deletion(s), and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)) at any one or more of the 37 positions of CNP37. Non-limiting examples of substitutions that can be made in CNP37, based on the numbering of CNP22, include K4R, G5S, G5R, G8S, K10R, G15S, S16Q, M17N, G19R, and combinations thereof. In an embodiment, the CNP37 derivatives contain a substitution of Met17 to a natural (e.g., asparagine) or unnatural amino acid or peptidomimetic, designed in part to avoid oxidation of the sulfur atom of methionine. In another embodiment, the CNP37 variants contain substitution(s) of Lys8, Lys10, Lys14 and/or Lys15 (based on numbering

from the N-terminus of CNP37) to non-basic natural or unnatural amino acid(s) or peptiomimetic(s), designed in part to reduce albumin binding.

In addition or alternatively to amino acid addition(s), deletion(s) and/or substitution(s), the CNP37 derivatives can be conjugated at the N-terminus, C-terminus, and/or an internal site to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bone-targeting peptide domains), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), amino acid sequences comprising one or more amino acids (e.g., osteocrin "NPR-C inhibitor" fragment), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids and natural fatty acids), and combinations thereof.

In one embodiment, the CNP variants are modified CNP37 peptides having mutation(s)/substitution(s) at the furin cleavage site (underlined) to improve *in vivo* resistance to the furin protease, and/or containing glycine (underlined) at the N-terminus to improve plasma stability and prevent pyroglutamate formation. Such CNP37 variants include but are not limited to:
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (**Analog CS**) (SEQ ID NO: 71); GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (**Analog CT**) (SEQ ID NO: 72); GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CU**) (SEQ ID NO: 73); GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (**Analog CW**) (SEQ ID NO: 74); and
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**Analog DB**) (SEQ ID NO: 75).

In another embodiment, the CNP variants comprise CNP22 or variants thereof conjugated at the N-terminus and/or C-terminus to moiet(ies) that facilitate translocation of the variants across a cell membrane or cell barrier. In one embodiment, the CNP variants are conjugated at the N-terminus and/or C-terminus to peptide sequence(s) that facilitate transport of the variants across a cell membrane or cell barrier, including via active peptide transporters..

In a further embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to chemical moieties such as, e.g., natural and/or synthetic polymers, to increase the total mass of the modified CNP peptide to characterized by the ranges

described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the chemical moieties are biocompatible hydrophilic or water-soluble natural (e.g., peptides, carbohydrates) or synthetic (*e.g.*, PEG (or PEO)) polymers.

In a particular embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to PEG (or PEO) polymers to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa. Pegylation of CNP22 or a variant thereof is designed, *inter alia*, to reduce immunogenicity and improve half-life by reducing renal clearance and increasing protease resistance. A PEG moiety can be attached to the N- and/or C-terminus of CNP22 or any variant described herein, including but not limited to CNP-17 (the Cys6-Cys22 cyclized portion of CNP22), CNP37, and variants of CNP17, CNP22 or CNP37 having N- and/or C-terminal amino acid extension(s), amino acid substitution(s) and/or amino acid deletion(s). In an embodiment, the Lys4 and/or Lys10 residues of CNP17, CNP22 or CNP37, or variants thereof, are substituted with a natural or unnatural amino acid (e.g., Arg, Gly, Ser, Gln, Glu or Cit) or peptidomimetic that does not contain a reactive primary amine on a side chain, to preclude any potential PEGylation of these lysine residues. In one embodiment, the Lys4 and/or Lys10 residues of the CNP peptides are substituted with Arg. In another embodiment, the Lys10 residue is not substituted with Arg.

In a further embodiment, CNP variants (including CNP22 and variants thereof) having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of such PEGylated CNP variants include PEO24-CNP27(Arg4), PEO12-CNP27(Arg4), PEO24-GANRR-CNP22, PEO12-GANRR-CNP22, PEO24-CNP27(Pro4), PEO12-CNP27(Pro4), PEO24-GANPR-CNP22, PEO12-GANPR-CNP22, PEO24-GANQQ-CNP22, PEO12-GANQQ-CNP22, PEO24-ER-CNP22(K4R), PEO12-ER-CNP22(K4R), PEO24-ER-CNP22, PEO12-ER-CNP22, PEO24-R-CNP22(K4R), PEO12-R-CNP22(K4R), PEO24-R-CNP22, and PEO12-R-CNP22, wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In one embodiment, the PEG (or PEO) polymer is conjugated to the N-terminus of the CNP variants.

The invention contemplates use of hydrophilic or water soluble polymers (e.g., PEG) that can vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP22 (2.2kDa), CNP17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, or variants having amino acid substitutions, additions and/or deletions such as, e.g., CNP27(Arg4), CNP27(Pro4), R-CNP22, R-CNP22(K4R), ER-CNP22 and ER-CNP22(K4R). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) moiety conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG moiety conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The invention also encompasses negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds (Caliceti, Adv. Drug Deliv. Rev., 55: 1261-77 (2003); Perlman, J. Clin. Endo. Metab., 88: 3227-35 (2003); Pitkin, Antimicrob. Ag. Chemo., 29: 440-444 (1986); Vehaskari, Kidney Int'l, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

In an embodiment, the invention encompasses CNP22 or variants thereof conjugated via NHS- or aldehyde-based chemistry to PEG (or PEO) of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In still another embodiment, the terminal hydroxyl group of the PEG polymer is capped with a

non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In a further embodiment, the invention provides CNP variants having one or more peptide bonds or peptide bond isosteres that have reduced susceptibility to cleavage by peptidases including neutral endopeptidase (NEP). NEP is a membrane-bound zinc-dependent endopeptidase that cleaves a substrate peptide bond at the amino end of large hydrophobic residues. Thus, modification of a peptide bond at a cleavage site for NEP to an unnatural peptide or non-peptide bond may preclude or decrease the efficiency of NEP cleavage.

For ANP and CNP, NEP cleavage is reported to occur first at the Cys6-Phe7 bond within the cyclized region, then elsewhere throughout the remainder of the structures. For BNP, cleavage is reported to occur first at the peptide N-terminus, then within the cyclic structure. Although the primary NEP cleavage site on CNP is reported to be the Cys6-Phe7 bond, when wtCNP22 was exposed to NEP digestion for 2.5 minutes *in vitro*, all possible sites were unexpectedly hydrolyzed, with the Cys6-Phe7 and Gly8-Leu9 peptide bonds being slightly most labile, as described in Example 2.

Substrate specificity of NEP is primarily determined by two substrate-binding subsites, S1' and S2' (Oefner et al., J. Mol. Biol. 296:341-349 (2000)). The S1' site accepts a large hydrophobic P1' residue of which the N-terminal peptide bond is subjected to hydrolysis (e.g., Phe, Leu, Ile and Met). The S2' site generally prefers a smaller residue, termed P2' (e.g., Gly or Ser). In the case of CNP, Phe7 is reported to be the preferred P1' residue for the NEP S1' site, while Gly8 is the preferred P2' residue for the S2' site. Because these two subsites can together accommodate only a certain total side chain size, any increase in the total size of the P1'-P2' residues of CNP can potentially disrupt NEP binding. For example, addition of a chloride atom at the 3-position of the P1' Phe7 aromatic ring (i.e., 3-Cl-Phe7) can potentially modify (e.g., destabilize) interactions between CNP and the NEP cleavage sites, for example at the S1' subsite. Addition of a tertiary butyl group to the smaller P2' residue Gly8 (i.e., tBu-Gly8) can potentially disrupt the interaction between CNP and the S2' subsite.

Accordingly, in one embodiment, CNP variants of the invention include CNP having an increase in the size of the P1'-P2' residues, such as Phe7-Gly8, to interfere with substrate recognition at the active site, thereby reducing susceptibility to NEP cleavage. Natural amino acids, unnatural amino acids and/or peptidomimetic moieties are substituted for one or more

large P1' hydrophobic residues, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, and/or for one or more smaller P2' residues, including but not limited to Cys6, Gly8, Gly15, Ser16 and Gly19.

The invention encompasses CNP variants comprising at least one modified amino acid and/or at least one modified peptide bond, at at least one residue involved in substrate recognition and/or cleavage by NEP, wherein the modified amino acids and modified peptide bonds can be natural amino acids, unnatural amino acids, peptidomimetics and/or peptide bond isosteres. In one embodiment, the NEP cleavage site on CNP between Cys6 and Phe7 is modified. In a related embodiment, the peptide bond (-C(=O)-NH-) between Cys6 and Phe7 is replaced with one of the following peptide bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, or

-NHC(=O)NH-.

In another embodiment, the CNP variants are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 90), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein (Wang et al., Adv. Drug Delivery Rev., 57: 1049-76 (2005));

polymeric and non-polymeric molecules that reduce renal clearance such as, e.g., negatively charged PEGs; and natural polymers (e.g., those containing amino acids, fatty acids and/or carbohydrates) and synthetic polymers (e.g., PEGs) that increase resistance of the CNP variant to NEP degradation by increasing the total mass of the CNP variant to the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa;

(b) and (c) may be the wild-type Cys6 and Phe7, another natural amino acid or an unnatural amino acid, or may contain a peptide bond isostere as described herein to increase resistance to NEP cleavage; and

(d) may be the wild-type Gly8, or may be a larger natural or unnatural (e.g., t-Bu-Gly) amino acid or peptidomimetic to reduce binding to NEP.

In one embodiment, such CNP variants contain at least one modified amino acid at (b), (c) and/or (d).

Other peptide bonds within CNP may be cleaved even if CNP22 or a variant thereof has an NEP-resistant peptide bond or peptide bond isostere at Cys6-Phe7, including the Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17, and Gly19-Leu20 bonds. Therefore, the invention encompasses CNP variants having peptide bond isostere(s) at one or more other NEP cleavages sites in addition to the Cys6-Phe7 bond, wherein the peptide bond isosteres include those described herein.

In another embodiment, the invention encompasses CNP variants having a cysteine analog at Cys6 and/or Cys22, including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid. In an embodiment, such CNP variants have a cyclic domain formed by a disulfide bond between the wild-type Cys6 or analog and Cys22 or analog.

In yet another embodiment, one or more residues of CNP22 or a variant thereof, up to all the residues, are substituted with a D-amino acid. Substitution of an L-amino acid with a D-amino acid essentially moves the side chain about 120 degrees from its original position, thereby potentially disrupting the binding of the CNP peptide to NEP. In a specific embodiment, L-Phe at Phe7 is substituted with its D-enantiomer, D-Phe.

In still another embodiment, a beta amino acid such as, e.g., 3-amino-2-phenylpropionic acid (or 2-phenyl-beta-alanine), replaces the wild-type alpha-amino acid Phe7. Use of a beta-amino acid effectively increases the peptide backbone length by one

methylene unit. Protease resistance can result from the change in substrate conformation or the increased distance between amino acid side chains.

Non-limiting examples of variants of CNP22 having an unnatural alpha-amino acid, a beta-amino acid or a peptide bond isostere include:

GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (**Analog A**) (SEQ ID NO: 56),
GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (**Analog B**) (SEQ ID NO: 57),
GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC (**Analog E**) (SEQ ID NO:136),
GLSKGCF-(tBu-Gly)-LKLDRIGSMSGLGC (**Analog F**) (SEQ ID NO: 58),
GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (**Analog G**) (SEQ ID NO:137), and
GLSKGC-[$NHCH_2CH(Ph)CO$]-GLKLDRIGSMSGLGC (**Analog H**, formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59).

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-Gly_{15}-Ser_{16}-(i)$_{17}$-Ser_{18}-Gly_{19}-(j)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein; polymeric and non-polymeric moieties that reduce renal clearance such as, e.g., negatively charged PEGs; polymers containing, e.g., amino acids, hydrophobic acids, and/or carbohydrates; and synthetic hydrophilic polymers such as, e.g., PEGs;

(a) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP cleavage, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-$(i)_{15}$-Ser_{16}-$(j)_{17}$-Ser_{18}-Gly_{19}-$(k)_{20}$-Gly_{21}-Cys_{22}-(z)(SEQ ID NO:143), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieities comprising, e.g., PEGs, amino acids, carbohydrates, and/or hydrophobic acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) is selected from the group consisting of Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln and Ser;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

To improve the delivery of the CNP variants to the target sites of bone-related disorders (e.g., skeletal dysplasias), the CNP variants can be attached (e.g., at the N-terminus and/or C-terminus) to bone- or cartilage-targeting moieties. Non-limiting examples of bone- or cartilage-targeting moieties include bisphosphonates; hydroxyapatite; glucosarnine; collagen (e.g., collagen type X); polyAsp; polyGlu; and amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein.

In addition to being less susceptible to NEP cleavage, the CNP variants potentially have reduced affinity to the NPR-C clearance receptor, while retaining CNP functionality. Besides NEP-mediated degradation, the half-life of CNP22 is influenced by the clearance receptor, NPR-C, which shares 58% sequence homology with the extracellular peptide-binding domain of NPR-B. CNP22 binds tightly to not only NPR-B (7-30 pM affinity), but also NPR-C (11-140 pM) (Bennett, B.D. et al., J. Biol. Chem., 266: 23060-67 (1991); Koller K.J. & Goeddel, D.V., Circulation, 86: 1081-88 (1992); Suga, S. et al., Endocrinology, 130: 229-39 (1992)). Even though the NPR-B crystal structure has yet to be reported, sequence homology as well as similarities between the NPR-C and NPR-A crystal structures (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-

31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)) suggest that NPR-B likely assumes a similar overall structural fold.

Therefore, an NPR-B homology model was built based on structure-based sequence alignment and crystallographic structures of the following related systems: CNP bound to NPR-C, ANP bound to NPR-A, and ANP bound to NPR-C (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)). Based on observations that the receptor appears to determine the bound peptide conformation, and that NPR-B most closely resembles NPR-A with respect to primary structure and functional properties, the NPR-B/CNP homology model was built with the NPR-A/ANP crystal structure as a model. Published signaling data of CNP variants (U.S. Patent No. 5,434,133 and US Patent Application Publication No. 2004/0138134 A1), and of functional ANP variants that no longer bind to NPR-C (Cunningham, EMBO 13(11) 2508-15, 1994) were used to refine and interpret the NPR-B/CNP model.

The present invention encompasses CNP variants designed for improved NPR-B selectivity based on a homology-based structural model of the NPR-B/CNP complex. Combining the experimental and computational structure data of natriuretic peptides bound to the various receptors with the published functional data, CNP variants were generated that continue to bind to NPR-B, but can potentially have reduced affinity to the NPR-C clearance receptor. For example, NPR-C has a unique insertion in a loop structure in the peptide-binding site, placing its loop residues closer to such peptide residues as CNP Gly8 (or ANP Gly9), compared to respective loop residues in NPR-A and NPR-B. Earlier studies indicated that the G9T mutation in ANP contributes to reduce affinity to NPR-C, thereby improving NPR-A selectivity (Cunningham, EMBO J., 13(11): 2508-15 (1994)). Accordingly, CNP variants were generated to replace the corresponding Gly8 residue with a larger residue (Ser, Val, Thr or Asn) to disrupt the CNP binding to NPR-C without affecting its binding to NPR-B. Further, one or more mutations were introduced at the C-terminal end of CNP, encompassing Gly15 to Gly21, which is predicted to interact with receptor-specific residues, based on the detailed structural analyses of the receptor/peptide complexes. For example, a G19R mutation in CNP22 does not result in a significant loss of NPR-B signaling activity. This mutation, however, cannot be modeled into the available crystal structure of NPR-C/CNP without altering the conformations of neighboring residues. These observations

suggest that the G19R mutation may selectively disrupt the binding of CNP to a particular receptor, such as NPR-C.

In an embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility and thereby increase receptor specificity. Comparative analyses of crystal structures of ANP bound to NPR-C and NPR-A (Ogawa, H. et al., J. Biol. Chem., 279: 28625-31 (2004); He, X.-L., J. Mol. Biol., 361: 698-714 (2006)) indicate that the conformational flexibility of ANP may play an important role in determining the receptor selectivity.

In one embodiment, functional CNP variants with potentially reduced affinity to NPR-C have one or more of the following amino acid substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R. In an embodiment, the CNP variants have multipoint substitutions at positions 1, 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and/or 21, and may optionally have modifications at any of the other positions in the peptide sequence of the variant.

In a further embodiment, the CNP variants described herein may be conjugated to moieties, up to a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, at the N-terminus, the C-terminus and/or an internal site, to facilitate bone/cartilage targeting, reduce NPR-C and renal clearance, increase resistance to NEP degradation, and/or improve CNP functionality. In one embodiment, the CNP variants are not conjugated to a polymeric moiety at a site within the cyclic region (corresponding to Cys6 to Cys22 of CNP22). Non-limiting examples of polymeric or non-polymeric moieties that can be conjugated to the CNP variants include synthetic bone-targeting compounds such as, e.g., bisphosphonates; bone/cartilage targeting peptide sequences such as, e.g., polyAsp and polyGlu; peptide sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein; peptide sequences derived from the functional domains of bone morphogenic proteins such as, e.g., BMP2, BMP3, BMP5, BMP7 and BMP8a; peptide sequences derived from polypeptides of natriuretic origin such as, e.g., NPPC, ANP and BNP; other natural polymeric or non-polymeric moieties such as, e.g., carbohydrates, fatty acids and phospholipids; biocompatible synthetic hydrophilic polymers such as, e.g., PEG (or PEO); hydrophobic polymeric or non-polymeric moieties such as, e.g., heptanoic acid and pentanoic acid; and combinations thereof.

The CNP variants described herein can have substantially similar or better functional activity than CNP22, e.g, with respect to stimulation of cGMP production and signaling. In one embodiment, the CNP variants *in vitro* stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM). In certain embodiments, the variants retain at least about 50%, 60%, 70%, 80%, 90%, 95% or 100% of the cGMP-stimulation activity of wild-type CNP22. In another embodiment, the CNP variants have improved cGMP-stimulation activity compared to CNP22.

Optionally excluded from the present invention are any of the natriuretic (e.g., CNP) peptides, fragments and variants specifically disclosed, and any of the natriuretic (e.g., CNP) peptides, fragments and variants actually produced, in any of the prior publications referenced herein, including but not limited to, U.S. 5,434,133, U.S. 6,034,231, U.S. 6,020,168, U.S. 6,743,425, U.S. 7,276,481, WO 94/20534, WO 02/047871, WO 2005/098490, WO 2004/047871, EP 0497368, EP 0466174, and Furuya et al., Biochem. Biophys. Res. Comm. 183: 964-969 (1992)). All such documents are incorporated by reference herein in their entirety.

In one embodiment, the present invention optionally excludes wild-type CNP53, wild-type CNP22, wild-type CNP17, wild-type BNP, and wild-type ANP. In another embodiment, the invention optionally excludes fragments of NPPC or CNP53 which are produced by proteolytic cleavage *in vivo*. In yet another embodiment, optionally excluded from the invention are the following truncated fragments of wild-type CNP53: CNP50, CNP46, CNP44, CNP39, CNP29 and CNP27.

In an embodiment, the present invention optionally excludes the peptides of SEQ ID NOs 1-4 and 6-71 as specifically disclosed in US 7,276,481. In another embodiment, the invention optionally excludes peptides of SEQ ID NO 5, disclosed generically in US 7,276,481, wherein such peptides are variants of CNP17 having at least one natural amino acid substitution at Leu9, Lys10, Leu11, Ser16, Met17, Gly19, and/or Leu20. In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof contain N-Me-Phe7, or N-Me-Phe7 and N-Me-Leu11. In a further embodiment, the invention optionally excludes CNP17 variants of SEQ ID NO 5, as disclosed in US 7,276,481, which are fused or conjugated to growth hormone (GH), insulin-like growth factor 1 (IGF-1), or thyroid hormone (TH). In yet another embodiment, optionally excluded are CNP22 variants in which CNP22 is fused to GH, IGF-1 or TH, or attached to GH, IGF-1 or TH via a linker (e.g., a peptide linker). In still another embodiment, optionally excluded are

CNP17 variants in which CNP17 or variants thereof are conjugated to biotin or fluorescein at the N-terminus or the C-terminus.

In a further embodiment, the present invention optionally excludes the peptides of Compound Nos. 1-27, and SEQ ID NOs 1-17, 22-24, 30, 31 and 40-42 as specifically disclosed in US 5,434,133. In another embodiment, the invention optionally excludes peptides of SEQ ID NOs 18-21 and 25-29, disclosed generically in US 5,434,133. In still another embodiment, the invention optionally excludes the peptides of SEQ ID NOs 1-4 and 9 as specifically disclosed in WO 94/20534.

In some embodiments, however, the invention still encompasses methods of use of the natriuretic (e.g., CNP) peptides, fragments and variants optionally excluded herein, as well as pharmaceutical compositions (including sterile pharmaceutical compositions) comprising such natriuretic (e.g., CNP) peptides, fragments and variants.

C. Synthesis and Purification of CNP Variants

In one embodiment, the CNP variants can be produced via recombinant expression using techniques commonly known in the art. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. (1989)); DNA Cloning: A Practical Approach, Volumes I and II, D. N. Glover, Ed. (1985); and Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Recombinant CNP variant polynucleotides and polypeptides are expressed in an expression vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or *in vitro* expression system. Expression vectors include all those known in the art, including without limitation cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide. The expression vector is inserted into an appropriate host cell for expression of the polynucleotide and polypeptide via transformation or transfection using techniques known in the art. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1989.

Host cells used to produce CNP variants as described herein can be bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells. The mammalian cells include, but

are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), chondrocytes and other bone-derived cells, and precursors of these somatic cell types. Host cells containing the CNP variant DNA or RNA are cultured under conditions appropriate for growth of the cells, expression of the DNA or RNA and identification/selection of cells expressing the CNP variant.

In another embodiment, CNP variants are synthesized and purified according to the methods of Atherton and Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

Peptides can be synthesized based on, e.g., the following peptide sequence of CNP: G^1LS(K or R)$GC^6F^7G^8L$(K or R or Nle or 6-OH-Nle)$LDRIGSMSGLGC^{22}$.

Exemplary CNP variants include but are not limited to:

Analog A (GLSKGC(CH2NH)FGLKLDRIGSMSGLGC) (SEQ ID NO: 56) was made by converting the backbone "-C=O" group of C^6 to a "-CH_2" group;

Analog B (GLSKGC(N-Me-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 57) was made by converting the backbone "-NH" group of F^7 to an "-N-CH_3" group;

Analog E (GLSKGC(D-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO:136) was made using D-Phe at F^7;

Analog F (GLSKGCF(tBu-Gly)LKLDRIGSMSGLGC) (SEQ ID NO: 58) was made using a tertiary-butyl-Gly at G^8;

Analog G (GLSKGC(3-Cl-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO:137) was made by adding a chloride atom to a meta position of the phenyl ring of F^7 (similar variants can be generated by making ortho, meta and/or para substitutions of the phenyl ring of Phe7 with Cl, F, Br, OH and/or CH_3); and

Analog H (GLSKGC[$NHCH_2CH$(Ph)CO]GLKLDRIGSMSGLGC) (SEQ ID NO: 59) was made using (±)-3-(amino)-2-phenylpropionic acid at F^7.

Examples of CNP variants having, e.g., amino acid extensions, substitutions with natural or unnatural amino acids or peptide bond isosteres, and/or conjugations to polymers or hydrophobic moieties, include without limitation:

Analog J	C6-CH2-NH, N-Me-L9, N-Me-L20 (SEQ ID NO: 91)
Analog K	N-Me-L9, N-Me-L20 (SEQ ID NO: 92)
Analog L	N-Me-L9, N-Me-L11, N-Me-L20 (SEQ ID NO: 93)
Analog M	N-Me-L9, N-Me-L11 (SEQ ID NO: 94)
Analog Z	K4R, F7Y (SEQ ID NO: 95)
Analog AA	K4R, G8V (SEQ ID NO: 96)
Analog AB	K4R, G8S (SEQ ID NO: 97)
Analog AC	K4R, G8T (SEQ ID NO: 98)
Analog AD	K4R, L9T (SEQ ID NO: 99)
Analog AE	K4R, G15R (SEQ ID NO: 100)
Analog AF	K4R, G15Cit (SEQ ID NO: 101)
Analog AG	K4R, M17V (SEQ ID NO: 102)
Analog AH	K4R (SEQ ID NO: 35)
Analog AJ	K4R, L20V (SEQ ID NO: 103)
Analog AK	K4R, L20t-Bu-Ala (SEQ ID NO: 104)
Analog AT	G1E, K4E (SEQ ID NO: 105)
Analog AV	G1E, K4E - pentanoic acid (attached at the N-terminus) (SEQ ID NO: 106)
Analog AW	G1E, K4E - heptanoic acid (attached at the N-terminus) (SEQ ID NO: 107)
Analog AX	CNP17 (delta N-term) (SEQ ID NO: 2)
Analog AY	GANRR-CNP22(K4R) (CNP27(Arg4)) (SEQ ID NO: 36)
Analog AZ	R-CNP22(K4R) (SEQ ID NO: 41)
Analog BB	G1E - heptanoic acid (attached at the N-terminus) (SEQ ID NO: 108)
Analog BC	G1E - pentanoic acid (attached at the N-terminus) (SEQ ID NO: 109)
Analog BF	K4R, K10Cit (SEQ ID NO: 110)
Analog BG	K4R, K10Q (SEQ ID NO: 111)
Analog BH	K4R, K10R (SEQ ID NO: 112)
Analog BJ	K4R, G15N (SEQ ID NO: 113)
Analog BK	K4R, G15S (SEQ ID NO: 114)
Analog BL	CNP-37 (SEQ ID NO: 60)
	CNP-53 (SEQ ID NO: 4)
Analog CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)
Analog CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)
Analog CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)
Analog CD	SPKMVQGSG-CNP17-KVLRRH (N- and C-terminal BNP tails) (SEQ ID NO: 68)
Analog CE	GERAFKAWAVARLSQ-CNP22 (HSA-CNP22) (SEQ ID NO: 81)
Analog CF	GQPREPQVYTLPPS-CNP22 (SEQ ID NO: 79)
	PEG(24K)-CNP22
	PEG(20K)-CNP22
	PEG(5K)-CNP22
	PEG(2K)-CNP22
	PEG(2K)-CNP17
	PEG(1K)-CNP27(Arg4)
	PEG(1K)-CNP22
	PEO4-(PEO12)3(branched)-CNP22
	PEO12-CNP22

PEO12-CNP27(Arg4)
PEO24-CNP27(Arg4); and

SEQ ID NOs: 1 to 6 and 34 to 144, and variants thereof that comprise up to 1, 2, 3, 4, or 5 further modifications.

In one embodiment, the CNP variants are cyclized via formation of a disulfide bond between Cys^6 and Cys^{22}. Cys^6 can be a cysteine analog such as, e.g., homocysteine or penicillamine. In a further embodiment, the CNP variants can be cyclized by a covalent bond formed head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail. In an embodiment, the covalent bond is formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus of the peptide (referred to as "terminal" amino acids in this context). In another embodiment, the covalent bond is formed between the side chains of the two terminal amino acids. In yet another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of the two terminal amino acids.

Head-to-tail cyclization of the terminal amine to the terminal carboxyl group can be carried out using a number of methods, e.g., using p-nitrophenyl ester, 2,4,5-trichlorophenyl ester, pentafluorophenyl ester, the azide method, the mixed anhydride method, HATU, a carbodimide (e.g., DIC, EDC or DCC) with a catalyst such as HOBt, HONSu or HoAt, or on-resin cyclization.

In addition, the cyclic structure can be formed via a bridging group involving the side chains of amino acid residues of the CNP variant and/or the terminal amino acid residues. A bridging group is a chemical moiety that allows cyclization of two portions of the peptide. Non-limiting examples of bridging groups include amides, thioethers, thioesters, disulfides, ureas, carbamates, sulfonamides, and the like. A variety of methods are known in the art for incorporation of units having such bridging groups. For example, a lactam bridge (i.e., a cyclic amide) can be formed between the N-terminal amino group or an amino group on a side chain and the C-terminal carboxylic acid or a carboxyl group on a side chain, e.g., the side chain of lysine or ornithine and the side chain of glutamic acid or aspartic acid. A thioester can be formed between the C-terminal carboxyl group or a carboxyl group on a side chain and the thiol group on the side chain of cysteine or a cysteine analog.

Alternatively, a cross link can be formed by incorporating a lanthionine (thio-dialanine) residue to link alanine residues that are covalently bonded together by a thioether bond. In another method, a cross-linking agent, such as a dicarboxylic acid (e.g., suberic acid

(octanedioic acid)), can link the functional groups of amino acid side chains, such as free amino, hydroxyl, and thiol groups.

Enzyme-catalyzed cyclization can also be used. For example, it has been reported that the thioesterase domain of tyrocidine synthetase can be used to cyclize a thioester precursor, a subtilisin mutant can be utilized to cyclize peptide glycolate phenylalanylamide esters, and the antibody ligase 16G3 can be employed to cyclize a p-nitrophenylester. For a review of peptide cyclization, see Davies, J. Peptide Sci., 9: 471-501 (2003), incorporated herein by reference in its entirety.

In certain embodiments, the final cyclized product has a purity of at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99%.

D. Chemically Modified CNP Variants

Chemical modification of CNP22 or variants thereof can potentially impart advantageous properties to the modified CNP peptides, such as increased stability and half-life and reduced immunogenicity (for a general discussion of chemical modification of therapeutic proteins, see Pharmazie, 57(1): 5-29 (2002)). For example, attaching natural or synthetic, polymeric or non-polymeric moieties (e.g., PEG) to CNP peptides, to increase the total mass of the CNP peptides to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa, can reduce the susceptibility of the modified peptides to *in vivo* cleavage by exopeptidases and/or endopeptidases (e.g., NEP). In addition to PEGylation, glycosylation and other chemical derivatization procedures, e.g., modification by phosphorylation, amidation, carboxylation, acetylation, methylation, and creation of acid-addition salts, amides, esters and N-acyl derivatives, may also mask potentially immunogenic regions and/or proteolytically sensitive regions (Science, 303: 480-482 (2004)).

Examples of chemical modifications include, without limitation, the polymer addition method of Bednarsaki and the cross-linking method of Altus Corporation for improving stability and protease resistance and reducing immunogenicity. Bednarsaki showed that polymer addition can improve protein temperature stability (J. Am. Chem. Soc., 114(1): 378-380 (1992)), and Altus Corporation found that glutaraldehyde cross-linking can improve enzyme stability.

Chemical modification of polypeptides can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (e.g., based on wild-type macromolecule reactivity-directed derivatization and/or site-selective modification using

a combination of site-directed mutagenesis and chemical modification) or, alternatively, using expressed protein ligation methods (Curr. Opin. Biotechnol., 13(4): 297-303 (2002)).

Pegylated CNP Variants

In one embodiment, for increased stability (e.g., resistance to NEP degradation), CNP22 or variants thereof (including those having amino acid additions, substitutions and/or deletions) are conjugated to hydrophilic, natural or synthetic polymers, to increase the total mass of the modified CNP peptides to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6, 7 or higher kDa. In certain embodiments, the added hydrophilic polymers have a total mass of about 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, or about 5 kDa.

In an embodiment, the hydrophilic polymers are water-soluble so that the CNP peptides conjugated thereto do not precipitate out in an aqueous (e.g., physiological) environment. Further, the hydrophilic polymers are biocompatible, i.e., do not cause injury, toxicity or an immunological reaction *in vivo*.

The hydrophilic polymers can be branched or unbranched. In one embodiment, the hydrophilic polymers are not branched.

Various sites of conjugation of CNP22 or variants thereof to a hydrophilic polymer are possible, including but not limited to: (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site (e.g., Lys4); (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site. In one embodiment, CNP22 or variants thereof are conjugated to a hydrophilic polymer only at the N-terminus. In another embodiment, conjugation is only at an internal site (e.g., Lys4). In yet another embodiment, conjugation is at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP peptides are not conjugated to a hydrophilic polymer at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). If conjugation to a hydrophilic polymer is based on bond formation with a reactive primary amino group on the CNP peptide, conjugation at an internal site (e.g., Lys4 and/or Lys10) can be prevented by substitution of Lys4 and/or Lys10 with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

Non-limiting examples of hydrophilic polymers include polymers formed from carboxylic acid-bearing monomers (e.g., methacrylic acid (MA) and acrylic acid (AA)), polyvinyl alcohols, polymers formed from hydroxyl-bearing monomers (e.g., hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), hydroxypropyl methacrylamide, and 3-trimethylsilylpropyl methacrylate (TMSPMA)), polyalkylene oxides, polyoxyethylated polyols (e.g., glycerol), poly(ethylene glycol) (PEG), poly(propylene glycol), mono-C_1-C_{10} alkoxy-PEGs (e.g., monomethoxy-PEG), tresyl monomethoxy-PEG, aryloxy-PEGs, PEG acrylate (PEGA), PEG methacrylate, PEG propionaldehyde, bis-succinimidyl carbonate PEG, copolymers of 2-methacryloyloxyethyl-phosphorylcholine (MPC) and N-vinyl pyrrolidone (VP), hydroxy functional poly(N-vinyl pyrrolidone) (PVP), SIS-PEG (SIS is polystyrene-polyisobutylene-polystyrene block copolymer), polystyrene-PEG, polyisobutylene-PEG, PCL-PEG (PCL is polycaprolactone), PLA-PEG (PLA is polylactic acid), PMMA-PEG (PMMA is poly(methyl methacrylate)), PDMS-PEG (PDMS is polydimethyloxanone), PVDF-PEG (PVDF is polyvinylidene fluoride), PLURONIC™ surfactants (polypropylene oxide-co-polyethylene glycol), poly(tetramethylene glycol), poly(L-lysine-g-ethylene glycol) (PLL-g-PEG), poly(L-lysine-g-hyaluronic acid) (PLL-g-HA), poly(L-lysine-g-phosphoryl choline) (PLL-g-PC), poly(L-lysine-g-vinyl pyrrolidone) (PLL-g-PVP), poly(ethylimine-g-ethylene glycol) (PEI-g-PEG), poly(ethylimine-g-hyaluronic acid) (PEI-g-HA), poly(ethylimine-g-phosphoryl choline) (PEI-g-PC), poly(ethylimine-g-vinyl pyrrolidone) (PEI-g-PVP), PLL-co-HA, PLL-co-PC, PLL-co-PVP, PEI-co-PEG, PEI-co-HA, PEI-co-PC, PEI-co-PVP, cellulose and derivatives thereof (e.g., hydroxyethyl cellulose), dextran, dextrins, hyaluronic acid and derivatives thereof (e.g., sodium hyaluronate), elastin, chitosan, acrylic sulfate, acrylic sulfonate, acrylic sulfamate, methacrylic sulfate, methacrylic sulfonate, methacrylic sulfamate, polymers and copolymers thereof, and polymers and copolymers of combinations thereof.

In a particular embodiment, the hydrophilic polymer is poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO). As used herein, the term "PEG" or "PEO" encompasses all the forms of PEG, branched and unbranched, which can be used to derivatize polypeptides, including without limitation mono-(C_1-C_{10}) alkoxy-PEGs and aryloxy-PEGs.

In one embodiment, the PEG-CNP conjugates comprise a PEG (or PEO) polymer of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet

another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In a further embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl, so that the PEG polymer terminates in an alkoxy group. In an embodiment, the PEG polymer is not branched. In another embodiment, CNP22 or variants thereof are conjugated to a PEG polymer only at the N-terminus.

PEGs and PEOs potentially include molecules with a distribution of molecular weights, i.e., they are potentially polydispersed, depending on the manner in which they are prepared. The size/mass distribution of a polymeric preparation can be characterized statistically by its weight average molecular weight (M_w) and its number average molecular weight (M_n), the ratio of which is called the polydispersity index (M_w/M_n). M_w and M_n can be measured by mass spectroscopy. PEG-CNP variants conjugated to a PEG moiety larger than 1.5 kDa may exhibit a range of molecular weights due to the polydispersed nature of the parent PEG molecule. For example, in the case of mPEG2K (Sunbright ME-020HS, NOF Co.), the molecular masses of the PEG molecules are distributed over a range from about 1.5 kDa to about 3 kDa, with a polydispersity index of 1.036. By contrast, the PEGs conjugated to CNP22 or variants thereof using $MS(PEG)_n$ reagents (n = 4, 8, 12 or 24, denoted as, e.g., "PEO12" or "PEO24") from Pierce Biotechnology (Rockford, Illinois) are monodispersed, having discrete chain length and defined molecular weight.

Methods for generating polypeptides comprising a PEG moiety are known in the art (see, e.g., US Patent 5,824,784). Methods for preparing PEGylated CNP peptides generally comprise the steps of (a) reacting CNP22 or a variant thereof with a PEGylation reagent under conditions suitable for attaching PEG to the CNP peptide (e.g., at the N-terminus), and (b) obtaining the reaction product(s). Because pegylating a CNP peptide might significantly alter its binding to NPR-B, depending on the size of the PEG moiety and the location of PEGylation, different kinds of PEG and PEGylation reaction conditions can be explored. The chemistry that can be used for PEGylation of a CNP peptide includes acylation of reactive primary amine(s) of the peptide using the NHS-ester of methoxy-PEG (O-[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates any charge of the original primary amine. PEG-CNP peptides designated with the symbol "PEO12" or

"PEO24", as well as those designated with the symbol "PEG1K", "PEG2K", "PEG5K" or "PEG20K", are PEGylated via reaction of a primary amino group on the peptide with an NHS ester-activated, methoxy-end capped PEG reagent. PEG-CNP variants can also be prepared by other methods, e.g., via reductive amination involving a primary amino group on the peptide and a PEG aldehyde, such as, e.g., PEG-propionaldehyde, or mono-C_1-C_{10} alkoxy or aryloxy derivatives thereof (see U.S. Patent 5,252,714).

Unlike ribosome protein synthesis, synthetic peptide synthesis proceeds from the C-terminus to the N-terminus. Accordingly, Boc-PEG (containing tert-butyloxycarbonyl (Boc)) is one method to attach PEG to the C-terminus of a peptide (R. B. Merrifield, J. Am. Chem. Soc., 85(14): 2149-2154 (1963)). Alternatively, Fmoc (fluorenylmethoxycarbonyl) chemistry can be employed (E. Atherton and R.C. Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

The present methods for preparing PEG-CNP variants provide a substantially homogenous mixture of polymer-protein conjugates. After purification, discrete PEG-CNP preparations are sufficiently pure for *in vitro* and *in vivo* testing of biological properties. As demonstrated herein, certain PEG-CNP variants exhibit reduced susceptibility to NEP cleavage and substantially similar or better functionality (e.g., stimulation of cGMP production).

As described herein, PEGylation reactions of CNP22 or variants thereof, using appropriate PEGylation reagent/CNP peptide ratios and reaction conditions, provide PEG-CNP derivatives. The nature and extent of PEGylation can be determined using, e.g., PAGE and HPLC analysis. In certain embodiments, at least about 50%, 60%, 70%, 80%, 90%, 95% or 99% of CNP22 or variants thereof are mono-PEGylated at the N-terminus. To optimize the beneficial effects of PEGylation on the biological properties of a CNP peptide, the polymer length, conformation (e.g., branched or linear), and/or functionalization (e.g., adding a negatively charged group) of a PEG moiety can be varied. PEGylated CNP variants are tested for NEP resistance, pharmacokinetics and bioactivity (e.g., the ability to bind to NPR-B and stimulate the generation of cGMP). PEGylated CNP variants that show improved NEP resistance and at least about 50% of the cGMP-stimulating activity of CNP22 can be further tested, e.g., *in vitro* in a rat chondrosarcoma cell-based achondroplasia model and *in vivo* in a murine achondroplasia animal model.

E. Methods of Using CNP Variants, Pharmaceutical Compositions of CNP Variants, and Routes of Administration

Methods of Using CNP Variants

Bone-Related Disorders

Fibroblast growth factors (FGFs) play important roles in bone formation, and mutations in FGF receptor genes (FGFR 1, 2 and 3) give rise to a variety of inherited skeletal malformations (Curr. Biol., 5: 500-507 (1995)). In particular, activating mutations in FGFR-3 are responsible for disorders of the long bones, including achondroplasia, the most common form of human genetic dwarfism (Nature, 371: 252-254 (1994); Cell, 78: 335-342 (1994)), the milder disorder hypochondroplasia (Ann. N.Y. Acad. Sci., 785: 182-187 (1996)), and the more severe and neonatal lethal thanatophoric dysplasia (TD) types I and II (Hum. Mol. Genet., 5: 509-512 (1996); Nat. Genet., 9: 321-328 (1995)). Mouse models overexpressing FGF-2, and consequentially activating FGFR-3, show shortened long bones and macrocephaly (Mol. Biol. Cell, 6: 1861-73 (1995)). Consistent with this model, mice deficient in FGFR-3 show remarkable skeletal overgrowth with wider growth plates (Nature Genet., 12: 390-397 (1996)).

Complementary experiments with CNP, NPR-B and NPR-C suggest a link between the peptide ligand, the corresponding receptors, and bone growth. Activation of NPR-B by elevated plasma concentrations of CNP in transgenic mice causes skeletal overgrowth (Nat. Med., 10: 80-86 (2004)) histologically similar to that of the growth plate cartilage of FGFR-3 knockout mice (Nat. Genet., 4: 390-397 (1996)). In NPR-C knockout mice, NPR-C-mediated clearance of CNP should be eliminated; consistent with this prediction, the knockout animals show elongated long bones and elongated vertebrae with kyphosis (Proc. Natl. Acad. Sci. USA 96: 7403-08 (1999)). Conversely, CNP knockout mice are dwarfed with shorter long bones and vertebrae, a phenotype histologically similar to that of achondroplasia, and have increased mortality as a result of malocclusion and pulmonary restriction from the small rib cage (Proc. Natl. Acad. Sci. USA, 98: 4016-4021 (2001)). Consistent with the proposed role of CNP as an activator of NPR-B, the NPR-B knockout mouse has the same dwarfed skeletal phenotype and increased mortality as the CNP knockout mouse (Proc. Natl. Acad. Sci USA, 101: 17300-05 (2004)). Furthermore, in a mouse model of achondroplasia with activated FGFR-3 in the cartilage, targeted overexpression of CNP in chondrocytes counteracts dwarfism (Yasoda et al., Nat. Med., 10: 80-86 (2004)). Additionally, CNP has been show to play a role in regulating endochondral bone growth and chondrocyte activity, including but

not limited to chondrocyte proliferation and differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promotion of endochondral ossification (Yasoda et al., Nat. Med., 10: 80-86 (2004)). These results suggest that activation of the CNP/NPR-B system is a potential therapeutic strategy for treatment of human achondroplasia.

By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the invention are potentially useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia.

The CNP variants can also potentially be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP variants can potentially be useful in treating osteoarthritis.

Vascular Smooth Muscle Disorders

CNP and other vasoactive peptides (including ANP, BNP and urodilatin) have vasodilator and diuretic properties and play an important role in cardiovascular homeostasis

(J. Cardiovasc. Pharmacol., 117: 1600-06 (1998); Kidney Int., 49: 1732-37 (1996); Am. J. Physiol., 275: H1826-1833 (1998)). CNP is widely distributed in the cardiovascular system, especially in high concentration in vascular endothelial cells (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998)). CNP is a potent relaxant of vascular smooth muscle, particularly in the coronary circulation (Biochem. Biophys. Res. Commun., 205: 765-771 (1994)), and is an inhibitor of smooth muscle cell proliferation (Biochem. Biophys. Res. Commun., 177: 927-931 (1991)). Although the vasodilator effect of CNP is less potent than that of ANP (about 1:100) (Hypertens. Res., 21: 7–13 (1998); Am. J. Physiol., 275: L645–L652 (1998)), CNP mRNA is increased in response to shear stress (FEBS Lett., 373: 108-110 (1995)) and plasma levels of CNP are elevated in inflammatory cardiovascular pathologies (Biochem. Biophys. Res. Commun., 198: 1177-1182 (1994)). CNP has been shown to suppress inflammation through inhibition of macrophage infiltration in injured carotid arteries of rabbits (Circ. Res., 91: 1063–1069 (2002)) and to directly inhibit cardiac fibroblast proliferation through an NPR-B/cGMP-dependent pathway (Endocrinology, 144: 2279–2284 (2003)).

The cardiovascular actions of CNP are mediated via activation of the NPR subtypes, NPR-B and NPR-C (Endocrinology, 130: 229-239 (1992)), the latter accounting for 95% of NPRs expressed *in vivo* (Science, 293: 1657-1662 (2001)). The CNP/NPR-B pathway leads to elevation of cGMP, a well-established secondary messenger in the cardiovascular system. NPR-C's 37-amino acid portion from the C-terminus has a consensus sequence that interacts with the heterotrimeric G protein G_i (J. Biol. Chem., 274: 17587-17592 (1999)), which has been shown to regulate adenylate cyclase and phospholipase C activity (J. Biol. Chem., 276: 22064-70 (2001); Am. J. Physiol., 278: G974-980 (2000); J. Biol. Chem., 271: 19324-19329 (1996)). CNP mediates smooth muscle hyperpolarization and relaxation via activation of NPR-C and the opening of a G protein-regulated, inwardly rectifying K^+ channels (Proc. Natl. Acad. Sci. USA, 100: 1426-1431 (2003)). Likewise, CNP has important anti-proliferative effects in cardiac fibroblasts and, through interaction with NPR-C, regulates local blood flow and systemic blood pressure by hyperpolarizing smooth muscle cells (R. Rose and W. Giles, J. Physiol. 586: 353-366 (2008)).

By binding to NPR-B on vascular smooth muscle cells, CNP22 stimulates the production of cGMP, which acts as an intracellular secondary messenger to cause ultimately the relaxation of blood vessels. Based on the hypotensive actions of CNP, the CNP variants of the invention are potentially useful for treating hypertension, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute and chronic renal insufficiency, and so on.

In addition, activation of cGMP signaling suppresses the growth of vascular smooth muscle cells. Accordingly, the CNP variants of the invention can potentially be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis.

The studies described above suggest that CNP may be a potential therapeutic candidate for vascular smooth muscle relaxation and remodeling. Pharmacological effects of CNP concerning certain disorders have been attributed, in part, to vasoprotective effects rather than to vasodilator activity (Am. J. Respir. Crit. Care Med., 170: 1204-1211 (2004)). Therefore, the CNP variants of the present invention can potentially be used to treat conditions, e.g., vascular smooth muscle disorders, in which CNP may have a vasoprotective effect, including without limitation inducing smooth muscle relaxation and inhibiting infiltration of macrophages into cardiac tissue. In one embodiment, the CNP variants are used to treat heart failure, including but not limited to acute decompensated heart failure and acute congestive heart failure.

Pharmaceutical Compositions of CNP Variants

A further embodiment of the present invention is directed to pharmaceutical compositions comprising an effective amount of a CNP variant, optionally another biologically active agent, and one or more pharmaceutically acceptable excipients, diluents, stabilizers, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions can include diluents of various buffer content (*e.g.*, Tris-HCl, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Polysorbate 20, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimerosol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The use of excipients, diluents, carriers and so on in the formulation of pharmaceutical compositions is known in the art; see, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)), which is incorporated herein by reference in its entirety. An effective amount of an active ingredient is a therapeutically, prophylactically, or diagnostically effective amount, which can readily be determined by a person skilled in the art by taking into consideration such factors as body weight, age, and therapeutic goal.

The compositions comprising a CNP variant can also include a buffering agent to maintain the pH of a solution within a desired range. Exemplary agents include sodium

acetate, sodium phosphate, and sodium citrate. Mixtures of these buffering agents can also be used. The amount of buffering agent useful in the composition depends largely on the particular buffer used and the desired pH of the solution. For example, acetate is a more efficient buffer at pH 5 than pH 6, so less acetate may be used in a solution at pH 5 than at pH 6. In one embodiment, the pH range for the compositions of the present invention is from about pH 3 to about pH 7.5.

The compositions of the present invention can further include an isotonicity-adjusting agent to render the solution isotonic and more compatible for injection. In an embodiment, the agent is sodium chloride within a concentration range of 0-150 mM.

The therapeutic methods described herein use pharmaceutical compositions comprising a CNP variant and one or more pharmaceutically acceptable excipients, diluents, carriers and/or vehicles, and optionally other therapeutic and/or prophylactic ingredients. Non-limiting examples of excipients include liquids such as water, saline, glycerol, polyethylene glycol, hyaluronic acid, ethanol, cyclodextrins, modified cyclodextrins (*i.e.*, sufobutyl ether cyclodextrins), *etc.* Suitable excipients for non-liquid formulations are also known to those of skill in the art.

Pharmaceutically acceptable salts can be used in the compositions of the present invention and include, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients and salts is available in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, (Easton, Pennsylvania (1990)).

Auxiliary substances, such as wetting or emulsifying agents, biological buffering substances, surfactants, and the like, may also be present in vehicles. A biological buffer can be virtually any solution which is pharmacologically acceptable and which provides the formulation with the desired pH, *i.e.*, a pH in the physiologically acceptable range. Examples of buffer solutions include saline, phosphate buffered saline, Tris buffered saline, Hank's buffered saline, and the like.

The pharmaceutical compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the composition can take the form of, e.g., a tablet, capsule, or softgel capsule, or can be an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or

more commonly used carriers such as lactose and corn starch. Lubricating agents, such as magnesium stearate or sodium stearyl fumarate, can also be added. When liquid suspensions are used, the active agent can be mixed with emulsifying and suspending agents. If desired, flavoring, coloring and/or sweetening agents can also be added to solid and liquid formulations. Other optional components for incorporation into oral formulations include, but are not limited to, preservatives, suspending agents, thickening agents, and the like.

For solid compositions, conventional nontoxic solid carriers include, e.g., pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can be prepared, e.g., by dissolving, dispersing, suspending, *etc.*, an active agent and optional pharmaceutical adjuvants in an excipient, such as, e.g., water, saline, aqueous dextrose, glycerol, ethanol, and the like, to form a solution or suspension. If desired, the pharmaceutical compositions can also contain minor amounts of nontoxic auxiliary substances such as, e.g., wetting agents, emulsifying agents, pH buffering agents, tonicifying agents, and the like, e.g., sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, *etc.* Methods of preparing solid and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, e.g., Remington's Pharmaceutical Sciences, referenced above).

Parenteral formulations can be prepared in conventional forms, e.g., as liquid solutions or suspensions, solid forms suitable for solubilization or suspension in liquid prior to injection, or as emulsions. For example, sterile injectable suspensions can be formulated according to techniques known in the art using suitable carriers, dispersing agents, wetting agents, suspending agents, and the like. The sterile injectable formulations can also be a sterile injectable solution or a suspension in a nontoxic, parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils, fatty esters and/or polyols can be employed as solvents or suspending media.

The invention also provides containers, e.g., vials, tubes, bottles, and the like, comprising the sterile injectable compositions. Kits comprising such compositions may also include instructions for use, and optionally dispensing devices, such as aerosol dispensing device, syringe, and/or needle. In some embodiments, the composition is prepackaged within a syringe.

The pharmaceutical compositions can be formulated as a slow release, controlled release or sustained release system for maintaining a relatively constant level of dosage over a desired time period such as, e.g., 1 week, 2 weeks or 1 month. Slow release, controlled release and sustained release compositions can be prepared using, e.g., biodegradable polymeric systems, and can take the form of, e.g., microparticles, as is known in the art.

Dosages

As used herein, the term "therapeutically effective amount" of an active agent (e.g., a CNP variant) refers to an amount that provides therapeutic benefit to a patient. The amount may vary from one individual to another and may depend upon a number of factors, including the overall physical condition of the patient. A therapeutically effective amount of a CNP variant can be readily ascertained by one skilled in the art, using publicly available materials and procedures. For example, the amount of a CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (214 females and 189 males), which list height for age, head circumference, and segmental growth (Horton WA et al., Standard growth curves for achondroplasia, J. Pediatr., 93: 435-8 (1978)). CDC charts can be used to assess weight for age and weight for height or BMI for age. Secondary outcomes with courses that are more chronic in nature can also be measured.

Having a longer serum half-life than wild-type CNP22, the CNP variants can potentially be administered less frequently than CNP22. The dosing frequency for a particular individual may vary depending upon various factors, including the disorder being treated and the condition and response of the individual to the therapy. In certain embodiments, a pharmaceutical composition containing a CNP variant is administered to a subject about one time per day, one time per two days, one time per three days, or one time per week. In one embodiment, for treatment of bone-related disorders (e.g., skeletal dysplasias, including achondroplasia), a daily or weekly dose of a CNP variant is administered to patients until and/or through adulthood.

The CNP variants described herein can be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) and conditions (e.g., vascular smooth muscle disorders) in which CNP can provide a vasoprotective effect. The safety and therapeutic efficacy of the CNP variants can be determined by standard pharmacological procedures in cell cultures or

experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

Data obtained from cell culture assays and animal studies can be used to formulate a range of dosage for use in humans. The dosage normally lies within a range of circulating concentrations that include the ED_{50}, with little or minimal toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The therapeutically effective dose can be determined from cell culture assays and animal studies.

Modes of Administration

The CNP variants, or pharmaceutical compositions comprising them, can be administered to subjects in various ways such as, e.g., by injection subcutaneously, intravenously, intra-arterially, intraperitoneally, intramuscularly, intradermally, or intrathecally. In an embodiment, the CNP variants are administered by a single subcutaneous, intravenous, intra-arterial, intraperitoneal, intramuscular, intradermal or intrathecal injection once a day. The CNP variants can also be administered by direct injection at or near the site of disease. Further, the CNP variants can be administered by implantation of a depot at the target site of action (e.g., an abnormal or dysplasic bone). Alternatively, the CNP variants can be administered under the tongue (e.g., sublingual tablet) or by inhalation into the lungs (e.g., inhaler or aerosol spray), by delivery into the nasal cavity (e.g., intranasal spray), by delivery into the eye (e.g., eye drop), or by transdermal delivery (e.g., by means of a patch on the skin). The CNP variants may also be administered orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides), microemulsions, and the like. It would be clear to one skilled in the art that the compositions can also be administered by other modes, and determination of the most effective mode of administration of the compositions is within the skill of the skilled artisan.

Depending on the intended mode of administration, the pharmaceutical compositions can be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, creams, ointments, lotions or the

like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions include an effective amount of a CNP variant and one or more pharmaceutically acceptable excipients, carriers or diluents (e.g., buffers), and may optionally include other ingredients such as, e.g., adjuvants. In addition to a CNP variant, the composition also can optionally contain another active agent, which may enhance the effects of the CNP variant or may exert other pharmacological effects in addition to those of the CNP variant.

In general, the CNP variants can be administered as pharmaceutical formulations including those suitable for oral (including buccal and sub-lingual), rectal, nasal, topical, pulmonary, vaginal or parenteral (including intramuscular, intraarterial, intrathecal, subcutaneous and intravenous) administration, or in a form suitable for administration by inhalation or insufflation. One manner of administration is subcutaneous using a convenient daily or weekly dosage regimen, which can be adjusted according to the degree of affliction. A further method of administration is by osmotic pump or mini-pump, which allows for controlled, continuous delivery of the pharmaceutical composition over a pre-determined period.

A further method of administration is by osmotic pump (e.g., an Alzet pump) or mini-pump, which allows for controlled, continuous delivery of the pharmaceutical composition over a pre-determined period. The osmotic pump or mini-pump can be implanted subcutaneously, or near the target site (e.g., the long bones of limbs, the epiphyses, etc.).

As explained above, the CNP variants can potentially be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis. For local delivery of a CNP variant to the diseased bodily vessel (e.g., blood vessel), the CNP variant can be delivered by means of a medical device (e.g., a stent) implanted at the diseased site. In one embodiment, the CNP variant is impregnated in a polymeric matrix or polymeric coating disposed over a stent. In another embodiment, the CNP variant is contained in reservoirs or channels formed in the body of a stent and covered by a porous polymeric membrane or layer through which the CNP variant can diffuse. The polymeric matrix, coating, membrane or layer can comprise at least one biodegradable (e.g., hydrophilic) polymer, as is known in the art. In a further embodiment, the CNP variant can be contained in micropores in the body of a stent. The CNP variant can be delivered from a stent by burst release, pulse release, controlled release or sustained release, or a combination thereof. For example, the stent can locally deliver the CNP variant

to the diseased site in a burst release followed by a sustained release. Sustained release can be over a period up to about 2 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

Combination Therapy

In one embodiment, a CNP variant can be used in combination with one or more other active agents useful for treating, ameliorating or preventing CNP-responsive conditions or disorders such as, e.g., bone-related disorders (e.g., skeletal dysplasias) and vascular smooth muscle disorders. The other active agent(s) can enhance the effects of the CNP variant or exert other pharmacological effects in addition to those of the CNP variant. Non-limiting examples of active agents that can be used in combination with the CNP variants described herein are other natriuretic peptides (e.g., BNP) and inhibitors (e.g., antagonists) of peptidases (e.g., NEP and furin), NPR-C and tyrosine kinases (e.g., FGFR-3). By preventing NEP cleavage of the CNP variant, an NEP inhibitor can prolong the half-life of the variant. Examples of NEP inhibitors include, without limitation, thiorphan and candoxatril. Co-use of an NPR-C inhibitor can also prolong the half-life of the CNP variant via inhibition of the variant's clearance by NPR-C. A non-limiting example of an NPR-C inhibitor is the fragment FGIPMDRIGRNPR (SEQ ID NO: 82), which would be released at the target site (e.g., bone growth plate) upon proteolytic cleavage of the FGIPMDRIGRNPR-CNP22 chimera (Analog CZ) (SEQ ID NO: 82) or similar chimeras comprising variants of CNP22 (e.g., those containing amino acid substitution(s), addition(s), and/or deletion(s) relative to CNP22). Co-use of a tyrosine kinase inhibitor can accentuate the effects of a CNP therapy by inhibiting the tyrosine kinase receptor FGFR-3, a negative regulator of chondrocyte and bone growth. Non-limiting examples of tyrosine kinase inhibitors include those disclosed in U.S. 6,329,375 and 6,344,459.

To achieve the appropriate therapeutic outcome in the combination therapies, one would generally administer to the subject the CNP composition and other therapeutic(s) in a combined amount effective to produce the desired therapeutic outcome (*e.g.*, restored bone growth). This process may involve administering the CNP composition and other therapeutic agent(s) at the same time. Simultaneous administration can be achieved by administering a single composition or pharmacological protein formulation that includes both the CNP variant and other therapeutic agent(s). Alternatively, the other therapeutic agent(s) can be taken separately at about the same time as a pharmacological formulation (e.g., tablet,

injection or drink) of the CNP variant. The CNP variant can also be formulated into a foodstuff such as brownies, pancakes, or cake, suitable for ingestion.

In other alternatives, administration of the CNP variant can precede or follow administration of the other therapeutic agent(s) by intervals ranging from minutes to hours. In embodiments where the other therapeutic agent(s) and the CNP composition are administered separately, one would generally ensure that the CNP variant and the other therapeutic agent(s) are administered within an appropriate time of one another so that both the CNP variant and the other therapeutic agent(s) can exert, synergistically or additively, a beneficial effect on the patient. For example, one can administer the CNP composition within about 0.5-6 hours (before or after) of the other therapeutic agent(s). In one embodiment, the CNP composition is administered within about 1 hour (before or after) of the other therapeutic agent(s).

Identifying and Monitoring Patient Populations

Protocols can be established to identify subjects suitable for CNP therapy and to determine whether a given patient is responsive to CNP therapy. For example, for treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements *in utero* and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

Additional aspects and details of the invention will be apparent from the following examples, which are intended to be illustrative rather than limiting.

F. Examples

Example 1
Synthesis of CNP Variants

CNP variants were prepared using the methods described herein. Substitutions with natural or unnatural amino acids or peptidomimetics were made, as indicated in **Tables 1-3** (shown in Example 2), at the respective amino acid residues in the wild-type sequence of CNP22. In certain variants, additional amino acids were added to the N-terminal and/or C-terminal ends of the whole or a portion of the wild-type CNP22 sequence (see **Table 3**).

Also prepared were CNP variants in which a PEG (or PEO) moiety was conjugated to the N-terminus of CNP22 or variants thereof (see **Table 4**, shown in Example 2). PEGylation reagents can be obtained from the commercial sources shown in Table 5.

Table 5

Vendor	Product Name	Name	MW (Da)	PEGylation Reagent
NOF	Sunbright ME-200CS	mPEG20K	20,000	CH3(CH2CH2O)450-(CH2)5COO-NHS
NOF	Sunbright ME-050CS	mPEG5K	5,000	CH3(CH2CH2O)110-(CH2)5COO-NHS
Pierce	(Methyl-PEG12)3-PEG4-NHS Ester	(mPEG12)3-PEG4	2,400	[CH3(CH2CH2O)12]3-(CH2CH2O)4-NHCO(CH2)3-COO-NHS
NOF	Sunbright ME-020HS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO-NHS
NOF	Sunbright ME-020CS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO-NHS
NOF	Sunbright ME-010HS	mPEG1K	1,000	CH3(CH2CH2O)23-CO(CH2)2COO-NHS
Pierce	Methyl PEG24-NHS Ester	MS(PEG)24	1,200	CH3(CH2CH2O)24-(CH2)2COO-NHS
Pierce	EZ-Link NHS-PEG12-Biotin	PEO12-Biotin	940	Biotin-(CH2CH2O)12-(CH2)2COO-NHS
Pierce	Methyl PEG12-NHS Ester	MS(PEG)12	690	CH3(CH2CH2O)12-(CH2)2COO-NHS
Pierce	EZ-Link NHS-PEG4-Biotin	PEO4-biotin	590	Biotin-(CH2CH2O)4-(CH2)2COO-NHS
Pierce	Mono(lactosylamido) mono(succinimidyl)suberate	LSS	590	
Pierce	EZ-link NHS-LC-LC-Biotin	LC-LC-Biotin	570	
Pierce	EZ-link NHS-LC-Biotin	LC-Biotin (LC = long chain)	450	
Pierce	EZ-link NHS-Biotin	Biotin	340	

The PEG (also called PEO) polymers purchased from Pierce Biotechnology (Rockford, Illinois) are monodispersed – i.e., they contain a single discrete polymer of a particular molecular weight. By contrast, the PEG polymers purchased from NOF (Nippon Oil and Fat) are polydispersed – i.e., they contain a mixture of polymers having a distribution of molecular weights.

To PEGylate CNP22 or variants thereof, reaction and purification conditions are optimized for each PEG-CNP conjugate. According to a general PEGylation procedure,

reaction mixtures contain about 1 mM CNP22 or a variant thereof, and about 1 to 5 mM NHS-activated PEG in potassium phosphate buffer, pH between about 5.0 and 6.5. To mono-PEGylate selectively at the peptide N-terminus and minimize PEGylation at an internal site (e.g., Lys4 of CNP22), the PEGylation reaction can be conducted under more acidic conditions (e.g., at a pH between about 5.5 and 6.5) to protonate selectively and hence deactivate the more basic primary amino group on the lysine side chain. After about 1 to 3 hours of incubation at room temperature, the PEGylation reaction is quenched by addition of aqueous glycine buffer. Reaction products are then separated by reverse-phase HPLC, optimized for each PEG-CNP conjugate. Fractionation samples are speedvacced to dryness, and reconstituted/formulated in 1 mM HCl. Identification and purity of each PEG-CNP product are determined by liquid chromatography-mass spectrometry (LC/MS).

Example 2

Cleavage of CNP Variants by Neutral Endopeptidase *In Vitro*

To determine the effects of amino acid substitutions, amino acid extensions, backbone modifications, side chain modifications and PEGylation on the susceptibility of CNP variants to neutral endopeptidase (NEP) cleavage, peptide cleavage assays were carried out using an *in vitro* assay that monitored disappearance of the non-cleaved CNP variant.

Recombinant human NEP (1 ug/mL final concentration) was added to 100 uM CNP variant diluted in 0.1 M Tris, pH 7. The reaction mixture was incubated at 37 °C for various periods of time, and the reaction was quenched with EDTA (10 mM final) followed by heat denaturation. The reaction mixture was reduced and then the reaction products were analyzed using HPLC and mass spectroscopy. The half-life of the CNP variant was calculated based on the disappearance of intact CNP variant over time. The results for digested CNP variants were compared to a parallel wtCNP22 digestion and normalized to the results for 100 uM CNP22 digested by 1 mg/mL NEP ($t_{1/2}$ = 80 min).

Table 1 lists the half-lives, based on the *in vitro* NEP cleavage assay, of various CNP variants having backbone or side chain modifications. Removal of three of the six NEP cleavage sites in Analog L nevertheless resulted in a significantly shorter half-life. The greatest resistance to NEP cleavage was exhibited by Analog N, which contains the D-enantiomer of all 22 amino acids of CNP22, and by Analog M, which has an N-methylated amide bond at both Leu9 and Leu11. However, both Analogs N and M failed to stimulate production of cGMP (see below).

Concerning Analogs A, B, E, F, G and H, half-lives were determined to be about 1.5- to about 2.5-fold longer for analog E and G. All of these six analogs showed resistance or improved resistance to cleavage at the Cys6-Phe7 bond relative to wtCNP22 (data not shown). The rank order of analog resistance to NEP at 1ug/ml, based on half-life, is Analog G (3-Cl-Phe) ≥ Analog E (D-Phe) > Analog H ("beta-2 Phe"), Analog B (N-Me-Phe), and Analog F (t-Bu-Gly) = wtCNP22 > Analog A (Cys-CH_2-NH). Analogs E and G have about 1.5 times longer half-life in comparison to wtCNP22. Besides resistance to cleavage of the Cys6-Phe7 bond, Analogs B, E, F, G and H also exhibited resistance to cleavage of the Gly8-Leu9 bond in the presence of 1 ug/mL NEP (data not shown). These results indicate that CNP variants having backbone or side chain modifications between Cys6 and Gly8 can be resistant to NEP cleavage of the Cys6-Phe7 bond and/or Gly8-Leu9 bond, but do not necessarily have improved overall resistance to NEP or a longer half-life than CNP22. The results seem to be contrary to reports in the literature that NEP first cleaves at the Cys6-Phe7 bond of CNP22 and then elsewhere.

Table 1

Analog	Backbone and Side Chain Modifications	cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10 nM	1 uM	($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46±10	100±13	80[2]
N	D-CNP22 (all D-amino acids) (SEQ ID NO: 115)	2	1	>>160
A	CNP22, C6-CH2-NH (reduced carbonyl) (SEQ ID NO: 56)	6	66	55
B	CNP22, N-methyl-F7 (methylated amide) (SEQ ID NO: 57)	2	38	80
BD	CNP22, N-methyl-L9 (SEQ ID NO: 116)	2	8	ND
BN	CNP22, N-methyl-L11 (SEQ ID NO: 117)	10	51	ND
BE	CNP22, N-methyl-L20 (SEQ ID NO: 118)	2	5	ND
M	CNP22, N-methyl-L9, N-methyl-L11 (SEQ ID NO: 94)	1	11	>>160
K	CNP22, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 92)	1	1	80
L	CNP22, N-methyl-L9, N-methyl-L11, N-methyl-L20 (SEQ ID NO: 93)	18	10	30
J	CNP22, C6-CH2-NH, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 91)	ND	ND	50
E	CNP22, D-F7 (D-Phe) (SEQ ID NO: 136)	2	6	130
H	CNP22, Beta-2-F7 (3-amino-2-phenylpropionyl) (SEQ ID NO: 57)	2	2	80

G	CNP22, 3-chloro-F7 (SEQ ID NO: 137)	17	93	135
F	CNP22, t-butyl-G8 (SEQ ID NO: 58)	2	18	80
V	CNP22, K4G, 3,4-dichloro-F7 (SEQ ID NO: 119)	ND	ND	68
X	CNP22, K4G, 3-methyl-F7 (SEQ ID NO: 120)	ND	ND	68
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 2 lists the half-lives, based on the *in vitro* NEP cleavage assay, of various CNP variants having substitutions with natural and/or unnatural amino acids. The greatest resistance to NEP cleavage was shown by Analog BK, which has K4R and G15S substitutions, and Analog BJ, which has K4R and G15N substitutions.

Table 2

Analog	Specificity Mutations	cGMP Response relative to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10nM	1uM	($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46±10	100±13	80
AH	CNP22, K4R (SEQ ID NO: 35)	59	121	80
BP	CNP22, K4R, G5S (SEQ ID NO: 121)	45	ND	ND
BO	CNP22, K4R, G5R (SEQ ID NO: 122)	18	80	ND
P	CNP22, K4G (SEQ ID NO: 123)	ND	ND	68
Z	CNP22, K4R, F7Y (SEQ ID NO: 95)	2	18	ND
AB	CNP22, K4R, G8S (SEQ ID NO: 97)	26±26	86±17	ND
AA	CNP22, K4R, G8V (SEQ ID NO: 96)	3	25	ND
AC	CNP22, K4R, G8T (SEQ ID NO: 98)	11±2	66±16	80
AD	CNP22, K4R, L9T (SEQ ID NO: 99)	4	68	ND
BH	CNP22, K4R, K10R (SEQ ID NO: 112)	12	80	ND
BF	CNP22, K4R, K10Cit (SEQ ID NO: 110)	6	33	ND
BG	CNP22, K4R, K10Q (SEQ ID NO: 111)	9	45	ND
BY	CNP22, K4R, K10S (SEQ ID NO: 124)	16	53	ND
BK	CNP22, K4R, G15S (SEQ ID NO: 114)	13±1	71±11	≥160
BJ	CNP22, K4R, G15N (SEQ ID NO: 113)	4	41	150
AE	CNP22, K4R, G15R (SEQ ID NO: 100)	0.3	0.3	ND

AF	CNP22, K4R, G15Cit (SEQ ID NO: 101)	1.4	2	ND
BZ	CNP22, K4R, S16Q (SEQ ID NO: 125)	42	116	ND
BX	CNP22, K4R, M17N (SEQ ID NO: 126)	40±2	103±17	ND
AG	CNP22, K4R, M17V (SEQ ID NO: 102)	10	65	ND
BQ	CNP22, K4R, G19S (SEQ ID NO: 127)	21	63	ND
BR	CNP22, K4R, G19R (SEQ ID NO: 128)	22±6	84±10	ND
AJ	CNP22, K4R, L20V (SEQ ID NO: 103)	0.2	8	ND
AK	CNP22, K4R, L20t-butyl-Ala (SEQ ID NO: 104)	1	21	ND
AT	CNP22, G1E, K4E (SEQ ID NO: 105)	11	54	60
BS	CNP22, K4R, L20R (SEQ ID NO: 129)	11	8	ND
BT	CNP22, K4R, G21S (SEQ ID NO: 130)	7	39	ND
BU	CNP22, K4R, G21T (SEQ ID NO: 131)	6	21	ND
BW	CNP22, K4R, G21R (SEQ ID NO: 132)	20	21	ND
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 3 lists the half-lives, based on the *in vitro* NEP cleavage assay, of CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions. Analogs AZ, CC, CF, BL, CS, CK and CL were most resistant to NEP degradation.

Table 3

	N- and C-Terminal Modifications	**cGMP Response rel. to 1 uM CNP22[1]**		**NEP Cleavage**
	Natriuretic Peptide	**10nM**	**1uM**	**($t_{1/2}$, min)**
	CNP22	46±10	100±13	80
BC	Pentanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 109)	ND	ND	ND
BB	Heptanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 108)	32±4	84±19	45-65
AV	Pentanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 106)	ND	ND	120
AW	Heptanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 107)	ND	ND	<20
AX	CNP17 (delta N-term) (SEQ ID NO: 2)	18	69	ND
AZ	R-CNP22, K4R (SEQ ID NO: 41)	54±11	106±15	≥160

BA	ER-CNP22, K4R (SEQ ID NO: 39)	38±10	113±10	90
AY	GANRR-CNP22, K4R (CNP-27) (SEQ ID NO: 36)	59±9	105±20	65
CH	GANQQ-CNP22, K4R (SEQ ID NO: 69)	44±8	95±11	ND
CI	GANPR-CNP22, K4R (SEQ ID NO: 37)	50±1	105±12	ND
CG	GANSS-CNP22, K4R (SEQ ID NO: 70)	27±1	88±1	95
CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)	24	76	ND
CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)	36	84	ND
CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)	34	101	>160
CF	GQPREPQVYTLPPS-CNP22 (IgG1(Fc) fragment) (SEQ ID NO: 79)	23±9	72±19	>160
BL	QEHPNARKYKGANKK-CNP22 (CNP37) (SEQ ID NO: 60)	43±15	97±27	>>160
CE	GERAFKAWAVARLSQ-CNP22 (HSA fragment) (SEQ ID NO: 81)	15	87	ND
CY	GQHKDDNPNLPRGANPR-CNP22 (HSA fragment) (SEQ ID NO: 80)	ND	ND	ND
CQ	GHHSHEQHPHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 76)	16	95	ND
CX	GHHSHEQHPHGANPR-CNP22 (HRGP fragment) (SEQ ID NO: 78)	ND	ND	ND
CS	GQEHPNARKYKGANPK-CNP22 (modified CNP37) (SEQ ID NO: 129)	19	61	>>160
CT	GQEHPNARKYKGANQK-CNP22 (modified CNP37) (SEQ ID NO: 130)	60	121	ND
CU	GQEHPNARKYKGANQQ-CNP22 (modified CNP37) (SEQ ID NO: 131)	9	57	ND
	GQEHPNARKYKGANKK-CNP22 (modified CNP37) (SEQ ID NO: 132)	ND	ND	ND
CW	GQEHPNARKYKGANKP-CNP22 (modified CNP37) (SEQ ID NO: 74)	ND	ND	ND
CR	GAHHPHEHDTHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 128)	14±5	77±12	ND
CZ	FGIPMDRIGRNPR-CNP22 (osteocrin "NPR-C inhibitor") (SEQ ID NO: 82)	ND	ND	ND
DA	GKRTGQYKLGSKTGPGPK-CNP22 (FGF2 "heparin-binding domain" fragment) (SEQ ID NO: 83)	ND	ND	ND
CK	GQPREPQVYTGANQQ-CNP22, K4R (IgG1 fragment) (SEQ ID NO: 84)	2	32	≥160
CL	GVPQVSTSTGANQQ-CNP22, K4R (HSA fragment) (SEQ ID NO: 85)	3	35	>160
CN	GQTHSSGTQSGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 87)	12	115	ND

CM	GQPSSSSQSTGANQQ-CNP22, K4R (fibronectin) (SEQ ID NO: 86)	ND	ND	ND
CO	GSTGQWHSESGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 88)	2	33	ND
CP	GSSSSSSSSSGANQQ-CNP22, K4R (zinc finger) (SEQ ID NO: 89)	ND	ND	ND
CD	SPKMVQGSG-CNP17-KVLRRH ("BNP tails") (SEQ ID NO: 109)	25	102	ND
CJ	RSSCFGGRIDRIGAC ("C-ANP4-23", ANP-derived) (SEQ ID NO: 133)	ND	ND	ND
	CNP22, K4R, K10R, N.-term.--N-term. dimer / disuccinimidyl glutarate (SEQ ID NO: 134)	19	44	ND
	CNP22, K4R, K10R, N-term.--N.-term. dimer / Bis-PEO5 (SEQ ID NO: 135)	19	41	ND
BM	CNP53 (SEQ ID NO: 4)	61	101	>>160
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 4 lists the half-lives, based on the *in vitro* NEP cleavage assay, of CNP variants conjugated to PEG (or PEO) polymers at the N-terminus. All the PEGylated CNP variants tested and shown in Table 4 displayed resistance or enhanced resistance to NEP cleavage except for PEO12-CNP27(Pro4) (i.e., PEO12-GANPR-CNP22(K4R)), which had the same half-life as wtCNP22. Interestingly, N-terminal PEGylation of CNP22 having a K4G substitution does not seem to confer significant improvement in NEP resistance. For example, PEG2K-CNP22(K4G) was only slightly more resistant to NEP cleavage than CNP22 (data not shown), whereas PEG2K-CNP22 had a much longer half-life *in vitro* than CNP22.

Table 4

N-Terminal PEGylation		**cGMP Response rel. to 1 uM CNP22[1]**		**NEP Cleavage**
Natriuretic Peptide	**PEG**	**10nM**	**1uM**	**($t_{1/2}$, min)**
CNP22		46±10	100±13	80
CNP22	PEG20K	0	15	>>160

CNP22	PEG5K	8±1	20±7	>>160
CNP22	PEG2K	6±2	32±4	>>160
CNP22	PEO4-$(PEO12)_3$ (branched)	17±1	52±6	>>160
CNP22	PEO24 (1.2 kDa)	8±5	46±10	>>160
CNP22	PEG1K	15±3	68±17	>>160
CNP22	PEO12 (0.6 kDa)	12±7	57±18	≥160
CNP22	(PEO12)-Biotin	19	81	140
CNP22, K4G	(PEO12)-Biotin	10	27	100
CNP22, K4R	PEO24	15	56	ND
CNP22, K4R	PEO12	13	44	ND
CNP-17	PEG2K	5	50	>160
R-CNP22, K4R	PEO24	15±2	75±12	ND
R-CNP22, K4R	PEO12	23±2	93±19	≥160
ER-CNP22, K4R	PEO24	6±2	60±10	ND
ER-CNP22, K4R	PEO12	20±1	92±25	ND
GANRR-CNP22, K4R (CNP27)	PEG2K	15±2	45±18	ND
GANRR-CNP22, K4R (CNP27)	PEO24	28±9	82±18	>>160
GANRR-CNP22, K4R (CNP27)	PEG1K	15±0.4	56±23	>>160
GANRR-CNP22, K4R (CNP27)	PEO12	40±2	99±13	>>160
GANQQ-CNP22, K4R	PEO24	16±13	73±30	ND
GANQQ-CNP22, K4R	PEO12	30	78	ND
GANPR-CNP22, K4R	PEO24	ND	ND	ND
GANPR-CNP22, K4R	PEO12	ND	ND	80
GANSS-CNP22, K4R	PEO24	8±5	46±21	ND
GANSS-CNP22, K4R	PEO12	8±0.3	52±13	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Figure 1 shows the NEP resistance profile of five N-terminal PEGylated conjugates of CNP22. The CNP22 peptides conjugated to PEG (or PEO) polymers of increasing mass exhibited increasing resistance to NEP degradation. In particular, PEO24-CNP22, PEG2K-CNP22 and PEG5K-CNP22 were resistant to NEP degradation over the assay period of 160 minutes.

Figure 2 displays the NEP resistance profile of CNP variants CNP37, CNP53 and CNP27(Arg4) (i.e., GANRR-CNP22(K4R)) having an N-terminal amino acid extension. As can be clearly seen, both CNP37 and CNP53 were resistant to NEP degradation in this *in vitro* assay, whereas CNP27(Arg4) had the same lability to NEP hydrolysis as CNP22.

Figure 3 depicts the NEP resistance profile of CNP17 and CNP27(Arg4) conjugated to a PEG (or PEO) moiety at the N-terminus. PEGylation of CNP27(Arg4) greatly improved the NEP resistance of this CNP variant, with PEO24-CNP27(Arg4) being completely resistant to NEP cleavage over the assay period of 160 minutes. Increasing the mass of the PEO moiety from about 0.6 kDa (PEO12) to about 1.2 kDa (PEO24) improved the NEP resistance of PEGylated CNP27(Arg4). PEGylation of CNP27(Arg4) to a monodispersed PEO24 moiety rather than a polydispersed PEG1K moiety also improved NEP resistance. Finally, although both PEO24-CNP27(Arg4) and PEG2K-CNP17 have a similar total mass (keeping in mind that PEG2K is polydispersed), the former displayed significantly better NEP resistance.

Example 3

CNP Variant Stimulation of cGMP Production in NIH3T3 Cells

To determine the functional activity of CNP variants, the production of GMP was measured in NIH3T3 cells exposed to the CNP variants. Murine NIH3T3 cells express endogenously the CNP signaling receptor, NPR-B, which shares 98% protein sequence identity with human NPR-B. NIH3T3 cells were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and antibiotics at 37° C with 5% CO_2. Twenty four to 48 hours prior to signaling, cells were passaged to 12-well plates with a density of 2-5 x 10^5 cells per well at the time of the assay. CNP variants were resuspended in 1 mM HCl to a stock concentration of 1 mg/mL (455 uM for wtCNP22) and subsequently diluted to a 30 uM working stock solution with phosphate-buffered saline (PBS). Ten-fold serial dilutions were prepared in phosphate-buffered saline. Culture medium was removed from the cells and replaced with 0.4 mL PBS/Dulbecco's modified Eagle medium (50/50, v/v) containing 0.75 mM isobutylmethylxanine. Plates were incubated at 37 °C, 5% CO_2 for 15 minutes before addition of 0.2 mL CNP variant in PBS and continued incubation at 37 °C for 15 minutes. Reactions were stopped by the addition of 0.2 mL lysis buffer supplied with the CatchPoint cGMP assay kit (Molecular Devices), and

cGMP production was determined with the CatchPoint cGMP Assay (Molecular Devices). All stimulation experiments were performed in duplicate.

Tables 1-4 summarize the ability of CNP variants having backbone or side chain modifications, amino acid substitutions, N-terminal amino acid extensions, and/or N-terminal PEGylation, respectively, to stimulate cGMP production in NIH3T3 cells. In all four tables, the values for cGMP production in NIH3T3 cells exposed to 10 nM or 1 uM CNP variant are normalized to cell number and cGMP production in the presence of 1 uM wtCNP22.

Regarding the results in **Table 1**, only Analog G having 3-Cl-Phe at position 7 displayed substantially the same NPR-B stimulation activity at 1 uM as wtCNP22. With respect to **Table 2**, various CNP variants with amino acid substitutions, including Analogs BO, AB, BH, BK, BZ, BX and BR, showed substantially similar NPR-B stimulation activity as wtCNP22. Worthy of note is that Analog AH, which has a K4R substitution, stimulated significantly greater cGMP production than wtCNP22.

Considering the results in **Table 3**, many CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions, exhibited comparable NPR-B stimulation activity as wtCNP22. The functional CNP variants include Analog BB, which is CNP22(G1E) attached to heptanoic acid at the N-terminus, and Analog CD, which is the cyclic domain of CNP22 ("CNP17" retaining the Cys6 to Cys22 sequence) conjugated to the N-terminal and C-terminal "tails" of BNP. **Figure 4** illustrates that CNP27(Arg4) (i.e., GANRR-CNP22(K4R)), CNP37 and CNP53 all had equivalent NPR-B stimulation activity as wtCNP22 in the *in vitro* assay.

Of note from Table 3 is that among the CNP variants assayed for both CNP functionality and NEP resistance, Analog AZ (R-CNP22(K4R)), Analog CA, Analog CB, Analog CC, Analog CF, Analog BL (CNP37), Analog CE, Analog CS and Analog CD (CNP17 having N- and C-terminal BNP tails) all had comparable NPR-B stimulation activity as CNP22 while being much more resistant to NEP cleavage than CNP22.

With regard to the results in **Table 4**, eight N-terminal PEGylated CNP variants at 1 uM stimulated cGMP production to at least 70% of the level achieved by wtCNP22. Several interesting aspects appear in Table 4. First, N-terminal PEGylation of CNP27(Arg4) with a monodispersed PEO polymer (PEO12 is about 0.6 kDa, PEO24 about 1.2 kDa) resulted in significantly better NPR-B functionality than that with a polydispersed PEG polymer (PEG1K has a polymer number average molecular weight (M_n) of around 1 kDa,

PEG2K around 2 kDa) (see also **Figure 5**). Second, N-terminal PEGylation of wtCNP22 with a polydispersed PEG polymer of increasing M_n (PEG1K, PEG2K, PEG5K and PEG20K) or with a monodispersed PEO polymer of greater mass (PEO12 and PEO24) correspondingly decreased the NPR-B activation ability of the CNP variants (see also **Figure 6**). Third, PEO24-CNP27(Arg4), having the N-terminal GANRR extension, stimulated significantly greater cGMP production than PEO24-CNP22 and PEO24-CNP22(K4R). Also of note is that among the N-terminal PEGylated CNP variants assayed for both CNP functionality and NEP resistance, PEO12-R-CNP22(K4R), PEO12-CNP27(Arg4) and PEO24-CNP27(Arg4) all had substantially the same NPR-B stimulation activity as CNP22 while being much more resistant to NEP degradation than CNP22.

Example 4

Binding Specificity for NPR-A, NPR-B and NPR-C

Signaling Competition Assay

To determine the binding specificity of CNP variants for the clearance receptor NPR-C, a signaling competition assay is carried out. An expression plasmid for human NPR-C (purchased from OriGene) is transiently expressed in NIH3T3 cells. Twenty-four hours after transfection, NIH3T3 cells are processed for the NPR-B/cGMP stimulation assay described in Example 3. Addition of wtCNP22 is expected to bind to both the endogenous NPR-B and the transiently expressed NPR-C, resulting in decreased cGMP production and a shift in the dose-response curve to the right. CNP variants having reduced affinity for NPR-C are expected not to induce a shift, or to induce a smaller shift, in the dose-response curve.

Alternatively, the relative affinities of CNP variants for NPR-A, NPR-B and NPR-C are determined in a signaling competition assay (U.S. Patent 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)). HEK293T cells, or another suitably transfectable cell line (e.g., HeLa cells, HEK293 cells, etc) are transiently transfected with expression plasmids for NPR-A (OriGene) or NPR-B (cDNA cloned with standard molecular biology techniques into pcDNA3.1 (Invitrogen)) with or without NPR-C (OriGene).

Determination of Binding Affinities (K_i) for NPR-A, NPR-B and NPR-C

The binding affinities (K_i) of CNP variants for NPR-A, NPR-B and NPR-C are determined in a heterologous competition binding assay (U.S. Patent 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)). Membranes from HEK293 cells, or another suitably transfectable cell line (e.g., HeLa cells), expressing human NPR-A, NPR-B or NPR-C are prepared for radio-labeled ligand binding assays. Membrane preparations are diluted in an appropriate buffer and varying concentrations of wtCNP22 or CNP variant (competitor) are added with I^{125}-labeled wtCNP22 (Bachem). Samples are incubated at room temperature to allow for ligand/receptor equilibration and bound peptide is separated from free peptide by filtration through PVDF filter membranes. Filters are washed before the addition of scintillant and counting by a scintillation counter. Binding is measured in duplicate for each concentration of competitor peptide. CNP variant affinity (K_i, equilibrium dissociation constant) and B_{max} (receptor number) are calculated by non-linear regression analysis and/or the Cheng-Prusoff equation.

Example 5

Effect of CNP Variants on the Growth of Rat Chondrosarcoma (RCS) Cells and cGMP Production in RCS Cells

To assess the ability of CNP variants to stimulate bone growth, skeletal dysplasia is simulated in cell culture by treating rat chondrosarcoma (RCS) cells with fibroblast growth factor 2 (FGF-2), which activates fibroblast growth factor receptor 3 (FGFR-3) and induces growth arrest (Krejci et al., J. Cell Sci., 118(21):5089-5100 (2005)).

Optimal CNP treatment parameters are determined by varying CNP concentration (0.05, 0.1, 0.2 and 0.5 uM), and treatment duration and interval (continuous; 2.5 min, 10 min, 30 min, 1 hr, 2 hr, 4 hr and 8 hr once a day; 2.5 min, 10 min, 30 min, 1 hr, 2 hr and 4 hr twice a day). After 72 hours, cells are counted using an automated cell counter, and the amount of extracellular matrix is estimated using alcian blue staining.

RCS cells are then treated with a CNP variant using the optimal conditions determined from the growth experiments with wtCNP22. The concentration of cGMP is measured by competitive ELISA for untreated RCS cells, RCS cells treated with CNP, and RCS cells treated with the CNP variant. Cell growth and matrix synthesis resulting from treatment with the CNP variant are also monitored and compared to those resulting from CNP treatment.

To assess the effect of CNP variants in a human cell culture system, primary re-differentiated human chondrocytes in alignate beads are treated with wtCNP22 and CNP variants, and cGMP concentration is determined by competitive ELISA as a measure of effective CNP signaling.

The methods described herein can be employed to assess the ability of CNP variants to stimulate cGMP production in and growth of rat chondrosarcoma cells *in vitro*.

Example 6

Dose Response Study in Rat Chondrosarcoma Cells

The tyrosine kinase receptor fibroblast growth factor receptor 3 (FGFR-3), a negative regulator of chondrocyte growth, is contitutively on in achondroplasia subjects. Continuous exposure of rat chondrosarcoma (RCS) cells to fibroblast growth factor 2 (FGF-2) simulates achondroplasia in cell culture by activating FGFR-3 and inducing growth arrest (Krejci et al., J. Cell Sci., 118(21): 5089-5100 (2005)). To determine the dose of CNP variant and frequency of dosing that stimulate sufficient growth of bone cells, a dose response study was performed using the RCS cell assay as described in Example 5.

RCS cells were seeded at 10 x 10^3 cells per well in 24-well plates, grown for 24 hr, treated for 72 hr, and then counted. RCS cells were continuously exposed to FGF-2 (5 ng/mL) to simulate a constitutively active FGFR-3, which induced cell growth arrest (see bar #5 in **Figure 7**). Wild-type CNP22 (0.2 uM) was cultured continuously (72 hr), 1 hr daily or 2 hr daily. All stimulants were changed daily. Continuous exposure of RCS cells to 0.2 uM CNP22 in the presence of 5.0 ng/mL FGF-2 partially reversed FGF2-induced growth arrest, leading to the growth of approximately 200 x 10^3 cells per well (bar #6 in Figure 7), compared to approximately 100 x 10^3 cells per well in the absence of CNP22 (bar #5 in Figure 7).

Both 1 hr exposure to CNP22 (0.2 uM) once a day and 2 hr exposure to CNP22 (0.2 uM) once a day achieved about 84% of the effect of continuous CNP22 (0.2 uM) exposure on chondrocyte growth (bars #7 and 8 in Figure 7). These results demonstrate that continuous exposure of growth-arrested chondrocytes to CNP22 is not required for reversal of cell growth arrest. Furthermore, histological and cell morphological analysis of the extracellular matrix showed that CNP22 treatment antagonized FGF2-mediated loss of chondrosarcoma extracellular matrix and increased matrix synthesis (data not shown).

Similar dose response studies can be conducted with the CNP variants described herein to determine their effective dose for reversing FGF2-induced growth arrest of RCS cells.

Example 7

Ex Vivo Studies on Stimulation of Tibial and Femoral Growth

Mouse tibial organ culture model has been used to demonstrate the efficacy of wild-type CNP22 in stimulating longitudinal bone growth. Treatment of wild-type tibiae with CNP22 at 10^{-8}, 10^{-7} or 10^{-6} M for 6 days increased longitudinal growth by 31%, 40% and 42%, respectively. Histological evaluation also showed expansion of the hypertrophic zone, e.g., an increase in the number and size of hypertrophic chondrocytes in the growth plate (Agoston et al., BMC Dev. Biol. 7:18 (2007)). Similar findings were observed in tibiae isolated from $FGFR3^{Ach}$ mice (Yasoda et al., Nat. Med. 10: 80-86 (2004)).

To determine the efficacy of CNP variants in stimulating longitudinal bone growth, CNP variants are tested in a mouse organ culture model of endochondral bone growth in wild-type and $FGFR3^{wt/Ach}$ (heterozygote) mice. In brief, the pharmacological activity of wild-type CNP22 and CNP variants is compared in an organ culture model of embryonic or neonatal mouse tibiae, isolated from wild-type and $FGFR3^{wt/Ach}$ littermates. Overall bone growth and histological changes within the growth plate are assessed. Conditioned culture medium is assessed for CNP, as well as biomarkers of intracellular signaling (cGMP), cartilage metabolism (type II collagen, other collagens, aggrecan, chondroitin sulfate), bone metabolism (bone alkaline phosphatase, osteocalcin, type I collagen [C-telopeptide, N-telopeptide]), and inflammation (interleukin-1, interleukin 6, interleukin-11). In addition, the effects of various treatment regimens of wild-type CNP22 are compared to determine the optimal conditions to enhance longitudinal bone growth.

Effective CNP variants are identified by their ability to stimulate production of cGMP, and bone growth as measured by increased longitudinal bone length and expansion of the cells in the hypertrophic zone of the growth plate.

The efficacy of wtCNP22, CNP37 and PEO24-CNP27(Arg4) (i.e., PEO24-GANRR-CNP22(K4R)) in stimulating longitudinal femoral growth was evaluated in the mouse organ culture model. For these experiments femora were isolated from 2-3 day old wild-type mice and cultured in alphaMEM supplemented with 0.2% BSA, 0.5 mM L-glutamine, 40 units

penicillin/mL and 40 ug streptomycin/mL, for 8 days in the presence of vehicle, CNP22 or CNP variants. The treatment commenced at day 0 and was repeated every two days thereafter, as the medium was changed. Bones were measured prior to treatment and every two days thereafter, using a dissection microscope fitted with a 1 cm eye-piece reticule. Conditioned medium was used for biomarker analysis. At day 8 bones were fixed in 4% paraformaldehyde for 24 hr, decalcified in 5% formic acid for 24 hrs, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and stained with Alcian Blue for 30 min (pH 2.5; MasterTech) and van Gieson (EM Sciences) for 5 min. Alcian Blue stains cartilage blue and van Gieson stains osteoid (unminerlized bone) red. Stained sections were visualized and photographed by brightfield microscopy. The thickness of the hypertrophic region of the growth plate cartilage was determined by image analysis.

Figure 8 illustrates the effect of wtCNP22, CNP37 and PEO24-CNP27(Arg4) on longitudinal growth of 3-day old wild-type femurs treated with the CNP peptides every two days. The results were normalized to measurements prior to treatment (day 0). The studies were performed in triplicate (vehicle) or quadruplicate (CNP peptides). As shown in Figure 8, CNP37 and PEO24-CNP27(Arg4), as well as CNP22, were effective in stimulating longitudinal femoral growth, with the N-terminal PEGylated CNP variant being the most effective.

Example 8

Serum/Plasma Stability of CNP Variants *In Vitro*

In preparation for pharmacokinetics (PK) studies, the stability of CNP variants in serum and/or plasma is evaluated.

Briefly, the analyte is isolated by the removal of serum or plasma proteins by either a 2% trichloroacetic acid precipitation or a 1:3 serum:acetonitrile precipitation. The precipitation mixture is vortexed at 14,000 rpm for five minutes, and a portion of the supernatant is removed and diluted with water prior to transfer to a silanized autosampler vial for analysis. Serum extracts are then analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) with electrospray ionization mass spectrometry (ESI-MS). A single mass (m/z), shown to be specific for the CNP variant, is monitored for quantitation purposes.

Initially, analytical stability and recovery is determined. Analytical (RP-HPLC and ESI-MS) parameters are optimized through the analysis of matrix standards (serum extracts fortified with analyte post-precipitation). After optimization, analytical recovery is determined by spiking serum samples at known concentrations and comparison of the analyte response to that of matrix standards prepared at similar concentrations. Analyte stability in serum extracts is also determined to assure no significant losses occur after serum precipitation and prior to actual analysis. To test the effect of freezing on serum stability, a two-cycle freeze/thaw study is also performed. In this study a serum sample is spiked with CNP variant and analyzed prior to freezing overnight at -20 °C. The sample is then thawed at room temperature and re-analyzed. The process is repeated for a second freeze/thaw cycle.

Serum stability of CNP variant is determined by spiking of serum/plasma samples with CNP variant at a concentration of 10 ug/mL. The sample is placed in a 37 °C water bath for a period of three hours. At 30 minute intervals duplicate aliquots of serum are removed and analyzed. If rapid losses of analyte are evident (> 50% in 30 minutes), the study may be repeated with 10 minute timepoints.

In an exemplary method for determining the stability of CNP variants in murine plasma, a mixture of CNP variant (10 uL of a stock solution of about 2.5-5.0 mg/mL), heparinized murine plasma (50 uL, Bioreclamation, CD-1 Lith Hep 62231), and 5 M NaCl (10 uL) is incubated at 37 °C and 5% CO_2 for 0-5 hr, and then quenched with 10x protease inhibitor cocktail (15 uL, Sigma P2714). For extraction, 150 uL of MeOH/0.1% FA is added to 85 uL of the reaction mixture, and the resulting mixture is vortexed for 1 min and then centrifuged at 15 °C for 15 min. 75 uL of the supernatant is added to 300 uL of aqueous 0.1% FA. A small portion of the resulting mixture is subjected to analysis by LC/MS.

Example 9

Pharmacokinetics and cGMP Production in Rats and Mice

Studies were conducted in normal rats to evaluate the pharmacokinetics (PK) profile of CNP22 and certain CNP variants and the time courses of plasma cGMP concentration after single intravenous (i.v.) or subcutaneous (s.c.) administration of the CNP peptides. Plasma CNP immunoreactivity was determined by using a competitive radioimmunoassay (RIA) with an anti-CNP rabbit polyclonal antibody. Plasma cGMP concentration was determined by RIA using a commercially available kit (YAMASA cyclic GMP Assay kit, YAMASA Corporation).

Normal male rats, 7-8 weeks of age, were used. Recombinant wild-type CNP22, CNP37 and PEO24-CNP27(Arg4) (i.e., PEO24-GANRR-CNP22(K4R)) were evaluated. A dosage of 20 nmol/kg of each CNP peptide as a solution in 5% mannitol was intravenously injected once into the tail, or a dosage of 50 nmol/kg of each CNP peptide as a solution in 0.03 mol/L acetic acid buffer solution, pH 4.0, containing 1%(w/v) benzyl alcohol and 10% (w/v) sucrose, was subcutaneously injected once into the back.

Plasma CNP immunoreactivity was determined by the competitive RIA using anti-CNP rabbit polyclonal antibody. Standard and QC samples were prepared. Fifty uL of the standard, QC and assay samples were added, respectively, to test tubes containing 50 uL of RIA buffer. Diluted anti-CNP rabbit polyclonal antibody (100 uL) was added to the tubes. All tubes were kept at 4 °C overnight. ^{125}I-[Tyr^{0}]-CNP22 solution (100 uL) and rabbit IgG solution (100 uL) were added and left at approximately 4 °C overnight. One milliliter of anti-rabbit IgG goat serum containing 10% polyethylene glycol was added, vortexed and left at approximately 4 °C for at least 1 hour, and then the insoluble fraction was precipitated by centrifugation. After aspiration of the supernatant, the amount of radiation (gamma line) in the sediment was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma cGMP concentrations in the sample at 5, 30, 60 and 90 minutes after i.v. dosing, or at 5, 30, 60, 120 and 180 minutes after s.c. dosing, were determined by the competitive RIA using anti-cGMP monoclonal antibody. Standard samples were prepared. 100 uL of the assay samples (standard solutions for the calibration curve or the diluted plasma samples for cGMP determination) were transferred to test tubes. Then 100 uL of anti-cGMP monoclonal antibody solution and 100 uL of ^{125}I-labeled succinyl cGMP tyrosine methyl ester solution were added to the tubes, respectively. All tubes were kept at 4 °C overnight. After the addition of 500 uL dextran charcoal solution, the tubes were vortexed and then placed on ice for 10 minutes. The reaction mixture was centrifuged and 500 uL of the supernatant was transferred from each sample to a new test tube. The amount of radiation (gamma line) in the supernatant was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma CNP immunoreactivity was employed for pharmacokinetics (PK) analysis. PK analysis was performed using WINNONLIN® Professional (Pharsight Corporation). The PK profiles of CNP22, CNP37 and PEO24-CNP27(Arg4) after i.v. administration were calculated using PK parameters such as concentration at 0 hour (C_0: extrapolation, pmol/mL),

total body clearance (CL_{tot}: mL/min/kg), distribution volume at steady state (V_{dss}: mL/kg), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min). The PK profiles of the CNP peptides after s.c. administration were calculated using PK parameters such as maximum plasma concentration (C_{max}: pmol/mL), time to reach C_{max} (T_{max}: min), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min).

In plasma spike recovery experiments, the RIA detected CNP22, CNP37 and PEO24-CNP27(Arg4) similarly (data not shown).

Procedures similar to those described above are employed to study in mice the PK profiles of CNP22 and variants thereof and their ability to stimulate cGMP production.

The PK profiles of CNP22, CNP37 and PEO24-CNP27(Arg4) after i.v. administration in three rats are illustrated in **Figure 9**. As shown by Figure 9, CNP37 and PEO24-CNP27(Arg4) had a much longer half-life and a much greater bioavailability than CNP22. The half-life, $T_{1/2}$ (min), is 1.42 (± 0.45) for CNP22, 22.3 (± 1.5) for PEO24-CNP27(Arg4), and 49.5 (± 28.0) for CNP37. The area under the curve, AUC (pmol'min/mL), is 320 (± 54) for CNP22, 1559 (± 568) for CNP37, and 2084 (± 424) for PEO24-CNP27(Arg4).

The PK profiles of the three CNP peptides after s.c. administration in three rats are depicted in **Figure 10**. Compared to CNP22, PEO24-CNP27(Arg4) had a much longer half-life (78.1 min (± 16.4) vs. 10.0 (± 5.0)) and a much greater bioavailability (60% (± 6%) vs. 19% (± 9%)).

The time courses of plasma cGMP concentrations after i.v. administration of the three CNP peptides in three rats are displayed in **Figure 11**. Figure 11 clearly demonstrates that i.v. administration of CNP37 and PEO24-CNP27(Arg4) resulted in much higher plasma levels of cGMP at 30, 60 and 90 minutes than i.v. administration of CNP22.

The time profiles of plasma cGMP concentrations after s.c. administration of the three CNP peptides in three rats are shown in **Figure 12**. Subcutaneous administration of PEO24-CNP27(Arg4) and CNP37 also resulted in substantially higher plasma concentrations of cGMP than s.c. administration of CNP22, with the difference relative to CNP22 increasing over time for PEO24-CNP27(Arg4), but decreasing over time for CNP37.

The rat studies indicate that compared to wtCNP22, the CNP variants CNP37 and PEO24-CNP27(Arg4) had a substantially longer half-life *in vivo*, had a substantially greater bioavailability *in vivo*, and stimulated substantially higher levels of cGMP production *in vivo*

for an extended period of time. The resistance of CNP37 and PEO24-CNP27(Arg4) to NEP degradation correlates to a longer plasma half-life *in vivo*, which in turn correlates to prolonged NPR-B/cGMP signaling *in vivo*. These results show that unlike CNP22, the CNP variants of the invention, administered by i.v. or s.c. injection (e.g., once daily), can potentially be effective in treating CNP-responsive conditions or disorders, such as bone-related disorders and vascular smooth muscle disorders..

Example 10

Pharmacokinetics Study in Mice

To determine CNP variants having increased NEP resistance, for efficacy study in FGFR3^{ach} mice (see Example 11), a pharmacokinetics (PK) study is carried out that compares the pharmacokinetics properties of CNP variants to wild-type CNP22. The FGFR3^{ach} mouse is a mutant mouse model of achondroplasia, containing a single transgene on a background of FVB mice.

Wild-type CNP22 or variant thereof is administered as a single intravenous (i.v.) dose in 6-week old wild-type FVB mice. Exemplary PK studies were conducted using wtCNP22. Six-week old FVB/N mice were intravenously administered wtCNP22 in a single dose at 100 nmol/kg. Mean plasma levels of CNP22 were calculated, and the estimated half-life of CNP22 was determined to be from 0.76 min to 1.03 min.

CNP variants displaying greater resistance to NEP degradation are expected to exhibit increased serum concentrations over time and a longer half-life *in vivo*.

Example 11

Efficacy in Mouse Model of Achondroplasia

The efficacy of CNP22 and variants thereof in enhancing growth and correcting achondroplasia is tested in a mouse model of achondroplasia, using a strain of transgenic mice expressing the mutated human FGFR-3 gene (FGFR3^{ach}) (Wang et al., Proc. Natl Acad. Sci. U S A, 96(8): 4455–4460 (1999); Naski et al., Development USA, 125: 4977-4988 (1998); U.S. Patent Nos. 6,265,632 and 6,136,040).

At 3 weeks of age, FGFR3^{ach} mice and their wild-type littermates are anesthetized by 2.5% Avertin to have lateral whole-body X-ray images taken by Faxitron, and randomized by body weight into the following treatment groups (n=8/group): (1) wild-type/vehicle, (2)

FGFR3^{ach}/vehicle, (3) FGFR3^{ach} /CNP22, (4) FGFR3^{ach} /first CNP variant, (5) FGFR3^{ach} /second CNP variant, and (6) FGFR3^{ach} /third CNP variant. Mice undergo once daily subcutaneous administration of designated test article for 5 weeks. Satellite groups (n=3) are used to confirm exposure of each test article following a single subcutaneous administration on Day 1.

On Day 37, all mice are sacrificed by terminal anesthesia and whole-animal photographs and X-ray images by Faxitron are taken. Left and right tibia, femur, humerus, and ulna are collected and measured using a digital caliper. The left portions of each bone are processed for histology, and the right portions are snap-frozen for archival. Samples obtained from the bones are used to evaluate and compare the effects of CNP22 and CNP variants on endochondral bone growth.

Example 12

Clinical Evaluation of CNP Variants

The following example provides guidance on the parameters to be used for the clinical evaluation of compositions comprising CNP22 or variants thereof in the therapeutic methods of the present invention. As discussed herein, CNP22 or variants thereof will be used in the treatment of disorders responsive to CNP, including disorders of the bone and vascular smooth muscle. Clinical trials will be conducted which will provide an assessment of doses of CNP22 or variants thereof for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 24 weeks to collect sufficient safety information on about 100 evaluable patients. The initial dose for the trials will vary from about 0.001 to about 1.0 mg/kg/week. In the event that the initial dose in this range does not produce a significant direct clinical benefit, the dose should be increased within this range or beyond this range as necessary, and maintained for an additional minimal period of, but not necessarily limited to, 24 weeks to establish safety and to evaluate efficacy further.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (including kidney and liver functions), urinalysis, and CBC with differential. In addition, other parameters relevant to clinical benefit will be monitored. The present example also includes the determination of pharmacokinetic parameters of CNP22 or variants thereof, including absorption, distribution, metabolism, excretion, and half-life and

bioavailability in the blood. It is anticipated that such analyses will help relate dose to clinical response.

Methods

Patients will undergo a baseline medical history and physical exam, and a standard set of clinical laboratory tests (including CBC, Panel 20, CH50, and UA). The patients will be followed closely with weekly visits to the clinic. The patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Diagnosis and Inclusion Criteria

The patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder. A specific example of a potentially CNP-responsive, bone-related disorder is achondroplasia, which may be confirmed by genetic testing and other evidence of an FGFR-3 mutation or dysfunction. The ideal age range of achondroplasia patients will be from infant (< 1 year of age) to pre-adolescent (< 13 years of age). A patient will be excluded from this study if the patient is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

Safety

Therapy with CNP22 or variants thereof will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

It has been shown that compared to wild-type CNP22, certain CNP variants of the invention are much more resistant to NEP degradation *in vitro*, have a much longer plasma half-life and bioavailability in rats, stimulate a much higher level of cGMP production in rats, and induce a greater amount of bone growth of the femur from wild-type mice. Furthermore, it has been shown that short duration dose regimen treatments with CNP22 are nearly as effective as continuous CNP22 treatment in reversing FGF2-induced arrest of chondrocyte growth *in vitro*. These results demonstrate the potential use of CNP variants of the invention

in treating CNP-responsive conditions or disorders such as, e.g., bone-related disorders and vascular smooth muscle disorders.

It is understood that every embodiment of the invention described herein may optionally be combined with any of the other embodiments described herein. Every patent literature and every non-patent literature cited herein are incorporated herein by reference in their entirety.

Numerous modifications and variations to the invention, as set forth in the embodiments and illustrative examples described herein, are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.

WHAT IS CLAIMED IS:

1. A variant of C-type natriuretic peptide (CNP) having a total mass in the range from about 2.6 kDa to about 7.0 kDa, and having the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-$(i)_{15}$-Ser_{16}-$(j)_{17}$-Ser_{18}-Gly_{19}-$(k)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 50) wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of bone- or cartilage-targeting moieties; moieties that reduce renal clearance; hydrophilic polymers; amino acid sequences comprising one or more amino acids; carbohydrates; hydrophobic acids; and combinations thereof;

(a) may be the wild-type Lys at that position or may be replaced with a natural amino acid, an unnatural amino acid or a peptidomimetic that does not have a reactive primary amino group on a side chain;

(b) is selected from the group consisting of Cys and peptide bond isosteres;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl, or wherein the benzene ring of the Phe analog is replaced with another aryl group or with a heteroaryl group;

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres;

(f) may be the wild type Lys at that position or may be replaced with a natural amino acid, an unnatural amino acid or a peptidomimetic that does not have a reactive primary amino group on a side chain;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres; and

wherein the CNP variant is not CNP-53.

2. The CNP variant of claim 1, wherein:

(b) is Cys;

(c) is Phe;

(d) is Gly;

(e) is Leu;

(g) is Leu;

(h) is Ile;

(i) is Gly;

(j) is Met; and

(k) is Leu.

3. The CNP variant of any of the preceding claims, wherein (a) and (f) independently are Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu.

4. The CNP variant of claim 3, wherein (a) is Arg and (f) is Lys or Arg.

5. The CNP variant of any of the preceding claims, wherein the peptide bond isosteres are selected from the group consisting of:

$-CH_2-NH-$;

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl;

$-C(=O)-NH-CH_2-$;

$-CH_2-S-$;

$-CH_2-S(O)_n-$, where n is 1 or 2;

$-CH_2-CH_2-$;

-CH=CH-;

-C(=O)-CH_2-;

-CH(CN)-NH-;

-CH(OH)-CH_2-;

-O-C(=O)-NH-; and

-NHC(=O)NH-.

6. A variant of C-type natriuretic peptide (CNP) having a total mass in the range from about 2.6 kDa to about 7.0 kDa, and having the formula:

(x)-(y)-Cys_6-Phe_7-Gly_8-Leu_9-$(f)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 142), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of bone- or cartilage-targeting moieties; moieties that reduce renal clearance; hydrophilic polymers; amino acid sequences comprising one or more amino acids; carbohydrates; hydrophobic acids; and combinations thereof;

(y) may be absent or may comprise from 1 to 5 amino acids from the sequence Gly_1-Leu_2-Ser_3-Lys_4-Gly_5, corresponding to positions 1 to 5 of CNP-22, wherein any of these five amino acids may optionally be substituted with a natural amino acid or an unnatural amino acid; and

(f) may be the wild-type Lys at that position or may be replaced with a natural amino acid, an unnatural amino acid or a peptidomimetic that does not have a reactive primary amino group on a side chain; and

wherein the CNP variant is not CNP-53.

7. The CNP variant of claim 6, wherein (y) comprises Arg rather than Lys at the position corresponding to Lys4 of CNP-22.

8. The CNP variant of claim 6 or 7, wherein (f) is Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu.

9. The CNP variant of any of the preceding claims, wherein the bone- or cartilage-targeting moieties are selected from the group consisting of bisphosphonates; hydroxyapatite; glucosarnine; collagen type X; polyAsp; polyGlu; and amino acid sequences derived from bone-targeting domains of osteocrin, osteopontin, osteocalcin, and sialoprotein.

10. The CNP variant of any of the preceding claims, wherein the moieties that reduce renal clearance are hydrophilic polymers comprising at least one functional group that is negatively charged under physiological conditions.

11. The CNP variant of claim 10, wherein the moieties that reduce renal clearance are poly(ethylene glycol) (PEG) polymers comprising functional group(s) that generate carboxylate group(s), sulfate group(s), phosphate group(s), or combinations thereof, under physiological conditions.

12. The CNP variant of any of the preceding claims, wherein the hydrophilic polymers are poly(ethylene glycol) (PEG) polymers or derivatives thereof.

13. The CNP variant of claim 12, wherein the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.4 kDa to about 2.5 kDa.

14. The CNP variant of claim 13, wherein the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.6 kDa to about 1.5 kDa.

15. The CNP variant of any of claims 12 to 14, wherein the PEG polymers or derivatives thereof are monodispersed.

16. The CNP variant of any of the preceding claims, wherein the amino acid sequences comprising one or more amino acids are derived from natriuretic polypeptides or non-natriuretic polypeptides, and wherein the amino acid sequences may optionally comprise amino acid addition(s), deletion(s), and/or substitution(s) relative to the wild-type sequences of the natriuretic polypeptides or non-natriuretic polypeptides.

17. The CNP variant of claim 16, wherein the natriuretic polypeptides are selected from the group consisting of CNP-22, CNP-53, Natriuretic Peptide Precursor C (NPPC), atrial natriuretic peptide (ANP), Natriuretic Peptide Precursor A (NPPA), brain natriuretic peptide (BNP), and Natriuretic Peptide Precursor B (NPPB).

18. The CNP variant of claim 16, wherein the non-natriuretic polypeptides are selected from the group consisting of albumin, immunoglobulins, histine-rich glycoproteins (HRGPs), fibronectin, fibrinogen, zinc finger-containing polypeptides, fibroblast growth factor 2 (FGF-2), and bone morphogenetic proteins (BMPs).

19. The CNP variant of claim 18, wherein the amino acid sequences comprising one or more amino acids are derived from the functional domains of BMP1, BMP2, BMP3, BMP5, BMP7 and BMP8a.

20. The CNP variant of any of the preceding claims, wherein the amino acid sequences comprising one or more amino acids comprise from 1 to 40 amino acids.

21. The CNP variant of claim 20, wherein the amino acid sequences comprise from 1 to 20 amino acids.

22. The CNP variant of any of the preceding claims, wherein the hydrophobic acids are selected from the group consisting of natural fatty acids and linear or branched, saturated or unsaturated C_5-C_{12} carboxylic acids.

23. The CNP variant of any of the preceding claims, wherein (x) is an amino acid sequence comprising from 1 to 20 amino acids.

24. The CNP variant of claim 23, wherein (x) does not comprise two consecutive basic amino acids at the positions immediately preceding the position corresponding to Gly1 of CNP-22.

25. The CNP variant of claim 23 or 24, wherein the N-terminus of (x) ends in Gly.

26. The CNP variant of any of the preceding claims, wherein a PEG polymer or a derivative thereof is conjugated to the N-terminus of the variant, which may or may not comprise an (x) group containing an amino acid sequence of one or more amino acids.

27. The CNP variant of any of the preceding claims, wherein the variant comprises an Arg residue immediately preceding the position corresponding to Gly1 of CNP-22.

28. A variant of C-type natriuretic peptide 22 (CNP-22) (SEQ ID NO: 1) that retains CNP-22 activity and exhibits at least a 5-fold longer half-life in circulation compared to CNP-22, wherein the variant:

has a total mass in the range from about 2.6 kDa to about 7.0 kDa;

comprises an amino acid sequence at least 70% homologous to amino acids 6 to 22 of SEQ ID NO: 1; and

is not CNP-53.

29. The CNP variant of claim 28, which comprises at least 5 consecutive amino acids of albumin, fibrinogen, an immunoglobulin, or a natriuretic peptide.

30. The CNP variant of claim 29, wherein the natriuretic peptide is ANP, BNP, or CNP-53.

31. The CNP variant of claim 30, which comprises at least 5 consecutive amino acids of amino acids 1-32 of CNP-53 (SEQ ID NO: 4).

32. The CNP variant of claim 28, which comprises a hydrophilic polymer.

33. The CNP variant of claim 32, wherein the hydrophilic polymer is PEG.

34. A variant of C-type natriuretic peptide (CNP) selected from the group consisting of:

the CNP variants listed in Tables 1, 2, 3 and 4 in the written description (SEQ ID NOs: 1 to 6 and SEQ ID NOs: 34 to 144);

R-CNP22;

ER-CNP22;

GANQQ-CNP22;

GANRR-CNP22;

GANPR-CNP22;

GANSS-CNP22;

PEO12-R-CNP22;

PEO24-R-CNP22;

PEO12-ER-CNP22;

PEO24-ER-CNP22;

PEO12-GANQQ-CNP22;

PEO24-GANQQ-CNP22;

PEO12-GANRR-CNP22;

PEO24-GANRR-CNP22;

PEO12-GANPR-CNP22;

PEO24-GANPR-CNP22;

PEO12-GANSS-CNP22; and

PEO24-GANSS-CNP22;

wherein the CNP variant is not CNP-17, CNP-22, CNP-53 or ANP.

35. The CNP variant of claim 34, which is selected from the group consisting of:

R-CNP22(K4R) (Analog AZ) (SEQ ID NO: 41);

AAWARLLQEHPNA-CNP22 (Analog CA) (SEQ ID NO: 61);

AAWARLLQEHPNAR-CNP22 (Analog CB)(SEQ ID NO: 62);

DLRVDTKSRAAWAR-CNP22 (Analog CC) (SEQ ID NO: 63);

GQPREPQVYTLPPS-CNP22 (Analog CF) (SEQ ID NO: 79);

QEHPNARKYKGANKK-CNP22 (CNP37, Analog BL) (SEQ ID NO: 60);

GERAFKAWAVARLSQ-CNP22 (Analog CE) (SEQ ID NO: 81);

GHHSHEQHPHGANQQ-CNP22 (Analog CQ) (SEQ ID NO: 76);

GQEHPNARKYKGANPK-CNP22 (Analog CS) (SEQ ID NO: 71);

GQEHPNARKYKGANQK-CNP22 (Analog CT) (SEQ ID NO: 130);

GAHHPHEHDTHGANQQ-CNP22 (Analog CR) (SEQ ID NO: 77);

GQTHSSGTQSGANQQ-CNP22(K4R) (Analog CN) (SEQ ID NO: 87);

SPKMVQGSG-CNP17-KVLRRH (Analog CD) (SEQ ID NO: 68);

PEG1K-CNP22 (SEQ ID NO: 1);

PEO12-CNP22 (SEQ ID NO: 1);

PEO12-R-CNP22(K4R) (SEQ ID NO: 41);

PEO24-R-CNP22(K4R) (SEQ ID NO: 41);

PEO12-ER-CNP22(K4R) (SEQ ID NO: 39);

PEO24-ER-CNP22(K4R) (SEQ ID NO: 39);

PEG1K-GANRR-CNP22(K4R) (SEQ ID NO: 36);

PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36);

PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36);

PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37);

PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37);

PEO12-GANQQ-CNP22(K4R) (SEQ ID NO: 69); and

PEO24-GANQQ-CNP22(K4R) (SEQ ID NO: 69).

36. A variant of C-type natriuretic peptide (CNP) having a total mass in the range from about 2.6 kDa to about 7.0 kDa, which comprises:

(a) a hydrophilic polymer, and

(b) any one of CNP22 (SEQ ID NO: 1), GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), ER-CNP22(K4R) (SEQ ID NO: 39), R-CNP22(K4R) (SEQ ID NO: 41), or GANQQ-CNP22(K4R) (SEQ ID NO: 69),

wherein the hydrophilic polymer is attached to the peptide at the N-terminus, the C-terminus or an internal site, or a combination thereof.

37. The CNP variant of any of the preceding claims, which has a cyclic structure formed by a disulfide bond between the residues at the positions corresponding to Cys6 and Cys22 of CNP-22.

38. The CNP variant of any of the preceding claims, which has a pI in the range from about 8.0 to about 10.5.

39. The CNP variant of claim 38, which has a pI in the range from about 8.5 to about 10.0.

40. The CNP variant of any of the preceding claims, which has a total mass in the range from about 2.8 kDa to about 6.0 kDa.

41. The CNP variant of any of the preceding claims, which has at least a two-fold longer half-life than wild-type CNP-22 in an *in vitro* assay evaluating susceptibility to neutral endopeptidase (NEP) cleavage.

42. The CNP variant of any of the preceding claims, which *in vitro* stimulates the production of at least about 50% of the cGMP level produced under the same concentration of wild-type CNP-22.

43. A pharmaceutical composition comprising a CNP variant according to any of the preceding claims, and a pharmaceutically acceptable excipient, carrier or diluent.

44. Use of a CNP variant according to any of claims 1 to 42, in the preparation of a medicament for the treatment of a skeletal dysplasia selected from the group consisting of achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata,

Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia.

45. The use of claim 44, wherein the skeletal dysplasia is achondroplasia.

46. Use of a CNP variant according to any of claims 1 to 42, in the preparation of a medicament for the treatment of a vascular smooth muscle disorder selected from the group consisting of hypertension, restenosis, arteriosclerosis, acute decompensated heart failure, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute renal insufficiency, and chronic renal insufficiency.

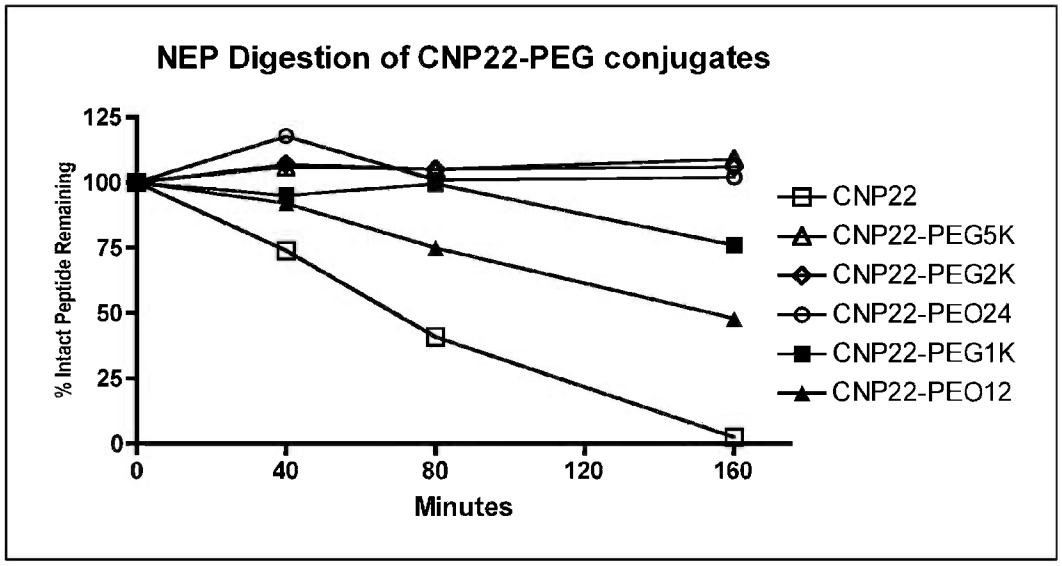


FIGURE 1

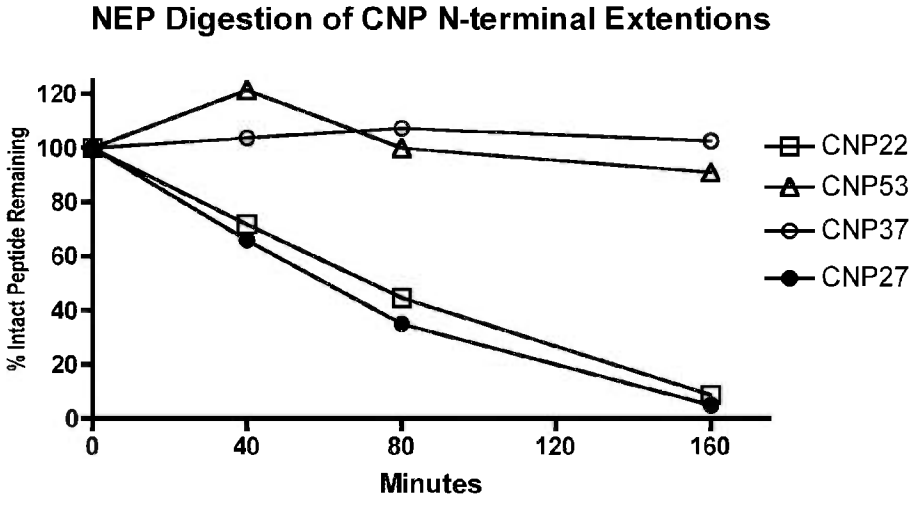


FIGURE 2

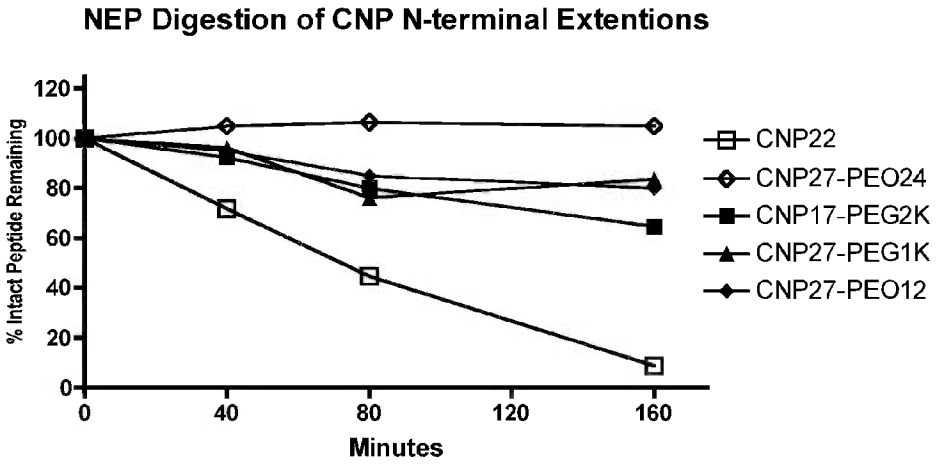


FIGURE 3

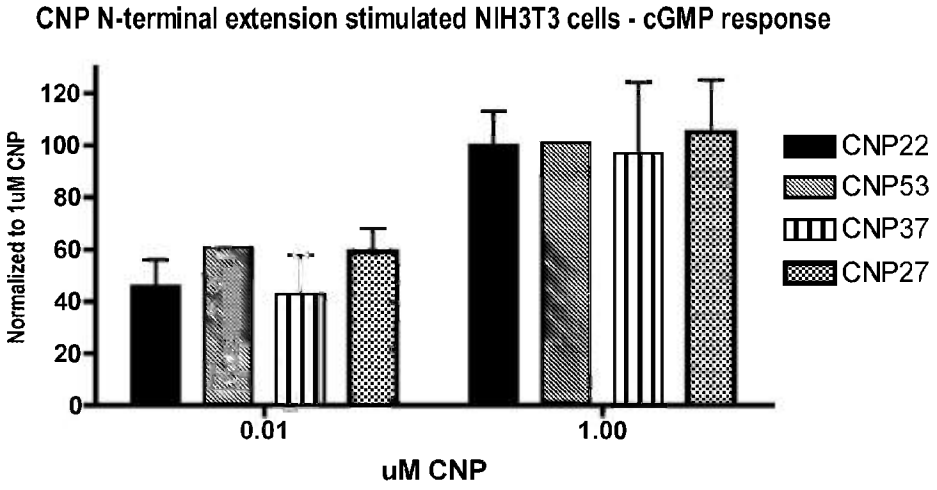


FIGURE 4

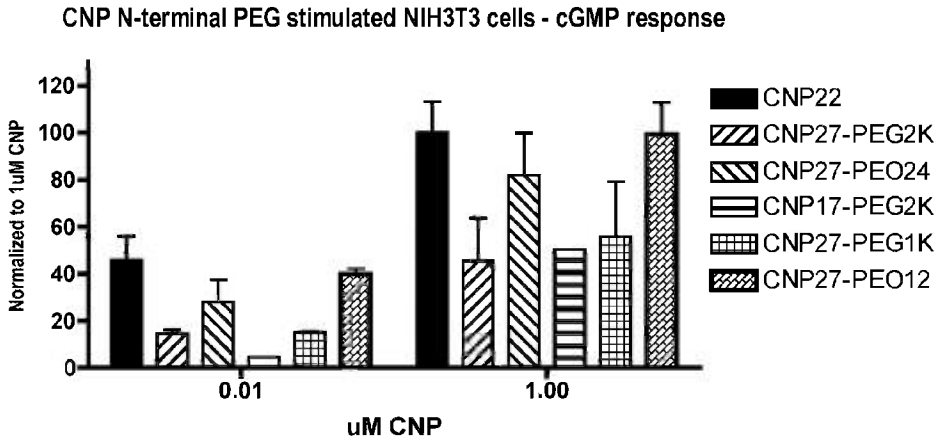


FIGURE 5

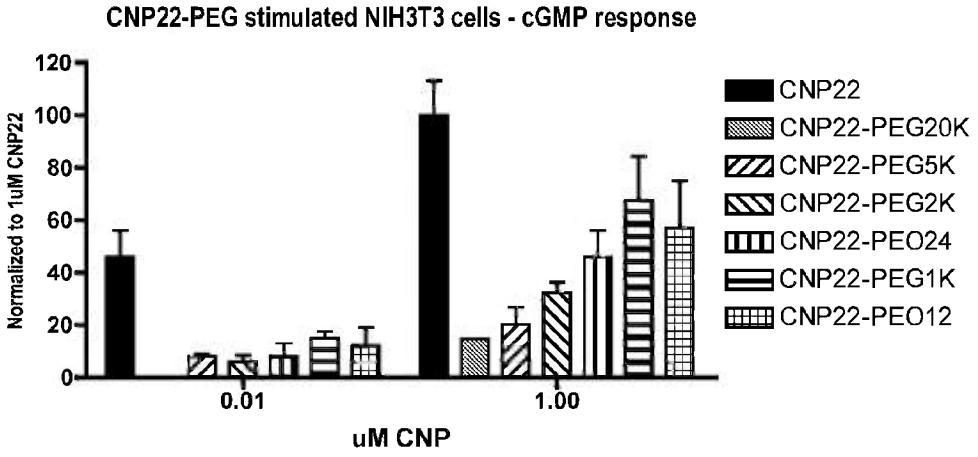
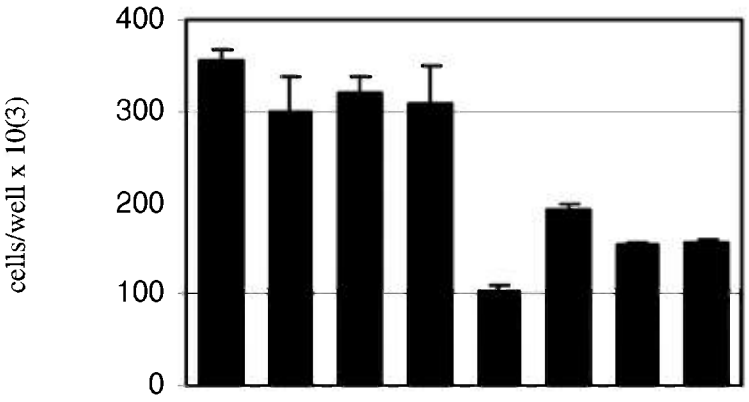


FIGURE 6



Bars from left to right:

1	control (no CNP, no FGF2)
2	CNP (0.2 uM) continuous
3	CNP (0.2 uM) 1 hr once daily
4	CNP (0.2 uM) 2 hr once daily
5	FGF2 (5 ng/mL) continuous
6	FGF2 + CNP (0.2 uM) continuous
7	FGF2 + CNP (0.2 uM) 1 hr daily
8	FGF2 + CNP (0.2 uM) 2 hr daily

FIGURE 7

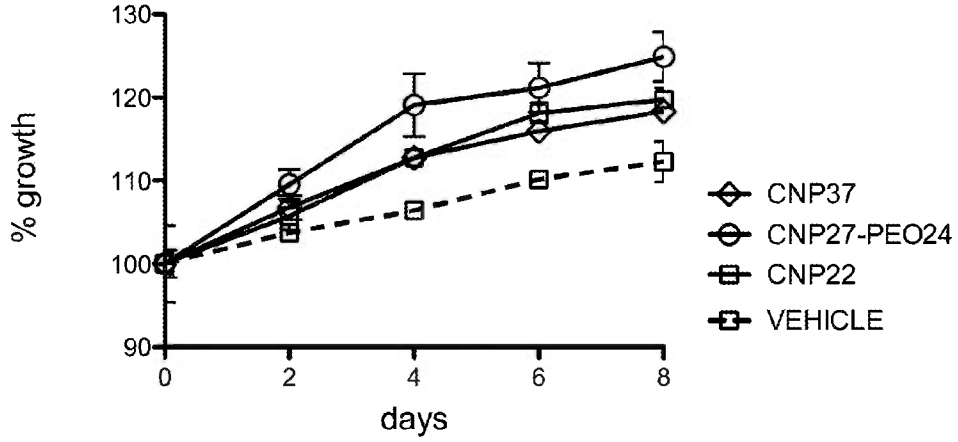


FIGURE 8

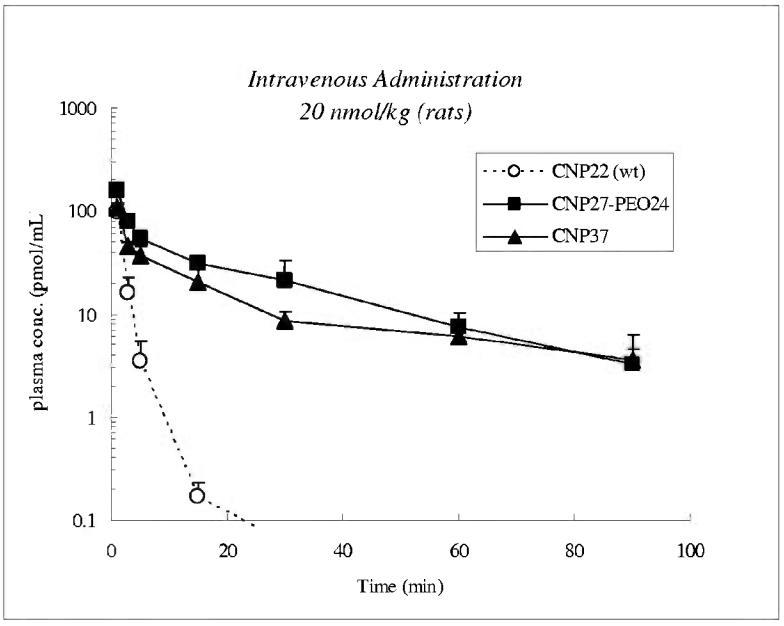


FIGURE 9

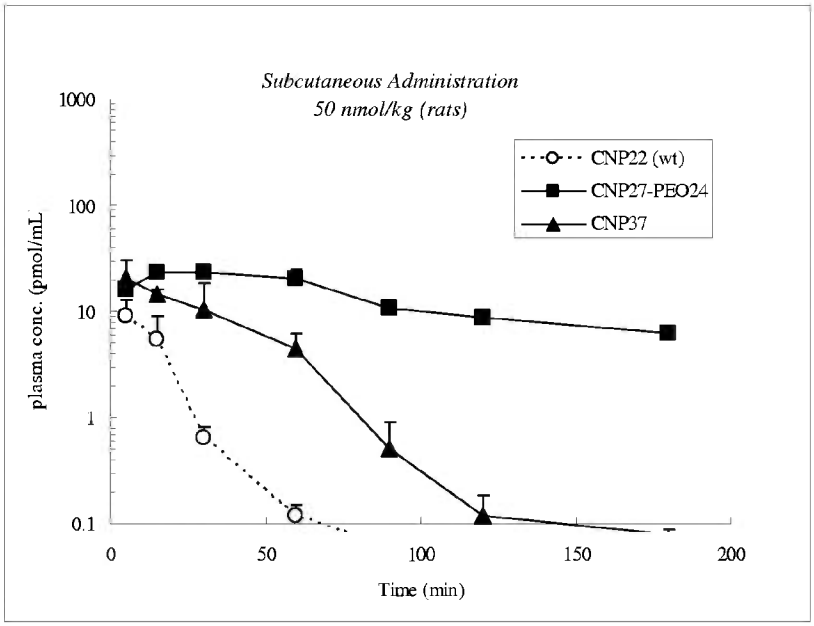


FIGURE 10

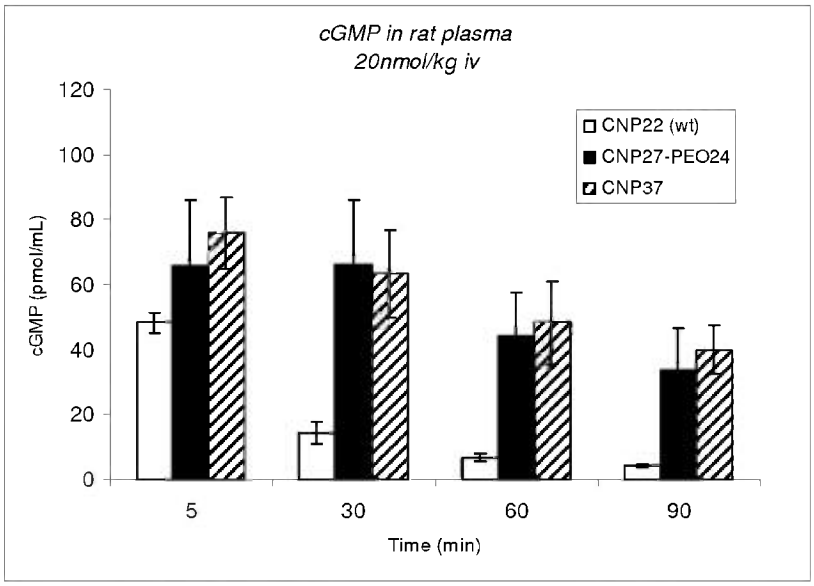


FIGURE 11

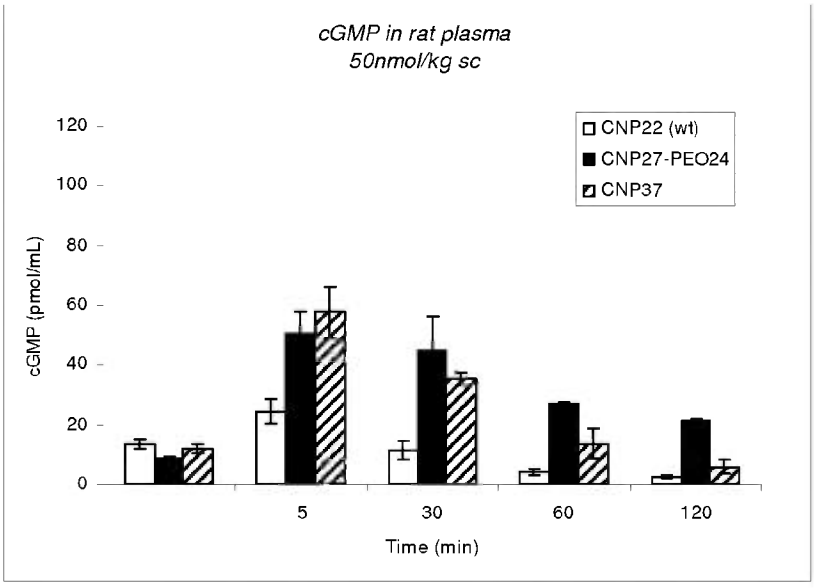


FIGURE 12

Publication Number: WO 2010/135541 A2
Publication Date: Nov 25, 2010

Abstract

The present disclosure provides variants of C-type natriuretic peptide (CNP), pharmaceutical compositions comprising CNP variants, and methods of making CNP variants. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders, such as skeletal dysplasias (e.g., achondroplasia), and vascular smooth muscle disorders (e.g., restenosis and arteriosclerosis).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 November 2010 (25.11.2010)

PCT

(10) International Publication Number
WO 2010/135541 A2

(51) International Patent Classification:
A61K 38/16 (2006.01) *A61K 38/10* (2006.01)
A61K 49/00 (2006.01) *C07K 14/00* (2006.01)
G01N 33/53 (2006.01) *C07K 7/00* (2006.01)
C12Q 1/42 (2006.01) *A61P 9/00* (2006.01)
C12P 21/06 (2006.01) *A61P 9/12* (2006.01)
C12N 1/21 (2006.01) *A61P 13/12* (2006.01)
G01N 33/68 (2006.01) *A61P 19/00* (2006.01)
A61K 38/00 (2006.01)

(21) International Application Number:
PCT/US2010/035586

(22) International Filing Date:
20 May 2010 (20.05.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/180,112 20 May 2009 (20.05.2009) US
61/254,563 23 October 2009 (23.10.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

(57) Abstract: The present disclosure provides variants of C-type natriuretic peptide (CNP), pharmaceutical compositions comprising CNP variants, and methods of making CNP variants. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders, such as skeletal dysplasias (e.g., achondroplasia), and vascular smooth muscle disorders (e.g., restenosis and arteriosclerosis).

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VARIANTS OF C-TYPE NATRIURETIC PEPTIDE

Cross-Reference to Related Applications

The present application claims the priority and benefit of US Provisional Application No. 61/254,563, filed on October 23, 2009, and US Provisional Application No. 61/180,112, filed on May 20, 2009, the disclosure of each of which is incorporated herein by reference in its entirety.

Field of the Disclosure

The field of the disclosure relates, in general, to variants of C-type natriuretic peptide (CNP), compositions comprising CNP variants, methods of making CNP variants, and methods of using CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as skeletal dysplasias (e.g., achondroplasia) and vascular smooth muscle disorders.

Background of the Disclosure

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP) (Genbank Accession No. NP_006163, for the ANP precursor protein, NPPA), brain natriuretic peptide (BNP) (GenBank Accession No. NP_002512, for the BNP precursor protein, NPPB), and C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)). These small, single chain peptides (ANP, BNP, CNP) have a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. ANP and BNP bind to and activate the natriuretic peptide receptor A (NPR-A), also termed guanalyl cyclase A (GC-A), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Likewise, CNP interacts with NPR-B (GC-B) to stimulate the generation of cGMP (J. Hypertens., 10: 1111-1114 (1992)). A third type of receptor, NPR-C, binds each of the natriuretic peptides with high affinity and functions primarily to capture the peptides from the extracellular compartment and deposit the peptides into lysosomes, where they are degraded (Science, 238: 675-678 (1987)). ANP and BNP are produced primarily within the muscle cells of the heart, and are believed to have important roles in cardiovascular homeostasis (Science, 252: 120-123 (1991)). CNP is expressed more widely, including in the

central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). The predominant CNP form in cartilage is unknown. Both CNP-53 and CNP-22 bind similarly to NPR-B. Furthermore, they both induce cGMP production in a dose-dependent and similar fashion (VT Yeung, Peptides, 17: 101-106 (1996)).

Natural CNP gene and polypeptide have been previously described. U.S. Patent No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Patent No. 6,034,231 discloses the human gene and polypeptide of proCNP (126 amino acids) and the human CNP-53 gene and polypeptide.

Clearance of CNP from the extracellular space occurs through the action of membrane-bound neutral endopeptidase (NEP), which rapidly degrades CNP (Biochem. J., 291 (Pt 1): 83-88 (1993)), and through NPR-C, which binds to and deposits CNP into lysosomes, where CNP is degraded. CNP has been shown to have an *in vivo* half-life of 2.6 min in the normal human (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). The low plasma concentration of CNP (J. Bone Moner. Res., 19 (Suppl.1)S20 (2004)) and its co-expression with NPR-B in a number of tissues suggests that CNP functions primarily through an autocrine/paracrine mechanism.

As stated above, CNP binds to and activates natriuretic peptide receptor B (NPR-B), also termed guanylyl cyclase B (GC-B), resulting in higher intracellular cyclic guanosine monophosphate (cGMP) levels. Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. Accordingly, elevated or depressed levels of any of the components in this pathway may lead to aberrant bone growth. For example, knockout of either CNP or NPR-B in mouse models

results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, *et al.*, J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, *et al.*, Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. Both chondrocyte proliferation and differentiation appear to be disturbed, leading to remarkably short growth plate cartilage (P. Krejci et al., J. Cell Sci. 118: 5089-5100 (2005)). Endochondral ossification is the process that governs longitudinal long-bone growth. There are four zones of the growth plate – resting, proliferative, hypertrophic and zone of calcification. In the growth plate, NPR-B is expressed by proliferative cells while NPR-C is expressed by hypertrophic cells (Yamashite et al., J. Biochem. 127: 177-179 (2000)). In normal endochondral bone growth, chondrocytes organize in columns and proliferate in the proliferative zone of the growth plate. These columns are disorganized in achondroplasia patients. Additionally, the hypertrophic zone is where the cells become large and eventually apoptose (lyse), leading to osteocyte invasion and mineralization. The hypertrophic chondrocytes and the overall size of the zone are much smaller in achondroplasia patients than in normal patients. CNP is an agonist for NPR-B, a positive regulator of chondrocyte and bone growth. Downstream signaling of CNP/NPR-B inhibits the FGFR-3 pathway at the level of mitogen-activated protein kinase (MAP K). Inhibition at MAP K promotes proliferation and differentiation of the chondrocytes in the proliferative and hypertrophic zones of the growth plate, resulting in bone growth.

In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

Therapeutic use of CNP is currently limited by its short plasma half-life, which has been shown to be 2.6 minutes *in vivo* in humans (J Clin. Endocrinol. Metab., 78: 1428-35 (1994)). To increase CNP concentration above intrinsic levels (about 5 pM) typically found

in human plasma, continuous infusion has been necessary in all human and animal studies using systemically administered CNP. A CNP variant having a longer *in vivo* serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a sustainable therapeutic strategy. Two mechanisms by which the half-life of CNP is reduced in human plasma are degradation by neutral endopeptidase (NEP) and clearance by natriuretic peptide receptor C (NPR-C) (Growth Horm. & IGF Res., 16: S6-S14 (2006)). Modifications of peptides reportedly can improve resistance to endopeptidase and exopeptidase cleavage (Amino Acids, 30: 351-367 (2006); Curr. Opin. Biotech., 17: 638-642 (2006)).

The biological activities of various analogs and derivatives of CNP have been evaluated. By substituting S-methyl Cys in place of both Cys_6 and Cys_{22}, cyclization of the peptide via a Cys6-Cys22 disulfide linkage was reportedly shown to be important for the activity of CNP in stimulating cGMP formation (Biochem. Biophys. Res. Comm., 183: 964-969 (1992), also using alanine scanning to identify amino acids important for CNP functionality). A significant additional enhancement of activity reportedly results from the combined presence of the amino acids Leu_9, Lys_{10}, and Leu_{11}. U.S. Patent No. 5,434,133 describes CNP analogs comprising CNP-22 with substitutions at amino acid position 6, 7, 9, 11, or 22, wherein the amino acid is selected from Cys or Pmp (pentacyclomercaptopropionic acid) at position 6, Phe, 4-chloro-Phe, 4-fluoro-Phe, 4-nitro-Phe, or Cha (3-cyclohexyl-Ala) at position 7, Gly, Val, Aib, or tLeu at position 9, Leu or Ile at position 11, and Cys or Pmp at position 22.

U.S. Patent Publication No. 2004/0138134 (now US Patent 7,276,481) describes CNP variants comprising amino acids Cys_6 to Cys_{22} of CNP-22 ("CNP-17") which include at least one substitution for another natural amino acid at position 9, 10, 11, 16, 17, 19, or 20, CNP variants with insertions and deletions, such as addition of a His residue at the reported primary site of NEP cleavage, between Cys_6 and Phe_7, and methods of using such variants for increasing the size of a bone growth plate in abnormal bone and elongation of an abnormal bone. However, no significant gains in activity as measured by cGMP production were obtained for these variants, and activity was diminished for nearly all of the variants, as observed in an *in vitro* cell-based method (Example 7). Further no supportive data, such as for example *in vitro* stability or *in vivo* determination of improved pharmacokinetics (PK) were provided to substantiate the asserted NEP resistance and NPR-C resistance of the CNP analogs. U.S. Patent No. 6,743,425 discloses substances for treating achondroplasia which

activate NPR-B/GC-B and are peptides or low molecular weight compounds, including the C-type natriuretic peptides CNP-22 and CNP-53. PCT Publication No. WO 94/20534 discloses a chimera of CNP-22 and the 5-amino acid C-terminus of ANP designated as the vasonatrin peptide (VNP), a limited number of amino acid substitutions and cyclic chimeric peptides that result from formation of a disulfide or double bond.

Approaches for improving the half-life of other natriuretic peptide family members include decreasing the affinity of ANP for NPR-C (U.S. Patent No. 5,846,932), utilizing pentapeptide antagonists of NPR-C (WO 00/61631), and co-administering NEP inhibitors such as thiorphan and candoxatril (Clin. Exp. Pharma. Physiol., 25: 986-991 (1997), Hyperten., 30: 184-190 (1997)). WO 2004/047871 describes conjugates of BNP and BNP variants to polyalkylene glycol moieties, sugar moieties, polysorbate moieties, polycationic moieties, and other hydrophilic polymer moieties that reportedly exhibit improved half-life in circulation and reportedly are useful for the treatment of acute congestive heart failure.

There have been no published reports, however, on a successful strategy for making CNP resistant to NEP while retaining its functionality.

Summary of the Disclosure

The present disclosure relates to variants of C-type natriuretic peptide (CNP) which are useful in the treatment of bone-related disorders (e.g., achondroplasia) and vascular smooth muscle disorders. The disclosure encompasses CNP variants having increased serum half-life, e.g. as a result of reduced ability to bind to neutral endopeptidase (NEP), greater resistance to proteolysis by NEP and/or reduced affinity to the clearance natriuretic peptide receptor C (NPR-C), while retaining the functionality of CNP.

The wild-type sequence of human CNP-22 (referred to herein as "hCNP22", "wtCNP22" or "CNP22") is set forth below:

(N-terminus) Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 1).

Positions 6 to 22 of CNP22 form a cyclic domain by means of a disulfide bond between Cys6 and Cys22. The 17-amino acid cyclic structure has been shown to be important for binding of CNP to NPR-B (Schiller, Biochem. Biophys. Res. Commun., 138: 880-886 (1986)). The amino acid sequence of positions 6 to 22 of CNP22 is referred to herein as "CNP17" (SEQ ID NO: 2).

CNP is susceptible to NEP cleavage at a number of sites: Cys6-Phe7, Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. In one embodiment, the disclosure encompasses a CNP variant that is (1) modified to increase its overall size or molecular weight, e.g., to a range from about 2.6 kDa or 2.8 kDa to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 6.4 kDa, or to about 7 kDa, 7.2 kDa or about 8.2 kDa, and/or (2) modified at certain amino acid positions to reduce its susceptibility to NEP cleavage at 1, 2, 3, 4, 5 or all 6 of the sites listed above. The size or molecular weight of the CNP variant can be increased by various means, e.g., by conjugating additional amino acids and/or other kinds of chemical (e.g., natural or synthetic polymeric) groups to the peptide sequence at, e.g., the N-terminus, the C-terminus and/or side chain(s), and/or by using natural amino acids, unnatural amino acids, and/or peptidomimetics with bulkier side chains. The CNP variant is optionally further conjugated to other functional or structural moieties. Optionally in combination with any of the embodiments described herein, mutation(s) (e.g., substitution(s), addition(s), and/or deletion(s)) may be introduced to certain position(s) of CNP22 to reduce the CNP variants' affinity to NPR-C. Further modifications may be made without affecting NEP resistance or CNP activity, e.g., conservative substitutions, or other modifications known in the art.

In one embodiment, the CNP variant is represented by the general formula: (x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 5), wherein:

the CNP variant comprises one or more modified amino acids, which may result in modified peptide bonds (e.g., through use of peptide bond isosteres), at a position corresponding to one or more of the following CNP residues: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21; and

(x) and (z) independently may be absent or may be an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, ANP, BNP) or a non-natriuretic polypeptide (e.g., human serum albumin (HSA), IgG, etc.).

In an embodiment, the CNP variant includes: (1) a modification at an amino acid position corresponding to one of positions 6, 7 or 8 (Cys6, Phe7 or Gly8) of CNP22, (2) optionally deletion, addition and/or substitution of any or all of the amino acids at positions 1-5 (Gly1, Leu2, Ser3, Lys4, and Gly5) and (3) optionally up to 1, 2, 3, 4, 5, 6 ,7, 8, 9 or 10 further modifications (deletions, additions and/or substitutions) at positions corresponding to

positions 6-22, of which 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 may be conservative substitutions or other substitutions described herein or known in the art.

It is understood that a reference to a particular amino acid position by number (e.g. position 7 of CNP22) refers to the corresponding amino acid position in any CNP variant, even if the number of the position in that CNP variant has changed due to preceding insertions or deletions. For example, a reference to "position 7" or "Phe7" would mean the corresponding position 2 for a CNP variant in which the first five amino acids had been deleted. Similarly, a reference to "position 7" would mean the corresponding position 8 for a CNP variant in which one amino acid had been added to the N-terminus.

In any of the embodiments described herein, the CNP variant may be cyclized through a covalent bond between positions corresponding to 6 and 22 of CNP22. It is contemplated that the covalent bond is formed using any methods known in the art. In another embodiment, the CNP variant may be cyclized through a covalent bond formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus (referred to as "terminal" amino acids for this purpose) of the peptide. In one embodiment, the covalent bond is formed between the side chains of the two terminal amino acids or the amino acids at positions corresponding to 6 and 22 of CNP22. In another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of each terminal amino acid. For example, head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail bonds are possible for the covalent bond formed between the terminal amino acids or between the amino acids at positions corresponding to 6 and 22 of CNP22.

In one embodiment, the disclosure provides a CNP variant having reduced affinity to NEP, and/or greater resistance to cleavage by NEP and/or increased *in vivo* serum half-life, while retaining functionality of CNP (e.g., stimulation of cGMP production). NEP preferably recognizes substrates smaller than about 3 kDa, due to the limited size of its active site cavity (Oefner, J. Mol. Biol., 296: 341-349 (2000)). In an embodiment, the CNP variants are modified to increase their overall molecular weight to a range from about 2.6 or 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa, e.g., by adding about 0.6 to about 5 kDa of amino acids, hydrophilic or water-soluble polymers, hydrophobic acids (including fatty acids), and/or carbohydrates. In specific exemplary embodiments, the CNP variants have a molecular weight between about 2.6 kDa and about 7 kDa, or between about 2.8 kDa and 6 kDa, or between about 2.8 kDa and about 5.8 kDa. In certain embodiments, at least about

0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa are added, to increase the total molecular weight of the CNP variant, e.g., to a range from about 2.6 or 2.8 kDa up to about 4 kDa, 4.2 kDa, 4.4 kDa, 4.6 kDa, 4.8 kDa, 5 kDa, 5.2 kDa, 5.4 kDa, 5.6 kDa, 5.8 kDa, 6 kDa, 6.2 kDa, 7.2 kDa, 8.2 kDa or higher. In some embodiments, such CNP variants comprise an amino acid sequence at least about 70%, 75%. 80%, 85%, 90%, or 95% identical or homologous to amino acids 6-22 of CNP22. In other embodiments, such CNP variants comprise a substitution, insertion or deletion of 1, 2, 3, 4, 5, 6 or 7 amino acids with another natural or unnatural amino acid or peptidomimetic. While both conservative and non-conservative substitutions or insertions are envisioned at any position, introduction of modifications may commence, e.g., by conservative substitutions in regions that have been identified in the art as involved in CNP activity or NPR-B binding, while non-conservative substitutions may be made in those regions that have been previously shown to be tolerant of modification.

In another embodiment, the CNP variants comprise a CNP having an intact cyclized portion between Cys6 and Cys22, and N-terminal and/or C-terminal tails that contain about 1-40, 1-20, 5-40, 5-35, 10-35, 15-35, 5-31, 10-31, or 15-31 amino acids and are fragments derived from a CNP polypeptide and/or a non-CNP polypeptide. In an embodiment, such CNP variants have a molecular weight in a range from about 2.8 kDa to about 4, 4.6, 5, 5.2, 5.8, 6, 6.4 or 7 kDa. Non-limiting examples of such CNP variants include wild-type CNP22 or CNP22 with one or more amino acid substitutions (e.g., a K4R substitution), having an N-terminal and/or C-terminal extension derived from natriuretic peptide precursor sequences (e.g., ANP, BNP or CNP) from human or other species, a natriuretic peptide precursor C (NPPC) variant with amino acid substitutions, additions and/or deletions (e.g., the CNP variants may be truncations of CNP-53 which result in peptides with a molecular weight between about 2.8 kDa and 5.8 kDa), or other non-CNP polypeptides such as, e.g., serum albumin or IgG protein (e.g., the CNP variants may be CNP chimeras containing fragments of serum albumin or IgG from human or other species).

In one embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG, also called polyethylene oxide (PEO)), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may each be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

Gly-Ala-Asn-Lys-Lys (SEQ ID NO:7),

Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 8),

Gly-Ala-Asn-Pro-Arg (SEQ ID NO: 9),

Gly-Ala-Asn-Gln-Gln (SEQ ID NO: 10),

Gly-Ala-Asn-Ser-Ser (SEQ ID NO: 11),

Gly-Ala-Asn-Arg-Gln (SEQ ID NO: 12),

Gly-Ala-Asn-Arg-Met (SEQ ID NO: 13),

Gly-Ala-Asn-Arg-Thr (SEQ ID NO: 14),

Gly-Ala-Asn-Arg-Ser (SEQ ID NO: 15),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala (SEQ ID NO: 16),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg (SEQ ID NO: 17),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg (SEQ ID NO: 18),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 19),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg, (SEQ ID NO: 20)

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 21), and

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 22).

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

Ser-Leu-Arg-Arg-Ser-Ser (SEQ ID NO: 23);

Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 24);

Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 25);

Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 26);

Lys-Val-Leu-Arg-Arg-Tyr (SEQ ID NO: 27);

Lys-Val-Leu-Arg-Arg-His (SEQ ID NO: 28);

Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg (SEQ ID NO: 29);

Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr (SEQ ID NO: 30);

Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln (SEQ ID NO: 31); and

Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser (SEQ ID NO: 32).

In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently may be conjugated to an amino acid extension containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acids. In one embodiment, the amino acid extension is derived from NPPC, CNP53, ANP or BNP. In a specific embodiment, the amino acid extension is Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 33). In a related embodiment, this 15-amino acid extension is added to the N-terminus to provide a CNP variant of the formula Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 34).

In one embodiment, the CNP variants comprise wtCNP22 or a variant thereof (e.g., one having addition(s), deletion(s), and/or substitution(s) such as, e.g., a K4R substitution) (SEQ ID NO: 35) conjugated at the N-terminus and/or C-terminus to a hydrophilic polymer (e.g., PEG) to increase their overall molecular size to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. Such CNP variants are optionally further conjugated at the N-terminus and/or C-terminus to a polymeric group comprising, e.g., amino acids, carbohydrates, hydrophobic acids and/or phospholipids, a non-limiting example of which is an N-terminal amino acid extension containing 1 to 35, or 5 to 31, amino acids. In an embodiment, a hydrophilic polymeric (e.g., PEG) moiety of at least about 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 or 1.8 kDa, or up to about 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8 or 5 kDa, is added to the N-terminus and/or C-terminus of wtCNP22 or a variant thereof.

As shown herein, conjugation of a hydrophilic or water-soluble PEG (or PEO) polymer of about 0.6 kDa or more to CNP22 or variants thereof generally increases resistance to NEP cleavage markedly. However, addition of PEG, even as small as 0.6 kDa, to wtCNP22 may reduce CNP functionality (e.g., stimulation of cGMP signaling), and addition of greater than about 2 or 3 kDa of PEG to CNP22 or variants thereof may reduce CNP functional activity in a size-dependent manner. But CNP functionality (at least comparable to that of wtCNP22) is retained when a PEG (or PEO) polymer of about 0.6 kDa to about 1.2 kDa, or potentially to about 2 kDa, is conjugated to a CNP variant having an N-terminal amino acid extension in which at least one relatively large amino acid that may potentially be positively charged under physiological conditions (e.g., arginine) immediately precedes the position corresponding to Gly1 of CNP22, such as, e.g., GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), ER-CNP22 (SEQ ID NO: 38), ER-CNP22(K4R) (SEQ ID NO: 39), R-CNP22 (SEQ ID NO: 40) and R-CNP22(K4R) (SEQ ID NO: 41).

Accordingly, in one embodiment, PEGylated CNP variants comprise at the N-terminus of CNP22 or a variant thereof (e.g., one having a K4R substitution) an amino acid extension containing at least 1, 2, 3, 4 or 5 amino acids, wherein the PEG polymer is conjugated to the N-terminus of the amino acid-extended CNP variant to result in a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP cleavage. In an embodiment, for enhanced CNP functionality, such pegylated, amino acid-extended CNP variants contain at least one relatively large natural or unnatural amino acid that may potentially be positively charged

under physiological conditions, immediately preceding the position corresponding to Gly1 of CNP22. In a specific embodiment, the pegylated, amino acid-extended CNP variants contain at least one arginine residue immediately preceding the position corresponding to Gly1 of CNP22.

In addition to CNP variants conjugated at the N-terminus and/or C-terminus to a hydrophilic or water-soluble polymer such as, e.g., PEG (or PEO), the disclosure encompasses CNP variants conjugated to such a polymer at an internal site. For purposes of brevity here, PEG (or PEO) will be used as a representative example of a hydrophilic or water-soluble polymer. Various sites of PEGylation of a CNP variant are possible, including but not limited to: (1) PEGylation only at the N-terminus; (2) PEGylation only at the C-terminus; (3) PEGylation only at an internal site (e.g., Lys4); (4) PEGylation at both the N-terminus and the C-terminus; (5) PEGylation at the N-terminus and an internal site; and (6) PEGylation at the C-terminus and an internal site. For increased resistance to NEP degradation and retention of CNP functionality, in certain embodiments the total mass of PEGylatedCNP variants is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6 or 7 kDa. In a particular embodiment, CNP17, CNP22, CNP37 (defined below) or variants thereof (including those having amino acid additions, substitutions and/or deletions) are PEGylated only at the N-terminus. In another embodiment, the CNP variants are PEGylated only at an internal site (e.g., Lys4). In yet another embodiment, the CNP variants are PEGylated at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP variants are not PEGylated at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). To prevent PEGylation at an internal site, Lys4 and/or Lys10 can be substituted with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG molecules) that may vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and

length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP-22 (2.2kDa), CNP-17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22, or variants with amino acid substitutions, additions and/or deletions, for example, GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), R-CNP22 (SEQ ID NO: 40), R-CNP22(K4R) (SEQ ID NO: 41), ER-CNP22 (SEQ ID NO: 38) and ER-CNP22(K4R) (SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) group conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG group conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure further contemplates negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds.

In a related embodiment, the disclosure contemplates PEG-CNP conjugates comprising NHS- or aldehyde-based PEG of the formula $(CH_2CH_2O)_n$, wherein n is an integer from 12 to 50, and the PEG polymer is up to about 2.5 kDa in molecular weight. In a specific embodiment, n is 12 or 24. In an embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In an additional embodiment, the PEG polymers or derivatives thereof have a polymer number-average molecular weight in the range from about 0.4 kDa to about 2.5 kDa or from about 0.6 kDa to about 1.5 kDa.

In a further embodiment, the wtCNP or CNP variant peptide is conjugated to a moiety including, e.g., bisphosphonates, carbohydrates, hydrophobic acids (including fatty acids) or amino acid sequences. Such amino acid sequences include for example polyAsp or polyGlu useful in bone/cartilage targeting, or can be derived from bone proteins with elucidated bone-targeting domains or derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc. In embodiments described herein where CNP22 or a variant thereof is attached to a bone- or cartilage-targeting moiety, such a

moiety is designed to promote getting the modified CNP peptide to chondrocytes of bone growth plates, where the peptide can bind and activate NPR-B on the chondrocytes.

In another embodiment, the disclosure provides CNP variants with a peptide bond that is less susceptible to cleavage by peptidases including NEP. The disclosure encompasses a CNP variant comprising at least one modified residue at a site of endopeptidase cleavage. In one embodiment, the Cys6-Phe7 peptide bond (-C(=O)-NH-) at an NEP cleavage site in CNP can be replaced with anyone of the following peptide-bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, and

-NHC(=O)NH-.

In another embodiment, Phe7 is substituted with its enantiomer D-Phe. In yet another embodiment, D-enantiomers are introduced to one or more, up to all 22, positions within wtCNP-22. In a further embodiment, a beta amino acid such as 3-amino-2-phenylpropionic acid is substituted for Phe7, which increases the length of the backbone while reducing the length of the side chain. In yet another embodiment, the disclosure contemplates Cys analogs at the Cys6 position including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid.

Even in the presence of a NEP-resistant bond between Cys6 and Phe7, other peptide bonds can be hydrolyzed by NEP, including Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20. Accordingly, the disclosure encompasses CNP analogs containing peptide bond isosteres at multiple locations in the backbone of the CNP analogs. In one embodiment, CNP analogs or variants comprise modifications at more than one peptidase

cleavage site. In a further embodiment, such variant comprises a CNP with substitutions at amino acid residues important in binding to the NEP active site, thereby increasing resistance to NEP degradation. One or more NEP-binding residues, including but not limited to Gly8, Gly15, Ser18, Gly19 and/or Gly21, are replaced with larger-size natural or unnatural amino acid residues to reduce affinity to the NEP active site. In yet another embodiment, one or more hydrophobic residues essential in NEP recognition, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, are substituted with natural or unnatural amino acids and/or peptidomimetics that decrease NEP binding. In yet another embodiment, one to five of the first five amino acids of CNP can be deleted or substituted with any other natural amino acids or unnatural amino acids or peptidomimetics, or one or more natural or unnatural amino acids or peptidomimetics can be added to any one or to all of the first five positions of CNP.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-$(a)_4$-Gly_5-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone proteins with elucidated bone-targeting domains, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; polymeric or non-polymeric molecules that reduce renal clearance such as, e.g., charged PEG molecules; and extensions comprising, e.g., polymers (e.g., PEGs), carbohydrates, hydrophobic acids (including fatty acids), and/or amino acids, and wherein such amino acid extensions can contain, e.g., from 1 to 31, or 1 to 35, or 5 to 35, or 10 to 35, or 15 to 35 amino acid residues, and can be derived from NPPC, ANP, BNP, other non-CNP (poly)peptides such as, e.g., serum albumin or IgG, or variants of the aforementioned polypeptides having substitutions, additions and/or deletions, or combinations thereof;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone or cartilage targeting such as for example polyAsp and polyGlu, amino acid sequences from bone-targeting domains of bone proteins such as, e.g.,

osteopontin, osteocalcin and sialoprotein, and amino acid sequences derived from non-CNP (poly)peptides such as, e.g., ANP or BNP;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In one embodiment, the CNP variants comprise a modification at one or more of positions 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and/or 20, and may optionally have modifications at any of the other positions disclosed herein.

In embodiments described herein where CNP22 or variants thereof can be attached to a hydrophobic acid, the CNP peptides can be attached to one or hydrophobic acids. Non-limiting examples of hydrophobic acids include straight-chain or branched, saturated or unsaturated C_5-C_{12} carboxylic acids (e.g., pentanoic acid, heptanoic acid, etc.) and natural fatty acids. The hydrophobic acids can be attached to the N-terminus, the C-terminus, and/or the side chain of one or more amino acid residues. In one embodiment, the hydrophobic acids are conjugated to the N-terminus. In an embodiment, conjugation of CNP22 or a variant thereof to a hydrophobic acid is designed, *inter alia*, to promote non-specific interaction between the modified CNP peptide and serum albumin, thereby increasing the size of the CNP peptide and protecting it from cleavage by proteases such as, e.g., NEP. The interaction between the hydrophobic acid-conjugated CNP peptide and albumin is designed to be not too strong, so that the modified CNP peptide can diffuse through cartilage, get to chondrocytes of bone growth plates, and bind and activate NPR-B.

In a further embodiment, the disclosure provides CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), comprise at least one modified amino acid at position $(b)_6$, $(c)_7$ and/or $(d)_8$, and are represented by the general formula:

$(x)\text{-}Gly_1\text{-}Leu_2\text{-}Ser_3\text{-}(a)_4\text{-}Gly_5\text{-}(b)_6\text{-}(c)_7\text{-}(d)_8\text{-}(e)_9\text{-}(f)_{10}\text{-}(g)_{11}\text{-}Asp_{12}\text{-}Arg_{13}\text{-}(h)_{14}\text{-}Gly_{15}\text{-}Ser_{16}\text{-}(i)_{17}\text{-}Ser_{18}\text{-}Gly_{19}\text{-}(j)_{20}\text{-}Gly_{21}\text{-}Cys_{22}\text{-}(z)$ (SEQ ID NO: 47), wherein:

(x) may be absent or may be a peptide sequence containing one to five amino acids which is derived from a natriuretic polypeptide (e.g. NPPC, CNP, ANP or BNP) or a non-natriuretic polypeptide as described herein (e.g., HSA, IgG, a bone-targeting protein, etc.);

(z) may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as, e.g., polyAsp and polyGlu; bone proteins with bone-targeting domains and derivatives thereof, such as fusion proteins or peptides sequences of osteopontin, osteocalcin, and sialoprotein; molecules that reduce renal clearance, such as, e.g., charged PEGs; and molecules that increase resistance of CNP to NEP-mediated degradation, as described herein;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) may be Cys or descarboxy cysteine, or the $(b)_6$-$(c)_7$ peptide bond (-C(=O)-NH-) may be replaced with any one of the following peptide bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, or

-NHC(=O)NH-;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; peptide bond isosteres of Phe such as N-alkylated derivatives of Phe wherein the N-

alkyl group is selected from the group consisting of methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro-phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) is any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amino group on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the disclosure encompasses CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of

wtCNP22 (e.g., 1 uM), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased resistance to NEP degradation, and are represented by the general formula:

(x)-(y)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 48), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is polyethylene glycol (PEG), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or FGF2;

(y) may be absent or may be one or more amino acids from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (corresponding to positions 1 to 5 of CNP22) (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution);

(h) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (h) is not Arg; and

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG).

In an embodiment, (x), (y) and (z) together contain from about 10 to about 40, or from about 15 to about 35 amino acids. In another embodiment, (x) is an amino acid sequence comprising from 1 to 40 amino acids, or from 1 to 20 amino acids.

Further contemplated are CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM), and comprise the sequence:

(y)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22} (SEQ ID NO: 138), wherein:

(y) comprises one or more amino acids selected from Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1) and/or substitutions at one or more of those positions using natural or unnatural amino acids (e.g., K4R substitution), and further comprises a hydrophilic or water soluble polymer of molecular weight from about 0.6 kDa to about 5 kDa. In an embodiment, the hydrophilic or water-soluble polymer is conjugated to the N-terminus of such amino acid-extended CNP variant. In a particular embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In yet another embodiment, the disclosure provides CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM), wherein the CNP variants comprise an N-terminal and/or C-terminal peptide extension containing from 1 to 15 amino acids, and are conjugated to a hydrophilic or water soluble polymer. In an embodiment, the peptide extension contains from 5 to 10 amino acids. In a specific embodiment, the peptide extension contains 5 amino acids. In another specific embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO).

In a still further embodiment, the CNP variants of the disclosure *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced underthe same concentration of wtCNP22 (e.g. 1 uM), and comprise at least a 15 amino acid fragment derived from natriuretic peptide precursor C (NPPC), wherein the fragment is at least 70% homologous to a sequence from wild type NPPC containing the same number of amino acid residues.

In still another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

(x)-$(b)_6$-$(c)_7$-$(d)_8$-$(e)_9$-$(f)_{10}$-$(g)_{11}$-Asp_{12}-Arg_{13}-$(h)_{14}$-Gly_{15}-Ser_{16}-$(i)_{17}$-Ser_{18}-Gly_{19}-$(j)_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 49), wherein:

(x) may be absent (i.e., the N-terminus ends with an -NH_2 group) or may be selected from the group consisting of a sequence of 1, 2, 3, 4 or 5 amino acids from the peptide Gly_1-Leu_2-Ser_3-Lys_4-Gly_5 (SEQ ID NO: 1); amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; bone-targeting domains from bone

proteins such as for example osteopontin, osteocalcin or sialoprotein; molecules that reduce renal clearance such as hydrophilic or water-soluble polymers, including but not limited to charged PEG molecules; and moieties comprising PEG, carbohydrates, hydrophobic acids, amino acids, or combinations thereof, wherein such moieties can be amino acid extensions including but not limited to amino acid sequences derived from NPPC or non-CNP (poly)peptides such as, e.g., BNP, ANP, serum albumin or IgG;

(z) may be absent or may be selected from the group consisting of amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting proteins, such as for example osteopontin, osteocalcin or sialoprotein; and amino acid sequences derived from NPPC or non-CNP (poly)peptides, as described herein;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Val, Ser, Thr and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-

hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa for increased NEP resistance, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-(i)$_{15}$-Ser_{16}-(j)$_{17}$-Ser_{18}-Gly_{19}-(k)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 50), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone/cartilage targeting such as for example polyAsp or polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as for example fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieties comprising, e.g., hydrophilic polymers (e.g., PEG), carbohydrates, hydrophobic acids, and/or amino acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

In a further embodiment, the CNP variants can have amino acid substitution(s) at one or more of any of positions 1 to 22 of CNP22. In one embodiment, Gly1 is substituted with Arg or Glu. In another embodiment, Lys4 is replaced with Arg. In still another embodiment, Gly5 is substituted with Arg, Gln or Ser. In yet another embodiment, Gly15 is substituted with Ser, Asn, Arg or Cit. In a further embodiment, Gly19 is substituted with Ser, Arg or Asn. In yet another embodiment, Gly21 is substituted with Ser, Thr, or Arg.

In one embodiment, the CNP variant is selected from the group consisting of GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (formed using descarboxy-Cys) (SEQ ID NO: 56), GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO: 57), GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC(SEQ ID NO:136), GLSKGCF-(tBuG)-LKLDRIGSMSGLGC (SEQ ID NO: 58), GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (SEQ ID NO:137), and GLSKGC-[$NHCH_2CH(Ph)CO$]-GLKLDRIGSMSGLGC (formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59). In a further embodiment, a disulfide bond exists between Cys6, descarboxy-Cys or another sulfhydryl-containing cysteine analog at the Cys6 position, and Cys22 of any CNP variant described herein.

In another embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-53**) (SEQ ID NO: 4);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-37**, **Analog BL**) (SEQ ID NO: 60);

AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (**Analog CA**) (SEQ ID NO: 61);

AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (**Analog CB**) (SEQ ID NO: 62);

DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (**Analog CC**) (SEQ ID NO: 63);

RGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 40);

ERGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 38);

GANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 64);

GANRRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 65);

GANPRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 66);

GANSSGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 67);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144) (sometimes designated "CNP27-HSA" or "HSA-CNP27" in the Examples and figures);

SPKMVQGSG-CNP17-KVLRRH (**Analog CD**) (SEQ ID NO: 68) (CNP17 having N-terminal and C-terminal tails derived from BNP).

In a further embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:

GANRRGLSRGCFGLKLDRIGSMSGLGC (**Analog AY**) ((SEQ ID NO: 36);

GANPRGLSRGCFGLKLDRIGSMSGLGC (**Analog CI**) (SEQ ID NO: 37);

RGLSRGCFGLKLDRIGSMSGLGC (**Analog AZ**) (SEQ ID NO: 41);

ERGLSRGCFGLKLDRIGSMSGLGC (**Analog BA**) (SEQ ID NO: 39);

GANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CH**) (SEQ ID NO: 69); and

GANSSGLSRGCFGLKLDRIGSMSGLGC (**Analog CG**) (SEQ ID NO: 70).

In one embodiment, CNP variants having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 65), PEO12-GANRR-CNP22(SEQ ID NO: 65), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22(SEQ ID NO: 66), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22(K4R) (SEQ ID NO: 39), PEO12-ER-CNP22(K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 38), PEO12-ER-CNP22(SEQ ID NO: 38), PEO24-R-CNP22(K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 40), and PEO12-R-CNP22(SEQ ID NO: 40), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In an embodiment, the PEG (or PEO) polymer is attached to the N-terminus of the CNP variants.

Additional CNP variants include, but are not limited to, derivatives of CNP37 having mutation(s) at the furin cleavage site (underlined), designed to improve *in vivo* resistance to the furin protease, and/or having glycine (underlined) preceding glutamine, designed to prevent pyroglutamine formation, including but not limited to:

GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (**An. CS**) (SEQ ID NO: 71);

GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (**An. CT**) (SEQ ID NO: 72);

GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (**An. CU**) (SEQ ID NO: 73);
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (**An. CW**) (SEQ ID NO: 74);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37, **An. DB**) (SEQ ID NO: 75);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 145).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CQ**) (histidine-rich glycoprotein (HRGP) fragment-CNP22 chimera) (SEQ ID NO: 76);
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CR**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 77);
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CX**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 78);
GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (**Analog CF**) ($IgG_1(F_c)$ fragment-CNP22 chimera) (SEQ ID NO: 79);
GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CY**) (human serum albumin (HSA) fragment-CNP22 chimera) (SEQ ID NO: 80);
GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (**Analog CE**) (HSA fragment-CNP22 chimera) (SEQ ID NO: 81);
FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CZ**) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera) (SEQ ID NO: 82); and
GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (**Analog DA**) (FGF2 "heparin-binding domain" fragment-CNP22 chimera) (SEQ ID NO: 83).

In a further embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22(K4R)"), including but not limited to:
GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CK**) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera) (SEQ ID NO: 84);
GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CL**) (HSA fragment-CNP22(K4R) chimera) (SEQ ID NO: 85
GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CM**) (fibronectin fragment-CNP22(K4R) chimera) (SEQ ID NO: 86);

GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CN**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 87);

GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CO**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 88); and

GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CP**) (zinc finger fragment-CNP22(K4R) chimera) (SEQ ID NO: 89).

In yet another embodiment, the CNP variants are chimera, or fusion proteins, comprising a CNP peptide or variant, and a cleavable peptide or protein, or peptide tag. Exemplary cleavable proteins or peptides include, but are not limited to, histidine (e.g., hexa-His) tags; TAF12: human transcription factor TAF12; KSI: ketosteroid isomerase; MBP: maltose-binding protein; ß-Gal: ß-galactosidase; GST: glutathione-S-transferase; Trx: thioredoxin; CBD: chitin binding domain; BMPM: BMP-2 mutation, SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly(Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

In yet another embodiment, the CNP variant may be a monomer or a dimer. In a related embodiment the monomers of dimeric CNP variants can be attached N-terminus to N-terminus via a linker or no linker, N-terminus to C-terminus via a linker or no linker, or C-terminus to C-terminus via a linker or no linker.

Chimera comprising an IgG fragment and CNP22 or a variant thereof are designed for, *inter alia*, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, *inter alia*, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, *inter alia*, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin,

fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other advantageous features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In any of the embodiments disclosed herein, the CNP variants may have substantially the same or better biological activity than wild-type CNP22. For example, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, e.g., with respect to interaction with NPR-B (GC-B) to stimulate the generation of cGMP. Alternatively, or in addition, the CNP variants may retain at least 50%, 60%, 70%, 80%, 90%, 95% or more of the activity of wild-type CNP22, or may have greater activity than CNP22, with respect to regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation, chondrocyte differentiation, inhibition of the mitogen activated protein (MAP) kinase/ MEK (Raf-1) kinase signaling pathway, and promoting endochondral ossification. In any of the embodiments described herein, the CNP variants may comprise an amino acid

sequence that is at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or more identical or homologous to amino acids 6-22 or 1-22 of wild-type CNP22.

In a further embodiment, the disclosure provides variants of CNP22 having less affinity to the NPR-C clearance receptor while retaining the ability to bind and activate NPR-B. The present disclosure encompasses variants that were, or can be, generated from a homology-based structural model of the NPR-B/CNP complex as described in the Detailed Description. In another embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility, which may increase their specificity for binding to NPR-B over NPR-C. Variants of CNP having potentially reduced affinity to the NPR-C include but are not limited to those having one or more of the following substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R.

In yet another embodiment, the CNP variants have a modification and/or substitution at one or more of positions 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and 21, and may optionally have modifications and/or substitutions at any of the other positions disclosed herein. In a further embodiment, the CNP variants can optionally have conjugation(s) or extension(s), e.g., at the N- and/or C-terminus to facilitate bone/cartilage targeting, reduce renal clearance, and/or increase resistance to NEP degradation. Such conjugation(s) or extension(s) can comprise molecules or sequences formed or derived from, e.g., polyAsp, polyGlu, bone- or cartilage-targeting peptides, osteopontin, osteocalcin, sialoprotein, PEGs, carbohydrates, hydrophobic acids, NPPC or non-CNP (poly)peptides, or combinations thereof.

In still another embodiment, the CNP variants are prepared by standard solid-phase peptide synthesis methods with natural or unnatural amino acid(s) or peptidomimetic(s) being substituted and/or added where appropriate. In another embodiment, the CNP variants are produced by recombinant synthesis processes, e.g., via fusion proteins containing a tag or carrier protein, wherein use of the tag or carrier protein facilitates, e.g., detection, isolation and/or purification of the fusion protein, and selective chemical or proteolytic cleavage of the tag or carrier protein from the fusion protein provides the target CNP variant. In a further embodiment, PEGylation of the CNP variants occurs following, or part of, chemical or biological synthesis with the conjugation reaction being performed by NHS- or aldehyde-based chemistry or other chemistry known in the art. In another embodiment, the CNP variants comprise a disulfide bond. In a related embodiment, the disulfide bond forms a

cyclic peptide. In a particular embodiment, the disulfide bond is formed between cysteine residues at positions corresponding to positions 6 and 22 of CNP22.

It is further contemplated that the CNP variants can be conjugated to a hydrophobic polymeric or non-polymeric moiety, such as, e.g., heptanoic acid, pentanoic acid, or fatty acids. The hydrophobic moiety can be conjugated to the side chain of an amino acid residue, including but not limited to a lysine, a serine, a cysteine or a threonine, or can be attached to the N-terminus and/or C-terminus of the CNP variant.

In an embodiment, the CNP variants as described herein have a pI in the range from about 8 to about 10.5 or from about 8.5 to about 10.

In a further embodiment, the disclosure provides a pharmaceutical composition comprising a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the compositions are sterile pharmaceutical compositions suitable for parenteral injection. In some embodiments, the compositions comprise substantially pure CNP variant, e.g. at least about 90% or 95% pure. In some embodiments, the compositions contain less than about 5%, 4%, 3%, 2%, 1% or 0.5% contaminants, such as other human proteins, porcine proteins, or CNP53 or fragments thereof (other than the desired CNP variant). In certain embodiments, the sterile composition is administered to a subject for treating or preventing any of the CNP-responsive conditions or disorders disclosed herein.

CNP variants of the disclosure advantageously retain CNP activity and exhibit increased serum half-life. Retention of CNP activity can be shown, for example, as retention of desired *in vivo* biological effect, or retention of at least about 50%, 60%, 70%, 80%, 90%, 95% or at least about 100% of the cGMP stimulating activity of CNP22, under the same concentration (e.g., 1 uM of CNP peptide or greater than the ED80). In some embodiments, CNP variants exhibit at least about 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or 40-fold increase in serum half-life compared to CNP22.

In a related embodiment, the CNP variants described herein have increased NEP resistance and exhibit increased half-life compared to wild-type CNP22. In one embodiment, the half-life of the CNP variants is increased by about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or about 100% compared to wild-type CNP22.

In certain embodiments, the CNP variants described herein increase cGMP production *in vitro*, increase cGMP production *in vivo*, increase *in vivo* the level of one or more

biomarkers associated with cartilage or bone formation or growth, increase resistance to NEP cleavage *in vitro*, increase plasma or serum half-life *in vivo*, increase bioavailability *in vivo*, or increase the length of particular bones *in vivo*, or effect combinations of such increases, by about 1.5-fold, about 2-fold, about 2.5-fold, about 3-fold, about 3.5-fold, about 4-fold, about 4.5-fold, or about 5-fold or more compared to wild-type CNP22.

In yet another embodiment, the disclosure provides methods of treating conditions or disorders responsive to CNP, comprising administering a therapeutically effective amount of a CNP variant or a composition comprising the same to a subject in need thereof. In one embodiment, disorders responsive to CNP are disorders of bone growth, including but not limited to skeletal dysplasias and inherited skeletal malformations such as disorders associated with fibroblast growth factor receptor 3 (FGFR-3) mutations. In a specific embodiment, the disorder associated with FGFR-3 mutation(s) is achondroplasia. In another embodiment, the disorders responsive to CNP are disorders associated with vascular smooth muscle cells and tissues. In a further embodiment, the CNP variants are useful for increasing the size of the growth plate of a bone (e.g., a limb bone). In another embodiment, the CNP variants are useful for elongating a bone or increasing long bone growth. In still another embodiment, the CNP variants are useful for enhancing matrix production, proliferation and differentiation of chondrocytes.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750 or 800 ug/kg, or about 1 mg/kg, 1.25 mg/kg, 1.5 mg/kg, 2 mg/kg or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

In another embodiment, the CNP variants are administered in a single treatment or in multiple doses. The multiple doses may be administered daily, or in multiple doses over the

course of treatment. In certain embodiments, it is contemplated that the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months or as deemed appropriate by a treating physician.

In certain embodiments, administration of the CNP variant is adjusted to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered subcutaneously, intravenously, or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

In additional embodiments, the disclosure provides a method of treating a CNP-responsive condition or disorder, comprising administering a CNP peptide or variant to a subject, and monitoring the level of at least one bone- or cartilage-associated biomarker in the subject (e.g., in a biological sample from the subject), wherein an increase or decrease in the level of the bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In some embodiments, when the level of a biomarker increases in association with bone or cartilage formation or growth, an increase in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject. In other embodiments, when the level of a biomarker decreases in association with bone or cartilage formation or growth, a decrease in the level of that biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject.

In further embodiments, the therapeutic method further comprises adjusting the amount (or dose) or frequency of administration of the CNP peptide or variant, wherein:

(i) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a

target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(ii) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker increases in association with bone or cartilage formation or growth; or

(iii) the amount (or dose) or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is above a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth; or

(iv) the amount (or dose) or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is below a target level, where the level of the biomarker decreases in association with bone or cartilage formation or growth.

It is contemplated that the target level of a biomarker refers to the level or range of levels of the biomarker that is associated with therapeutic effect in the subject and/or beneficial effect in alleviating or ameliorating symptoms of the disorder or condition. In certain embodiments, a level of a biomarker above or below a target level may be deleterious to the subject.

In other embodiments, the disclosure contemplates a method for assessing the effect of administration of a CNP peptide or variant on bone or cartilage formation or growth. In one embodiment, the method provides for assaying or measuring the level of at least one bone- or cartilage-associated biomarker in a subject that has been administered a CNP peptide or variant in order to assess the effect of the CNP peptide or variant on bone and cartilage formation and growth *in vivo*. In a related embodiment, an increase in the level of the at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP peptide or variant has a positive effect on bone or cartilage formation or growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (*e.g*, endogenous level of CNP-22 or CNP-53), cGMP, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, propeptides

of collagen type II and fragments thereof, collagen type II and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In further embodiments, the disclosure contemplates a method for assessing the effect of a CNP peptide or variant on the level of at least one bone- or cartilage-associated biomarker in a subject, comprising assaying or measuring the level of the bone- or cartilage-associated biomarker in a biological sample from a subject that has been administered a CNP peptide or variant. In some embodiments, the method further comprises administering the CNP peptide or variant to the subject before assaying or measuring the level of the bone- or cartilage-associated biomarker.

In certain embodiments, the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP (*e.g*, endogenous level of CNP-22 or CNP-53), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In some embodiments of methods (e.g., therapeutic, diagnostic and assay methods) relating to bone- or cartilage-associated biomarkers, the CNP peptide or variant is CNP-22, CNP-53, or any of the CNP peptides and variants described herein. In certain embodiments of such methods, the CNP peptide or variant is not CNP-22 or CNP-53.

In other embodiments, the disclosure provides a method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein, under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides. In a related embodiment, the host cell is transformed with an expression vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein.

In one embodiment, the vector is a plasmid. In still another embodiment, the plasmid is selected from the group consisting of pET-21a, pJexpress, pET-31b, pET-15b, pET-32a, pET-41a, pMAL, pQE-30, pET-SUMO, pET-22b, and pTYB11.

In certain embodiments, the cleavable peptide or protein comprises a polypeptide that is selected from the group consisting of a histidine tag, human transcription factor TAF12,

ketosteroid isomerase, maltose-binding protein, ß-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof.

In a related embodiment, the cleavable peptide or protein is cleaved by a cleaving agent. In some embodiments, the cleaving agent is selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease. Additional exemplary cleaving agents include, but are not limited to, palladium, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enteropeptidase, Kex 2 protease, Omp T protease, subtilisin, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. In a related embodiment, the disclosure contemplates isolating the expressed fusion polypeptide from the host cell or culture medium. In a further embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

In one embodiment, the disclosure provides a bacterial host cell comprising an expression vector, said vector comprising a polynucleotide encoding a CNP variant peptide linked to a polynucleotide encoding a cleavable peptide or protein. In some embodiments, the cleavable peptide or protein is selected from the group consisting of a histidine tag, human transcription factor TAF12, ketosteroid isomerase, maltose-binding protein, ß-galactosidase, glutathione-S-transferase, thioredoxin, chitin binding domain, and BMP-2 mutation, or fragments thereof.

In another embodiment, the host cell is a bacteria, such as *E. coli*. In a related embodiment, the *E. coli* cell is selected from the group consisting of BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In still a further embodiment, the host cell comprises a vector as described above. In some embodiments, the host cell is transformed with the vector prior to cell culture.

In certain embodiments, it is contemplated that the host cell is cultured in a medium under conditions suitable for expression of a fusion polypeptide encoded by the polynucleotides. In one embodiment, the fusion polypeptide is expressed as a soluble protein or as inclusion body. In a related embodiment, the expressed fusion polypeptide is isolated from the host cell or culture medium. In still another embodiment, the isolated fusion polypeptide is contacted with a cleaving agent as described herein.

Brief Description of the Figures

Figure 1 shows expression of CNP fusion proteins in *E. coli* (Fig. 1A: Coomassie blue stain, Fig 1B: Western blot). M: protein marker; T: total cell lysates; S: soluble supernatants; P: 2 ug CNP22; KSI: KSI-CNP(M/N) fusion protein expression (insoluble); N: Un-induced KSI-CNP fusion protein total lysates; KSI': KSI-P-CNP fusion protein expression (insoluble); Trx: Trx-P-CNP fusion protein expression (soluble); MBP: MBP-P-CNP fusion protein expression (soluble); TAF: TAF-P-CNP fusion protein expression (insoluble) (BL21); TAF': TAF-P-CNP fusion protein expression (insoluble) BL21(DE3), where CNP is Gly-CNP37.

Figure 2 shows formic acid cleavage of TAF-CNP inclusion bodies. M: protein marker; 1: Gly-CNP37 positive control; 2: uncleaved TAF-CNP inclusion bodies; 3: 2% formic acid cleaved TAF-CNP inclusion bodies, where CNP is Gly-CNP37.

Figures 3A-E depict expression of CNP fusion proteins in *E. coli*. M: protein marker; Tu: total un-induced cell lysates; Su: un-induced soluble supernatants; T: total induced cell lysates; S: soluble supernatants; C1: CNP22; C: Gly-wtCNP37 ("CNP38"); P: insoluble pellets. **A**: KSI: KSI-CNP38(M/N) fusion protein expression (Insoluble); KSI': KSI-Pro-CNP38 (Pro-Gly-wtCNP37 is designated "Pro-CNP38") fusion protein expression (Insoluble); Trx: Trx-Pro-CNP38 fusion expression (Soluble); MBP: MBP-Pro-CNP38 fusion protein expression (Soluble); TAF: TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21 cell; TAF': TAF-Pro-CNP38 fusion protein expression (Insoluble) from BL21(DE3)cell. **B**: TAF-Pro-CNP37 and BMP-Pro-CNP37 fusion protein expression. **C**: BMP-Pro-CNP38 fusion protein and BMP protein expression. **D**: TAF-Pro-HSA-CNP ("Pro-GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 188) is designated "Pro-HSA-CNP") fusion protein expression. **E**: TAF-Pro-CNP38 fusion protein and TAF protein expression.

Figures 4A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. **A**: 50 % formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-

cleaved TAF-Pro-CNP38 inclusion bodies; 25°C, 37°C, 42°C, 55°C: TAF-Pro-CNP38 inclusion bodies were cleaved in 50% formic acid at 25°C, 37°C, 42°C or 55°C for 24 hours. 37°C-S and 55°C-S: soluble supernatants from the cleavage reactions at 37°C and 55°C neutralized with 10N NaOH and centrifuged at 14,000 rpm for 15 minutes. **B**: 10 % and 2% formic acid cleavage of TAF-Pro-CNP38 inclusion bodies. M: protein marker; U: un-cleaved TAF-Pro-CNP38 inclusion bodies; C: formic acid cleaved TAF-Pro-CNP38; S: soluble supernatant after centrifuged at 14,000 rpm for 5 minutes without neutralization; P: insoluble pellet after centrifuged at 14,000 rpm for 5 minutes without neutralization. **C**: LC/MS analysis of 2% and 10% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

Figures 5A-C depict formic acid cleavage of TAF-Pro-CNP38 inclusion bodies at different temperature and time of formic acid cleavage. M: protein marker; C: Gly-wtCNP37 ("CNP38") positive control; U: un-cleaved TAF-Pro-CNP38 inclusion bodies. **A**: 2% formic acid cleaved TAF-Pro-CNP38 at 42°C, 55°C or 70°C for 6, 24 or 48 hours. **B**: 2% formic acid cleaved TAF-Pro-CNP38 at 55°C, 60°C, 65°C or 70°C for 17 or 24 hours. **C**: LC/MS analysis of 2% formic acid cleaved products from TAF-Pro-CNP38 inclusion bodies.

Figure 6 is an SDS-PAGE (Coomassie blue stain) for the expression of a TAF-CNP34 fusion protein. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; S: supernatant; TI: total cell lysates induced; SI: supernatant induced.

Figure 7 is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38, TAF(C/A & 10D/10E)-Pro-CNP38, and TAF-Pro-CNP53, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

Figure 8 is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-CNP34 and TAF-Pro-CNP53. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

Figure 9 is an LC/MS chromatogram showing the peak for CNP-34 after formic acid cleavage of TAF-CNP34.

Figure 10 is an LC/MS chromatogram showing the peak for Pro-CNP53 after formic acid cleavage of TAF-Pro-CNP53.

Figure 11 is an SDS-PAGE (Coomassie blue stain) for the expression of fusion proteins TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; C: control [Gly-CNP37 ("CNP38")]; T: total cell lysates; TI: total cell lysates induced; S: supernatant; SI: supernatant induced.

Figure 12 is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF(4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

Figure 13 is an SDS-PAGE (Coomassie blue stain) of the products of formic acid cleavage of the fusion proteins TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, where "Pro-CNP38" denotes Pro-Gly-CNP37. M: protein marker; P: positive control [Gly-CNP37 ("CNP38")]; U: uncleaved; C: cleaved; CS: cleaved supernatant; CP: cleaved pellet.

Figure 14 is a Western blot, using an anti-CNP antibody, of a TAF-Pro-CNP38 fusion protein produced in a 10L fermentation of BL21(DE3) cells, where the cells were induced at OD_{600} = 64 and hour 17 and "Pro-CNP38" denotes Pro-Gly-CNP37.

Figure 15 is an SDS-PAGE (Coomassie blue stain) of eluate fractions from SP-Sepharose cation-exchange column chromatography of a cruder Pro-Gly-CNP37 ("Pro-CNP38") product. A: TAF-Pro-CNP38 inclusion body (IB) in water; B: IB in formate; C: IB in formate, neutralized; D: neutralized pellet; E: neutralized supernatant; F: TMAE Hi-CAP load; G: TMAE Hi-CAP flow through/SP-Sepharose load; H: SP-Sepharose flow through; SP-Sepharose eluate fractions 1-47: 10 uL/lane.

Figure 16 shows the degree of resistance of N-terminal PEGylated CNP22 conjugates to neutral endopeptidase (NEP) *in vitro*.

Figure 17 depicts the degree of NEP resistance of CNP variants having an amino acid extension at the N-terminus ["CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36)].

Figure 18 illustrates the degree of NEP resistance of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36).

Figure 19 illustrates the degree of NEP resistance of wtCNP22 and CNP variants Gly-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA" in the figures) (SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12" in the figures) (SEQ ID NO: 36).

Figure 20 shows the ability of CNP variants having an N-terminal amino acid extension to stimulate cGMP production in NIH3T3 cells *in vitro*. The results are relative to the level of cGMP produced in the presence of 1 uM CNP22. "CNP27" is GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figure 21 displays the ability of N-terminal PEGylated CNP17 and GANRR-CNP22(K4R) ("CNP27") (SEQ ID NO: 36) to stimulate cGMP production in NIH3T3 cells.

Figure 22 illustrates the effects of N-terminal PEGylation of CNP22 on cGMP production.

Figure 23 illustrates the cGMP production induced by wtCNP22 and CNP variants Gly-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA") (SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) in NIH3T3 cells.

Figures 24A and **B** show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in *in vitro* signaling competition assays.

Figure 25 demonstrates that exposure of rat chondrosarcoma cells to CNP22 1 hour once daily or 2 hours once daily has substantially similar effectiveness in reversing FGF2-induced arrest of chondrocyte growth as continuous exposure to CNP22.

Figures 26A and B show results from a dose response study of CNP22 effects on FGF2-arrested rat chondrosarcoma (RCS) cells.

Figures 27A-D show that addition of CNP22 to RCS cells arrested by FGF2 increases matrix synthesis and partly inhibits FGF2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into or decrease from matrix. Panels A and C, synthesis; B and D, degradation; A and B, ^{35}S measurement; C and D, ^{3}H measurement. Statistically significant differences are highlighted (ANOVA; *$p<0.05$, **$p<0.01$).

Figures 28A-C show the levels of aggrecan and fibronectin production (mRNA , panels A and C, and protein, panel B) in RCS cells cultured with FGF2 and CNP22.

Figure 29 shows the efficacy of CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24" in the figures) (SEQ ID NO: 36) in stimulating longitudinal growth of wild-type femur in an *ex vivo* mouse organ model.

Figure 30 shows longitudinal bone growth of 2-3 day-old wild-type mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means ±SEM (n=8).

Figure 31 shows longitudinal bone growth of 2-3 day old achondroplastic $FGFR3^{ach}$ mouse tibias treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means ±SEM (n=7-8).

Figure 32 shows longitudinal bone growth of 2-3 day-old wild-type mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means ±SEM (n=8).

Figure 33 shows longitudinal bone growth of 2-3 day-old $FGFR3^{ach}$ mouse femurs treated with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) every two days. Results are normalized to measurements prior to treatment (day 0). Data is represented as means ±SEM (n=3-7).

Figures 34A-I depict CNP37 biodistribution in $FGFR3^{ach}$ mouse femurs treated *ex vivo* every two days. Panels A-C illustrate distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

Figures 35A-C depict the cellularity of proliferating columns in growth plates after treatment of wild-type and $FGFR3^{ach}$ mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell numbers per column after treatment, (C) morphological studies after treatment. The panels in Fig 35C(i) to C(vi). correspond to the sample order set out in Fig. 35B. Data is represented as means ±SEM (n=4-8).

Figures 36A-C depict chondrocyte hypertrophy after *ex vivo* treatment of wild-type and $FGFR3^{ach}$ mouse femurs with CNP22 or CNP37 every two days for 8 days. (A) no treatment, (B) cell size after treatment, (C) morphological studies after treatment. The panels

in Fig 36C(i) to C(vi) correspond to the sample order set out in Fig. 36B. Data is represented as means ±SEM (n=4-9).

Figures 37A-I depict the biodistribution of CNP37 in FGFR3^{ach} mouse tibias treated *in vivo*. Panels A-C show distribution in distal femurs, panels D-F illustrate distribution in articular chondrocytes and panels G-I illustrate distribution in hypertrophic chondrocytes.

Figures 38A-C illustrate the *in vivo* effects of CNP37 on FGFR3^{ach} mouse tibia growth plate: (A) total growth plate thickness, (B) proliferating zone thickness, and (C) hypertrophic zone thickness. Data is represented as means ±SEM (n=7-15).

Figure 39 shows cGMP levels in conditioned media from wild-type mouse femurs treated *ex vivo* with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) ($p < 0.01$).

Figure 40 shows cGMP levels in conditioned media from FGFR3^{ach} mouse femurs treated *ex vivo* with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p < 0.01$).

Figure 41 depicts cGMP levels in conditioned media from wild-type mouse tibias treated *ex vivo* with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p < 0.01$).

Figure 42 shows cGMP levels in conditioned media from FGFR3^{ach} mouse tibias treated *ex vivo* with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p < 0.01$).

Figure 43 demonstrates that *ex vivo* exposure of wild-type and FGFR3^{ach} mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II in the conditioned media ($p < 0.05$).

Figure 44 depicts the hypertrophic region of femoral bones isolated from wild-type mice and FGFR3^{Y367C} mice (a mouse model of severe achondroplasia) and treated *ex vivo* with vehicle or 1 uM Pro-Gly-CNP37 ("ProCNP38") for 6 days, demonstrating that Pro-Gly-CNP37 treatment resulted in increase in bone growth and expansion in the growth plate.

Figure 45 shows that CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) intravenously (i.v.) administered to rats have a much longer half-life and a much greater bioavailability in the plasma than CNP22.

Figure 46 illustrates that PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) subcutaneously (s.c.) administered to rats also has a much longer half-life and a much greater bioavailability in the plasma than CNP22.

Figure 47 demonstrates that i.v. administered CNP37 and PEO24-GANRR-CNP22(K4R) stimulate a much greater level of cGMP production in rats than CNP22.

Figure 48 shows that s.c. administered PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and, to a lesser extent, CNP37 are substantially more effective in stimulating cGMP production in rats than CNP22.

Figure 49 shows body weight measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figure 50 shows tail length measurements of wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figure 51 illustrates the effect of treatment of $FGFR3^{ach}$ mice with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on body length ($p=0.02$, 1-tailed t-test, unequal variance).

Figure 52 shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on tail length in $FGFR3^{ach}$ mice.

Figures 53A and **B** show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of distal long bones (A, ulna; B, tibia) in $FGFR3^{ach}$ mice ($p<0.01$, one-tailed t-test, unequal variance).

Figures 54A and **B** show the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on the length of proximal bones (A, humerus; B, femur) in $FGFR3^{ach}$ mice ($p<0.01$, one-tailed t-test, unequal variance).

Figure 55 illustrates that CNP37 administration corrects rhizomelia (disproportion of the length of the proximal limbs) as assessed by the femur:tibia ratio observed in $FGFR3^{ach}$ mice ($p<0.01$, one-tailed t-test, unequal variance).

Figure 56 shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on head length in $FGFR3^{ach}$ mice ($p<0.01$ one-tailed t-test, unequal variance).

Figure 57 shows that treatment of FGFR3^{ach} mice with CNP37 increases the size of the external auditory meatus (EAM) (P=0.03, one-tailed t-test, unequal variance).

Figure 58 shows the effect of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on spinal length in achondroplasic mice, expressed as extension of vertebral bodies (e.g., lumbar vertebra 5).

Figure 59 shows that treatment of FGFR3^{ach} mice with CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) results in increased cGMP plasma levels 15-min post-dose.

Figure 60 illustrates the serum levels of cleaved collagen type II in FGFR3^{ach} mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figure 61 shows the serum levels of osteocalcin in FGFR3^{ach} mice treated 5 weeks with CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figure 62 shows cGMP plasma levels 15-min post-dose from wild-type mice treated with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) ($p < 0.05$).

Figure 63 shows the serum levels of cleaved collagen type II in wild-type mice treated 5 weeks with Gly-CNP37 or PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36).

Figures 64-66 display the levels of cGMP, cleaved collagen type II and alkaline phosphatase after administrion of vehicle or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 ("Pro-CNP38") to wild-type mice.

Figures 67 and **68** depict cleaved collagen type II and total alkaline phosphatase levels after administrion of vehicle or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens.

Figure 69 illustrates the relative increase in body length of Pro-Gly-CNP37 ("Pro-CNP38") and vehicle treated animals in two separate studies (S1 and S2) at Day 37 vs Day 1.

Figures 70A and B show changes in bone mineral density (A) and bone mineral content (B) after administration of Pro-Gly-CNP37 ("Pro-CNP38").

Figure 71 shows plasma cGMP levels 15 min after the last subcutaneous dose of CNP variant, Day 36, where Gly-wtCNP37 is "CNP38", Pro-Gly-wtCNP37 is "Pro-CNP38", and GHKSEVAHRFK-wtCNP27(SEQ ID NO: 144) is "HSA-CNP27" in Figures 71-73.

Figure 72 shows serum levels of cleaved collagen type II from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

Figure 73 shows serum levels of alkaline phosphatase from mice treated with Gly-CNP37, Pro-Gly-CNP37 or GHKSEVAHRFK-CNP27 (SEQ ID NO: 144).

Figures 74A and **B** depict desensitization of the cGMP response after acute (A) or chronic (**B**) treatment with 1 uM Gly-CNP37.

Figure 75A demonstrates that daily treatment of wild-type mice with 200 nmol/kg of Pro-Gly-CNP37 ("Pro-CNP38") for 8 days did not desensitize the cGMP response. **Figure 75B** shows that treatment of the mice with 200 nmol/kg of Pro-Gly-CNP37 once a day for two consecutive days potentiated the cGMP response.

Figures 76A-D show that treatment of wild-type mice with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in distal femurs (cartilage and bone) (A), femoral cortices (bone) (**B**), ear pinna (cartilage) (C), and kidney (D). **Figures 76E-H** show that liver (E), heart (F), lung (G) and brain (H) tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points.

Figures 77-82 show results from an on-going study in normal juvenile cynomolgus monkeys subcutaneously injected daily with vehicle or 10 or 36 ug/kg of Pro-Gly-CNP37. Both doses of Pro-Gly-CNP37 have increased growth plate width (**Figure 77**), increased right and left tibia lengths (**Figures 78A** and **B**), increased leg length (**Figure 79**), increased arm length (**Figure 80**), increased body length (**Figure 81**), and increased the serum level of alkaline phosphatase (**Figure 82**).

Figure 83 depicts the observed plot of degradation rate constant (K_{obs}) vs. pH at pH 3-8 and 5 °C, 25 °C and 40 °C for Gly-CNP37 formulations.

Detailed Description of the Disclosure

The present disclosure relates to novel variants of CNP having reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, pharmaceutical compositions comprising such CNP variants, and methods of using such CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as achondroplasia and disorders associated with vascular smooth muscle cells and tissues.

A. Definitions

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below.

As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood that the term "about" or "approximately" applies to each one of the numerical values in that series.

The terms "ambient temperature" and "room temperature" are used interchangeably herein and refer to the temperature of the surrounding environment (*e.g.*, the room in which a reaction is conducted or a composition is stored). In certain embodiments, ambient temperature or room temperature is a range from about 15 °C to about 28 °C, or from about 15 °C to about 25 °C, or from about 20 °C to about 28 °C, or from about 20 °C to about 25 °C, or from about 22 °C to about 28 °C, or from about 22 °C to about 25 °C. In other embodiments, ambient temperature or room temperature is about 15 °C, 16 °C, 17 °C, 18 °C, 19 °C, 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, 25 °C, 26 °C, 27 °C or 28 °C.

Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present disclosure may employ, unless otherwise indicated, certain conventional methods of synthetic organic chemistry, mass spectrometry, preparative and analytical chromatography, protein chemistry, biochemistry, recombinant DNA technology and pharmacology, within the skill of the art. See, *e.g.*, T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S.

Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

"Polypeptide" and "protein" refer to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof, linked *via* peptide bonds or peptide bond isosteres. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The terms "polypeptide" and "protein" are not limited to a minimum length of the product. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms "polypeptide" and "protein" also include postexpression modifications of the polypeptide or protein, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present disclosure, a "polypeptide" can include "modifications," such as deletions, additions, substitutions (which may be conservative in nature or may include substitutions with any of the 20 amino acids that are commonly present in human proteins, or any other naturally or non-naturally-occurring or atypical amino acids), and chemical modifications (e.g., addition of or substitution with peptidomimetics), to the native sequence. These modifications may be deliberate, as through site-directed mutagenesis, or through chemical modification of amino acids to remove or attach chemical

moieties, or may be accidental, such as through mutations arising with hosts that produce the proteins or through errors due to PCR amplification.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Conservative substitution" refers to substitution of an amino acid in a polypeptide with a functionally, structurally or chemically similar natural or unnatural amino acid. In one embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1), Alanine (A) Serine (S), Threonine (T);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

In another embodiment, the following groups each contain natural amino acids that are conservative substitutions for one another:

(1) Glycine (G), Alanine (A);

(2) Aspartic acid (D), Glutamic acid (E);

(3) Asparagine (N), Glutamine (Q);

(4) Arginine (R), Lysine (K);

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Alanine (A);

(6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); and

(7) Serine (S), Threonine (T), Cysteine (C).

In a further embodiment, amino acids may be grouped as set out below.

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence backbone orientation: Gly, Pro; and

(6) aromatic: Trp,Tyr, Phe, His.

In one embodiment, the peptides or polypeptides described herein are generated via recombinant means, using a polynucleotide encoding a CNP variant. The disclosure thus encompasses polynucleotides encoding any of the CNP variants described herein, host cells or vectors comprising such polynucleotides, optionally linked to expression control sequences, and methods of using such polynucleotides, vectors or host cells to produce CNP variants of the disclosure. CNP variants expressed by such polynucleotides may be produced by methods including growing host cells in culture medium under conditions suitable for expression of the polynucleotide encoding a CNP variant, and isolating the expression product from the host cells or culture medium. Actual expression products may vary slightly from the encoded protein product depending on any post-translational processing.

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), the nucleotide sequence also encompasses an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T." "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Expression control sequence" refers to a nucleotide sequence that regulates the expression of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive), enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, *e.g.*, a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (*e.g.*, promoter, origin of replication, ribosome-binding site, *etc.*) as well.

"Chimera" as used herein refers to a polynucleotide or polypeptide comprising at least two heterologous polynucleotide or polypeptide sequences (i.e. derived from different sources or not associated with each other as a naturally-occurring sequence) which are directly or indirectly attached or linked together using techniques commonly known in the art, e.g., recombinant expression or chemical crosslinking. In one embodiment, the heterologous sequence can comprise a protein or peptide directly or indirectly linked to a CNP peptide or variant, including proteins or peptides which are cleavable from the CNP peptide or variant. In a related embodiment, CNP variants are chimera as described herein.

In certain embodiments, chimeras include CNP fusion proteins comprising a cleavable carrier protein or peptide tag. The term "cleavable carrier protein" or "cleavable peptide tag" refers to a peptide or polypeptide sequence that may be fused, directly or indirectly via a linker, to a heterologous polypeptide sequence, and is removable from the heterologous sequence using an agent that cleaves or separates the cleavable peptide or polypeptide from the heterologous polypeptide or protein. In some embodiments, the cleavable carrier protein or peptide tag improves generation, purification and/or detection of the fusion protein or the heterologous polypeptide. Exemplary cleavable carrier proteins and peptide tags include, but are not limited to, human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), ß-galactosidase (ß-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, c-Myc, poly(His), poly(Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and fragments thereof.

A "cleaving agent" is an agent that is useful to cleave or separate, e.g., a cleavable peptide or polypeptide from a heterologous polypeptide or protein. Exemplary cleaving agents include, but are not limited to, palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, proTEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K,

pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

The terms "identical" and percent "identity", in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially homologous" or "substantially identical", in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In certain embodiments, the substantial homology or identity exists over regions of the sequences that are at least about 25, 50, 100 or 150 residues in length. In another embodiment, the sequences are substantially homologous or identical over the entire length of either or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math., 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection. One example of a useful algorithm is PILEUP, which uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol., 35: 351-360 (1987) and is similar to the method described by

Higgins & Sharp, CABIOS, 5: 151-153 (1989). Another algorithm useful for generating multiple alignments of sequences is Clustal W (Thompson *et al.*, Nucleic Acids Research, 22: 4673-4680 (1994)). An example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm (Altschul *et al.*, J. Mol. Biol., 215: 403-410 (1990); Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA, 89: 10915 (1989); Karlin & Altschul, Proc. Natl. Acad. Sci. USA, 90: 5873-5787 (1993)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

A further indication that two nucleic acid sequences or polypeptides are substantially homologous or identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two polypeptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

"Substantially pure" or "isolated" means an object species is the predominant species present (*i.e.*, on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. In one embodiment, a substantially pure composition means that the species of interest comprises at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or more of the macromolecular species present in the composition on a molar or weight basis. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (*e.g.*, BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In an embodiment, the compounds of the disclosure are substantially pure or isolated. In another embodiment, the compounds of the disclosure are substantially pure or isolated with respect to the macromolecular starting materials used in their production. In yet another embodiment, the pharmaceutical compositions of the disclosure comprise a substantially pure or isolated CNP variant admixed with one or more pharmaceutically acceptable excipients, carriers or diluents, and optionally with another biologically active agent.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) and which has not been intentionally modified by man in the laboratory is naturally occurring. In one embodiment, a "naturally occurring" substance is of human origin.

"Wild-type" (wt) is a term referring to the natural form, including sequence, of a polynucleotide, polypeptide or protein in a species. A wild-type form is distinguished from a mutant form of a polynucleotide, polypeptide or protein arising from genetic mutation(s).

In one embodiment, a first polypeptide that is an "analog" or "variant" or "derivative" of a second polypeptide is a polypeptide having at least about 50%, 60% or 70% sequence homology, but less than 100% sequence homology, with the second polypeptide. Such analogs, variants or derivatives may be comprised of non-naturally occurring amino acid residues, including without limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues. Such analogs, variants or derivatives may also be composed of one or a plurality of D-amino acid residues, and may also contain peptidomimetics or peptide bond isosteres such as non-peptide linkages between two or more amino acid or peptidomimetic residues. In another embodiment, a first polypeptide is an "analog", "variant" or "derivative" of a second polypeptide if the first polypeptide is not a known cleavage product of the second polypeptide or is not a known precursor of the second polypeptide, even if the first polypeptide has 100% sequence homology to the second polypeptide or has a wild-type sequence.

In an embodiment, the term "derived from" as used herein refers to a polypeptide or peptide sequence that is based on a wild type or naturally occurring polypeptide or peptide sequence and can have one or more deletions, additions, and/or substitutions with natural amino acids, unnatural amino acids or peptidomimetics. In one embodiment, the derivative sequence shares at least about 40%, 50%, 60% or 70%, but less than 100%, sequence similarity to the wild-type or naturally occurring sequence. In another embodiment, the derivative may be a fragment of a polypeptide, wherein the fragment is substantially homologous (e.g., at least about 70%, 75%, 80%, 85%, 90%, or 95% homologous) to the wild-type polypeptide over a length of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids. In still another embodiment, a polypeptide is "derived from" a wild-type polypeptide if it has a moiety (e.g., a polymer such as, e.g., PEG) directly or indirectly

attached to it which is not present on the wild-type polypeptide, even if both polypeptides share 100% homology in their amino acid sequence.

As used herein, an "NPPC-derived" polypeptide refers to a polypeptide derived from the natriuretic peptide precursor C (NPPC) polypeptide, which is a single chain 126-amino acid pre-pro polypeptide, and which upon cleavage ultimately results in wtCNP22. Removal of the signal peptide from NPPC yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP, or CNP-22). Therefore, CNP22 itself is an "NPPC-derived" polypeptide by virtue of being derived from NPPC. In one embodiment, an NPPC-derived polypeptide is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that an NPPC-derived peptide may comprise from about 1 to about 53, or 1 to 37, or 1 to 35, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, an NPPC-derived peptide may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Bone- or cartilage-associated biomarker" or "bone- or cartilage-associated marker" refers to a growth factor, enzyme, protein, or other detectable biological substance or moiety whose level is increased or decreased in association with, e.g., cartilage turnover, cartilage formation, cartilage growth, bone resorption, bone formation, bone growth, or combinations thereof. Such biomarkers may be measured before, during and/or after administration of a CNP variant as described herein. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I and fragments thereof, collagen type I and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, and alkaline phosphatase. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine. In some embodiments, the biomarkers are measured in blood, plasma or serum from animals undergoing efficacy/pharmacodynamic *in vivo* studies and/or from the conditioned media of *ex vivo* studies.

In certain embodiments, the level of at least one bone- or cartilage-associated biomarker is measured and the amount or frequency of administration of CNP variant administered to a subject can be adjusted according to the level of the biomarker measured.

In some embodiments, the level of biomarker is "below a target level" or "above a target level." A target level of a biomarker is a level or range of levels of the biomarker at which a therapeutic effect is observed in the subject receiving the CNP variant. In certain embodiments, the target level of a biomarker for a subject having a CNP-responsive disorder or condition is the level or range of levels of the biomarker observed in a normal, non-affected subject. In other embodiments, to indicate a therapeutic effect, the target level of a biomarker need not be equivalent to the level or range of levels of the biomarker observed in a normal subject, but can be within, e.g., 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% or 5% of the "normal" level or range of levels of the biomarker observed in a non-affected subject.

For example, if the level of a biomarker increases in association with bone or cartilage formation or growth, the target level of the biomarker indicating a therapeutic effect may be higher than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be lower than the "normal" level(s), at about the "normal" level(s), or above the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In one embodiment, if the level of a biomarker is below a target level, it indicates an inadequate therapeutic effect, which may require an increase in the amount or frequency of administration of CNP variant administered. In a related embodiment, if the biomarker is above a target level, it indicates that more CNP variant than necessary has been administered, which may require a decrease in the amount or frequency of administration of the CNP variant administered.

As another example, if the level of a biomarker decreases in association with bone or cartilage formation or growth, the target level of the biomarker indicating a therapeutic effect may be lower than the level of the biomarker in patients suffering from a CNP-responsive disorder who have not been administered a CNP variant, and may optionally be higher than the "normal" level(s), at about the "normal" level(s), or below the "normal" level(s) of the biomarker in subjects not suffering from that disorder. In such a case, the converse of the above adjustments in CNP variant amount and frequency of administration may apply.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition

comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a compound of the disclosure calculated in an amount sufficient to produce the desired effect, optionally in association with a pharmaceutically acceptable excipient, diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present disclosure depend

on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

"Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

The terms "polyethylene glycol", "PEG", "polyethylene oxide" and "PEO" are used interchangeably herein unless indicated otherwise. A CNP peptide (CNP22 or a variant thereof) conjugated via an amino group to a "PEOn" polymer associated with the number n, in general has the formula: $CH_3–[–O–CH_2CH_2–]_n–C(=O)–NHR$, where n is the number of ethylene oxide units and R denotes the rest of the peptide. The "PEOn" polymer can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. Such a "PEOn" (e.g., PEO12 or PEO24) polymer is monodispersed, i.e., is a single discrete polymer of a particular molecular weight. Similarly, a CNP peptide conjugated via an amino group to a "PEGnK" polymer associated with the number nK, in general has the formula: $CH_3–[–O–CH_2CH_2–]_p–C(=O)–NHR$, where p is an integer greater than 1. The "PEGnK" polymer also can optionally have an alkylene group, $(CH_2)_m$, where m is an integer from 1 to 5, between the carbonyl carbon and the repeating ethylene oxide units. However, such a "PEGnK" (e.g., PEG1K, PEG2K,

PEG5K or PEG20K) polymer is polydispersed, i.e., contains a mixture of polymers having a distribution of molecular weights, where the number nK denotes the polymer number-average molecular weight (M_n) in kilo Daltons. For example, "PEG2K" conjugated to a CNP peptide denotes a polydispersed PEG polymer having a polymer number-average molecular weight of around 2 kDa.

When a range of the mass of a polymer (e.g., PEG) is given (e.g., in units of kDa), the range refers to a range of polymer number-average molecular weights, not to a range of molecular weights of multiple polymers in a polydispersed mixture, unless expressly indicated otherwise.

The term "halogen", "halide" or "halo" refers to fluorine, chlorine, bromine, and/or iodine.

The term "alkyl" refers to a linear or branched saturated monovalent hydrocarbon radical, wherein the alkyl may optionally be substituted with one or more substituents Q as described herein. In certain embodiments, the alkyl is a linear saturated monovalent hydrocarbon radical that has 1 to 20 (C_{1-20}), 1 to 15 (C_{1-15}), 1 to 12 (C_{1-12}), 1 to 10 (C_{1-10}), or 1 to 6 (C_{1-6}) carbon atoms, or a branched saturated monovalent hydrocarbon radical of 3 to 20 (C_{3-20}), 3 to 15 (C_{3-15}), 3 to 12 (C_{3-12}), 3 to 10 (C_{3-10}), or 3 to 6 (C_{3-6}) carbon atoms. As used herein, linear C_{1-6} and branched C_{3-6} alkyl groups are also referred as "lower alkyl." Examples of alkyl groups include, but are not limited to, methyl, ethyl, propyl (including all isomeric forms, including n-propyl and isopropyl), butyl (including all isomeric forms, including n-butyl, isobutyl, sec-butyl and tert-butyl), pentyl (including all isomeric forms), and hexyl (including all isomeric forms). For example, C_{1-6} alkyl refers to a linear saturated monovalent hydrocarbon radical of 1 to 6 carbon atoms or a branched saturated monovalent hydrocarbon radical of 3 to 6 carbon atoms.

The term "alkoxy" refers to an –O-alkyl group. In certain embodiments, an alkoxy group may optionally be substituted with one or more substituents Q as described herein.

The term "haloalkyl" refers to an alkyl group that is substituted with one or more halide atoms. In certain embodiments, a haloalkyl group is substituted with one, two, three, four, five or six halide atoms. In certain embodiments, a haloalkyl group may optionally be substituted with one or more additional substituents Q as described herein.

The term "cycloalkyl" refers to a cyclic saturated bridged and/or non-bridged monovalent hydrocarbon radical, which may be optionally substituted with one or more

substituents Q as described herein. In certain embodiments, the cycloalkyl has from 3 to 20 (C_{3-20}), from 3 to 15 (C_{3-15}), from 3 to 12 (C_{3-12}), from 3 to 10 (C_{3-10}), or from 3 to 7 (C_{3-7}) carbon atoms. Examples of cycloalkyl groups include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, decalinyl, and adamantyl.

The term "heterocyclyl" or "heterocyclic" refers to a monocyclic non-aromatic ring system or a multicyclic ring system that contains at least one non-aromatic ring, wherein one or more of the non-aromatic ring atoms are heteroatoms independently selected from O, S, or N, and the remaining non-aromatic ring atoms are carbon atoms. In certain embodiments, the heterocyclyl or heterocyclic group has from 3 to 20, from 3 to 15, from 3 to 10, from 3 to 8, from 4 to 7, or from 5 to 6 ring atoms. In certain embodiments, the heterocyclyl is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include a fused or bridged ring system, and in which the nitrogen or sulfur atoms may be optionally oxidized, the nitrogen atoms may be optionally quaternized, and some rings may be partially or fully saturated, or aromatic. The heterocyclyl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. Examples of heterocyclic groups include, but are not limited to, acridinyl, azepinyl, benzimidazolyl, benzindolyl, benzoisoxazolyl, benzisoxazinyl, benzodioxanyl, benzodioxolyl, benzofuranonyl, benzofuranyl, benzonaphthofuranyl, benzopyranonyl, benzopyranyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, benzothiadiazolyl, benzothiazolyl, benzothiophenyl, benzotriazolyl, benzothiopyranyl, benzoxazinyl, benzoxazolyl, benzothiazolyl, β-carbolinyl, carbazolyl, chromanyl, chromonyl, cinnolinyl, coumarinyl, decahydroisoquinolinyl, dibenzofuranyl, dihydrobenzisothiazinyl, dihydrobenzisoxazinyl, dihydrofuryl, dihydropyranyl, dioxolanyl, dihydropyrazinyl, dihydropyridinyl, dihydropyrazolyl, dihydropyrimidinyl, dihydropyrrolyl, dioxolanyl, 1,4-dithianyl, furanonyl, furanyl, imidazolidinyl, imidazolinyl, imidazolyl, imidazopyridinyl, imidazothiazolyl, indazolyl, indolinyl, indolizinyl, indolyl, isobenzotetrahydrofuranyl, isobenzotetrahydrothienyl, isobenzothienyl, isochromanyl, isocoumarinyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolidinyl, isothiazolyl, isoxazolidinyl, isoxazolyl, morpholinyl, naphthyridinyl, octahydroindolyl, octahydroisoindolyl, oxadiazolyl, oxazolidinonyl, oxazolidinyl, oxazolopyridinyl, oxazolyl, oxiranyl, perimidinyl, phenanthridinyl, phenathrolinyl, phenarsazinyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, 4-piperidonyl, pteridinyl, purinyl, pyrazinyl, pyrazolidinyl, pyrazolyl, pyridazinyl, pyridinyl, pyridopyridinyl, pyrimidinyl, pyrrolidinyl,

pyrrolinyl, pyrrolyl, quinazolinyl, quinolinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuryl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydrothienyl, tetrazolyl, thiadiazolopyrimidinyl, thiadiazolyl, thiamorpholinyl, thiazolidinyl, thiazolyl, thienyl, triazinyl, triazolyl, and 1,3,5-trithianyl. In certain embodiments, a heterocyclic group may optionally be substituted with one or more substituents Q as described herein.

The term "aryl" refers to a monocyclic aromatic group or a multicyclic monovalent aromatic group that contain at least one aromatic hydrocarbon ring. In certain embodiments, the aryl has from 6 to 20 (C_{6-20}), from 6 to 15 (C_{6-15}), or from 6 to 10 (C_{6-10}) ring atoms. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, fluorenyl, azulenyl, anthryl, phenanthryl, pyrenyl, biphenyl, and terphenyl. Aryl also refers to bicyclic or tricyclic carbon rings, where at least one of the rings is aromatic and the others may be saturated, partially unsaturated, or aromatic, for example, dihydronaphthyl, indenyl, indanyl, and tetrahydronaphthyl (tetralinyl). In certain embodiments, an aryl group may optionally be substituted with one or more substituents Q as described herein.

The term "heteroaryl" refers to a monocyclic aromatic group or a multicyclic aromatic group that contain at least one aromatic ring, wherein at least one aromatic ring contains one or more heteroatoms independently selected from O, S, and N. Each ring of a heteroaryl group can contain one or two O atoms, one or two S atoms, and/or one to four N atoms, provided that the total number of heteroatoms in each ring is four or less and each ring contains at least one carbon atom. The heteroaryl may be attached to the main structure at any heteroatom or carbon atom which results in the creation of a stable compound. In certain embodiments, the heteroaryl has from 5 to 20, from 5 to 15, or from 5 to 10 ring atoms. Examples of monocyclic heteroaryl groups include, but are not limited to, pyrrolyl, pyrazolyl, pyrazolinyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, thiadiazolyl, isothiazolyl, furanyl, thienyl, oxadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, and triazinyl. Examples of bicyclic heteroaryl groups include, but are not limited to, indolyl, benzothiazolyl, benzoxazolyl, benzothienyl, quinolinyl, tetrahydroisoquinolinyl, isoquinolinyl, benzimidazolyl, benzopyranyl, indolizinyl, benzofuranyl, isobenzofuranyl, chromonyl, coumarinyl, cinnolinyl, quinoxalinyl, indazolyl, purinyl, pyrrolopyridinyl, furopyridinyl, thienopyridinyl, dihydroisoindolyl, and tetrahydroquinolinyl. Examples of tricyclic heteroaryl groups include, but are not limited to, carbazolyl, benzindolyl, phenanthrollinyl, acridinyl, phenanthridinyl, and xanthenyl. In certain embodiments, a heteroaryl group may optionally be substituted with one or more substituents Q as described herein.

The term "optionally substituted" is intended to mean that a group, including alkyl, alkoxy, haloalkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, may be substituted with one or more substituents Q (in one embodiment, one, two, three or four substituents Q), where each Q is independently selected from the group consisting of cyano, halo, oxo, nitro, C_{1-6} alkyl, C_{1-6} alkoxy, halo-C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, heteroaryl, $-C(O)R^e$, $-C(O)OR^e$, $-C(O)NR^fR^g$, $-C(NR^e)NR^fR^g$, $-OR^e$, $-OC(O)R^e$, $-OC(O)OR^e$, $-OC(O)NR^fR^g$, $-OC(=NR^e)NR^fR^g$, $-OS(O)R^e$, $-OS(O)_2R^e$, $-OS(O)NR^fR^g$, $-OS(O)_2NR^fR^g$, $-NR^fR^g$, $-NR^eC(O)R^f$, $-NR^eC(O)OR^f$, $-NR^eC(O)NR^fR^g$, $-NR^eC(=NR^h)NR^fR^g$, $-NR^eS(O)R^f$, $-NR^eS(O)_2R^f$, $-NR^eS(O)NR^fR^g$, $-NR^eS(O)_2NR^fR^g$, $-SR^e$, $-S(O)R^e$, $-S(O)_2R^e$, and $-S(O)_2NR^fR^g$, wherein each R^e, R^f, R^g, and R^h is independently hydrogen, C_{1-6} alkyl, C_{3-7} cycloalkyl, heterocyclyl, C_{6-14} aryl, or heteroaryl; or R^f and R^g, together with the N atom to which they are attached, form heterocyclyl.

B. CNP Variants

The use of CNP22 as a therapeutic is limited by its short half-life in plasma (J. Clin. Endocrinol. Metab., 78: 1428-35 (1994)). In human plasma, the concentration of CNP22 typically is less than five picomolar. CNP22 is degraded and cleared from circulation by NEP and NPR-C in humans (Growth Hormone & IGF Res., 16: S6-S14). In all human and animal studies using systemically administered CNP22, continuous infusion has been used to increase the CNP22 concentration in the subjects. A CNP peptide having a longer half-life and at least a similar level of functionality would be beneficial to a CNP-based therapeutic strategy. CNP variants are also disclosed in related International Application No. PCT/US08/84270, specifically incorporated herein by reference.

The present disclosure provides CNP variants which have reduced affinity to NEP and/or NPR-C, and reduced susceptibility to cleavage by NEP and/or clearance by NPR-C, but which have substantially similar or better functionality than wild-type CNP22. Reduced susceptibility of CNP variants to cleavage by NEP and/or clearance by NPR-C would increase the plasma or serum half-life of the variants, thereby increasing the opportunity for the variants to distribute to the target tissues and sites and effectuate the desired pharmacological effects. In certain embodiments, the CNP variants described herein have reduced susceptibility to cleavage by NEP and/or clearance by NPR-C *in vitro* or *in vivo* by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, and have increased plasma or serum half-life *in vivo* by at least about 1.5-fold, 2-

fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold compared to wtCNP22, while retaining at least about 50%, 60%, 70%, 80%, 90% or 100% of the functionality of wtCNP22, or having at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, or 5-fold greater functionality than wtCNP22. CNP functionality can be evaluated in terms of, e.g., the level of one or more biomarkers (e.g., cGMP) associated with cartilage or bone formation or growth in an *in vitro* or *in vivo* study, the length of particular bones in an *ex vivo* or *in vivo* study, etc.

Natural substrates of NEP are small and natriuretic peptides (about 2.2 to about 3.2kDa) are the largest of the natural substrates. According to X-ray crystallographic analyses, the NEP active-site is buried deep inside a central cavity, effectively restricting the size of substrate molecules to no more than about 3 kDa (Oefner et al., J. Mol. Biol., 296: 341-349 (2000)). Based on NPR-B signaling studies, variants of CNP-22, such as CNP-17 (retaining only the cyclic domain, Cys6 – Cys22, of CNP22) and CNP-53 (CNP-22 with a 31-amino acid extension at the N-terminus), can still bind and activate NPR-B similarly to the 2.2 kDa wtCNP-22. Accordingly, the disclosure encompasses CNP variants conjugated to a natural (e.g., peptide) and/or synthetic (e.g., PEG) polymer at the N-terminus and/or C-terminus of CNP22 or variants thereof, which exhibit increased NEP resistance but retain the ability to bind and activate the NPR-B signaling receptor.

In one embodiment, the disclosure encompasses CNP variants represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:139), or

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO:140), wherein:

(x) and (z) each independently are a natural polymer (e.g., a peptide sequence containing at least one amino acid) and/or a synthetic polymer (e.g., PEG) as described herein, such that the total mass of the CNP variant is characterized by the ranges described generally herein, e.g., in the range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the residues from Cys6 to Cys22 form a cyclic portion. In an embodiment, (x) and/or (z) comprise an amino acid extension derived from NPPC or a non-CNP polypeptide (e.g., ANP, BNP, IgG, etc.), wherein the extension contains 1 to 40, 1 to 35, 1 to 31, 5 to 35, 5 to 31 or 5 to 15 amino acids. In another embodiment, the CNP variants comprise one or

more modifications and/or substitutions with another natural amino acid, an unnatural amino acid, a peptidomimetic and/or a peptide bond isostere at one or more of the following positions of CNP22: Gly1, Lys4, Gly5, Cys6, Phe7, Gly8, Leu9, Lys10, Leu11, Ile14, Gly15, Ser16, Met17, Gly19, Leu20 and Gly21.

In another embodiment, CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, are represented by the general formula:

(x)-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 141), or

(x)-Gly_1-Leu_2-Ser_3-$(b)_4$-Gly_5-Cys_6-Phe_7-Gly_8-Leu_9-$(h)_{10}$-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 6), wherein:

(x) is a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG (or PEO), and a non-limiting example of a natural polymeric group is an amino acid sequence containing from 1 to 35 amino acids and derived from NPPC or variants thereof with substitutions and/or deletions, ANP, BNP, or other non-CNP (poly)peptides such as, e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, osteocrin or fibroblast growth factor 2 (FGF2);

(z) may be absent or may be a synthetic or natural polymeric group, or a combination thereof, wherein a non-limiting example of a synthetic polymeric group is PEG, and a non-limiting example of a natural polymeric group is an amino acid sequence derived from a natriuretic polypeptide (e.g., NPPC, CNP, ANP or BNP) or non-natriuretic polypeptide (e.g., serum albumin or IgG); and

(b) and (h) independently may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or any natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Glu or Ser. In one embodiment, (b) is Arg. In another embodiment, for improved NEP resistance, (b) is not Gly. In yet another embodiment, (h) is not Arg.

Non-limiting examples of amino acid sequences derived from NPPC or variants thereof include:

Arg,

Glu-Arg,

Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 7),

Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 8),

Gly-Ala-Asn-Pro-Arg (SEQ ID NO: 9),

Gly-Ala-Asn-Gln-Gln (SEQ ID NO: 10),

Gly-Ala-Asn-Ser-Ser (SEQ ID NO: 11),

Gly-Ala-Asn-Arg-Gln (SEQ ID NO: 12),

Gly-Ala-Asn-Arg-Met (SEQ ID NO: 13),

Gly-Ala-Asn-Arg-Thr (SEQ ID NO: 14),

Gly-Ala-Asn-Arg-Ser (SEQ ID NO: 15),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala (SEQ ID NO: 16),

Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg (SEQ ID NO: 17),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg (SEQ ID NO: 18),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO:19),

Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg (SEQ ID NO:20),

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Lys-Lys (SEQ ID NO: 21), and

Asp-Leu-Arg-Val-Asp-Thr-Lys-Ser-Arg-Ala-Ala-Trp-Ala-Arg-Leu-Leu-Gln-Glu-His-Pro-Asn-Ala-Arg-Lys-Tyr-Lys-Gly-Ala-Asn-Arg-Arg (SEQ ID NO: 22).

Non-limiting examples of amino acid sequences derived from non-CNP polypeptides such as, e.g., ANP, BNP, serum albumin and IgG include:

Ser-Leu-Arg-Arg-Ser-Ser (SEQ ID NO: 23);

Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 24);

Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 25);

Met-Val-Gln-Gly-Ser-Gly (SEQ ID NO: 26);

Lys-Val-Leu-Arg-Arg-Tyr (SEQ ID NO: 27);

Lys-Val-Leu-Arg-Arg-His (SEQ ID NO: 28);

Gly-Gln-His-Lys-Asp-Asp-Asn-Pro-Asn-Leu-Pro-Arg (SEQ ID NO: 29);

Gly-Val-Pro-Gln-Val-Ser-Thr-Ser-Thr (SEQ ID NO: 30);

Gly-Glu-Arg-Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg-Leu-Ser-Gln (SEQ ID NO: 31);

Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser (SEQ ID NO: 32).

In an embodiment, the N-terminal (x) group and/or the C-terminal (z) group of any of the CNP variants having an (x) and/or (z) group, as described herein, independently comprise

an amino acid sequence that contains a small number of, if any, acidic natural or unnatural amino acids (e.g., Asp or Glu). In another embodiment, (x) and/or (z) are enriched in basic natural or unnatural amino acids (e.g., Lys, Arg or His) to maintain an alkaline pI similar to the pI of CNP22 (pI 8.9). In one embodiment, the pI of the CNP variants is in the range from about 8 to about 10.5, designed so that the CNP variants can diffuse more readily through the extracellular matrix surrounding chondrocytes of bone growth plates. In narrower embodiments, the pI of the CNP variants is from about 8.5 to about 10.5, or from about 8.5 to about 10, or from about 9 to about 10.

In yet another embodiment, (x) and/or (z) are enriched in polar natural or unnatural amino acids, designed for increased aqueous solubility. In still another embodiment, (x) and/or (z) contain a small number of, if any, hydrophobic natural or unnatural amino acids (e.g., Ala, Val, Leu, Ile or Met).

In a further embodiment, the N-terminus of the CNP variants terminates in at least one glycine residue, designed for increased serum half-life. In a related embodiment, to prevent pyroglutamine formation, the N-terminus of CNP variants terminates in a glycine residue if it would otherwise terminate in glutamine. In one embodiment, the (x) group contains an amino acid extension whose N-terminus terminates in at least one glycine residue. In another embodiment, (x) and/or (z) do not contain two adjacent basic natural or unnatural amino acids (e.g., Lys-Lys or Arg-Arg), designed to reduce susceptibility to cleavage by the protease furin. In an embodiment, (x) does not contain two adjacent basic amino acids immediately preceding the position corresponding to Gly1 of CNP22.

In still another embodiment, the (x) group and/or the (z) group of the CNP variants comprise an amino acid sequence derived from NPPC (e.g., derived from CNP53). In an embodiment, (x) comprises an amino acid sequence derived from the N-terminal tail of ANP or BNP. In another embodiment, (z) comprises an amino acid sequence derived from the C-terminal tail of ANP or BNP. In a further embodiment, (x) and/or (z) comprise an amino acid sequence derived from a non-natriuretic polypeptide such as, e.g., IgG, human serum albumin (HSA), histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, and bone-targeting proteins (e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein).

In any embodiment described herein in which CNP22 or a variant thereof can have an N-terminal (x) group and/or a C-terminal (z) group, (x) and/or (z) independently can contain

an amino acid sequence derived from the functional domain of a bone morphogenetic protein (BMP). An N-terminal and/or C-terminal amino acid extension derived from the functional domain of a BMP can increase the NEP resistance, and hence the serum half-life of the CNP variant, by increasing the total mass of the CNP variant to characterized by the ranges described generally herein, e.g., a range from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa. In addition, since certain BMPs are growth factors and cytokines that induce the formation of bone and cartilage, a fragment derived from the functional domain of a BMP can promote chondrocyte, cartilage or bone growth by a mechanism distinct from activation of the guanylyl cyclase function of NPR-B by the cyclic domain of CNP22 or a variant thereof. Non-limiting examples of BMPs that promote bone formation and development, cartilage formation and development, and/or osteoblast differentiation include BMP1, BMP2, BMP3, BMP5, BMP7 and BMP8a. In an embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof independently are conjugated to an amino acid sequence derived from the last 140 amino acids in the C-terminal portion of BMP1, BMP2, BMP3, BMP5, BMP7 or BMP8a.

In one embodiment, the CNP variants contain an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, including but not limited to:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-53**) (SEQ ID NO: 4);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (**CNP-37, Analog BL**) (SEQ ID NO: 60);

AAWARLLQEHPNAGLSKGCFGLKLDRIGSMSGLGC (**Analog CA**) (SEQ ID NO: 61);

AAWARLLQEHPNARGLSKGCFGLKLDRIGSMSGLGC (**Analog CB**) (SEQ ID NO: 62);

DLRVDTKSRAAWARGLSKGCFGLKLDRIGSMSGLGC (**Analog CC**) (SEQ ID NO: 63);

RGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 40);

ERGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 38);

GANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 64);

GANRRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 65);

GANPRGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 66);

GANSSGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 67);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144); and

SPKMVQGSG-CNP17-KVLRRH (**Analog CD**) (CNP17 having N-terminal and C-terminal tails derived from BNP) (SEQ ID NO: 68).

In another embodiment, the CNP variants have a K4R substitution at position 4 of CNP22. Non-limiting examples of CNP(K4R) variants include:
GANRRGLSRGCFGLKLDRIGSMSGLGC (**Analog AY**) (SEQ ID NO: 36);
GANPRGLSRGCFGLKLDRIGSMSGLGC (**Analog CI**) (SEQ ID NO: 37);
RGLSRGCFGLKLDRIGSMSGLGC (**Analog AZ**) (SEQ ID NO: 41);
ERGLSRGCFGLKLDRIGSMSGLGC (**Analog BA**) (SEQ ID NO: 39);
GANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CH**) (SEQ ID NO: 69); and
GANSSGLSRGCFGLKLDRIGSMSGLGC (**Analog CG**) (SEQ ID NO: 70).

In further embodiments, the CNP variants are chimeras comprising CNP22, or a variant thereof having amino acid addition(s), deletion(s) and/or substitution(s), and a peptide fragment derived from a polypeptide or protein other than CNP, or the whole non-CNP polypeptide or protein, to the N-terminus of the CNP peptide, wherein CNP22 or the variant thereof may optionally have an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras comprise CNP22 or a variant thereof that has an N-terminal amino acid extension of one or more amino acid residues. In certain embodiments, the CNP chimeras contain lysine-lysine (KK) residues or GANKK residues immediately preceding the first position of CNP22 (Gly in the case of CNP22) or a variant thereof. In other embodiments, the CNP chimeras contain one or two residues different from lysine-lysine immediately preceding the first position of CNP22 or a variant thereof. Non-limiting examples of residues that can immediately precede the first position of CNP22 or a variant thereof include KP, PK, PR, PQ, QK, QQ, RR, SS, GANKP(SEQ ID NO: 200), GANPK (SEQ ID NO: 201), GANPR (SEQ ID NO: 9), GANPQ (SEQ ID NO: 202), GANQK(SEQ ID NO: 203), GANQQ (SEQ ID NO: 10), GANRR (SEQ ID NO: 8), and GANSS (SEQ ID NO: 11).

In another embodiment, the CNP variants are chimera comprising CNP22 and an N-terminal peptide fragment, including but not limited to:
GHHSHEQHPHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CQ**) (histidine-rich glycoprotein (HRGP) fragment-CNP22 chimera) (SEQ ID NO: 76);
GAHHPHEHDTHGANQQGLSKGCFGLKLDRIGSMSGLGC (**Analog CR**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 77);
GHHSHEQHPHGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CX**) (HRGP fragment-CNP22 chimera) (SEQ ID NO: 78);

GQPREPQVYTLPPSGLSKGCFGLKLDRIGSMSGLGC (**Analog CF**) ($IgG_1(F_c)$ fragment-CNP22 chimera) (SEQ ID NO: 79);

GQHKDDNPNLPRGANPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CY**) (human serum albumin (HSA) fragment-CNP22 chimera) (SEQ ID NO: 80);

GERAFKAWAVARLSQGLSKGCFGLKLDRIGSMSGLGC (**Analog CE**) (HSA fragment-CNP22 chimera) (SEQ ID NO: 81);

FGIPMDRIGRNPRGLSKGCFGLKLDRIGSMSGLGC (**Analog CZ**) (osteocrin "NPR C inhibitor" fragment-CNP22 chimera) (SEQ ID NO: 82); and

GKRTGQYKLGSKTGPGPKGLSKGCFGLKLDRIGSMSGLGC (**Analog DA**) (FGF2 "heparin-binding domain" fragment-CNP22 chimera) (SEQ ID NO: 83).

In yet another embodiment, the CNP variants are chimera comprising an N-terminal peptide fragment and CNP22 in which arginine is substituted for Lys4 of CNP22 ("CNP22(K4R)"), including but not limited to:

GQPREPQVYTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CK**) ($IgG_1(F_c)$ fragment-CNP22(K4R) chimera) (SEQ ID NO: 84);

GVPQVSTSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CL**) (HSA fragment-CNP22(K4R) chimera) (SEQ ID NO: 85);

GQPSSSSQSTGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CM**) (fibronectin fragment-CNP22(K4R) chimera) (SEQ ID NO: 86);

GQTHSSGTQSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CN**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 87);

GSTGQWHSESGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CO**) (fibrinogen fragment-CNP22(K4R) chimera) (SEQ ID NO: 88); and

GSSSSSSSSSGANQQGLSRGCFGLKLDRIGSMSGLGC (**Analog CP**) (zinc finger fragment-CNP22(K4R) chimera) (SEQ ID NO: 89).

Chimera comprising IgG and CNP22 or a variant thereof are designed for, *inter alia*, increased resistance to NEP degradation and reduced binding to serum albumin. CNP chimera comprising a surface fragment of HSA are designed for, *inter alia*, reduced immunogenicity and reduced binding to serum albumin. HRGP-CNP22 and HRGP-CNP22(K4R) chimera containing a cationic, histidine-rich, non-lysine, non-arginine sequence at the N-terminus are designed for, *inter alia*, increased stability to proteases. Chimera containing an osteocrin fragment are designed to release, upon protease (e.g., furin) cleavage, the osteocrin fragment at bone growth plates, where the fragment would inhibit the

clearance receptor NPR-C. With respect to chimera comprising an FGF2 heparin-binding fragment, heparin binding to the fragment is designed to protect the chimera from degradation, thereby providing a longer serum half-life. Chimera containing a fibronectin, fibrinogen, or zinc-finger fragment are designed for reduced binding to serum albumin, among other features.

Not intending to be bound by theory, a CNP variant of molecular weight from about 2.6 or 2.8 kDa to about 6 or 7 kDa which has increased resistance to NEP degradation and has similar or improved functionality (e.g., binding to NPR-B and stimulation of cGMP signaling) as compared to wtCNP22, may be more effective if it does not bind tightly to plasma proteins such as serum albumin. A CNP variant that does not bind tightly to plasma proteins (e.g., serum albumin) may be more effective in diffusing through cartilage, getting to chondrocytes of bone growth plates, and binding to and activating NPR-B for cGMP signaling. In one embodiment, CNP variants designed for reduced binding to plasma proteins (e.g., serum albumin) are chimeras comprising CNP22 or a variant thereof and a peptide fragment from IgG. In another embodiment, CNP variants designed for reduced binding to plasma proteins are chimeras comprising CNP22 or CNP22(K4R) and a fragment from a polypeptide (e.g., IgG, HSA, fibronectin, fibrinogen, a zinc finger-containing polypeptide, etc.). In yet another embodiment, CNP variants designed for reduced binding to plasma proteins comprise CNP22 or a variant thereof conjugated to a hydrophilic or water-soluble polymer. In one embodiment, the hydrophilic or water-soluble polymer is PEG (or PEO). In another embodiment, the hydrophilic or water-soluble polymer (e.g., PEG) is functionalized with one or more functional groups that impart a negative charge to the polymer under physiological conditions, such as, e.g, carboxyl, sulfate or phosphate groups, or a combination thereof.

In a further embodiment, CNP variants of the disclosure include truncated CNP peptides ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wild-type amino acid sequences derived from hCNP-53. Such truncated CNP peptides include:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-53) (SEQ ID NO: 4);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
GLSKGCFGLKLDRIGSMSGLGC (CNP-22) (SEQ ID NO: 1);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178); and
CFGLKLDRIGSMSGLGC (CNP-17) (SEQ ID NO: 2).
In certain embodiments, CNP variants do not include CNP-17, CNP-22 or CNP-53.

In another embodiment, the truncated CNP peptides ranging from hCNP-17 to hCNP-53 can contain amino acid addition(s), deletion(s) and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)), as described herein, at any one or more of the amino acid positions of the particular truncated CNP peptides. In yet another embodiment, the truncated CNP peptides having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can be conjugated at the N-terminus, C-terminus and/or internal site(s) to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bisphosphonates, bone- or cartilage-targeting peptide sequences (e.g., polyAsp, polyGlu), peptide sequences derived from bone-targeting domains of bone proteins (e.g., osteopontin, osteocalcin, sialoprotein)), peptide sequences derived from the functional domains of bone morphogenetic proteins (e.g., BMP2, BMP3, BMP5, BMP7, BMP8a), peptide sequences derived from natriuretic polypeptides (e.g., NPPC, ANP, BNP), peptide sequences derived from polypeptides of non-natriuretic origin (e.g., serum albumin, IgG, histidine-rich glycoproteins, fibronectin, fibrinogen, zinc finger-containing polypeptides, FGF-2, osteocrin), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at

bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids, natural fatty acids), phospholipids, and combinations thereof. In an embodiment, the truncated CNP peptides having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), and optionally conjugated to one or more moieties at the N-terminus, C-terminus and/or internal site(s), have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 kDa or 2.8 kDa to about 6 or 7 kDa.

In a further embodiment, the CNP variants are derivatives of CNP37, which is QEHPNARKYKGANKK-CNP22 (SEQ ID NO: 60). The CNP37 variants can contain amino acid addition(s), deletion(s), and/or substitution(s) with natural or unnatural amino acid(s) or peptidomimetic(s) (e.g., peptide bond isostere(s)) at any one or more of the 37 positions of CNP37. Non-limiting examples of substitutions that can be made in CNP37, based on the numbering of CNP22, include K4R, G5S, G5R, G8S, K10R, G15S, S16Q, M17N, G19R, and combinations thereof. In an embodiment, the CNP37 derivatives contain a substitution of Met17 to a natural (e.g., asparagine) or unnatural amino acid or peptidomimetic, designed in part to avoid oxidation of the sulfur atom of methionine. In another embodiment, the CNP37 variants contain substitution(s) of Lys8, Lys10, Lys14 and/or Lys15 (based on numbering from the N-terminus of CNP37) to non-basic natural or unnatural amino acid(s) or peptiomimetic(s), designed in part to reduce albumin binding.

In addition or alternatively to amino acid addition(s), deletion(s) and/or substitution(s), the CNP37 derivatives can be conjugated at the N-terminus, C-terminus, and/or an internal site to any of the moieties described herein, including but not limited to bone- or cartilage-targeting moieties (e.g., bone-targeting peptide domains), moieties that reduce renal clearance (e.g., negatively charged PEG moieties), hydrophilic polymers (e.g., PEG), amino acid sequences comprising one or more amino acids (e.g., osteocrin "NPR-C inhibitor" fragment), carbohydrates (e.g., carbohydrates recognized by receptors on the surface of cells at bone growth plates), hydrophobic acids (e.g., C_5-C_{12} carboxylic acids and natural fatty acids), and combinations thereof.

In one embodiment, the CNP variants are modified CNP37 peptides having mutation(s)/substitution(s) at the furin cleavage site (underlined), designed to improve *in vivo* resistance to the furin protease, and/or containing glycine (underlined) at the N-terminus, designed to improve plasma stability and prevent pyroglutamine formation. Such CNP37 variants include but are not limited to:
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (**An. CS**) (SEQ ID NO: 71);

GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (**An. CT**) (SEQ ID NO: 72);
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (**An. CU**) (SEQ ID NO: 73);
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (**An. CW**) (SEQ ID NO: 74);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37, **An. DB**) (SEQ ID NO: 75); and
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 145).

In a further embodiment, the CNP variants of the disclosure include CNP peptides and variants thereof that can be produced by the fusion protein process described herein. Non-limiting examples of CNP variants that can be produced by the fusion protein process described herein, using chemical or proteolytic cleavage or protein self-cleavage, include:
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-wtCNP53) (SEQ ID NO: 179);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-wtCNP37) (SEQ ID NO: 75);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP37) (SEQ ID NO: 60);
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC (HSA fragment-wtCNP27) (SEQ ID NO: 144);
GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP53(M48N)] (SEQ ID NO: 180);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);
GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);
GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-wtCNP53) (SEQ ID NO: 185);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-wtCNP37) (SEQ ID NO: 145);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-wtCNP37) (SEQ ID NO: 186);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (wtCNP34) (SEQ ID NO: 187);

P-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-wtCNP27) (SEQ ID NO: 188);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-wtCNP53) (SEQ ID NO: 190);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-wtCNP37) (SEQ ID NO: 191);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-wtCNP37) (SEQ ID NO: 192);

M-GHKSEVAHRFK-GANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-wtCNP27) (SEQ ID NO: 193);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

Other CNP variants, including truncated CNP peptides ranging from hCNP-17 to hCNP-53 and having wild-type sequences or amino acid addition(s), deletion(s) and/or substitution(s), can also be produced by the fusion protein process described herein, so long as the intended site of chemical or proteolytic cleavage of the fusion protein is not present within the amino acid sequence of the target CNP variant itself. As a non-limiting example, the fusion protein process described herein can be employed to produce truncated wtCNP34 using formic acid cleavage.

In additional embodiments, for any of the CNP peptides and CNP variants described herein that have asparagine (Asn/N) residue(s) and/or glutamine (Gln/Q) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln. Such substitution(s) are designed in part to minimize or avoid any potential deamidation of asparagine and/or glutamine. Non-limiting examples of CNP peptides and variants in which any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln, include wtCNP34, wtCNP37, Gly-wtCNP37, Pro-wtCNP37, Pro-Gly-wtCNP37, GHKSEVAHRFK-wtCNP27 (SEQ ID NO: 144), Pro-GHKSEVAHRFK-wtCNP27 (SEQ ID

NO: 188), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). In certain embodiments, an asparagine residue of the CNP peptides and CNP variants described herein is not substituted with glutamine, aspartic acid or glutamic acid. In certain embodiments, a glutamine residue of the CNP peptides and CNP variants described herein is not substituted with asparagine, aspartic acid or glutamic acid. As a non-limiting example, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 (PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC) (SEQ ID NO: 145) can independently be substituted with any other natural or unnatural amino acids, including glutamine, to avoid any potential deamidation of the asparagine residue(s) to aspartic acid or isoaspartic acid. In certain embodiments, asparagine residues 7 and/or 15 of Pro-Gly-wtCNP37 are not substituted with glutamine, aspartic acid or glutamic acid.

It is understood, however, that the present disclosure encompasses CNP variants in which any one or more, up to all, residues susceptible to deamidation or a deamidation-like reaction (e.g., isomerization) may be converted to other residue(s) via deamidation or a deamidation-like reaction to any extent, up to 100% conversion per converted residue. In certain embodiments, the disclosure encompasses CNP variants in which:

(1) any one or more, up to all, asparagine (Asn/N) residues may be converted to aspartic acid or aspartate, and/or to isoaspartic acid or isoaspartate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(2) any one or more, up to all, glutamine (Gln/Q) residues may be converted to glutamic acid or glutamate, and/or to isoglutamic acid or isoglutamate, via deamidation up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(3) any one or more, up to all, aspartic acid or aspartate (Asp/D) residues may be converted to isoaspartic acid or isoaspartate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(4) any one or more, up to all, glutamic acid or glutamate (Glu/E) residues may be converted to isoglutamic acid or isoglutamate via a deamidation-like reaction (also called isomerization) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue; or

(5) a combination of the above.

As a non-limiting example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Pro-Gly-wtCNP37 [PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue, as described above.

As a further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine and/or aspartic acid residues of Pro-Gly-wtCNP37 [PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC] (SEQ ID NO: 145) may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, and/or (2) isoaspartic acid/isoaspartate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As another example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of Gly-wtCNP37 [GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 75)] may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As yet another example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, glutamine, aspartic acid, and/or glutamic acid residues of wtCNP37 [QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 60)] may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) glutamic acid/glutamate and/or isoglutamic acid/isoglutamate, (3) isoaspartic acid/isoaspartate, and/or (4) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a further example, the present disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of an HSA-wtCNP27 chimera, GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 144), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

As a still further example, the disclosure encompasses CNP variants in which any one or more, up to all, asparagine, aspartic acid, and/or glutamic acid residues of a Pro-HSA-wtCNP27 chimera, PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 188), may be converted to (1) aspartic acid/aspartate and/or isoaspartic acid/isoaspartate, (2) isoaspartic acid/isoaspartate, and/or (3) isoglutamic acid/isoglutamate, respectively, via deamidation or a deamidation-like reaction up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% conversion per converted residue.

In addition, the present disclosure encompasses CNP variants in which any one or more, up to all, methionine (Met/M) residues may be oxidized to any chemically feasible oxidized form (e.g., sulfoxide and/or sulfone) up to about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% transformation per oxidized residue.

In another embodiment, the CNP variants comprise CNP22 or variants thereof conjugated at the N-terminus and/or C-terminus to moiet(ies) that facilitate translocation of the variants across a cell membrane or cell barrier. In one embodiment, the CNP variants are conjugated at the N-terminus and/or C-terminus to peptide sequence(s) that facilitate transport of the variants across a cell membrane or cell barrier, including via active peptide transporters.

In a further embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to chemical moieties such as, e.g., natural and/or synthetic polymers, to increase the total mass of the modified CNP peptide to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa. In one embodiment, the chemical moieties are biocompatible hydrophilic or water-soluble natural (e.g., peptides, carbohydrates) or synthetic (*e.g.*, PEG (or PEO)) polymers.

In a particular embodiment, the N-terminus and/or C-terminus of CNP22 or a variant thereof are conjugated to PEG (or PEO) polymers to result in a total mass characterized by

the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa. Pegylation of CNP22 or a variant thereof is designed, *inter alia*, to reduce immunogenicity and improve half-life by reducing renal clearance and increasing protease resistance. A PEG moiety can be attached to the N- and/or C-terminus of CNP22 or any variant described herein, including but not limited to CNP-17 (the Cys6-Cys22 cyclized portion of CNP22), CNP37, and variants of CNP17, CNP22 or CNP37 having N- and/or C-terminal amino acid extension(s), amino acid substitution(s) and/or amino acid deletion(s). In an embodiment, the Lys4 and/or Lys10 residues of CNP17, CNP22 or CNP37, or variants thereof, are substituted with a natural or unnatural amino acid (e.g., Arg, Gly, Ser, Gln, Glu or Cit) or peptidomimetic that does not contain a reactive primary amine on a side chain, to preclude any potential PEGylation of these lysine residues. In one embodiment, the Lys4 and/or Lys10 residues of the CNP peptides are substituted with Arg. In another embodiment, the Lys10 residue is not substituted with Arg.

In a further embodiment, CNP variants (including CNP22 and variants thereof) having a PEG (or PEO) moiety and an amino acid extension at the N-terminus contain arginine at the position immediately preceding the position corresponding to Gly1 of CNP22. Such PEGylated CNP variants are designed for increased resistance to NEP degradation, reduced binding to serum albumin, and enhanced CNP functional activity (e.g., activation of cGMP signaling). Non-limiting examples of PEGylated CNP variants include PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), PEO24-GANRR-CNP22(SEQ ID NO: 36), PEO12-GANRR-CNP22(SEQ ID NO: 36), PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37), PEO24-GANPR-CNP22(SEQ ID NO: 37), PEO12-GANPR-CNP22(SEQ ID NO: 37), PEO24-GANQQ-CNP22(SEQ ID NO: 64), PEO12-GANQQ-CNP22(SEQ ID NO: 64), PEO24-ER-CNP22(K4R) (SEQ ID NO: 39), PEO12-ER-CNP22(K4R) (SEQ ID NO: 39), PEO24-ER-CNP22(SEQ ID NO: 39), PEO12-ER-CNP22(SEQ ID NO: 39), PEO24-R-CNP22(K4R) (SEQ ID NO: 41), PEO12-R-CNP22(K4R) (SEQ ID NO: 41), PEO24-R-CNP22(SEQ ID NO: 41), and PEO12-R-CNP22(SEQ ID NO: 41), wherein PEO24 is a monodispersed 1.2 kDa PEG polymer and PEO12 is a monodispersed 0.6 kDa PEG polymer. In one embodiment, the PEG (or PEO) polymer is conjugated to the N-terminus of the CNP variants.

The disclosure contemplates use of hydrophilic or water soluble polymers (e.g., PEG) that can vary in type (e.g., homopolymer or copolymer; random, alternating or block

copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus and/or C-terminus, preferably not at any of the residues in the cyclized region of CNP (corresponding to residues 6-22 of CNP22)), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 3, 4 or 5 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP peptide by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. Such CNP variants can be generated using, e.g., wtCNP22 (2.2kDa), CNP17 retaining only the cyclized region (residues 6-22) of wtCNP22, CNP variants having an amino acid extension at the N-terminus and/or C-terminus of CNP22 or CNP17, or variants having amino acid substitutions, additions and/or deletions such as, e.g., GANRR-CNP22(K4R) (SEQ ID NO: 36), GANPR-CNP22(K4R) (SEQ ID NO: 37), R-CNP22(SEQ ID NO: 40), R-CNP22(K4R) (SEQ ID NO: 41), ER-CNP22(SEQ ID NO: 38) and ER-CNP22(K4R) (SEQ ID NO: 39). In an embodiment, the PEG-CNP variants having a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, contain a monodispersed, linear PEG (or PEO) moiety conjugated at the N-terminus and/or C-terminus via NHS- or aldehyde-based chemistry, or a two-arm or three-arm branched PEG moiety conjugated at the N-terminus and/or C-terminus via NHS-based chemistry. The disclosure also encompasses negatively charged PEG-CNP variants designed for reduced renal clearance, including but not limited to carboxylated, sulfated and phosphorylated compounds (Caliceti, Adv. Drug Deliv. Rev., 55: 1261-77 (2003); Perlman, J. Clin. Endo. Metab., 88: 3227-35 (2003); Pitkin, Antimicrob. Ag. Chemo., 29: 440-444 (1986); Vehaskari, Kidney Int'l, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

In another embodiment, the PEG (or PEO) moieties conjugated to the N-terminus, C-terminus and/or internal site(s) of CNP variants described herein contain one or more functional groups that are positively charged under physiological conditions. Such PEG moieties are designed, *inter alia*, to improve distribution of such PEGylated CNP variants to cartilage tissues. In one embodiment, such PEG moieties contain one or more primary, secondary or tertiary amino groups, quaternary ammonium groups, and/or other amine-containing (e.g., urea) groups.

In an embodiment, the disclosure encompasses CNP22 or variants thereof conjugated via NHS- or aldehyde-based chemistry to PEG (or PEO) of the formula $(CH_2CH_2O)_n$,

wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In still another embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl.

In a further embodiment, the disclosure provides CNP variants having one or more peptide bonds or peptide bond isosteres that have reduced susceptibility to cleavage by peptidases including neutral endopeptidase (NEP). NEP is a membrane-bound zinc-dependent endopeptidase that cleaves a substrate peptide bond at the amino end of large hydrophobic residues. Thus, modification of a peptide bond at a cleavage site for NEP to an unnatural peptide or non-peptide bond may preclude or decrease the efficiency of NEP cleavage.

For ANP and CNP, NEP cleavage is reported to occur first at the Cys6-Phe7 bond within the cyclized region, then elsewhere throughout the remainder of the structures. For BNP, cleavage is reported to occur first at the peptide N-terminus, then within the cyclic structure. Although the primary NEP cleavage site on CNP is reported to be the Cys6-Phe7 bond, when wtCNP22 was exposed to NEP digestion for 2.5 minutes *in vitro*, all possible sites were unexpectedly hydrolyzed, with the Cys6-Phe7 and Gly8-Leu9 peptide bonds being slightly most labile, as described in Example 2.

Substrate specificity of NEP is primarily determined by two substrate-binding subsites, S1' and S2' (Oefner et al., J. Mol. Biol. 296:341-349 (2000)). The S1' site accepts a large hydrophobic P1' residue of which the N-terminal peptide bond is subjected to hydrolysis (e.g., Phe, Leu, Ile and Met). The S2' site generally prefers a smaller residue, termed P2' (e.g., Gly or Ser). In the case of CNP, Phe7 is reported to be the preferred P1' residue for the NEP S1' site, while Gly8 is the preferred P2' residue for the S2' site. Because these two subsites can together accommodate only a certain total side chain size, any increase in the total size of the P1'-P2' residues of CNP can potentially disrupt NEP binding. For example, addition of a chloride atom at the 3-position of the P1' Phe7 aromatic ring (i.e., 3-Cl-Phe7) can potentially modify (e.g., destabilize) interactions between CNP and the NEP cleavage sites, for example at the S1' subsite. Addition of a tertiary butyl group to the

smaller P2' residue Gly8 (i.e., tBu-Gly8) can potentially disrupt the interaction between CNP and the S2' subsite.

Accordingly, in one embodiment, CNP variants of the disclosure include CNP having an increase in the size of the P1'-P2' residues, such as Phe7-Gly8, to interfere with substrate recognition at the active site, thereby reducing susceptibility to NEP cleavage. Natural amino acids, unnatural amino acids and/or peptidomimetic moieties are substituted for one or more large P1' hydrophobic residues, including but not limited to Phe7, Leu9, Leu11, Ile14, Met17 and Leu20, and/or for one or more smaller P2' residues, including but not limited to Cys6, Gly8, Gly15, Ser16 and Gly19.

The disclosure encompasses CNP variants comprising at least one modified amino acid and/or at least one modified peptide bond, at at least one residue involved in substrate recognition and/or cleavage by NEP, wherein the modified amino acids and modified peptide bonds can be natural amino acids, unnatural amino acids, peptidomimetics and/or peptide bond isosteres. In one embodiment, the NEP cleavage site on CNP between Cys6 and Phe7 is modified. In a related embodiment, the peptide bond (-C(=O)-NH-) between Cys6 and Phe7 is replaced with one of the following peptide bond isosteres:

$-CH_2-NH-$,

-C(=O)-N(R)-, where the amide group is alkylated with any of the following R groups: methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl,

$-C(=O)-NH-CH_2-$,

$-CH_2-S-$,

$-CH_2-S(O)_n-$, where n is 1 or 2,

$-CH_2-CH_2-$,

-CH=CH-,

$-C(=O)-CH_2-$,

-CH(CN)-NH-,

$-CH(OH)-CH_2-$,

-O-C(=O)-NH-, or

-NHC(=O)NH-.

In another embodiment, the CNP variants are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-Lys_4-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-Leu_9-Lys_{10}-Leu_{11}-Asp_{12}-Arg_{13}-Ile_{14}-Gly_{15}-Ser_{16}-Met_{17}-Ser_{18}-Gly_{19}-Leu_{20}-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 90), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein (Wang et al., Adv. Drug Delivery Rev., 57: 1049-76 (2005)); polymeric and non-polymeric molecules that reduce renal clearance such as, e.g., negatively charged PEGs; and natural polymers (e.g., those containing amino acids, fatty acids and/or carbohydrates) and synthetic polymers (e.g., PEGs) that increase resistance of the CNP variant to NEP degradation by increasing the total mass of the CNP variant to the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa;

(b) and (c) may be the wild-type Cys6 and Phe7, another natural amino acid or an unnatural amino acid, or may contain a peptide bond isostere as described herein to increase resistance to NEP cleavage; and

(d) may be the wild-type Gly8, or may be a larger natural or unnatural (e.g., t-Bu-Gly) amino acid or peptidomimetic to reduce binding to NEP.

In one embodiment, such CNP variants contain at least one modified amino acid at (b), (c) and/or (d).

Other peptide bonds within CNP may be cleaved even if CNP22 or a variant thereof has an NEP-resistant peptide bond or peptide bond isostere at Cys6-Phe7, including the Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17, and Gly19-Leu20 bonds. Therefore, the disclosure encompasses CNP variants having peptide bond isostere(s) at one or more other NEP cleavages sites in addition to the Cys6-Phe7 bond, wherein the peptide bond isosteres include those described herein.

In another embodiment, the disclosure encompasses CNP variants having a cysteine analog at Cys6 and/or Cys22, including but not limited to homocysteine, penicillamine, 2-mercaptopropionic acid, and 3-mercaptopropionic acid. In an embodiment, such CNP variants have a cyclic domain formed by a disulfide bond between the wild-type Cys6 or analog and Cys22 or analog.

In yet another embodiment, one or more residues of CNP22 or a variant thereof, up to all the residues, are substituted with a D-amino acid. Substitution of an L-amino acid with a D-amino acid essentially moves the side chain about 120 degrees from its original position,

thereby potentially disrupting the binding of the CNP peptide to NEP. In a specific embodiment, L-Phe at Phe7 is substituted with its D-enantiomer, D-Phe.

In still another embodiment, a beta amino acid such as, e.g., 3-amino-2-phenylpropionic acid (or 2-phenyl-beta-alanine), replaces the wild-type alpha-amino acid Phe7. Use of a beta-amino acid effectively increases the peptide backbone length by one methylene unit. Protease resistance can result from the change in substrate conformation or the increased distance between amino acid side chains.

Non-limiting examples of variants of CNP22 having an unnatural alpha-amino acid, a beta-amino acid or a peptide bond isostere include:

GLSKGC(CH_2NH)FGLKLDRIGSMSGLGC (**Analog A**) (SEQ ID NO: 56),
GLSKGC-(N-Me-Phe)-GLKLDRIGSMSGLGC (**Analog B**) (SEQ ID NO: 57),
GLSKGC-(D-Phe)-GLKLDRIGSMSGLGC (**Analog E**) (SEQ ID NO:136),
GLSKGCF-(tBu-Gly)-LKLDRIGSMSGLGC (**Analog F**) (SEQ ID NO: 58),
GLSKGC-(3-Cl-Phe)-GLKLDRIGSMSGLGC (**Analog G**) (SEQ ID NO:137), and
GLSKGC-[$NHCH_2CH(Ph)CO$]-GLKLDRIGSMSGLGC (**Analog H**, formed using 3-amino-2-phenylpropionic acid) (SEQ ID NO: 59).

In a further embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP degradation, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-Gly_{15}-Ser_{16}-(i)$_{17}$-Ser_{18}-Gly_{19}-(j)$_{20}$-Gly_{21}-Cys_{22}-(z) (SEQ ID NO: 46), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin, and sialoprotein; polymeric and non-polymeric moieties that reduce renal clearance such as, e.g., negatively charged PEGs; polymers containing, e.g., amino acids, hydrophobic acids, and/or carbohydrates; and synthetic hydrophilic polymers such as, e.g., PEGs;

(a) may be the wild-type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not

have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide-bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, heterocyclyl, C_{6-14} aryl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly (tBu-Gly), Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr and peptide-bond isosteres such as, e.g., N-Me-Leu;

(f) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (f) is not Arg;

(g) is selected from the group consisting of Leu and peptide-bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tBu-Gly, and peptide-bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(j) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Ser, Thr, Arg, and peptide-bond isosteres such as, e.g., N-Me-Leu.

In another embodiment, the CNP variants have a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, designed for increased resistance to NEP cleavage, and are represented by the formula:

(x)-Gly_1-Leu_2-Ser_3-(a)$_4$-Gly_5-(b)$_6$-(c)$_7$-(d)$_8$-(e)$_9$-(f)$_{10}$-(g)$_{11}$-Asp_{12}-Arg_{13}-(h)$_{14}$-(i)$_{15}$-Ser_{16}-(j)$_{17}$-Ser_{18}-Gly_{19}-(k)$_{20}$-Gly_{21}-Cys_{22}-(z)(SEQ ID NO:143), wherein:

(x) and (z) independently may be absent or may be selected from the group consisting of synthetic bone-targeting compounds such as, e.g., bisphosphonates; amino acid sequences useful in bone or cartilage targeting such as, e.g., polyAsp and polyGlu; amino acid sequences derived from bone-targeting domains of bone proteins and derivatives thereof, such as, e.g., fusion proteins or peptide sequences of osteopontin, osteocalcin, sialoprotein, etc.; moieties that reduce renal clearance, including but not limited to hydrophilic or water-soluble polymers such as, e.g., charged PEG molecules; and moieities comprising, e.g., PEGs, amino acids, carbohydrates, and/or hydrophobic acids;

(a) may be the wild type Lys at that position or may be replaced with a conservative amino acid substitution or a natural or unnatural amino acid or peptidomimetic that does not have a reactive primary amine on a side chain, including but not limited to Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln, Ser or Glu, wherein in one embodiment (a) is Arg;

(b) is selected from the group consisting of Cys and peptide bond isosteres between Cys6 and Phe7 such as, e.g., Cys-CH_2-NH;

(c) is selected from the group consisting of L-Phe; D-Phe; 3-amino-2-phenylpropionic acid; N-alkylated derivatives of Phe, wherein the N-alkyl group is methyl, ethyl, n-propyl, isopropyl, cyclopropyl, n-butyl, isobutyl, sec-butyl or tert-butyl; and Phe analogs, wherein one or more ortho-, meta-, and/or para- positions of the benzene ring of the Phe analog are substituted with one or more substituents selected from the group consisting of halogen, hydroxyl, cyano, straight or branched C_{1-6} alkyl, straight or branched C_{1-6} alkoxy, straight or branched halo-C_{1-6} alkyl, C_{3-10} cycloalkyl, C_{6-14} aryl, heterocyclyl and heteroaryl (examples include, but are not limited to, tyrosine, 3-chlorophenylalanine, 2,3-chloro-phenylalanine, 3-chloro-5-fluoro- phenylalanine, 2-chloro-6-fluoro-3-methyl-phenylalanine), or wherein the benzene ring of the Phe analog can be replaced with another aryl group (non-limiting

examples include 1- and 2-naphthylalanine) or with a heteroaryl group (non-limiting examples include pyridylalanine, thienylalanine and furylalanine);

(d) is selected from the group consisting of Gly, tert-butyl-Gly, Thr, Ser, Val and Asn;

(e) is selected from the group consisting of Leu, Ser, Thr, and peptide bond isosteres such as, e.g., N-Me-Leu;

(f) is selected from the group consisting of Lys, Arg, Gly, 6-hydroxy-norleucine, citrulline (Cit), Gln and Ser;

(g) is selected from the group consisting of Leu, Asn, and peptide bond isosteres such as, e.g., N-Me-Leu;

(h) is selected from the group consisting of Ile, tert-butyl-Gly (tBu-Gly), Asn, and peptide bond isosteres such as, e.g., N-Me-Ile;

(i) is selected from the group consisting of Gly, Arg, Ser and Asn;

(j) is selected from the group consisting of Met, Val, Asn, beta-Cl-Ala, 2-aminobutyric acid (Abu) and 2-amino-isobutyric acid (Aib); and

(k) is selected from the group consisting of Leu, norleucine (Nle), homoleucine (Hleu), Val, tert-butyl-Ala (tBu-Ala), Arg, Thr, Ser, and peptide bond isosteres such as, e.g., N-Me-Leu.

To improve the delivery of the CNP variants to the target sites of bone-related disorders (e.g., skeletal dysplasias), the CNP variants can be attached (e.g., at the N-terminus and/or C-terminus) to bone- or cartilage-targeting moieties. Non-limiting examples of bone- or cartilage-targeting moieties include bisphosphonates; hydroxyapatite; glucosarnine; collagen (e.g., collagen type X); polyAsp; polyGlu; and amino acid sequences derived from bone-targeting domains of bone proteins such as, e.g., osteocrin, osteopontin, osteocalcin, and sialoprotein.

In addition to being less susceptible to NEP cleavage, the CNP variants potentially have reduced affinity to the NPR-C clearance receptor, while retaining CNP functionality. Besides NEP-mediated degradation, the half-life of CNP22 is influenced by the clearance receptor, NPR-C, which shares 58% sequence homology with the extracellular peptide-binding domain of NPR-B. CNP22 binds tightly to not only NPR-B (7-30 pM affinity), but also NPR-C (11-140 pM) (Bennett, B.D. et al., J. Biol. Chem., 266: 23060-67 (1991); Koller K.J. & Goeddel, D.V., Circulation, 86: 1081-88 (1992); Suga, S. et al., Endocrinology, 130:

229-39 (1992)). Even though the NPR-B crystal structure has yet to be reported, sequence homology as well as similarities between the NPR-C and NPR-A crystal structures (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)) suggest that NPR-B likely assumes a similar overall structural fold.

Therefore, an NPR-B homology model was built based on structure-based sequence alignment and crystallographic structures of the following related systems: CNP bound to NPR-C, ANP bound to NPR-A, and ANP bound to NPR-C (He, X.-L. et al., Science, 293(5535): 1657-62 (2001); Ogawa, H. et al., J. Biol. Chem., 279(27): 28625-31 (2004); He, X.-L., J. Mol. Biol., 361(4): 698-714 (2006)). Based on observations that the receptor appears to determine the bound peptide conformation, and that NPR-B most closely resembles NPR-A with respect to primary structure and functional properties, the NPR-B/CNP homology model was built with the NPR-A/ANP crystal structure as a model. Published signaling data of CNP variants (U.S. Patent No. 5,434,133 and US Patent Application Publication No. 2004/0138134 A1), and of functional ANP variants that no longer bind to NPR-C (Cunningham, EMBO 13(11) 2508-15, 1994) were used to refine and interpret the NPR-B/CNP model.

The present disclosure encompasses CNP variants designed for improved NPR-B selectivity based on a homology-based structural model of the NPR-B/CNP complex. Combining the experimental and computational structure data of natriuretic peptides bound to the various receptors with the published functional data, CNP variants were generated that continue to bind to NPR-B, but can potentially have reduced affinity to the NPR-C clearance receptor. For example, NPR-C has a unique insertion in a loop structure in the peptide-binding site, placing its loop residues closer to such peptide residues as CNP Gly8 (or ANP Gly9), compared to respective loop residues in NPR-A and NPR-B. Earlier studies indicated that the G9T mutation in ANP contributes to reduce affinity to NPR-C, thereby improving NPR-A selectivity (Cunningham, EMBO J., 13(11): 2508-15 (1994)). Accordingly, CNP variants were generated to replace the corresponding Gly8 residue with a larger residue (Ser, Val, Thr or Asn) to disrupt the CNP binding to NPR-C without affecting its binding to NPR-B. Further, one or more mutations were introduced at the C-terminal end of CNP, encompassing Gly15 to Gly21, which is predicted to interact with receptor-specific residues, based on the detailed structural analyses of the receptor/peptide complexes. For example, a G19R mutation in CNP22 does not result in a significant loss of NPR-B signaling activity.

This mutation, however, cannot be modeled into the available crystal structure of NPR-C/CNP without altering the conformations of neighboring residues. These observations suggest that the G19R mutation may selectively disrupt the binding of CNP to a particular receptor, such as NPR-C.

In an embodiment, the CNP variants have substitution(s) at one or more Gly sites at positions 1, 5, 8, 15, 19 and 21, to reduce conformational flexibility and thereby increase receptor specificity. Comparative analyses of crystal structures of ANP bound to NPR-C and NPR-A (Ogawa, H. et al., J. Biol. Chem., 279: 28625-31 (2004); He, X.-L., J. Mol. Biol., 361: 698-714 (2006)) indicate that the conformational flexibility of ANP may play an important role in determining the receptor selectivity.

In one embodiment, functional CNP variants with potentially reduced affinity to NPR-C have one or more of the following amino acid substitutions: G1R, G1E, G5R, G5Q, G5S, F7Y, G8T, G8S, G8V, G8N, L9S, L9T, K10Cit, K10Q, K10S, I14N, G15R, G15S, G15N, G15Cit, S16Q, M17V, M17N, G19S, G19R, G19N, L20V, L20R, L20T, L20S, G21S, G21T and G21R. In an embodiment, the CNP variants have multipoint substitutions at positions 1, 5, 7, 8, 9, 10, 14, 15, 16, 17, 19, 20 and/or 21, and may optionally have modifications at any of the other positions in the peptide sequence of the variant.

In a further embodiment, the CNP variants described herein may be conjugated to moieties, up to a total mass characterized by the ranges described generally herein, e.g., from about 2.6 or 2.8 kDa to about 6 or 7 kDa, at the N-terminus, the C-terminus and/or an internal site, to facilitate bone/cartilage targeting, reduce NPR-C and renal clearance, increase resistance to NEP degradation, and/or improve CNP functionality. In one embodiment, the CNP variants are not conjugated to a polymeric moiety at a site within the cyclic region (corresponding to Cys6 to Cys22 of CNP22). Non-limiting examples of polymeric or non-polymeric moieties that can be conjugated to the CNP variants include synthetic bone-targeting compounds such as, e.g., bisphosphonates; bone/cartilage targeting peptide sequences such as, e.g., polyAsp and polyGlu; peptide sequences derived from bone-targeting domains of bone proteins such as, e.g., osteopontin, osteocalcin and sialoprotein; peptide sequences derived from the functional domains of bone morphogenetic proteins such as, e.g., BMP2, BMP3, BMP5, BMP7 and BMP8a; peptide sequences derived from polypeptides of natriuretic origin such as, e.g., NPPC, ANP and BNP; other natural polymeric or non-polymeric moieties such as, e.g., carbohydrates, fatty acids and phospholipids; biocompatible synthetic hydrophilic polymers such as, e.g., PEG (or PEO); hydrophobic polymeric or non-

polymeric moieties such as, e.g., heptanoic acid and pentanoic acid; and combinations thereof.

The CNP variants described herein can have substantially similar or better functional activity than CNP22, e.g., with respect to stimulation of cGMP production and signaling. In one embodiment, the CNP variants *in vitro* or *in vivo* stimulate the production of at least about 50% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM). In certain embodiments, the CNP variants retain at least about 50%, 60%, 70%, 80%, 90%, 95% or 100% of the cGMP-stimulation activity of wild-type CNP22 *in vitro* or *in vivo*. In another embodiment, the CNP variants have improved cGMP-stimulation activity compared to CNP22. In certain embodiments, the CNP variants *in vitro* or *in vivo* stimulate the production of at least about 110%, 120%, 130%, 140%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500% or more of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM).

Optionally excluded from the present disclosure are any of the natriuretic (e.g., CNP) peptides, fragments and variants specifically disclosed, and any of the natriuretic (e.g., CNP) peptides, fragments and variants actually produced, in any of the prior publications referenced herein, including but not limited to, U.S. 5,434,133, U.S. 6,034,231, U.S. 6,020,168, U.S. 6,743,425, U.S. 7,276,481, WO 94/20534, WO 02/047871, WO 2005/098490, WO 2004/047871, EP 0497368, EP 0466174, and Furuya et al., Biochem. Biophys. Res. Comm. 183: 964-969 (1992)). All such documents are incorporated by reference herein in their entirety.

In one embodiment, the present disclosure optionally excludes all known wild-type CNP-53, wild-type CNP-22, wild-type CNP-17, wild-type BNP, and wild-type ANP of human origin and non-human origin. For example, in an embodiment the disclosure optionally excludes human CNP-17, human CNP-22, chicken CNP-22 (corresponding to hCNP-22(Leu9Val)), trout and eel CNP-22 (corresponding to hCNP-22(Leu2Trp, Ser3Asn, Lys4Arg)), frog CNP22-I (corresponding to hCNP-22(Leu2Tyr, Lys4Arg, Leu9Val, Ser16Ala, Met17Phe)), frog CNP22-II (corresponding to hCNP-22(Leu2Thr, Ser16Ala)), human CNP-53, and pig and rat CNP-53 (corresponding to hCNP-53(Gln17His, Ala28Gly)). In another embodiment, the disclosure optionally excludes fragments of NPPC, proCNP and CNP-53 which are produced by proteolytic cleavage *in vivo* in humans and non-human animals. In yet another embodiment, optionally excluded from the disclosure are the

following truncated fragments of wild-type human CNP-53: CNP-50, CNP-46, CNP-44, CNP-39, CNP-30, CNP-29, CNP-28, CNP-27 and CNP-26.

In a further embodiment, the present disclosure optionally excludes CNP peptides and fragments thereof isolated or sought from the shark species *Triakis scyllia* and *Scyliorhinus canicula* (see, e.g., M. Takano *et al.*, Zool. Sci., 11: 451-454(1994)), including:
RLLKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-41) (SEQ ID NO: 204);
LKDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39) (SEQ ID NO: 205);
KDLSNNPLRFRGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38) (SEQ ID NO: 206); and
GPSRGCFGVKLDRIGAMSGLGC (CNP-22) (SEQ ID NO: 207).

In another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated or sought from the shark species *Lamna ditropis* (see, e.g., M. Takano *et al.*, Zool. Sci., 11: 451-454(1994)), including:
RLLKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-41) (SEQ ID NO: 208);
LKDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-39) (SEQ ID NO: 209);
KDLSNNPLRFKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-38) (SEQ ID NO: 210);
FKGRSKKGPSRGCFGVKLDRIGAMSGLGC (CNP-29) (SEQ ID NO: 211); and
GPSRGCFGVKLDRIGAMSGLGC (CNP-22) (SEQ ID NO: 212).

In still another embodiment, optionally excluded from the disclosure are CNP peptides and fragments thereof isolated from the shark species *Squalus acanthias* (see, e.g., M. Takano *et al.*, Zool. Sci., 11: 451-454(1994)), including:
RLLQDLSNNPLRFKGRSKKGPSRSCFGLKLDRIGAMSGLGC (CNP-41) (SEQ ID NO: 213); and
GPSRSCFGLKLDRIGAMSGLGC (CNP-22) (SEQ ID NO: 214).

In a further embodiment, the present disclosure optionally excludes CNP peptides isolated from medaka and puffer fish, designated "CNP-1", "CNP-2", "CNP-3" and "CNP-4" in K. Inoue *et al.*, Proc. Nat. Acad. Sci., 100(17): 10079-10084 (2003):
GWNRGCFGLKLDRIGSMSGLGC (medaka and puffer fish CNP-1) (SEQ ID NO: 215);
PMVAGGGCFGMKMDRIGSISGLGC (medaka CNP-2) (SEQ ID NO: 216);

GRSSMVGGRGCFGMKIDRIGSISGLGC (puffer fish CNP-2) (SEQ ID NO: 217);
GGMRSCFGVRLERIGSFSGLGC (medaka CNP-3) (SEQ ID NO: 218);
GGLRSCFGVRLARIGSFSGLGC (puffer fish CNP-3) (SEQ ID NO: 219);
GGSTSRSGCFGHKMDRIGTISGMGC (medaka CNP-4) (SEQ ID NO: 220); and
GGSSRSGCFGHKMDRIGTISGMGC (puffer fish CNP-4) (SEQ ID NO: 221).

In a still further embodiment, optionally excluded from the disclosure are CNP-39 isolated from platypus venom and the CNP-22 fragment thereof, designated "ovCNP-39" and "ovCNP-39(18-39)" in G. de Plater *et al.*, Toxicon., 36(6): 847-857 (1998):
LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGSTSGLGC (ovCNP-39) (SEQ ID NO: 222); and
GLSKGCFGLKLDRIGSTSGLGC (ovCNP-39(18-39)) (SEQ ID NO: 223).

In another embodiment, the present disclosure optionally excludes the following peptides as specifically disclosed in US 2007/0197434:
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ala-Met-Ser-Gly-Leu-Gly-Cys (SEQ ID NO: 224);
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Gln-Ser-Gly-Leu-Gly-Cys (SEQ ID NO: 225);
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Ala-Ser-Gly-Leu-Gly-Cys (SEQ ID NO: 226);
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys (SEQ ID NO: 227);
Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 228); and
Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (SEQ ID NO: 229).

In yet another embodiment, the disclosure optionally excludes peptides of SEQ ID NO:10, disclosed generically in US 2007/0197434, wherein such peptides are CNP-17 variants having certain natural amino acid substitution(s) at position(s) 4, 5, 6, 11, 12, 14 and/or 15.
In still another embodiment, optionally excluded from the disclosure are peptides corresponding to hCNP-53(Ser47Ala), hCNP-53(Met48Gln), hCNP-53(Met48Ala), and hCNP-53(C-term.)–Asn-Ser-Phe-Arg-Tyr.

In an embodiment, the present disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 6-71 as specifically disclosed in US 7,276,481. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NO 5, disclosed generically in US 7,276,481, wherein such peptides are variants of CNP17 having at least one natural amino acid substitution at Leu9, Lys10, Leu11, Ser16, Met17, Gly19, and/or Leu20. In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof contain N-Me-Phe7, or N-Me-Phe7 and N-Me-Leu11. In a further embodiment, the disclosure optionally excludes CNP17 variants of SEQ ID NO 5, as disclosed in US 7,276,481, which are fused or conjugated to growth hormone (GH), insulin-like growth factor 1 (IGF-1), or thyroid hormone (TH). In yet another embodiment, optionally excluded are CNP22 variants in which CNP22 is fused to GH, IGF-1 or TH, or attached to GH, IGF-1 or TH via a linker (e.g., a peptide linker). In still another embodiment, optionally excluded are CNP17 variants in which CNP17 or variants thereof are conjugated to biotin or fluorescein at the N-terminus or the C-terminus.

In a further embodiment, the present disclosure optionally excludes the peptides of Compound Nos. 1-27, and SEQ ID NOs 1-17, 22-24, 30, 31 and 40-42 as specifically disclosed in US 5,434,133. In another embodiment, the disclosure optionally excludes peptides of SEQ ID NOs 18-21 and 25-29, disclosed generically in US 5,434,133. In still another embodiment, the disclosure optionally excludes the peptides of SEQ ID NOs 1-4 and 9 as specifically disclosed in WO 94/20534.

In some embodiments, however, the disclosure still encompasses methods of use of the natriuretic (e.g., CNP) peptides, fragments and variants optionally excluded herein, as well as pharmaceutical compositions (including sterile pharmaceutical compositions) comprising such natriuretic (e.g., CNP) peptides, fragments and variants.

C. Synthesis and Purification of CNP Variants

In some embodiments, the CNP variants described herein are produced by recombinant expression, using certain techniques known in the art in certain embodiments. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. (1989)); DNA Cloning: A Practical Approach, Volumes I and II, D. N. Glover, Ed. (1985); and Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

In certain embodiments, the CNP variants described herein are produced by a recombinant process that comprises culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker. In some embodiments, the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant polypeptide linked to the polynucleotide encoding the cleavable peptide or protein. In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. The expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant.

Host cells used to produce CNP variants can be bacterial, yeast, insect, non-mammalian vertebrate, or mammalian cells. Bacterial cells include without limitation *E. coli* cell lines and strains. Non-limiting examples of *E. coli* cell lines and strains include BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41 [also called C41(DE3)], C43 [also called C43(DE3)], Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3). In an embodiment, CNP variants and CNP fusion proteins are produced using BL21(DE3) cells. Mammalian cells include, but are not limited to, hamster, monkey, chimpanzee, dog, cat, bovine, porcine, mouse, rat, rabbit, sheep and human cells. The host cells can be immortalized cells (a cell line) or non-immortalized (primary or secondary) cells and can be any of a wide variety of cell types, such as, but not limited to, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), ovary cells (e.g., Chinese hamster ovary or CHO cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), chondrocytes and other bone-derived cells, and precursors of these somatic cell types. Host cells containing the CNP variant DNA or RNA are cultured under conditions appropriate for growth of the cells, expression of the DNA or RNA and identification/selection of cells expressing the CNP variant.

In some embodiments, the host cells are grown or cultured for a time period at a temperature from about 10 °C to about 40 °C, or from about 20 °C to about 40 °C, or from about 30 °C to about 40 °C. In certain embodiments, the host cells are grown or cultured for

a time period at about 20 °C, 22 °C, 25 °C, 28 °C, 30 °C, 35 °C or 37 °C. In certain embodiments, the host cells are grown or cultured for a time period at about 35 °C or 37 °C.

Recombinant polynucleotides encoding CNP variant polypeptides (including CNP fusion proteins) are expressed in an expression vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or *in vitro* expression system. Expression vectors include all those known in the art, including without limitation cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide. The expression vector is inserted into an appropriate host cell, via transformation or transfection, for expression of the polynucleotide encoding the polypeptide (see, e.g., Sambrook et al. (*supra*)).

Non-limiting examples of expression vectors contemplated for production of CNP variants, including cleavable CNP fusion proteins, include pJexpress, pJexpress401, pJexpress404, pET-15b, pET-21a, pET-22b, pET-31b, pET-32a, pET-41a, pMAL, pMAL-c2X, pQE-30, pET-SUMO, and pTYB11. Expression of particular constructs can generate soluble CNP variants (including CNP fusion proteins) or insoluble CNP variants (including CNP fusion proteins) in the form of inclusion bodies.

In some embodiments, expression of the polynucleotide(s) encoding a CNP variant or CNP fusion protein is enhanced using an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible vector. In some embodiments, the host cells are grown or cultured for a time period at a temperature from about 10 °C to about 40 °C, or from about 20 °C to about 40 °C, or from about 30 °C to about 40 °C, in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 20 °C, 22 °C, 25 °C, 28 °C, 30 °C, 35 °C or 37 °C in the presence of IPTG. In certain embodiments, the host cells are grown or cultured for a time period at about 35 °C or 37 °C in the presence of 1 mM IPTG.

In further embodiments, the host cells are cultured with IPTG at a concentration from about 0.4 mM to about 2 mM, or from about 0.4 mM to about 1.5 mM, or from about 0.4 mM to about 1 mM. In certain embodiments, the IPTG is at a concentration of about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2 mM. In an embodiment, the concentration of IPTG is about 1 mM.

In certain embodiments, the CNP variants described herein are recombinantly expressed as fusion proteins comprising a CNP variant polypeptide and a cleavable carrier protein or cleavable tag (e.g., peptide tag), wherein the fusion protein comprises the CNP variant polypeptide directly linked to the cleavable carrier protein or tag or indirectly linked thereto via a linker. Use of a carrier protein or tag facilitates, e.g., detection, isolation and/or purification of the fusion protein. Cleavable carrier proteins and tags include, but are not limited to, histidine (e.g., hexa-His) tags; human transcription factor TAF12 (TAF12), TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E); ketosteroid isomerase (KSI); maltose-binding protein (MBP); ß-galactosidase (ß-Gal); glutathione-S-transferase (GST); thioredoxin (Trx); chitin binding domain (CBD); BMP-2, BMP-2 mutants, BMP-2(C/A); SUMO; and mutants and fragments thereof.

An expression construct can express a fusion protein comprising a CNP variant and a carrier protein or tag. The tag can be an amino acid sequence that confers a useful property to the fusion protein. In one embodiment, the tag is a ligand-binding domain that can be used to purify the fusion protein by applying the fusion protein to separation media containing the ligand. For example, a fusion protein comprising a glutathione-S-transferase (GST) domain can be applied to a chromatographic column containing glutathione-linked separation media. As another example, a fusion protein comprising maltose-binding protein (MBP) as a tag can be applied to separation media containing maltose. As a further example, a fusion protein comprising a polyhistidine tag may be applied to a nickel column, whereby chelation of the polyhistidine tag to the nickel column facilitates purification of the fusion protein. In another embodiment, the tag is a ligand. For example, a fusion protein can comprise glutathione as a tag and be applied to a chromatographic column containing glutathione-S-transferase-linked separation media. Non-limiting examples of carrier proteins and tags for use in fusion proteins include human transcription factor TAF12 (TAF12), ketosteroid isomerase (KSI), maltose-binding protein (MBP), ß-galactosidase (ß-Gal), glutathione-S-transferase (GST), thioredoxin (Trx), chitin-binding domain (CBD), BMP-2 mutation (BMPM), SUMO, CAT, TrpE, staphylococcal protein A, streptococcal proteins, starch-binding protein, cellulose-binding domain of endoglucanase A, cellulose-binding domain of exoglucanase Cex, biotin-binding domain, recA, Flag, poly(His), poly(Arg), poly(Asp), poly(Gln), poly(Phe), poly(Cys), green fluorescent protein, red fluorescent protein, yellow fluorescent protein, cyan

fluorescent protein, biotin, avidin, streptavidin, antibody epitopes, and mutants and fragments thereof.

To generate the target CNP variant, the carrier protein or tag can be cleaved from the fusion protein by means of chemical cleavage, protease cleavage, or protein self-cleavage. Exemplary chemical and proteolytic cleavage agents (cleavage sites in parenthesis) include, but are not limited to, formic acid (Asp-Pro), cyanogen bromide (CNBr) (Met-X), hydroxylamine (Asn-Gly), Factor Xa (IEGR-X) (SEQ ID NO: 230), Enterokinase (DDDDK-X) (SEQ ID NO: 231), ProTEV (EXXYXQ-G) (SEQ ID NO: 232), and SUMO protease. Due to the nature of the particular kinds of chemical cleavage, cleavage using formic acid may generate Pro-CNP, CNBr may generate CNP having Met-to-Asn substitution, and hydroxylamine may generate Gly-CNP. Alternatively, chemical or protease cleavage may be avoided by using particular constructs (e.g., pET-21a-CNP) that express CNP variants not as fusion proteins. Expression of pET-21a-CNP may produce Met-CNP. Or certain fusion proteins (e.g., those containing intein-CBD) can undergo self-cleavage to generate CNP.

In further embodiments, a fusion protein comprises a cleavable peptide linker between a CNP variant and a carrier protein or tag (e.g., peptide tag). In certain embodiments, the cleavable peptide linker is selected from the group consisting of Asp-Pro, Asn-Gly, Met-X, Val-Asp-Asp-Arg (SEQ ID NO: 233), Gly-Ser-Asp-Arg (SEQ ID NO: 234), Ile-Thr-Asp-Arg (SEQ ID NO: 235), Pro-Gly-Asp-Arg (SEQ ID NO: 236), Ile-Glu-Gly-Arg-X (SEQ ID NO: 230), Asp-Asp-Asp-Asp-Lys-X (SEQ ID NO: 231), Glu-X-X-Tyr-X-Gln-Gly (SEQ ID NO: 232), Ala-Phe-Leu-Gly-Pro-Gly-Asp-Arg (SEQ ID NO: 237), and MGSSHHHHHHSSGLVPRGSHTGDDDDKHMD (pET-15b linker) (SEQ ID NO: 95), where X denotes an amino acid. In some embodiments, the cleavable peptide linker is cleaved by a cleaving agent selected from the group consisting of palladium, cyanogen bromide (CNBr), formic acid, hydroxylamine, clostripain, thrombin, chymotrypsin, trypsin, trypsin-like proteases, carboxypeptidase, enterokinase (enteropeptidase), Kex 2 protease, Omp T protease, Factor Xa protease, subtilisin, proTEV, SUMO protease, V8 protease, HIV protease, rhinovirus protease, furilisin protease, IgA proteases, human Pace protease, collagenase, Nia protease, poliovirus 2Apro protease, poliovirus 3C protease, genenase, furin, elastase, Proteinase K, pepsin, rennin (chymosin), microbial aspartic proteases, papain, calpain, chymopapain, ficin (ficain), bromelain (bromelase), cathespisin B, caspases, thermolysin, Endoprotease Arg-C, Endoprotease Glu-C, Endoprotease Lys-C, kallikrein, and plasmin.

In certain embodiments, the cleavable carrier protein, tag (e.g., peptide tag) or peptide linker is cleaved using formic acid to release the CNP variant from the fusion protein. In some embodiments, the formic acid is at a concentration from about 1% to about 20%, or from about 1% to about 15%, or from about 2% to about 15%, or from about 1% to about 10%, or from about 2% to about 10%, or from about 1% to about 5%, or from about 2% to about 5%. In certain embodiments, the formic acid is at a concentration of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14% or 15%. In certain embodiments, the formic acid is at a concentration of about 2%, 5% or 10%.

In further embodiments, cleavage of the CNP fusion protein in the presence of formic acid is conducted at a temperature from about 20 °C to about 80 °C, or from about 30 °C to about 75 °C, or from about 40 °C to about 75 °C, or from about 50 °C to about 75 °C, or from about 50 °C to about 70 °C, or from about 55 °C to about 70 °C, or from about 50 °C to about 60 °C. In some embodiments, cleavage in the presence of formic acid is conducted at about 20 °C, 22 °C, 25 °C, 30 °C, 35 °C, 37 °C, 40 °C, 42 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C or 80 °C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 50 °C, 55 °C, 60 °C, 65 °C or 70 °C. In certain embodiments, cleavage in the presence of formic acid is conducted at about 55 °C or 70 °C.

In additional embodiments, cleavage of the CNP fusion protein in the presence of formic acid is performed for a time period from about 3 hr to about 48 hr, or from about 5 hr to about 48 hr, or from about 5 hr to about 36 hr, or from about 5 hr to about 24 hr, or from about 5 hr to about 18 hr, or from about 20 hr to about 24 hr, or for about 6 hr to about 10 hr. In certain embodiments, cleavage in the presence of formic acid is performed for about 5 hr, 6 hr, 12 hr, 15 hr, 18 hr, 20 hr or 24 hr.

In some embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2%, 5% or 10% formic acid at about 55 °C for about 20 hr to about 36 hr, or at about 60 °C for about 15 hr to about 24 hr, or at about 65 °C for about 10 hr to about 21 hr, or at about 70 °C for about 6 hr to about 18 hr. In certain embodiments, cleavage of the CNP fusion protein is conducted in the presence of about 2% formic acid at about 55 °C for about 20 hr to about 24 or 36 hr, or at about 60 °C for about 15 hr to about 24 hr, or at about 65 °C for about 10 hr to about 18 hr, or at about 70 °C for about 6 hr to about 10 hr.

The present disclosure provides mild conditions for cleavage of CNP fusion proteins using formic acid to afford high yields of CNP variants. The conditions described herein for

fusion protein cleavage using formic acid are also suitable for cleavage of fusion proteins comprising polypeptides or proteins other than CNP, where the fusion proteins contain an Asp-Pro peptide bond.

In further embodiments, soluble CNP fusion proteins or CNP fusion protein inclusion bodies are treated with a buffer and/or a detergent prior to chemical cleavage (e.g., using formic acid) or proteolytic cleavage of the CNP fusion protein. Non-limiting examples of buffers include B-PER II; diluted B-PER II (e.g., 1/20 dilution); B-PER; B-PER phosphate buffer; buffers containing Tris (e.g., 25 mM Tris, pH 7.5); buffers containing Tris and NaCl (e.g., 25 mM Tris, 150 mM NaCl, pH 7.9); and PBS. In an embodiment, the buffer is B-PER II. Non-limiting examples of detergents include octylsucrose, Triton X-100, Tween-20, NP-40, and CA-630. The detergent can be in a buffer (e.g., 1% detergent in 25 mM Tris buffer, pH 7.5). In certain embodiments, the detergent is Triton X-100 or CA-630.

It is understood that any of the methods and conditions described above may be used in combintion with any of the other methods and conditions described above to generate a CNP variant disclosed herein.

In other embodiments, the CNP variants described herein are synthesized using a peptide synthesizer and purified according to methods known in the art, e.g., according to the methods of Atherton and Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

Peptides can be synthesized based on, e.g., the following peptide sequence of CNP: G^1LS(K or R)$GC^6F^7G^8L$(K or R or Nle or 6-OH-Nle)$LDRIGSMSGLGC^{22}$.

Exemplary CNP variants include but are not limited to:

Analog A (GLSKGC(CH2NH)FGLKLDRIGSMSGLGC) (SEQ ID NO: 56) was made by converting the backbone "-C=O" group of C^6 to a "-CH_2" group;

Analog B (GLSKGC(N-Me-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO: 57) was made by converting the backbone "-NH" group of F^7 to an "-N-CH_3" group;

Analog E (GLSKGC(D-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO:136) was made using D-Phe at F^7;

Analog F (GLSKGCF(tBu-Gly)LKLDRIGSMSGLGC) (SEQ ID NO: 58) was made using a tertiary-butyl-Gly at G^8;

Analog G (GLSKGC(3-Cl-Phe)GLKLDRIGSMSGLGC) (SEQ ID NO:137) was made by adding a chloride atom to a meta position of the phenyl ring of F^7 (similar variants

can be generated by making ortho, meta and/or para substitutions of the phenyl ring of Phe7 with Cl, F, Br, OH and/or CH_3); and

Analog H (GLSKGC[$NHCH_2CH(Ph)CO$]GLKLDRIGSMSGLGC) (SEQ ID NO: 59) was made using (±)-3-(amino)-2-phenylpropionic acid at F^7.

Examples of CNP variants having, e.g., amino acid extensions, substitutions with natural or unnatural amino acids or peptide bond isosteres, and/or conjugations to polymers or hydrophobic moieties, include without limitation:

Analog J	C6-CH2-NH, N-Me-L9, N-Me-L20 (SEQ ID NO: 91)
Analog K	N-Me-L9, N-Me-L20 (SEQ ID NO: 92)
Analog L	N-Me-L9, N-Me-L11, N-Me-L20 (SEQ ID NO: 93)
Analog M	N-Me-L9, N-Me-L11 (SEQ ID NO: 94)
Analog Z	K4R, F7Y (SEQ ID NO: 95)
Analog AA	K4R, G8V (SEQ ID NO: 96)
Analog AB	K4R, G8S (SEQ ID NO: 97)
Analog AC	K4R, G8T (SEQ ID NO: 98)
Analog AD	K4R, L9T (SEQ ID NO: 99)
Analog AE	K4R, G15R (SEQ ID NO: 100)
Analog AF	K4R, G15Cit (SEQ ID NO: 101)
Analog AG	K4R, M17V (SEQ ID NO: 102)
Analog AH	K4R (SEQ ID NO: 35)
Analog AJ	K4R, L20V (SEQ ID NO: 103)
Analog AK	K4R, L20t-Bu-Ala (SEQ ID NO: 104)
Analog AT	G1E, K4E (SEQ ID NO: 105)
Analog AV	G1E, K4E - pentanoic acid (attached at the N-terminus) (SEQ ID NO: 106)
Analog AW	G1E, K4E - heptanoic acid (attached at the N-terminus) (SEQ ID NO: 107)
Analog AX	CNP17 (delta N-term) (SEQ ID NO: 2)
Analog AY	GANRR-CNP22(K4R) (SEQ ID NO: 36)
Analog AZ	R-CNP22(K4R) (SEQ ID NO: 41)
Analog BB	G1E - heptanoic acid (attached at the N-terminus) (SEQ ID NO: 108)
Analog BC	G1E - pentanoic acid (attached at the N-terminus) (SEQ ID NO: 109)
Analog BF	K4R, K10Cit (SEQ ID NO: 110)
Analog BG	K4R, K10Q (SEQ ID NO: 111)
Analog BH	K4R, K10R (SEQ ID NO: 112)
Analog BJ	K4R, G15N (SEQ ID NO: 113)
Analog BK	K4R, G15S (SEQ ID NO: 114)
Analog BL	CNP-37 (SEQ ID NO: 60)
	CNP-53 (SEQ ID NO: 4)
Analog CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)
Analog CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)
Analog CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)
Analog CD	SPKMVQGSG-CNP17-KVLRRH (N- and C-terminal BNP tails) (SEQ ID NO: 68)
Analog CE	GERAFKAWAVARLSQ-CNP22 (HSA-CNP22) (SEQ ID NO: 81)
Analog CF	GQPREPQVYTLPPS-CNP22 (SEQ ID NO: 79)
	PEG(24K)-CNP22
	PEG(20K)-CNP22

PEG(5K)-CNP22
PEG(2K)-CNP22
PEG(2K)-CNP17
PEG(1K)-GANRR-CNP22(K4R) (SEQ ID NO: 36)
PEG(1K)-CNP22
PEO4-(PEO12)3(branched)-CNP22
PEO12-CNP22
PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36)
PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36); and

SEQ ID NOs: 1 to 6 and 34 to 144, and variants thereof that comprise up to 1, 2, 3, 4, or 5 further modifications.

In one embodiment, the CNP variants are cyclized via formation of a disulfide bond between Cys^6 and Cys^{22}. Cys^6 can be a cysteine analog such as, e.g., homocysteine or penicillamine. In a further embodiment, the CNP variants can be cyclized by a covalent bond formed head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail. In an embodiment, the covalent bond is formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus of the peptide (referred to as "terminal" amino acids in this context). In another embodiment, the covalent bond is formed between the side chains of the two terminal amino acids. In yet another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of the two terminal amino acids.

Head-to-tail cyclization of the terminal amine to the terminal carboxyl group can be carried out using a number of methods, e.g., using p-nitrophenyl ester, 2,4,5-trichlorophenyl ester, pentafluorophenyl ester, the azide method, the mixed anhydride method, HATU, a carbodimide (e.g., DIC, EDC or DCC) with a catalyst such as HOBt, HONSu or HOAt, or on-resin cyclization.

In addition, the cyclic structure can be formed via a bridging group involving the side chains of amino acid residues of the CNP variant and/or the terminal amino acid residues. A bridging group is a chemical moiety that allows cyclization of two portions of the peptide. Non-limiting examples of bridging groups include amides, thioethers, thioesters, disulfides, ureas, carbamates, sulfonamides, and the like. A variety of methods are known in the art for incorporation of units having such bridging groups. For example, a lactam bridge (i.e., a cyclic amide) can be formed between the N-terminal amino group or an amino group on a side chain and the C-terminal carboxylic acid or a carboxyl group on a side chain, e.g., the side chain of lysine or ornithine and the side chain of glutamic acid or aspartic acid. A

thioester can be formed between the C-terminal carboxyl group or a carboxyl group on a side chain and the thiol group on the side chain of cysteine or a cysteine analog.

Alternatively, a cross link can be formed by incorporating a lanthionine (thio-dialanine) residue to link alanine residues that are covalently bonded together by a thioether bond. In another method, a cross-linking agent, such as a dicarboxylic acid (e.g., suberic acid (octanedioic acid)), can link the functional groups of amino acid side chains, such as free amino, hydroxyl, and thiol groups.

Enzyme-catalyzed cyclization can also be used. For example, it has been reported that the thioesterase domain of tyrocidine synthetase can be used to cyclize a thioester precursor, a subtilisin mutant can be utilized to cyclize peptide glycolate phenylalanylamide esters, and the antibody ligase 16G3 can be employed to cyclize a p-nitrophenylester. For a review of peptide cyclization, see Davies, J. Peptide Sci., 9: 471-501 (2003), incorporated herein by reference in its entirety.

In certain embodiments, the final cyclized product has a purity of at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99%.

D. Chemically Modified CNP Variants

Chemical modification of CNP22 or variants thereof can potentially impart advantageous properties to the modified CNP peptides, such as increased stability and half-life and reduced immunogenicity (for a general discussion of chemical modification of therapeutic proteins, see Pharmazie, 57(1): 5-29 (2002)). For example, attaching natural or synthetic, polymeric or non-polymeric moieties (e.g., PEG) to CNP peptides, to increase the total mass of the CNP peptides to the ranges described generally herein, e.g., a range from about 2.6 or 2.8 kDa to about 6 or 7 kDa, can reduce the susceptibility of the modified peptides to *in vivo* cleavage by exopeptidases and/or endopeptidases (e.g., NEP). In addition to PEGylation, glycosylation and other chemical derivatization procedures, e.g., modification by phosphorylation, amidation, carboxylation, acetylation, methylation, and creation of acid-addition salts, amides, esters and N-acyl derivatives, may also mask potentially immunogenic regions and/or proteolytically sensitive regions (Science, 303: 480-482 (2004)).

Examples of chemical modifications include, without limitation, the polymer addition method of Bednarsaki and the cross-linking method of Altus Corporation for improving stability and protease resistance and reducing immunogenicity. Bednarsaki showed that polymer addition can improve protein temperature stability (J. Am. Chem. Soc., 114(1): 378-

380 (1992)), and Altus Corporation found that glutaraldehyde cross-linking can improve enzyme stability.

Chemical modification of polypeptides can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (e.g., based on wild-type macromolecule reactivity-directed derivatization and/or site-selective modification using a combination of site-directed mutagenesis and chemical modification) or, alternatively, using expressed protein ligation methods (Curr. Opin. Biotechnol., 13(4): 297-303 (2002)).

Pegylated CNP Variants

In one embodiment, for increased stability (e.g., resistance to NEP degradation), CNP22 or variants thereof (including those having amino acid additions, substitutions and/or deletions) are conjugated to hydrophilic, natural or synthetic polymers, to increase the total mass of the modified CNP peptides to a range from about 2.6 kDa or 2.8 kDa to about 4, 5, 6, 7 or higher kDa. In certain embodiments, the added hydrophilic polymers have a total mass of about 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6, 2.8, 3, 3.2, 3.4, 3.6, 3.8, 4, 4.2, 4.4, 4.6, 4.8, or about 5 kDa.

In an embodiment, the hydrophilic polymers are water-soluble so that the CNP peptides conjugated thereto do not precipitate out in an aqueous (e.g., physiological) environment. Further, the hydrophilic polymers are biocompatible, i.e., do not cause injury, toxicity or an immunological reaction *in vivo*.

The hydrophilic polymers can be branched or unbranched. In one embodiment, the hydrophilic polymers are not branched.

Various sites of conjugation of CNP22 or variants thereof to a hydrophilic polymer are possible, including but not limited to: (1) only at the N-terminus; (2) only at the C-terminus; (3) only at an internal site (e.g., Lys4); (4) at both the N-terminus and the C-terminus; (5) at the N-terminus and an internal site; and (6) at the C-terminus and an internal site. In one embodiment, CNP22 or variants thereof are conjugated to a hydrophilic polymer only at the N-terminus. In another embodiment, conjugation is only at an internal site (e.g., Lys4). In yet another embodiment, conjugation is at the N-terminus and an internal site (e.g., Lys4). In still another embodiment, for better functionality the CNP peptides are not conjugated to a hydrophilic polymer at a site (e.g., Lys10) within the cyclic domain (corresponding to Cys6 to Cys22 of CNP22). If conjugation to a hydrophilic polymer is based on bond formation with a reactive primary amino group on the CNP peptide,

conjugation at an internal site (e.g., Lys4 and/or Lys10) can be prevented by substitution of Lys4 and/or Lys10 with a natural or unnatural amino acid or peptidomimetic that does not contain a reactive primary amino group on a side chain, such as, e.g., Gly, Ser, Arg, Asn, Gln, Asp, Glu or citrulline (Cit). In a particular embodiment, Lys4 and/or Lys10 are replaced with Arg. In another embodiment, Lys10 is not replaced with Arg.

Non-limiting examples of hydrophilic polymers include polymers formed from carboxylic acid-bearing monomers (e.g., methacrylic acid (MA) and acrylic acid (AA)), polyvinyl alcohols, polymers formed from hydroxyl-bearing monomers (e.g., hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), hydroxypropyl methacrylamide, and 3-trimethylsilylpropyl methacrylate (TMSPMA)), polyalkylene oxides, polyoxyethylated polyols (e.g., glycerol), poly(ethylene glycol) (PEG), poly(propylene glycol), mono-C_1-C_{10} alkoxy-PEGs (e.g., monomethoxy-PEG), tresyl monomethoxy-PEG, aryloxy-PEGs, PEG acrylate (PEGA), PEG methacrylate, PEG propionaldehyde, bis-succinimidyl carbonate PEG, copolymers of 2-methacryloyloxyethyl-phosphorylcholine (MPC) and N-vinyl pyrrolidone (VP), hydroxy functional poly(N-vinyl pyrrolidone) (PVP), SIS-PEG (SIS is polystyrene-polyisobutylene-polystyrene block copolymer), polystyrene-PEG, polyisobutylene-PEG, PCL-PEG (PCL is polycaprolactone), PLA-PEG (PLA is polylactic acid), PMMA-PEG (PMMA is poly(methyl methacrylate)), PDMS-PEG (PDMS is polydimethyloxanone), PVDF-PEG (PVDF is polyvinylidene fluoride), PLURONIC™ surfactants (polypropylene oxide-co-polyethylene glycol), poly(tetramethylene glycol), poly(L-lysine-g-ethylene glycol) (PLL-g-PEG), poly(L-lysine-g-hyaluronic acid) (PLL-g-HA), poly(L-lysine-g-phosphoryl choline) (PLL-g-PC), poly(L-lysine-g-vinyl pyrrolidone) (PLL-g-PVP), poly(ethylimine-g-ethylene glycol) (PEI-g-PEG), poly(ethylimine-g-hyaluronic acid) (PEI-g-HA), poly(ethylimine-g-phosphoryl choline) (PEI-g-PC), poly(ethylimine-g-vinyl pyrrolidone) (PEI-g-PVP), PLL-co-HA, PLL-co-PC, PLL-co-PVP, PEI-co-PEG, PEI-co-HA, PEI-co-PC, PEI-co-PVP, cellulose and derivatives thereof (e.g., hydroxyethyl cellulose), dextran, dextrins, hyaluronic acid and derivatives thereof (e.g., sodium hyaluronate), elastin, chitosan, acrylic sulfate, acrylic sulfonate, acrylic sulfamate, methacrylic sulfate, methacrylic sulfonate, methacrylic sulfamate, polymers and copolymers thereof, and polymers and copolymers of combinations thereof.

In a particular embodiment, the hydrophilic polymer is poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO). As used herein, the term "PEG" or "PEO"

encompasses all the forms of PEG, branched and unbranched, which can be used to derivatize polypeptides, including without limitation mono-(C_1-C_{10}) alkoxy-PEGs and aryloxy-PEGs.

In one embodiment, the PEG-CNP conjugates comprise a PEG (or PEO) polymer of the formula $(CH_2CH_2O)_n$, wherein n is an integer from about 6 to about 100, and the PEG polymer is from about 0.3 kDa to about 5 kDa. In another embodiment, n is an integer from about 12 to about 50, and the PEG polymer is from about 0.6 kDa to about 2.5 kDa. In yet another embodiment, n is from about 12 to about 24, and the PEG polymer is from about 0.6 kDa to about 1.2 kDa. In a further embodiment, the terminal hydroxyl group of the PEG polymer is capped with a non-reactive group. In a particular embodiment, the end-capping group is an alkyl group, e.g., a lower alkyl group such as methyl, so that the PEG polymer terminates in an alkoxy group. In an embodiment, the PEG polymer is not branched. In another embodiment, CNP22 or variants thereof are conjugated to a PEG polymer only at the N-terminus.

PEGs and PEOs potentially include molecules with a distribution of molecular weights, i.e., they are potentially polydispersed, depending on the manner in which they are prepared. The size/mass distribution of a polymeric preparation can be characterized statistically by its weight average molecular weight (M_w) and its number average molecular weight (M_n), the ratio of which is called the polydispersity index (M_w/M_n). M_w and M_n can be measured by mass spectroscopy. PEG-CNP variants conjugated to a PEG moiety larger than 1.5 kDa may exhibit a range of molecular weights due to the polydispersed nature of the parent PEG molecule. For example, in the case of mPEG2K (Sunbright ME-020HS, NOF Co.), the molecular masses of the PEG molecules are distributed over a range from about 1.5 kDa to about 3 kDa, with a polydispersity index of 1.036. By contrast, the PEGs conjugated to CNP22 or variants thereof using $MS(PEG)_n$ reagents (n = 4, 8, 12 or 24, denoted as, e.g., "PEO12" or "PEO24") from Pierce Biotechnology (Rockford, Illinois) are monodispersed, having discrete chain length and defined molecular weight.

Methods for generating polypeptides comprising a PEG moiety are known in the art (see, e.g., US Patent 5,824,784). Methods for preparing PEGylated CNP peptides generally comprise the steps of (a) reacting CNP22 or a variant thereof with a PEGylation reagent under conditions suitable for attaching PEG to the CNP peptide (e.g., at the N-terminus), and (b) obtaining the reaction product(s). Because PEGylating a CNP peptide might significantly alter its binding to NPR-B, depending on the size of the PEG moiety and the location of

PEGylation, different kinds of PEG and PEGylation reaction conditions can be explored. The chemistry that can be used for PEGylation of a CNP peptide includes acylation of reactive primary amine(s) of the peptide using the NHS-ester of methoxy-PEG (O-[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates any charge of the original primary amine. PEG-CNP peptides designated with the symbol "PEO12" or "PEO24", as well as those designated with the symbol "PEG1K", "PEG2K", "PEG5K" or "PEG20K", are PEGylated via reaction of a primary amino group on the peptide with an NHS ester-activated, methoxy-end capped PEG reagent. PEG-CNP variants can also be prepared by other methods, e.g., via reductive amination involving a primary amino group on the peptide and a PEG aldehyde, such as, e.g., PEG-propionaldehyde, or mono-C_1-C_{10} alkoxy or aryloxy derivatives thereof (see U.S. Patent 5,252,714).

Unlike ribosome protein synthesis, synthetic peptide synthesis proceeds from the C-terminus to the N-terminus. Accordingly, Boc-PEG (containing tert-butyloxycarbonyl (Boc)) is one method to attach PEG to the C-terminus of a peptide (R. B. Merrifield, J. Am. Chem. Soc., 85(14): 2149-2154 (1963)). Alternatively, Fmoc (fluorenylmethoxycarbonyl) chemistry can be employed (E. Atherton and R.C. Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, IRL Press (Oxford, England (1989)).

The present methods for preparing PEG-CNP variants provide a substantially homogenous mixture of polymer-protein conjugates. After purification, discrete PEG-CNP preparations are sufficiently pure for *in vitro* and *in vivo* testing of biological properties. As demonstrated herein, certain PEG-CNP variants exhibit reduced susceptibility to NEP cleavage and substantially similar or better functionality (e.g., stimulation of cGMP production).

As described herein, PEGylation reactions of CNP22 or variants thereof, using appropriate PEGylation reagent/CNP peptide ratios and reaction conditions, provide PEG-CNP derivatives. The nature and extent of PEGylation can be determined using, e.g., PAGE and HPLC analysis. In certain embodiments, at least about 50%, 60%, 70%, 80%, 90%, 95% or 99% of CNP22 or variants thereof are mono-PEGylated at the N-terminus. To optimize the beneficial effects of PEGylation on the biological properties of a CNP peptide, the polymer length, conformation (e.g., branched or linear), and/or functionalization (e.g., adding a negatively charged group) of a PEG moiety can be varied. PEGylated CNP variants are tested for NEP resistance, pharmacokinetics and bioactivity (e.g., the ability to bind to NPR-

B and stimulate the generation of cGMP). PEGylated CNP variants that show improved NEP resistance and at least about 50% of the cGMP-stimulating activity of CNP22 can be further tested, e.g., *in vitro* in a rat chondrosarcoma cell-based achondroplasia model and *in vivo* in a murine achondroplasia animal model.

E. Methods of Using CNP Variants, Pharmaceutical Compositions of CNP Variants, and Routes of Administration

Methods of Using CNP Variants

Bone-Related Disorders

Fibroblast growth factors (FGFs) play important roles in bone formation, and mutations in FGF receptor genes (FGFR 1, 2 and 3) give rise to a variety of inherited skeletal malformations (Curr. Biol., 5: 500-507 (1995)). In particular, activating mutations in FGFR-3 are responsible for disorders of the long bones, including achondroplasia, the most common form of human genetic dwarfism (Nature, 371: 252-254 (1994); Cell, 78: 335-342 (1994)), the milder disorder hypochondroplasia (Ann. N.Y. Acad. Sci., 785: 182-187 (1996)), and the more severe and neonatal lethal thanatophoric dysplasia (TD) types I and II (Hum. Mol. Genet., 5: 509-512 (1996); Nat. Genet., 9: 321-328 (1995)). Mouse models overexpressing FGF-2, and consequentially activating FGFR-3, show shortened long bones and macrocephaly (Mol. Biol. Cell, 6: 1861-73 (1995)). Consistent with this model, mice deficient in FGFR-3 show remarkable skeletal overgrowth with wider growth plates (Nature Genet., 12: 390-397 (1996)).

Complementary experiments with CNP, NPR-B and NPR-C suggest a link between the peptide ligand, the corresponding receptors, and bone growth. Activation of NPR-B by elevated plasma concentrations of CNP in transgenic mice causes skeletal overgrowth (Nat. Med., 10: 80-86 (2004)) histologically similar to that of the growth plate cartilage of FGFR-3 knockout mice (Nat. Genet., 4: 390-397 (1996)). In NPR-C knockout mice, NPR-C-mediated clearance of CNP should be eliminated; consistent with this prediction, the knockout animals show elongated long bones and elongated vertebrae with kyphosis (Proc. Natl. Acad. Sci. USA 96: 7403-08 (1999)). Conversely, CNP knockout mice are dwarfed with shorter long bones and vertebrae, a phenotype histologically similar to that of achondroplasia, and have increased mortality as a result of malocclusion and pulmonary restriction from the small rib cage (Proc. Natl. Acad. Sci. USA, 98: 4016-4021 (2001)). Consistent with the proposed role of CNP as an activator of NPR-B, the NPR-B knockout mouse has the same dwarfed skeletal

phenotype and increased mortality as the CNP knockout mouse (Proc. Natl. Acad. Sci USA, 101: 17300-05 (2004)). Furthermore, in a mouse model of achondroplasia with activated FGFR-3 in the cartilage, targeted overexpression of CNP in chondrocytes counteracts dwarfism (Yasoda et al., Nat. Med., 10: 80-86 (2004)). Additionally, CNP has been show to play a role in regulating endochondral bone growth and chondrocyte activity, including but not limited to chondrocyte proliferation and differentiation, inhibition of the mitogen activated protein (MAP) kinase/MEK (Raf-1) kinase signaling pathway, and promotion of endochondral ossification (Yasoda et al., Nat. Med., 10: 80-86 (2004)). These results suggest that activation of the CNP/NPR-B system is a potential therapeutic strategy for treatment of human achondroplasia.

By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia. Further, the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

In addition, the CNP variants are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

The CNP variants of the disclosure can also be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP variants are useful for countering the undesired effects of FGF-2 and increasing matrix synthesis in subjects suffering from arthritis, including osteoarthritis, thereby treating arthritis, including osteoarthritis.

Vascular Smooth Muscle Disorders

CNP and other vasoactive peptides (including ANP, BNP and urodilatin) have vasodilator and diuretic properties and play an important role in cardiovascular homeostasis (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998); Kidney Int., 49: 1732-37 (1996); Am. J. Physiol., 275: H1826-1833 (1998)). CNP is widely distributed in the cardiovascular system, especially in high concentration in vascular endothelial cells (J. Cardiovasc. Pharmacol., 117: 1600-06 (1998)). CNP is a potent relaxant of vascular smooth muscle, particularly in the coronary circulation (Biochem. Biophys. Res. Commun., 205: 765-771 (1994)), and is an inhibitor of smooth muscle cell proliferation (Biochem. Biophys. Res. Commun., 177: 927-931 (1991)). Although the vasodilator effect of CNP is less potent than that of ANP (about 1:100) (Hypertens. Res., 21: 7–13 (1998); Am. J. Physiol., 275: L645–L652 (1998)), CNP mRNA is increased in response to shear stress (FEBS Lett., 373: 108-110 (1995)) and plasma levels of CNP are elevated in inflammatory cardiovascular pathologies (Biochem. Biophys. Res. Commun., 198: 1177-1182 (1994)). CNP has been shown to suppress inflammation through inhibition of macrophage infiltration in injured carotid arteries of rabbits (Circ. Res., 91: 1063–1069 (2002)) and to directly inhibit cardiac fibroblast proliferation through an NPR-B/cGMP-dependent pathway (Endocrinology, 144: 2279–2284 (2003)).

The cardiovascular actions of CNP are mediated via activation of the NPR subtypes, NPR-B and NPR-C (Endocrinology, 130: 229-239 (1992)), the latter accounting for 95% of NPRs expressed *in vivo* (Science, 293: 1657-1662 (2001)). The CNP/NPR-B pathway leads to elevation of cGMP, a well-established secondary messenger in the cardiovascular system.

NPR-C's 37-amino acid portion from the C-terminus has a consensus sequence that interacts with the heterotrimeric G protein G_i (J. Biol. Chem., 274: 17587-17592 (1999)), which has been shown to regulate adenylate cyclase and phospholipase C activity (J. Biol. Chem., 276: 22064-70 (2001); Am. J. Physiol., 278: G974-980 (2000); J. Biol. Chem., 271: 19324-19329 (1996)). CNP mediates smooth muscle hyperpolarization and relaxation via activation of NPR-C and the opening of a G protein-regulated, inwardly rectifying K^+ channels (Proc. Natl. Acad. Sci. USA, 100: 1426-1431 (2003)). Likewise, CNP has important anti-proliferative effects in cardiac fibroblasts and, through interaction with NPR-C, regulates local blood flow and systemic blood pressure by hyperpolarizing smooth muscle cells (R. Rose and W. Giles, J. Physiol. 586: 353-366 (2008)).

By binding to NPR-B on vascular smooth muscle cells, CNP22 stimulates the production of cGMP, which acts as an intracellular secondary messenger to cause ultimately the relaxation of blood vessels. Based on the hypotensive actions of CNP, the CNP variants of the disclosure are useful for treating hypertension, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute and chronic renal insufficiency, and so on. In addition, activation of cGMP signaling suppresses the growth of vascular smooth muscle cells. Accordingly, the CNP variants of the disclosure can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis.

The studies described above suggest that CNP may be a potential therapeutic candidate for vascular smooth muscle relaxation and remodeling. Pharmacological effects of CNP concerning certain disorders have been attributed, in part, to vasoprotective effects rather than to vasodilator activity (Am. J. Respir. Crit. Care Med., 170: 1204-1211 (2004)). Therefore, the CNP variants of the present disclosure are useful for treating conditions, e.g., vascular smooth muscle disorders, in which CNP may have a vasoprotective effect, including without limitation inducing smooth muscle relaxation and inhibiting infiltration of macrophages into cardiac tissue. In one embodiment, the CNP variants are used to treat heart failure, including but not limited to acute decompensated heart failure and acute congestive heart failure. In another embodiment, the CNP variants are used to treat asthma, cardiomyopathy, and restenosis of coronary arteries (by increasing smooth muscle cell relaxation and decreasing proliferation of smooth muscle cells).

Pharmaceutical Compositions of CNP Variants

In additional embodiments, the disclosure provides pharmaceutical compositions comprising a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (*e.g.*, inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

In some embodiments, the compositions comprise the desired CNP variant in at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% purity. In certain embodiments, the compositions contain less than about 10%, 5%, 4%, 3%, 2%, 1% or 0.5% of macromolecular contaminants, such as other mammalian (*e.g.*, human) proteins and other CNP variants.

Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactants, emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (*e.g.*, water, ethanol); diluents of various buffer content (*e.g.*, Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (*e.g.*, Polysorbate 20, Polysorbate 80); anti-oxidants (*e.g.*, methionine, ascorbic acid, sodium metabisulfite); preservatives (*e.g.*, Thimerosol, benzyl alcohol, m-cresol); and bulking substances (*e.g.*, lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)), which is incorporated herein by reference in its entirety.

For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (*e.g.*, oil/water emulsions). A carrier can be a solvent or dispersing medium containing, *e.g.*, ethanol, a polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1

mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 1 mg/ml to about 10 mg/ml.

In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, and sodium citrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. For example, acetate is a more efficient pH buffer at pH 5 than pH 6, so less acetate may be used in a solution at pH 5 than at pH 6. In some embodiments, the buffering agent has a concentration of about 10 mM ± 5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0 ± 1.0.

In other embodiments, the compositions contain an isotonicity-adjusting agent to render the solution or suspension isotonic and more compatible for injection. Non-limiting examples of isotonicity agents include NaCl, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is NaCl. In certain embodiments, NaCl is in a concentration of about 160 ± 20 mM , or about 140 mM ± 20 mM, or about 120 ± 20 mM , or about 100 mM ± 20 mM, or about 80 mM ± 20 mM, or about 60 mM ± 20 mM.

In yet other embodiments, the compositions comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4% ± 0.2%, or about 1% ± 0.5%, or about 1.5% ± 0.5%, or about 2.0% ± 0.5%.

In still other embodiments, the compositions contain an anti-adsorbent (e.g., to mitigate adsorption of a CNP variant to glass or plastic). Anti-adsorbents include without limitation benzyl alcohol, Polysorbate 20, and Polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

In additional embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%. In other embodiments, when the stabilizer is methionine, the stabilizer is in a concentration from about 0.01% to about 0.5%, or from about 0.01% to about 0.2%. In still other embodiments, when the stabilizer is glycerin, the stabilizer is in a concentration from about 5% to about 100% (neat).

In further embodiments, the compositions contain an antioxidant. Exemplary anti-oxidants include without limitation methionine and ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP variant is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (*e.g.*, hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (*e.g.*, acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (*e.g.*, isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, (Easton, Pennsylvania (1990)).

The pharmaceutical compositions can be administered in various forms, such as tablets, capsules, granules, powders, solutions, suspensions, emulsions, ointments, and transdermal patches. The dosage forms of the compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the compositions can take the form of, *e.g.*, a tablet or capsule (including softgel capsule), or can be, *e.g.*, an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or more commonly used excipieints, diluents and carriers, such as mannitol, lactose, glucose, sucrose, starch, corn starch, sodium saccharin, talc, cellulose, magnesium carbonate, and lubricating agents (*e.g.*, magnesium stearate, sodium stearyl fumarate). If desired, flavoring, coloring and/or sweetening agents can be added to the solid and liquid formulations. Other optional ingredients for oral formulations include without limitation preservatives, suspending agents, and thickening agents. Oral formulations can also have an enteric coating to protect the CNP variant from the acidic

environment of the stomach. Methods of preparing solid and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, *e.g.*, Remington's Pharmaceutical Sciences, referenced above).

Formulations for parenteral administration can be prepared, *e.g.*, as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (*e.g.*, buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

Compositions comprising a CNP variant can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dexran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%. Exemplary anti-oxidants include, but are not limited to, methionine and ascorbic acid.

The disclosure also provides kits containing, *e.g.*, bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, *e.g.*, sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices,

such as aerosol or injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

As a non-limiting example, a kit can include syringes having a single chamber or dual chambers. For single-chamber syringes, the single chamber can contain a liquid CNP formulation ready for injection, or a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) that can be reconstituted into a solution or suspension for injection. For dual-chamber syringes, one chamber can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection.

As a further example, a kit can include one or more pen injector or autoinjector devices, and dual-chamber cartridges. One chamber of a cartridge can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection. A cartridge can comprise an amount of the CNP variant that is sufficient for dosing over a desired time period (e.g., 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, etc.). The pen injector or autoinjector can be adjusted to administer a desired amount of the CNP formulation from a cartridge.

In addition, pharmaceutical compositions comprising a CNP variant can be formulated as a slow release, controlled release or sustained release system for maintaining a relatively constant level of dosage over a desired time period, such as 1 week, 2 weeks, 3 weeks, 1 month, 2 months, or 3 months. Slow release, controlled release and sustained release formulations can be prepared using, *e.g.*, biodegradable polymeric systems {which can comprise, *e.g.*, hydrophilic polymers [*e.g.*, polylactide, polyglycolide, poly(lactide-glycolide)]}, and can take the form of, *e.g.*, microparticles, microspheres or liposomes, as is known in the art.

Dosages and Frequency of Dosing

As used herein, the term "therapeutically effective amount" of an active agent (e.g., a CNP variant) refers to an amount that provides therapeutic benefit to a patient. The amount may vary from one individual to another and may depend upon a number of factors, including the overall physical condition of the patient. A therapeutically effective amount of a CNP variant can be readily ascertained by one skilled in the art, using publicly available materials and procedures. For example, the amount of a CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (214 females and 189 males), which list height for age, head circumference, and segmental growth (Horton WA et al., Standard growth curves for achondroplasia, J. Pediatr., 93: 435-8 (1978)). CDC charts can be used to assess weight for age and weight for height or BMI for age. Secondary outcomes with courses that are more chronic in nature can also be measured.

Having a longer serum half-life than wild-type CNP22, the CNP variants can potentially be administered less frequently than CNP22. The dosing frequency for a particular individual may vary depending upon various factors, including the disorder being treated and the condition and response of the individual to the therapy. In certain embodiments, a pharmaceutical composition containing a CNP variant is administered to a subject about one time per day, one time per two days, one time per three days, or one time per week. In one embodiment, for treatment of bone-related disorders (e.g., skeletal dysplasias, including achondroplasia), a daily or weekly dose of a CNP variant is administered to patients until and/or through adulthood.

The CNP variants described herein can be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) and conditions (e.g., vascular smooth muscle disorders) in which CNP can provide a vasoprotective effect. The safety and therapeutic efficacy of the CNP variants can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

Data obtained from cell culture assays and animal studies can be used to formulate a range of dosage for use in humans. The dosage normally lies within a range of circulating

concentrations that include the ED_{50}, with little or minimal toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The therapeutically effective dose can be determined from cell culture assays and animal studies.

In certain embodiments, the CNP variants described herein are administered at a dose in the range from about 5 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP variants are administered at a dose of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variants are administered at a dose of about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 ug/kg, or about 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of CNP variants described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc.

The frequency of dosing/administration of a CNP variant for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The CNP variant can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the CNP variant is administered, in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week, 3 times per week, weekly, bi-weekly, every 3 weeks, monthly, every 6 weeks, every 2 months, every 3 months, or as deemed appropriate by the treating physician.

In some embodiments, a CNP variant is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP variant may be administered intravenously, subcutaneously or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1

week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

Modes of Administration

The CNP variants, or pharmaceutical compositions comprising them, can be administered to subjects in various ways such as, e.g., by injection subcutaneously, intravenously, intra-arterially, intraperitoneally, intramuscularly, intradermally, or intrathecally. In an embodiment, the CNP variants are administered by a single subcutaneous, intravenous, intra-arterial, intraperitoneal, intramuscular, intradermal or intrathecal injection once a day.

The CNP variants can also be administered by direct injection at or near the site of disease. Further, the CNP variants can be administered by implantation of a depot at the target site of action (e.g., an abnormal or dysplasic bone). Alternatively, the CNP variants can be administered sublingually under the tongue (e.g., sublingual tablet) or by inhalation into the lungs (e.g., inhaler or aerosol spray), by delivery into the nasal cavity (e.g., intranasal spray), by delivery into the eye (e.g., eye drop), or by transdermal delivery (e.g., by means of a patch on the skin). The CNP variants may also be administered orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

A further method of administration is by osmotic pump (e.g., an Alzet pump) or mini-pump (e.g., an Alzet mini-osmotic pump), which allows for controlled, continuous and/or slow-release delivery of the CNP variant or pharmaceutical composition over a pre-determined period. The osmotic pump or mini-pump can be implanted subcutaneously, or near the target site (e.g., the long bones of limbs, the epiphyses, etc.).

As explained above, the CNP variants can be used to treat conditions or diseases caused by the abnormal growth of vascular smooth muscle cells, including but not limited to restenosis and arteriosclerosis. For local delivery of a CNP variant to the diseased bodily vessel (e.g., blood vessel), the CNP variant can be delivered by means of a medical device

(e.g., a stent) implanted at the diseased site. In one embodiment, the CNP variant is impregnated in a polymeric matrix or polymeric coating disposed over a stent. In another embodiment, the CNP variant is contained in reservoirs or channels formed in the body of a stent and covered by a porous polymeric membrane or layer through which the CNP variant can diffuse. The polymeric matrix, coating, membrane or layer can comprise at least one biodegradable (e.g., hydrophilic) polymer, as is known in the art. In a further embodiment, the CNP variant can be contained in micropores in the body of a stent. The CNP variant can be delivered from a stent by burst release, pulse release, controlled release or sustained release, or a combination thereof. For example, the stent can locally deliver the CNP variant to the diseased site in a burst release followed by a sustained release. Sustained release can be over a period up to about 2 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

It will be apparent to one skilled in the art that the CNP variants or compositions thereof can also be administered by other modes. Determination of the most effective mode of administration of the CNP variants or compositions thereof is within the skill of the skilled artisan.

The CNP variants can be administered as pharmaceutical formulations suitable for, *e.g.*, oral (including buccal and sub-lingual), rectal, nasal, topical, pulmonary, vaginal or parenteral (including intramuscular, intraarterial, intrathecal, subcutaneous and intravenous) administration, or in a form suitable for administration by inhalation or insufflation. Depending on the intended mode of administration, the pharmaceutical formulations can be in the form of solid, semi-solid or liquid dosage forms, such as tablets, suppositories, pills, capsules, powders, liquids, suspensions, emulsions, creams, ointments, lotions, and the like. The formulations can be provided in unit dosage form suitable for single administration of a precise dosage. The formulations comprise an effective amount of a CNP variant, and one or more pharmaceutically acceptable excipients, carriers and/or diluents, and optionally one or more other biologically active agents.

Combination Therapy

In one embodiment, a CNP variant can be used in combination with one or more other active agents useful for treating, ameliorating or preventing CNP-responsive conditions or disorders such as, e.g., bone-related disorders (e.g., skeletal dysplasias) and vascular smooth muscle disorders. The other active agent(s) can enhance the effects of the CNP variant and/or exert other pharmacological effects in addition to those of the CNP variant. Non-limiting

examples of active agents that can be used in combination with the CNP variants described herein are other natriuretic peptides (e.g., BNP) and inhibitors (e.g., antagonists) of peptidases and proteases (e.g., NEP and furin), NPR-C and tyrosine kinases (e.g., FGFR-3). By preventing NEP cleavage of the CNP variant, an NEP inhibitor can prolong the half-life of the variant. Examples of NEP inhibitors include, without limitation, thiorphan and candoxatril. Co-use of an NPR-C inhibitor can also prolong the half-life of the CNP variant via inhibition of the variant's clearance by NPR-C. A non-limiting example of an NPR-C inhibitor is the fragment FGIPMDRIGRNPR (SEQ ID NO: 82), which would be released at the target site (e.g., bone growth plate) upon proteolytic cleavage of the FGIPMDRIGRNPR-CNP22 chimera (Analog CZ) (SEQ ID NO: 82) or similar chimeras comprising variants of CNP22 (e.g., those containing amino acid substitution(s), addition(s), and/or deletion(s) relative to CNP22). Co-use of a tyrosine kinase inhibitor can accentuate the effects of a CNP therapy by inhibiting the tyrosine kinase receptor FGFR-3, a negative regulator of chondrocyte and bone growth. Non-limiting examples of tyrosine kinase inhibitors include those disclosed in U.S. 6,329,375 and 6,344,459.

To achieve the appropriate therapeutic outcome in the combination therapies, one would generally administer to the subject the CNP composition and other therapeutic(s) in a combined amount effective to produce the desired therapeutic outcome (*e.g.*, restored bone growth). This process may involve administering the CNP composition and other therapeutic agent(s) at the same time. Simultaneous administration can be achieved by administering a single composition or pharmacological protein formulation that includes both the CNP variant and other therapeutic agent(s). Alternatively, the other therapeutic agent(s) can be taken separately at about the same time as a pharmacological formulation (e.g., tablet, injection or drink) of the CNP variant. The CNP variant can also be formulated into a foodstuff such as brownies, pancakes, or cake, suitable for ingestion.

In other alternatives, administration of the CNP variant can precede or follow administration of the other therapeutic agent(s) by intervals ranging from minutes to hours. In embodiments where the other therapeutic agent(s) and the CNP composition are administered separately, one would generally ensure that the CNP variant and the other therapeutic agent(s) are administered within an appropriate time of one another so that both the CNP variant and the other therapeutic agent(s) can exert, synergistically or additively, a beneficial effect on the patient. For example, one can administer the CNP composition within about 0.5-6 hours (before or after) of the other therapeutic agent(s). In one

embodiment, the CNP composition is administered within about 1 hour (before or after) of the other therapeutic agent(s).

Identifying and Monitoring Patient Populations

Protocols can be established to identify subjects suitable for CNP therapy and to determine whether a given patient is responsive to CNP therapy. For example, for treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements *in utero* and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

One CNP signaling marker is cGMP (guanosine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (*in vitro*) after CNP exposure, conditioned media from bone ex-plant studies (*ex vivo*) after CNP exposure, and in the plasma (*in vivo*) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover preceeds new bone formation.

A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type II collagen (cartilage-specific marker for cartilage formation), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic

in vivo studies and from the conditioned media of *ex vivo* studies, using commercially available kits.

In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP variant in order to monitor the effects of the CNP variant on bone and cartilage formation and growth *in vivo*. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP variant has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g, endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

In an embodiment, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP variant. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

F. Examples

Example 1

Synthesis of CNP Variants

CNP variants were prepared using the methods described herein. Substitutions with natural or unnatural amino acids or peptidomimetics were made, as indicated in **Tables 1-3** (shown in Example 3), at the respective amino acid residues in the wild-type sequence of CNP22. In certain variants, additional amino acids were added to the N-terminal and/or C-terminal ends of the whole or a portion of the wild-type CNP22 sequence (see **Table 3**).

Also prepared were CNP variants in which a PEG (or PEO) moiety was conjugated to the N-terminus of CNP22 or variants thereof (see **Table 4**, shown in Example 3). PEGylation reagents can be obtained from the commercial sources shown in Table 5.

Table 5

Vendor	Product Name	Name	MW (Da)	PEGylation Reagent
NOF	Sunbright ME-200CS	mPEG20K	20,000	CH3(CH2CH2O)450-(CH2)5COO-NHS
NOF	Sunbright ME-050CS	mPEG5K	5,000	CH3(CH2CH2O)110-(CH2)5COO-NHS
Pierce	(Methyl-PEG12)3-PEG4-NHS Ester	(mPEG12)3-PEG4	2,400	[CH3(CH2CH2O)12]3-(CH2CH2O)4-NHCO(CH2)3-COO-NHS
NOF	Sunbright ME-020HS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO-NHS
NOF	Sunbright ME-020CS	mPEG2K	2,000	CH3(CH2CH2O)45-(CH2)5COO-NHS
NOF	Sunbright ME-010HS	mPEG1K	1,000	CH3(CH2CH2O)23-CO(CH2)2COO-NHS
Pierce	Methyl PEG24-NHS Ester	MS(PEG)24	1,200	CH3(CH2CH2O)24-(CH2)2COO-NHS
Pierce	EZ-Link NHS-PEG12-Biotin	PEO12-Biotin	940	Biotin-(CH2CH2O)12-(CH2)2COO-NHS
Pierce	Methyl PEG12-NHS Ester	MS(PEG)12	690	CH3(CH2CH2O)12-(CH2)2COO-NHS
Pierce	EZ-Link NHS-PEG4-Biotin	PEO4-biotin	590	Biotin-(CH2CH2O)4-(CH2)2COO-NHS
Pierce	Mono(lactosylamido) mono(succininidyl)suberate	LSS	590	
Pierce	EZ-link NHS-LC-LC-Biotin	LC-LC-Biotin	570	
Pierce	EZ-link NHS-LC-Biotin	LC-Biotin (LC = long chain)	450	
Pierce	EZ-link NHS-Biotin	Biotin	340	

The PEG (also called PEO) polymers purchased from Pierce Biotechnology (Rockford, Illinois) are monodispersed – i.e., they contain a single discrete polymer of a particular molecular weight. By contrast, the PEG polymers purchased from NOF (Nippon Oil and Fat) are polydispersed – i.e., they contain a mixture of polymers having a distribution of molecular weights.

To PEGylate CNP22 or variants thereof, reaction and purification conditions are optimized for each PEG-CNP conjugate. According to a general PEGylation procedure,

reaction mixtures contain about 1 mM CNP22 or a variant thereof, and about 1 to 5 mM NHS-activated PEG in potassium phosphate buffer, pH between about 5.0 and 6.5. To mono-PEGylate selectively at the peptide N-terminus and minimize PEGylation at an internal site (e.g., Lys4 of CNP22), the PEGylation reaction can be conducted under more acidic conditions (e.g., at a pH between about 5.5 and 6.5) to protonate selectively and hence deactivate the more basic primary amino group on the lysine side chain. After about 1 to 3 hours of incubation at room temperature, the PEGylation reaction is quenched by addition of aqueous glycine buffer. Reaction products are then separated by reverse-phase HPLC, optimized for each PEG-CNP conjugate. Fractionation samples are speedvacced to dryness, and reconstituted/formulated in 1 mM HCl. Identification and purity of each PEG-CNP product are determined by liquid chromatography-mass spectrometry (LC/MS).

Example 2A

Recombinant Production of CNP Variants

CNP variants can be produced using recombinant technology. In certain embodiments, the CNP variants are produced as fusion proteins comprising a cleavable peptide, carrier protein or tag. Exemplary methods for recombinantly producing CNP fusion proteins are disclosed below.

Materials and Methods

Cloning of CNP fusion proteins into expression vectors

CNP DNA fragments were amplified using polymerase chain reaction (PCR) and the amplified PCR fragments were digested with Nde I and BamHI and cloned into pET21a vector (Novagen, Gibbstown, New Jersey). CNP fusion protein DNA was synthesized by DNA2.0 and cloned into different expression vectors (Table 6).

Table 6

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF-CNP	pJexpress401	TAF-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21; BL21(DE3)
pJexpress-KSI-	pJexpress404	KSI-CNP(M/N) inclusion bodies	CNBr (Met-X)	CNP(M/N)	BL21

CNP(M/N)					
pET-31b-KSI-CNP	pET-31b	KSI-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pET-32a-Trx-CNP	pET-32a	Trx-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)
pMAL-CNP	pMAL-c2X	MBP-CNP fusion protein (soluble)	Formic acid (Asp-Pro)	Pro-CNP	BL21(DE3)

CNP: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75); **TAF**: human transcription factor TAF12; **KSI**: ketosteroid isomerase; **MBP**: maltose-binding protein; **Trx**: thioredoxin

Expression of CNP fusion proteins in *E. coli*

CNP fusion protein expression plasmids were transformed into *E. coli* BL21 or BL21(DE3). Transformed cells were plated on LB plates containing 100 ug/ml carbeniciline or 50 ug/ml kanamycin and incubated overnight at 37 °C. One single colony was picked and cultured in 4 ml LB medium containing 100 ug/ml of carbeniciline or 50 ug/ml kanamycin at 37 °C with shaking. When an OD_{600} of bacterial culture reached 0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cell media and the media was incubated at 37 °C for 3 hours with shaking. For cell harvest, bacterial cells were centrifuged at 4000 rpm for 10 minutes and the cell pellets were stored at -80 °C. Cell pellets were lysed with B-PER II Bacterial Extraction Reagent (PIERCE, 0.4 ml per 4 ml of bacterial culture) and Benzonase Nuclease (Novagen, 0.025 U/ml) at room temperature for 10 minutes. Bacterial crude extract was reserved and centrifuged to obtain supernatant. Supernatant and crude extract were assayed for CNP fusion protein expression and solubility by SDS-PAGE and Western Blot.

Detection of CNP fusion protein expression with SDS-PAGE and Western blot

Ten uL of cell lysates or soluble supernatants was run on sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, California, NuPAGE 4-12% Bis-tris Gel, MES SDS buffer). The gel was stained using 20 ml Imperial Protein Stain (Thermo Fisher, Rockford, Illinois) at room temperature for 1 hour and de-stained with water. For Western blot, the protein was transferred to membrane with Gel blot (Invitrogen). The membrane was blocked in TBS buffer with 5% milk at room temperature for 1 hour. Rabbit anti-CNP22 antibody (1:2500 dilution) (Bachem, Torrance, California) was added to the membrane, which was then incubated at room temperature with shaking for 2 hours, and then the membrane was washed 3 times with TBS buffer.

Alkaline phosphate (AP) conjugated anti-rabbit IgG (1:5000 dilution) was added to the membrane, which was then incubated at room temperature with shaking for 1 hour, and then the membrane was washed 3 times with TBS buffer. Ten ml WESTERN BLUE® Stabilized Substrate (Promega, Madison, Wisconsin) was added to the membrane, which was then incubated at room temperature with shaking for 1 to 5 min, and then the membrane was washed with TBS buffer to remove excess stain.

Expression of TAF-CNP fusion protein in *E. coli BL21*

Cells (*E. coli* strain BL21) expressing TAF-CNP fusion protein were obtained from glycerol stock stored at -80° C and were grown in 4 ml LB medium containing 50 ug/ml of kanamycin at 37 °C overnight with shaking (250 rpm). Four ml of overnight grown cell culture was transferred to 200 ml LB medium containing 50 ug/ml of kanamycin and was grown at 37 °C with shaking (250 rpm). When the OD_{600} reached 0.6, IPTG was then added to a final concentration of 1 mM to induce protein expression at 37 °C with shaking (250 rpm) for 3 hours. Cells were then spun down at 3000 rpm for 10 minutes and the resulting cell pellet was frozen at -80 °C.

Purification of TAF-CNP inclusion bodies and formic acid cleavage

The cell pellet (from 200 ml culture) was resuspended in 25 ml of B-PER II buffer (PIERCE), the pellet was sonicated for 10 minutes (50%, 1 second, pause 2 seconds) on ice, centrifuged at 12000 rpm for 20 minutes at 4 °C, and then the pellet was resuspended in 25 ml 20x diluted B-PER II buffer. This was repeated until the supernatant became clear (3-5 times). One mL of resuspended TAF-CNP inclusion bodies was transferred to a 1.5 ml tube and centrifuged at 14000 rpm for 15 min. The supernatant was discarded and the pellet was dissolved with 10 ul of 88% formic acid, and then 490 ul of Millipore filtered water was added immediately. The pellet was mixed well by vortex and incubated at 55 °C for 20 to 24 hours (70 °C/6 hours are alternative conditions). The products of the formic acid cleavage were assayed by SDS-PAGE and LC/MS(C4RP).

LC/MS Sample Preparation

Inclusion bodies were isolated from about 8 mL of culture (about 1.5 OD) and the pellet was solubilized in 10 uL nt formate. Resolubilized pellet was immediately diluted to 2% or 10% final formate concentration (0.5 mL) and incubated at 55 °C for 21 hours (pH 2) (cloudiness was more evident in the 2% formate sample the next day). Both samples were

centrifuged at 15000 rpm for 2 minutes. Twenty uL of supernatant was injected into an LC/MS (C4 RP) apparatus.

Results

CNP fusion proteins were expressed in *E. coli*

All CNP fusion proteins were expressed in *E. coli* induced with 1 mM IPTG at 37 °C for 3 hours (**Figure 1**). The constructs pJexpress-TAF-CNP, pJexpress-KSI-CNP(M/N) and pET-31b-KSI-CNP were expressed as inclusion bodies, while the constructs pET-32a-Trx-CNP and pMAL-CNP were expressed as soluble fusion proteins. Western blot using anti-CNP22 antibody confirmed the expression of the CNP fusion proteins (**Figure 1**).

CNP was produced from TAF-CNP inclusion bodies by formic acid cleavage

TAF-CNP inclusion bodies were partially purified and treated with 2% formic acid at 55 °C for 20 to 24 hours (70 °C/6 hours are alternative conditions). The majority of TAF-CNP was cleaved and one extra band having similar size as Gly-CNP37 peptide appeared on SDS-PAGE (**Figure 2**). The cleaved sample was further analyzed by LC/MS(C4 RP). The LC/MS results showed that CNP was released in soluble form from TAF-CNP inclusion bodies after formic acid cleavage. LC/MS analysis indicated that formic acid cleavage of the CNP fusion proteins resulted in formation of cyclized Pro-Gly-CNP37 (MW = 4102). Calculation of protein amounts based on analysis suggested that about 60 ug of formic acid-generated CNP was produced from 8 mL of very low OD culture. From a small scale (e.g., about 8 mL) of low OD (e.g., 1.2 OD) cell culture, approximately 8 ug/ml CNP was produced, while fermentation of a larger scale (e.g., about 8 L) of higher OD (e.g., 38 OD) cell culture can produce approximately 1 mg/ml CNP.

Conclusion

Five expression constructs were generated to express CNP fusion proteins. Expression of all five constructs produced soluble (Trx and MBP) or insoluble (TAF and KSI) CNP fusion proteins. Approximately 1 mg/ml soluble CNP can be produced from TAF-CNP inclusion bodies by a simple formic acid cleavage procedure.

Example 2B

Production of Additional CNP variants in E. coli

Recombinant production of CNP variants was carried out as described in Example 2A. In this example, additional CNP constructs were generated with a QuikChange II XL site-directed mutagenesis kit (Stratagene) or were synthesized by DNA2.0. Additional CNP constructs and expression vectors are listed in Table 7.

Table 7

Construct	Vector	Product	Chemical cleavage	Final product	Expression *E. coli* strain
pJexpress-TAF(C/A)-Pro-CNP38	pJexpress401	TAF(C/A)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF(4D/4E)-Pro-CNP38	pJexpress401	TAF(4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF (C/A&4D/4E)-Pro-CNP38	pJexpress401	TAF(C/A&4D/4E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF (C/A&10D/10E)-Pro-CNP38	pJexpress401	TAF(C/A&10D/10E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	pJexpress401	TAF-NL-(C/A & 6D/6E)-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21(DE3)
pJexpress-BMP-Pro-CNP38	pJexpress401	BMP-Pro-CNP38 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP38	BL21; BL21(DE3)
pJexpress-TAF-Pro-CNP37	pJexpress401	TAF-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-BMP-Pro-CNP37	pJexpress401	BMP-Pro-CNP37 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP37	BL21; BL21(DE3)
pJexpress-TAF-Pro-HSA-CNP	pJexpress401	TAF-Pro-HSA-CNP inclusion bodies	Formic acid (Asp-Pro)	Pro-HSA-CNP	BL21
pJexpress-TAF	pJexpress401	TAF inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-BMP	pJexpress401	BMP inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF(C/A)	pJexpress401	TAF(C/A) inclusion bodies	N/A	N/A	BL21(DE3)

pJexpress-TAF(4D/4E)	pJexpress401	TAF(4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&4D/4E)	pJexpress401	TAF(C/A&4D/4E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF (C/A&10D/10E)	pJexpress401	TAF(C/A&10D/10E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-NL-(C/A & 6D/6E)	pJexpress401	TAF-NL-(C/A&6D/6E) inclusion bodies	N/A	N/A	BL21(DE3)
pJexpress-TAF-Pro-CNP53	pJexpress401	TAF-Pro-CNP53 inclusion bodies	Formic acid (Asp-Pro)	Pro-CNP53	BL21(DE3)
pJexpress-TAF-CNP34	pJexpress401	TAF-CNP34 inclusion bodies	Formic acid (Asp-Pro)	CNP34	BL21(DE3)

CNP38: GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Gly-CNP37 (SEQ ID NO: 75)];

Pro-CNP38: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [Pro-Gly-CNP37] (SEQ ID NO: 145);

CNP37: QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 60);

HSA-CNP: GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC [HSA-CNP27 (SEQ ID NO: 144)];

Pro-CNP53: PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIG-SMSGLGC (SEQ ID NO: 185);

CNP34: PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 163);

TAF-Pro-CNP38: MVLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAA-CQLARHRKSSTLEVKDVQLHLERQWNMWIMGSSHHHHHHSSGLVPRGSHT-GDDDDKHMDPGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 196);

TAF: human transcription factor TAF12 histone fold domain (HFD) and linker from pET-15b vector;

TAF-NL: TAF12 HFD without a linker;

TAF12 HFD: VLTKKKLQDLVREVCPNEQLDEDVEEMLLQIADDFIESVVTAACQLA-RHRKSSTLEVKDVQLHLERQWNMWI (SEQ ID NO: 197);

pET-15b linker: MGSSHHHHHHSSGLVPRGSHTGDDDDKHMD (SEQ ID NO: 195);

TAF(C/A): cysteine in TAF is changed to alanine;

TAF(D/E): aspartic acid in TAF is changed to glutamic acid (number indicates which amino acid residue is changed);

TAF(C/A & D/E): cysteine and aspartic acid in TAF are changed to alanine and glutamic acid, respectively;

BMP: bone morphogenetic protein 2 with seven C/A (cysteine to alanine) mutations;

KSI: ketosteroid isomerase; **MBP**: maltose-binding protein; **TRX**: thioredoxin

Results

All CNP fusion proteins were expressed in *E. coli* induced with 1 mM IPTG at 37°C for 3 hours. Constructs pJexpress-BMP-Pro-CNP38, pJexpress-TAF-Pro-CNP37, pJexpress-BMP-Pro-CNP37, pJexpress-Pro-HSA-CNP, pJexpress-TAF and pJexpress-BMP were expressed as inclusion bodies. A Western Blot with anti-CNP antibody was used to confirm the expression (**Figure 3**).

Pro-Gly-CNP37 ("Pro-CNP38") was produced from TAF-Pro-CNP38 inclusion bodies by formic acid cleavage

Formic acid is used as a denaturant for inclusion body proteins and can specifically cleave the peptide bond between Asp and Pro under optimized conditions. TAF-Pro-CNP inclusion bodies were partially purified as described above and treated with 50% formic acid at 25°C, 37°C, 42°C and 55°C for 24 hours. Most of the TAF-Pro-CNP38 was cleaved and one extra band with a similar size as the Gly-CNP37 ("CNP38") peptide showed on SDS-PAGE from the 37°C, 42°C and 55°C cleavages (**Figure 4A**). The cleavage reactions at 37°C and 55°C were neutralized with 10 M NaOH and centrifuged at 14,000 rpm for 15 minutes. The un-cleaved TAF-Pro-CNP38, TAF and other inclusion bodies precipitated in the pellets. The supernatants contained soluble Pro-CNP38 and were further analyzed by LC/MS. The LC/MS result showed the supernatants contained a mixture of nonspecific cleaved peptides generated by excess acidic hydrolysis.

When TAF-Pro-CNP inclusion bodies were treated with 2% and 10% formic acid at 55°C for 20 hours, the majority of TAF-Pro-CNP38 was cleaved and one extra band having a similar size as Gly-CNP37 was observed on SDS-PAGE (**Figure 4B**). The cleaved sample was further analyzed by LC/MS. The LC/MS analysis showed that correct Pro-CNP38 was released in soluble form from TAF-Pro-CNP38 inclusion bodies after formic acid cleavage. The yields of Pro-CNP38 from 2% and 10% formic acid cleavages were similar (**Figure 4C**).

Formic acid cleavage and neutralization for Pro-Gly-CNP37 ("Pro-CNP38") production and purification

Formic acid can dissolve and cleave TAF-Pro-CNP38, BMP-Pro-CNP38 and other inclusion bodies. The pH neutralization results in precipitation of insoluble contaminating proteins/peptides (un-cleaved TAF-Pro-CNP38 and BMP-Pro-CNP38, TAF, BMP and others). The soluble Pro-CNP38 stays in the supernatant after centrifugation. TAF-Pro-

CNP38 and BMP-Pro-CNP38 were cleaved in 2% formic acid at 55°C or 70°C for 24 hours. The cleavage reactions were neutralized with 1:1 ratio of 0.5 M Tris buffer and centrifuged at 14,000 rpm for 15 minutes. Results showed that the supernatants contained almost pure peptides and there was no observed recovery loss of Pro-CNP38 upon neutralization. This is a simple and efficient step for Pro-CNP38 purification.

Analysis of varying conditions for formic acid cleavage of TAF-Pro-CNP38 inclusion bodies for Pro-Gly-CNP37 ("Pro-CNP38") production

Formic acid can specifically cleave the peptide bond between Asp and Pro under optimized conditions. Non-specific cleavage of peptide bonds between Asp and any other amino acids or even non-specific cleavage of any peptide bond may occur if the formic acid cleavage conditions are not optimized. TAF-Pro-CNP38 inclusion bodies were cleaved with 2% formic acid at 42°C, 55°C or 70°C for 6, 24 or 48 hours. **Figure 5A** shows that TAF-Pro-CNP38 was cleaved completely at 70°C for 24 hours or at 55°C for 48 hours. The 70°C cleavage could be completed within 17 hours (**Figure 5B**). Although the 70°C/24h cleavage gave the highest yield, the non-specific cleavage products (for example, the peptide with molecular weight of 3142 generated from Pro-CNP38 by cleaving between peptide bond Asp and Arg) increased dramatically (**Figure 5C**).

The yield and purity of Pro-CNP38 production were improved when TAF-Pro-CNP38 inclusion bodies were purified or treated with B-PER II buffer before formic acid cleavage. Because B-PER II buffer contains detergent octylthioglucoside and is relatively expensive, other commonly used detergents or buffers were tested for large-scale Pro-CNP38 production. TAF-Pro-CNP38 inclusion bodies were re-suspended in different detergents (Octylsucrose; Triton x-100; Tween-20; NP-40; CA-630) or buffers (B-PER II; B-PER II 1/20 dilution; B-PER; B-PER phosphate buffer; 25 mM tris, 150 mM NaCl, pH 7.9; 25 mM tris, pH 7.5; filtered water; PBS) and incubated at room temperature (RT) for 24 hours. All detergents were 1% in 25 mM tris buffer, pH 7.5. After incubation in detergent or buffer, TAF-Pro-CNP38 inclusion bodies were cleaved by 2% formic acid at 55°C for 22 hours. Results showed that BPER II continued to exhibit good yield, and positive results were also obtained with CA630 and Triton X-100.

Pro-Gly-CNP37 ("Pro-CNP38") proteolytic cleavage products

One unidentified protease, possibly a membrane associated protease, cleaved the Pro-CNP38 peptide (produced from BL21 strain, MW 4102) into two peptides during Pro-CNP38

purification, resulting in peptides PGQEHPNAR (MW 1004) (SEQ ID NO: 198) and KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (MW 3115) (SEQ ID NO: 199). Not to be bound by theory, one possible reason why detergents may improve the yield and purity of Pro-CNP38 is that detergents can remove most, but possibly not all, of the unidentified protease. High temperature, basic pH and EDTA were tested to identify if these agents could inhibit the protease cleavage. TAF-Pro-CNP38 inclusion bodies were incubated at RT or 120°C for 2 hrs and centrifuged at 14000 rpm for 15 min. The pellets were re-suspended in 2% formic acid and incubated at 55°C or 70°C for 18 hours. A 1:1 ratio of 0.5 M Tris was added to neutralize the cleavage. The neutralized samples were centrifuged at 14000 rpm for 5 min and the supernatants were left at RT for 6 hrs or 22 hrs with or without 10 mM EDTA, pH 10. The proteolytic cleavage was assayed by LC/MS (**Table 8**).

Table 8. LC/MS result of Pro-CNP38 proteolytic cleavage

Sample		A210 Peak Area	Protein Conc. (mg/mL)		Percent MW4102	Percent MW3115
1	H 55C	942	0.04	*	91.8	8.2
2	H 70 0	2878	0.11	*	79.2	20.7
3	H 70 6H	2675	0.10	*	80.6	19.4
4	H 70 24	2741	0.11	*	79.2	20.7
5	H 70 EDTA	2385	0.09	*	80.8	19.2
6	H 70 pH 10	1917	0.07	*	81.2	18.8
7	55C	1291	0.05		61.1	38.9
8	70C 0	4533	0.18		96.8	3.2
9	70C 6H	4120	0.16		97.5	2.5
10	70C 24	4108	0.16		96.5	3.5
11	70C EDTA	4336	0.17		96.9	3.1
12	70C pH10	3425	0.13		97.5	2.4

All samples (8-12) cleaved at 70°C showed limited proteolytic cleavage (less than 4% cleavage of Pro-CNP38). Almost 40% of Pro-CNP38 was cleaved by protease when cleavage was carried out at 55°C (sample 7). Basic pH and EDTA did not influence the non-specific proteolytic cleavage. High temperature (120°C for 2 hrs) non-specifically cleaved Pro-CNP38.

It should be noted that the BL21(DE3) strain from Stratagene does not have the unidentified protease.

Production of Pro-Gly-CNP37 ("Pro-CNP38") and other CNP variants from different constructs:

Pro-CNP38 can be produced in large scale in *E. coli* by means of overexpression of a TAF-Pro-CNP38 fusion protein as inclusion bodies followed by formic acid cleavage of the fusion protein. Following the methods described herein, other TAF-CNP fusion proteins (TAF-CNP34 and TAF-Pro-CNP53) were expressed as inclusion bodies and then cleaved with formic acid to generate the CNP variants CNP34 and Pro-CNP53. **Figure 6** depicts the expression of TAF-CNP34, **Figure 7** the expression of TAF-Pro-CNP53, **Figure 8** the products of formic acid cleavage of TAF-CNP34 and TAF-Pro-CNP53, **Figure 9** the peak for CNP-34 in an LC/MS chromatogram, and **Figure 10** the peak for Pro-CNP53 in an LC/MS chromatogram.

Use of formic acid may result in unspecific cleavage(s) at peptide bond(s) other than the targeted Asp-Pro bond. To improve the purity and overall titer of the desired formic acid cleavage product, different residues of aspartic acid in TAF12 or fragments thereof were changed to glutamic acid. Moreover, one or more cysteine residues in TAF12 or fragments thereof were changed to alanine to prevent unspecific disulfide bond formation. All TAF-Pro-CNP38 fusion proteins having such mutations in TAF12 were expressed as inclusion bodies and cleaved with formic acid to produce Pro-CNP38. **Figure 7** shows the expression of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38, **Figure 11** the expression of TAF(C/A & 4D/4E)-Pro-CNP38 and TAF(4D/4E)-Pro-CNP38, **Figure 12** the products of formic acid cleavage of TAF(4D/4E)-Pro-CNP38 and TAF(C/A & 4D/4E)-Pro-CNP38, and **Figure 13** the products of formic acid cleavage of TAF-NL-(C/A & 6D/6E)-Pro-CNP38 and TAF(C/A & 10D/10E)-Pro-CNP38. **Table 9** summarizes the purity (prior to purification) and titer of Pro-CNP38 obtained from the various TAF-Pro-CNP38 constructs.

Table 9

Construct	Purity	Titer (ug/mL)
pJexpress-TAF-Pro-CNP38	32%	44
pJexpress-TAF(C/A)-Pro-CNP38	41%	50
pJexpress-TAF(4D/4E)-Pro-CNP38	36%	52
pJexpress-TAF(C/A & 4D/4E)-Pro-CNP38	42%	58
pJexpress-TAF(C/A & 10D/10E)-Pro-CNP38*	32%	26
pJexpress-TAF-NL-(C/A & 6D/6E)-Pro-CNP38	50%	55

*Cells with pJexpress-TAF(C/A&10D/10E)-Pro-CNP38 growed more slowly and the final cell density (OD_{600}) was lower compared to other TAF-Pro-CNP38 constructs.

Large-scale production of Pro-Gly-CNP37 ("Pro-CNP38") by fermentation and formic acid cleavage

BL21(DE3) cells comprising the pJexpress-TAF-CNP construct were grown in a 10 liter fermenter at 37 °C for about 16-17 hours until OD_{600} reached 64. The cells were then grown/cultured in the presence of 1 mM IPTG at about 35-37 °C, to induce expression of the TAF-Pro-CNP38 fusion protein, for about 7-8 hours until OD_{600} reached 160. The fermentation produced a titer of 9 g/L TAF-Pro-CNP38. **Figure 14** displays a Western blot of the TAF-Pro-CNP38 fusion protein produced in the fermentation.

A cell pellet recovered from 750 mL cell culture from the 10L fermentation was resuspended in phosphate-buffered saline, pH 7.4 (PBS) and lysed by three passes through a pressure homogenizer (10,000 bar). The resulting lysate was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The pellet fraction containing insoluble TAF-Pro-CNP38 fusion protein inclusion bodies was resuspended in 500 mL PBS with a rotostator. The suspension was centrifuged at 6,500 g for 10 minutes and the supernatant was discarded. The resulting inclusion body pellet was resuspended in 500 mL water with a rotostator and incubated at 55°C for 30 minutes. 250 mL 6% formic acid was added to the warmed inclusion body suspension for a final concentration of 2% formic acid and incubated at 55°C for 20-24 hours. 50 mL of 400mM Na_2HPO_4 was added after 20-24 hours to begin the neutralization of the formic acid cleavage reaction, and the resulting mixture was titrated with 50% w/v NaOH to pH 6.9-7.4 and left at room temperature for 30 minutes. Upon neutralization, a heavy precipitate formed and was removed by centrifugation at 6,500 g for 10 minutes. The supernatant was retained and contained on average 1.3 g/L culture of 80% pure Pro-CNP38. The majority of the *E.coli* and TAF-related proteins and peptides remained in the pellet.

The soluble Pro-CNP38 resulting from the formic acid cleavage and neutralized supernatant was 80% pure and contained a mixture of linear and cyclized Pro-CNP38 peptides along with other product-related impurities. The pH neutral supernatant containing Pro-CNP38 was sterile-filtered. Further purification by anion-exchange chromatography using a Fractogel TMAE Hi-CAP column (EMD Biosciences) to remove DNA, endotoxins, and peptide contaminants, which should bind to the column, was performed at pH 7-7.4. The

flow-through fraction contained partially cyclized Pro-CNP38. Cupric sulfate was added to the flow-through fraction to a final concentration of 10 uM and incubated at room temperature for 1 hour. The addition of Cu^{++} at neutral pH catalyzed the oxidation of free cysteine sulfhydryl groups on the peptide to form an intramolecular disulfide bond, resulting in 100% cyclized Pro-CNP38 and no detectable linear peptide. The conductivity of the solution was adjusted to < 15 mS/cm by the addition of water. Cation-exchange chromatography using an SP-Sepharose column (GE Healthcare) and a sodium phosphate buffer (pH 7) was then performed to remove any remaining DNA and endotoxins in the flow-through and further purify Pro-CNP38 to about 95-96% purity with < 0.5% non-product related impurities. **Figure 15** is an SDS-PAGE of eluate fractions from the SP-Sepharose column; the highest concentrations of Pro-CNP38 were found in fractions 22 to 30. Reverse-phase HPLC/MS analysis of non-pooled fraction 24 indicated the presence of 90% Pro-CNP38, 5% Pro-CNP38 with an oxidized methionine residue, 3% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17, and 1.6% Pro-CNP38 cleaved at the Asp-Arg bond in the cyclic domain. The yield of pure Pro-CNP38 peptide upon final purification was 0.9 g/L cell culture (36% total recovery).

Pro-CNP38 collected from five separate purifications was pooled for formulation. The pooled product contained 93.5% Pro-CNP38, 3.3% Pro-CNP38 with an oxidized methionine residue, 1.3% deamidated Pro-CNP38, and 1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. Samples were diluted with 50 mM sodium phosphate (pH 7) to a conductivity of 10 mS/cm and loaded onto a CM-Sepharose column (GE-Healthcare) for concentration and buffer exchange. The weak cation-exchange property of the CM-Sepharose resin allows peptides to dissociate from the column with weak acid solutions rather than typical salt gradients. Acid concentrations required for dissociation of Pro-CNP38 from the CM-Sepharose column depended upon column loading. At 50 mg Pro-CNP38 per mL of resin, 10 mM HCl was sufficient for Pro-CNP38 elution. At 9 mg Pro-CNP38 per mL of resin, 50 mM HCl was required for elution. When the loading was 9 mg Pro-CNP38 per mL of resin, Pro-CNP38 eluted in less than one column volume, which significantly concentrated the peptide. The eluate fraction contained 20.3 mg/mL of 95% pure Pro-CNP38, along with 3% Pro-CNP38 with an oxidized methionine residue, 1% deamidated Pro-CNP38, and < 1% Pro-CNP38 cleaved at the Gly-Cys bond to form CNP-17. The concentrated solution of Pro-CNP38 in weak acid is suitable for dilution into appropriate buffers for either liquid or lyophilized formulations.

Example 3

Cleavage of CNP Variants by Neutral Endopeptidase *In Vitro*

To determine the effects of amino acid substitutions, amino acid extensions, backbone modifications, side chain modifications and PEGylation on the susceptibility of CNP variants to neutral endopeptidase (NEP) cleavage, peptide cleavage assays were carried out using an *in vitro* assay that monitored disappearance of the non-cleaved CNP variant.

Recombinant human NEP (1 ug/mL final concentration) was added to 100 uM CNP variant diluted in 0.1 M Tris, pH 7. The reaction mixture was incubated at 37 °C for various periods of time, and the reaction was quenched with EDTA (10 mM final) followed by heat denaturation. The reaction mixture was reduced and then the reaction products were analyzed using HPLC and mass spectroscopy. The half-life of the CNP variant was calculated based on the disappearance of intact CNP variant over time. The results for digested CNP variants were compared to a parallel wtCNP22 digestion and normalized to the results for 100 uM CNP22 digested by 1 mg/mL NEP ($t_{1/2}$ = 80 min).

Table 1 lists the half-lives, based on the *in vitro* NEP cleavage assay, of various CNP variants having backbone or side chain modifications. Removal of three of the six NEP cleavage sites in Analog L nevertheless resulted in a substantially shorter half-life. Of tested CNP variants, the greatest resistance to NEP cleavage was exhibited by Analog N, which contains the D-enantiomer of all 22 amino acids of CNP22, and by Analog M, which has an N-methylated amide bond at both Leu9 and Leu11. However, both Analogs N and M failed to stimulate production of cGMP (see below).

Comparing the half-lives of Analogs A, B, E, F, G and H to one another, half-lives were determined to be about 1.5- to about 2.5-fold longer for Analogs E and G compared to those for Analogs A, B, F and H. All of these six analogs showed resistance or improved resistance to cleavage at the Cys6-Phe7 bond relative to wtCNP22 (data not shown). The rank order of analog resistance to NEP at 1ug/ml, based on half-life, is Analog G (3-Cl-Phe) $\geq$ Analog E (D-Phe) > Analog H ("beta-2 Phe"), Analog B (N-Me-Phe), and Analog F (t-Bu-Gly) = wtCNP22 > Analog A (Cys-CH_2-NH). Analogs E and G have about 1.5 times longer half-life in comparison to wtCNP22. Besides resistance to cleavage of the Cys6-Phe7 bond, Analogs B, E, F, G and H also exhibited resistance to cleavage of the Gly8-Leu9 bond in the presence of 1 ug/mL NEP (data not shown). These results indicate that CNP variants having backbone or side chain modifications between Cys6 and Gly8 can be resistant to NEP

cleavage of the Cys6-Phe7 bond and/or Gly8-Leu9 bond, but do not necessarily have improved overall resistance to NEP or a longer half-life than CNP22. The results seem to be contrary to reports in the literature that NEP first cleaves at the Cys6-Phe7 bond of CNP22 and then elsewhere.

Table 1

Analog	Backbone and Side Chain Modifications	cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10 nM	1 uM	($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80[2]
N	D-CNP22 (all D-amino acids) (SEQ ID NO: 115)	2	1	>> 160
A	CNP22, C6-CH2-NH (reduced carbonyl) (SEQ ID NO: 56)	6	66	55
B	CNP22, N-methyl-F7 (methylated amide) (SEQ ID NO: 57)	2	38	80
BD	CNP22, N-methyl-L9 (SEQ ID NO: 116)	2	8	ND
BN	CNP22, N-methyl-L11 (SEQ ID NO: 117)	10	51	ND
BE	CNP22, N-methyl-L20 (SEQ ID NO: 118)	2	5	ND
M	CNP22, N-methyl-L9, N-methyl-L11 (SEQ ID NO: 94)	1	11	>> 160
K	CNP22, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 92)	1	1	80
L	CNP22, N-methyl-L9, N-methyl-L11, N-methyl-L20 (SEQ ID NO: 93)	18	10	30
J	CNP22, C6-CH2-NH, N-methyl-L9, N-methyl-L20 (SEQ ID NO: 91)	ND	ND	50
E	CNP22, D-F7 (D-Phe) (SEQ ID NO: 136)	2	6	130
H	CNP22, Beta-2-F7 (3-amino-2-phenylpropionyl) (SEQ ID NO: 57)	2	2	80
G	CNP22, 3-chloro-F7 (SEQ ID NO: 137)	17	93	135
F	CNP22, t-butyl-G8 (SEQ ID NO: 58)	2	18	80
V	CNP22, K4G, 3,4-dichloro-F7 (SEQ ID NO: 119)	ND	ND	68
X	CNP22, K4G, 3-methyl-F7 (SEQ ID NO: 120)	ND	ND	68
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 2 lists the half-lives, based on the *in vitro* NEP cleavage assay, of various CNP variants having substitutions with natural and/or unnatural amino acids. Of tested variants, the greatest resistance to NEP cleavage was shown by Analog BK, which has K4R and G15S substitutions, and Analog BJ, which has K4R and G15N substitutions.

Table 2

Analog	Specificity Mutations	cGMP Response relative to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	10nM	1uM	($t_{1/2}$, min)
	CNP22 (SEQ ID NO: 1)	46 ± 10	100 ± 13	80
AH	CNP22, K4R (SEQ ID NO: 35)	59	121	80
BP	CNP22, K4R, G5S (SEQ ID NO: 121)	45	ND	ND
BO	CNP22, K4R, G5R (SEQ ID NO: 122)	18	80	ND
P	CNP22, K4G (SEQ ID NO: 123)	ND	ND	68
Z	CNP22, K4R, F7Y (SEQ ID NO: 95)	2	18	ND
AB	CNP22, K4R, G8S (SEQ ID NO: 97)	26 ± 26	86 ± 17	ND
AA	CNP22, K4R, G8V (SEQ ID NO: 96)	3	25	ND
AC	CNP22, K4R, G8T (SEQ ID NO: 98)	11 ± 2	66 ± 16	80
AD	CNP22, K4R, L9T (SEQ ID NO: 99)	4	68	ND
BH	CNP22, K4R, K10R (SEQ ID NO: 112)	12	80	ND
BF	CNP22, K4R, K10Cit (SEQ ID NO: 110)	6	33	ND
BG	CNP22, K4R, K10Q (SEQ ID NO: 111)	9	45	ND
BY	CNP22, K4R, K10S (SEQ ID NO: 124)	16	53	ND
BK	CNP22, K4R, G15S (SEQ ID NO: 114)	13 ± 1	71 ± 11	≥ 160
BJ	CNP22, K4R, G15N (SEQ ID NO: 113)	4	41	150
AE	CNP22, K4R, G15R (SEQ ID NO: 100)	0.3	0.3	ND
AF	CNP22, K4R, G15Cit (SEQ ID NO: 101)	1.4	2	ND
BZ	CNP22, K4R, S16Q (SEQ ID NO: 125)	42	116	ND
BX	CNP22, K4R, M17N (SEQ ID NO: 126)	40 ± 2	103 ± 17	ND
AG	CNP22, K4R, M17V (SEQ ID NO: 102)	10	65	ND
BQ	CNP22, K4R, G19S (SEQ ID NO: 127)	21	63	ND
BR	CNP22, K4R, G19R (SEQ ID NO: 128)	22 ± 6	84 ± 10	ND
AJ	CNP22, K4R, L20V (SEQ ID NO: 103)	0.2	8	ND
AK	CNP22, K4R, L20t-butyl-Ala (SEQ ID NO: 104)	1	21	ND
AT	CNP22, G1E, K4E (SEQ ID NO: 105)	11	54	60
BS	CNP22, K4R, L20R (SEQ ID NO: 129)	11	8	ND
BT	CNP22, K4R, G21S (SEQ ID NO: 130)	7	39	ND

BU	CNP22, K4R, G21T (SEQ ID NO: 131)	6	21	ND
BW	CNP22, K4R, G21R (SEQ ID NO: 132)	20	21	ND
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 3 lists the half-lives, based on the *in vitro* NEP cleavage assay, of CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions. Of the analogs tested, Analogs AZ, CC, CF, BL, CS, CK and CL, Pro-Gly-CNP37 and HSA-CNP27 were most resistant to NEP degradation.

Table 3

	N- and C-Terminal Modifications	cGMP Response rel. to 1 uM CNP22[1]		NEP Cleavage
	Natriuretic Peptide	**10nM**	**1uM**	**($t_{1/2}$, min)**
	CNP22	46 ± 10	100 ± 13	80
BC	Pentanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 109)	ND	ND	ND
BB	Heptanoic acid (N-term.)-CNP22, G1E (SEQ ID NO: 108)	32 ± 4	84 ± 19	45-65
AV	Pentanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 106)	ND	ND	120
AW	Heptanoic acid (N-term.)-CNP22, G1E, K4E (SEQ ID NO: 107)	ND	ND	< 20
AX	CNP17 (delta N-term) (SEQ ID NO: 2)	18	69	ND
	R-CNP22 (SEQ ID NO: 40)	ND	ND	ND
AZ	R-CNP22, K4R (SEQ ID NO: 41)	54 ± 11	106 ± 15	≥ 160
	ER-CNP22 (SEQ ID NO: 38)	ND	ND	ND
BA	ER-CNP22, K4R (SEQ ID NO: 39)	38 ± 10	113 ± 10	90
	GANRR-CNP22 (SEQ ID NO: 65)	ND	ND	ND
AY	GANRR-CNP22, K4R (SEQ ID NO: 36)	59 ± 9	105 ± 20	65
	GANQQ-CNP22 (SEQ ID NO: 64)	ND	ND	ND
CH	GANQQ-CNP22, K4R (SEQ ID NO: 69)	44 ± 8	95 ± 11	ND
	GANPR-CNP22 (SEQ ID NO: 66)	ND	ND	ND
CI	GANPR-CNP22, K4R (SEQ ID NO: 37)	50±1	105±12	ND
	GANSS-CNP22 (SEQ ID NO: 67)	ND	ND	ND
CG	GANSS-CNP22, K4R (SEQ ID NO: 70)	27 ± 1	88 ± 1	95

CA	AAWARLLQEHPNA-CNP22 (SEQ ID NO: 61)	24	76	ND
CB	AAWARLLQEHPNAR-CNP22 (SEQ ID NO: 62)	36	84	ND
CC	DLRVDTKSRAAWAR-CNP22 (SEQ ID NO: 63)	34	101	> 160
CF	GQPREPQVYTLPPS-CNP22 (IgG1(Fc) fragment) (SEQ ID NO: 79)	23 ± 9	72 ± 19	> 160
	PNARKYKGANKK-CNP22 (CNP34)	ND	ND	ND
BL	QEHPNARKYKGANKK-CNP22 (CNP37) (SEQ ID NO: 60)	43 ± 15	97 ± 27	>> 160
	PQEHPNARKYKGANKK-CNP22 (Pro-CNP37)	ND	ND	ND
CE	GERAFKAWAVARLSQ-CNP22 (HSA fragment) (SEQ ID NO: 81)	15	87	ND
CY	GQHKDDNPNLPRGANPR-CNP22 (HSA fragment) (SEQ ID NO: 80)	ND	ND	ND
CQ	GHHSHEQHPHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 76)	16	95	ND
CX	GHHSHEQHPHGANPR-CNP22 (HRGP fragment) (SEQ ID NO: 78)	ND	ND	ND
CS	GQEHPNARKYKGANPK-CNP22 (modified CNP37) (SEQ ID NO: 129)	19	61	>> 160
CT	GQEHPNARKYKGANQK-CNP22 (modified CNP37) (SEQ ID NO: 130)	60	121	ND
CU	GQEHPNARKYKGANQQ-CNP22 (modified CNP37) (SEQ ID NO: 131)	9	57	ND
DB	GQEHPNARKYKGANKK-CNP22 (Gly-CNP37) (SEQ ID NO: 75)	50 ± 14	98 ± 17	>> 160
	PGQEHPNARKYKGANKK-CNP22 (Pro-Gly-CNP37)	49 ± 6	103 ± 17	>> 160
CW	GQEHPNARKYKGANKP-CNP22 (modified CNP37) (SEQ ID NO: 74)	ND	ND	ND
CR	GAHHPHEHDTHGANQQ-CNP22 (HRGP fragment) (SEQ ID NO: 128)	14 ± 5	77 ± 12	ND
CZ	FGIPMDRIGRNPR-CNP22 (osteocrin "NPR-C inhibitor") (SEQ ID NO: 82)	ND	ND	ND
DA	GKRTGQYKLGSKTGPGPK-CNP22 (FGF2 "heparin-binding domain" fragment) (SEQ ID NO: 83)	ND	ND	ND
CK	GQPREPQVYTGANQQ-CNP22, K4R (IgG1(Fc) fragment) (SEQ ID NO: 84)	2	32	≥ 160
CL	GVPQVSTSTGANQQ-CNP22, K4R (HSA fragment) (SEQ ID NO: 85)	3	35	> 160
	GHKSEVAHRFKGANKK-CNP22 (HSA-CNP27) (SEQ ID NO: 144)	51 ± 9	109 ± 15	>> 160
	PGHKSEVAHRFKGANKK-CNP22 (Pro-HSA-CNP27)	32	107	ND
CN	GQTHSSGTQSGANQQ-CNP22, K4R (fibrinogen)	12	115	ND

	(SEQ ID NO: 87)			
CM	GQPSSSSQSTGANQQ-CNP22, K4R (fibronectin) (SEQ ID NO: 86)	ND	ND	ND
CO	GSTGQWHSESGANQQ-CNP22, K4R (fibrinogen) (SEQ ID NO: 88)	2	33	ND
CP	GSSSSSSSSSGANQQ-CNP22, K4R (zinc finger) (SEQ ID NO: 89)	ND	ND	ND
CD	SPKMVQGSG-CNP17-KVLRRH ("BNP tails") (SEQ ID NO: 68)	25	102	ND
CJ	RSSCFGGRIDRIGAC ("C-ANP4-23", ANP-derived) (SEQ ID NO: 133)	ND	ND	ND
	CNP22, K4R, K10R, N.-term.--N-term. dimer / disuccinimidyl glutarate (SEQ ID NO: 134)	19	44	ND
	CNP22, K4R, K10R, N-term.--N.-term. dimer / Bis-PEO5 (SEQ ID NO: 135)	19	41	ND
BM	CNP53 (SEQ ID NO: 4)	61	101	>> 160
	ANP	10	23	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Table 4 lists the half-lives, based on the *in vitro* NEP cleavage assay, of CNP variants conjugated to PEG (or PEO) polymers at the N-terminus. All the PEGylated CNP variants tested and shown in Table 4 displayed resistance or enhanced resistance to NEP cleavage except for PEO12-GANPR-CNP22(K4R), which had the same half-life as wtCNP22. N-terminal PEGylation of CNP22 having a K4G substitution does not seem to confer substantial improvement in NEP resistance. For example, PEG2K-CNP22(K4G) was only slightly more resistant to NEP cleavage than CNP22 (data not shown), whereas PEG2K-CNP22 had a much longer half-life *in vitro* than CNP22.

Table 4

N-Terminal PEGylation		**cGMP Response rel. to 1 uM CNP22[1]**		**NEP Cleavage**
Natriuretic Peptide	**PEG**	**10nM**	**1uM**	**($t_{1/2}$, min)**
CNP22		46 ± 10	100 ± 13	80
CNP22	PEG20K	0	15	>> 160

CNP22	PEG5K	8 ± 1	20 ± 7	>> 160
CNP22	PEG2K	6 ± 2	32 ± 4	>> 160
CNP22	PEO4-$(PEO12)_3$ (branched)	17 ± 1	52 ± 6	>> 160
CNP22	PEO24 (1.2 kDa)	8 ± 5	46 ± 10	>> 160
CNP22	PEG1K	15 ± 3	68 ± 17	> 160
CNP22	PEO12 (0.6 kDa)	12 ± 7	57 ± 18	160
CNP22	(PEO12)-Biotin	19	81	140
CNP22, K4G	(PEO12)-Biotin	10	27	100
CNP22, K4R	PEO24	15	56	ND
CNP22, K4R	PEO12	13	44	ND
CNP-17	PEG2K	5	50	> 160
R-CNP22, K4R (SEQ ID NO: 41)	PEO24	15 ± 2	75 ± 12	ND
R-CNP22, K4R (SEQ ID NO: 41)	PEO12	23 ± 2	93 ± 19	≥ 160
ER-CNP22, K4R (SEQ ID NO: 39)	PEO24	6 ± 2	60 ± 10	ND
ER-CNP22, K4R (SEQ ID NO: 39)	PEO12	20 ± 1	92 ± 25	ND
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG2K	15 ± 2	45 ± 18	ND
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO24	28 ± 9	82 ± 18	>> 160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEG1K	15 ± 0.4	56 ± 23	> 160
GANRR-CNP22, K4R (SEQ ID NO: 36)	PEO12	40 ± 2	99 ± 13	> 160
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO24	16 ± 13	73 ± 30	ND
GANQQ-CNP22, K4R (SEQ ID NO: 69)	PEO12	30	78	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO24	ND	ND	ND
GANPR-CNP22, K4R (SEQ ID NO: 37)	PEO12	ND	ND	80
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO24	8 ± 5	46 ± 21	ND
GANSS-CNP22, K4R (SEQ ID NO: 70)	PEO12	8 ± 0.3	52 ± 13	ND

[1] Stimulation of cGMP production in NIH3T3 cells by natriuretic peptide relative to cGMP production in the presence of 1 uM CNP22

[2] CNP22 NEP resistance $t_{1/2}$ averaged 80min. Due to variations in NEP catalytic activity between experiments, all CNP22 $t_{1/2}$ digestions were normalized to 80min and the difference coefficient was used to calculate analog $t_{1/2}$ in each experiment to obtain an adjusted $t_{1/2}$.

ND = Not Determined

Figure 16 shows the NEP resistance profile of five N-terminal PEGylated conjugates of CNP22. The CNP22 peptides conjugated to PEG (or PEO) polymers of increasing mass exhibited increasing resistance to NEP degradation. In particular, PEO24-CNP22, PEG2K-CNP22 and PEG5K-CNP22 were resistant to NEP degradation over the assay period of 160 minutes.

Figure 17 displays the NEP resistance profile of CNP variants CNP37 (Analog BL), CNP53 and GANRR-CNP22(K4R) (SEQ ID NO: 36) having an N-terminal amino acid extension. As can be clearly seen, both CNP37 and CNP53 were resistant to NEP degradation in this *in vitro* assay, whereas GANRR-CNP22(K4R) (SEQ ID NO: 36)had the same lability to NEP hydrolysis as CNP22.

Figure 18 depicts the NEP resistance profile of CNP17 and GANRR-CNP22(K4R) (SEQ ID NO: 36) conjugated to a PEG (or PEO) moiety at the N-terminus. PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) greatly improved the NEP resistance of this CNP variant, with PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) being completely resistant to NEP cleavage over the assay period of 160 minutes. Increasing the mass of the PEO moiety from about 0.6 kDa (PEO12) to about 1.2 kDa (PEO24) improved the NEP resistance of PEGylated GANRR-CNP22(K4R) (SEQ ID NO: 36). PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) to a monodispersed PEO24 moiety rather than a polydispersed PEG1K moiety also improved NEP resistance. Finally, although both PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and PEG2K-CNP17 have a similar total mass (keeping in mind that PEG2K is polydispersed), the former displayed substantially better NEP resistance.

NEP resistance assays were also performed on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144) and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). **Figure 19** shows that G-CNP37 and CNP27-HSA were completely resistant to NEP cleavage, and CNP27-PEO12 exhibited much greater stability to NEP degradation compared to wtCNP22.

Example 4

CNP Variant Stimulation of cGMP Production in NIH3T3 Cells

To determine the functional activity of CNP variants, the production of cGMP was measured in NIH3T3 cells exposed to the CNP variants. Murine NIH3T3 cells express endogenously the CNP signaling receptor, NPR-B, which shares 98% protein sequence identity with human NPR-B. NIH3T3 cells were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and antibiotics at 37° C with 5% CO_2. Twenty four to 48 hours prior to signaling, cells were passaged to 12-well plates with a density of 2-5 x 10^5 cells per well at the time of the assay. CNP variants were resuspended in 1 mM HCl to a stock concentration of 1 mg/mL (455 uM for wtCNP22) and subsequently diluted to a 30 uM working stock solution with phosphate-buffered saline

(PBS). Ten-fold serial dilutions were prepared in phosphate-buffered saline. Culture medium was removed from the cells and replaced with 0.4 mL PBS/Dulbecco's modified Eagle medium (50/50, v/v) containing 0.75 mM isobutylmethylxanine. Plates were incubated at 37 °C, 5% CO_2 for 15 minutes before addition of 0.2 mL CNP variant in PBS and continued incubation at 37 °C for 15 minutes. Reactions were stopped by the addition of 0.2 mL lysis buffer supplied with the CatchPoint cGMP assay kit (Molecular Devices), and cGMP production was determined with the CatchPoint cGMP Assay (Molecular Devices). All stimulation experiments were performed in duplicate.

Tables 1-4 summarize the ability of CNP variants having backbone or side chain modifications, amino acid substitutions, N-terminal amino acid extensions, and/or N-terminal PEGylation, respectively, to stimulate cGMP production in NIH3T3 cells. In all four tables, the values for cGMP production in NIH3T3 cells exposed to 10 nM or 1 uM CNP variant are normalized to cell number and cGMP production in the presence of 1 uM wtCNP22.

Regarding the results in **Table 1**, only Analog G having 3-Cl-Phe at position 7 displayed substantially the same NPR-B stimulation activity at 1 uM as wtCNP22. With respect to **Table 2**, various CNP variants with amino acid substitutions, including Analogs AH, BO, AB, BH, BZ, BX and BR, showed substantially similar NPR-B stimulation activity as wtCNP22.

Considering the results in **Table 3**, many CNP variants having N-terminal and/or C-terminal modifications, including amino acid extensions, exhibited comparable NPR-B stimulation activity as wtCNP22. The functional CNP variants include Analog BB, which is CNP22(G1E) attached to heptanoic acid at the N-terminus, and Analog CD, which is the cyclic domain of CNP22 ("CNP17" retaining the Cys6 to Cys22 sequence) conjugated to the N-terminal and C-terminal "tails" of BNP. **Figure 20** illustrates that GANRR-CNP22(K4R), CNP37 (SEQ ID NO: 36) (Analog BL) and CNP53 all had similar NPR-B stimulation activity as wtCNP22 in the *in vitro* assay.

Of note from Table 3 is that among the CNP variants assayed for both CNP functionality and NEP resistance, Analog AZ (R-CNP22(K4R)), Analog CC, Analog CF, Analog BL (CNP37), Analog DB (Gly-CNP37) and GHKSEVAHRFK-CNP27 (HSA-CNP27) (SEQ ID NO: 144) all had substantially similar NPR-B stimulation activity as CNP22 while being substantially more resistant to NEP cleavage than CNP22.

With regard to the results in **Table 4**, nine N-terminal PEGylated CNP variants at 1 uM stimulated cGMP production to at least about 70% of the level achieved by wtCNP22. Several noteworthy aspects appear in Table 4. First, N-terminal PEGylation of GANRR-CNP22(K4R) (SEQ ID NO: 36) with a monodispersed PEO polymer (PEO12 is about 0.6 kDa, PEO24 about 1.2 kDa) resulted in better NPR-B functionality than that with a polydispersed PEG polymer (PEG1K has a polymer number average molecular weight (M_n) of around 1 kDa, PEG2K around 2 kDa) (see also **Figure 21**). Second, N-terminal PEGylation of wtCNP22 with a polydispersed PEG polymer of increasing M_n (PEG1K, PEG2K, PEG5K and PEG20K) or with a monodispersed PEO polymer of greater mass (PEO12 and PEO24) correspondingly decreased the NPR-B activation ability of the CNP variants (see also **Figure 22**). Third, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), having the N-terminal GANRR (SEQ ID NO: 8) extension, stimulated greater cGMP production than PEO24-CNP22 and PEO24-CNP22(K4R). Also of note is that among the N-terminal PEGylated CNP variants assayed for both CNP functionality and NEP resistance, PEO12-R-CNP22(K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as CNP22 while being much more resistant to NEP degradation than CNP22.

Among the CNP variants listed in Tables 1-4 and assayed for both CNP functionality and NEP resistance, Analogs G, BK, AZ, CC, CF, BL and DB, Pro-Gly-CNP37, HSA-CNP27 (GHKSEVAHRFK-CNP27) (SEQ ID NO: 144), PEG1K-CNP22, (PEO12)-biotin-CNP22, PEO12-R-CNP22(K4R), PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36), and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) all had substantially similar NPR-B stimulation activity as wtCNP22 while being substantially more resistant to NEP cleavage than wtCNP22.

cGMP production assays were also carried out on wtCNP22 and CNP variants G-CNP37, GHKSEVAHRFK-wtCNP27 ("CNP27-HSA", SEQ ID NO: 144), wtCNP29 and PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36). **Figure 23** shows that CNP22 and all the CNP variants assayed induced production of similar cGMP levels at either low or high dose of CNP.

Example 5

Binding Specificity for NPR-A, NPR-B and NPR-C

Signaling Competition Assay

To determine the binding specificity of CNP variants for the clearance receptor NPR-C, a signaling competition assay is carried out. Expression plasmids for human NPR-B or NPR-C (each purchased from OriGene) are transiently transfected and receptors are expressed in HEK293T cells. Forty hours after transfection, NPR-B, NPR-C and native HEK293T cells are harvested, counted and plated at a ratio of 1:1 (NPR-B cells : competing cells (either NPR-C or native HEK293T cells)) in 12-well or 96-well plates. Twenty hours after plating, cells are processed for the NPR-B/cGMP stimulation assay described in Example 4. If present, the natriuretic clearance receptor, NPR-C, is expected to bind and internalize CNP, thereby reducing the overall CNP concentration available for signaling through NPR-B, resulting in decreased cGMP production and a shift in the dose-response curve to the right. A rightward shift in the dose-response curve has been verified for wtCNP22. CNP variants having reduced affinity for NPR-C are not expected to induce a shift, or are expected to induce a smaller shift, in the dose-response curve to the right. This signaling competition assay is similar to that previously described by Cunningham (U.S. Patent 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)).

The cGMP stimulation activity of wtCNP-22, Pro-Gly-wtCNP37 and ANP through NPR-B and NPR-A, and their selectivity for NPR-B vs. NPR-C and for NPR-A vs. NPR-C, were evaluated in signaling competition assays. NPR-A, NPR-B and NPR-C individually were transiently tranfected into HEK293T cells. Thirty hours after transfection, the cells were plated into 96-well plates: (A) 20,000 NPR-B cells + 20,000 mock transfected cells; (B) 20,000 NPR-B cells + 20,000 NPR-C cells; (C) 20,000 NPR-A cells + 20,000 mock transfected cells; and (D) 20,000 NPR-A cells + 20,000 NPR-C cells. Twenty hours after plating, the culture media was removed and replaced with serum-free media:PBS (1:1) + 0.75 uM IBMX for 15 minutes. For cGMP signaling through NPR-B, dose series for ANP, CNP-22 and Pro-Gly-CNP37 were added and incubated at 37 °C for 12 minutes before the assay was stopped by cell lysis. For cGMP signaling through NPR-A, dose series for CNP-22 and Pro-Gly-CNP37 were incubated at 37 °C for 12 minutes, while dose series for ANP were incubated at 37 °C for 6 minutes (because NPR-A appeared to be a "faster" guanylyl cyclase than NPR-B, the incubation time for ANP was shortened in order not to max out (use up all cellular GTP) too soon when signaling with ANP). **Figures 24A** and **B** show that CNP-22 and Pro-Gly-CNP37 ("Pro-CNP38") stimulated cGMP production through NPR-B with

similar dose-response curves, and to a much greater extent than through NPR-A, and exhibited a similar profile for NPR-B vs. NPR-C selectivity in the signaling competition assays.

Determination of Binding Affinities (K_i) for NPR-A, NPR-B and NPR-C

The binding affinities (K_i) of CNP variants for NPR-A, NPR-B and NPR-C are determined in a heterologous competition binding assay (U.S. Patent 5,846,932; B. Cunningham, EMBO J. 13(11): 2508-2515 (1994); H. Jin et al., J. Clin. Invest. 98(4): 969-976 (1996)). Membranes from HEK293 cells, or another suitably transfectable cell line (e.g., HeLa cells), expressing human NPR-A, NPR-B or NPR-C are prepared for radio-labeled ligand binding assays. Membrane preparations are diluted in an appropriate buffer and varying concentrations of wtCNP22 or CNP variant (competitor) are added with I^{125}-labeled wtCNP22 (Bachem). Samples are incubated at room temperature to allow for ligand/receptor equilibration and bound peptide is separated from free peptide by filtration through PVDF filter membranes. Filters are washed before the addition of scintillant and counting by a scintillation counter. Binding is measured in duplicate for each concentration of competitor peptide. CNP variant affinity (K_i, equilibrium dissociation constant) and B_{max} (receptor number) are calculated by non-linear regression analysis and/or the Cheng-Prusoff equation.

CNP variants exhibiting reduced affinity to NPR-C are expected to have reduced susceptibility to clearance by NPR-C and thus a longer plasma or serum half-life. Increased half-life of the CNP variants in circulation would increase the availability of the variants for therapeutic activity.

Example 6

Effect of CNP Variants on the Growth of Rat Chondrosarcoma (RCS) Cells and cGMP Production in RCS Cells

To assess the ability of CNP variants to stimulate bone growth, skeletal dysplasia is simulated in cell culture by treating rat chondrosarcoma (RCS) cells with fibroblast growth factor 2 (FGF-2), which activates fibroblast growth factor receptor 3 (FGFR-3) and induces growth arrest (Krejci et al., J. Cell Sci., 118(21):5089-5100 (2005)).

Optimal CNP treatment parameters are determined by varying CNP concentration (0.05, 0.1, 0.2 and 0.5 uM), and treatment duration and interval (continuous; 2.5 min, 10 min, 30 min, 1 hr, 2 hr, 4 hr and 8 hr once a day; 2.5 min, 10 min, 30 min, 1 hr, 2 hr and 4 hr twice

a day). After 72 hours, cells are counted using an automated cell counter, and the amount of extracellular matrix is estimated using alcian blue staining.

RCS cells are then treated with a CNP variant using the optimal conditions determined from the growth experiments with wtCNP22. The concentration of cGMP is measured by competitive ELISA for untreated RCS cells, RCS cells treated with CNP, and RCS cells treated with the CNP variant. Cell growth and matrix synthesis resulting from treatment with the CNP variant are also monitored and compared to those resulting from CNP treatment.

To assess the effect of CNP variants in a human cell culture system, primary re-differentiated human chondrocytes in alignate beads are treated with wtCNP22 and CNP variants, and cGMP concentration is determined by competitive ELISA as a measure of effective CNP signaling.

The methods described herein can be employed to assess the ability of CNP variants to stimulate cGMP production in and growth of rat chondrosarcoma cells *in vitro*.

Example 7

Dose Response Study in Rat Chondrosarcoma Cells

The tyrosine kinase receptor fibroblast growth factor receptor 3 (FGFR-3), a negative regulator of chondrocyte growth, is contitutively on in achondroplasia subjects. Stimulation of the FGFR-3 receptor with FGF-2 causes growth arrest by prolonged activation of Erk MAPK, and causes decreased matrix synthesis and loss of matrix, as well as a change in cell shape. Continuous exposure of rat chondrosarcoma (RCS) cells to fibroblast growth factor 2 (FGF-2) simulates achondroplasia in cell culture by activating FGFR-3 and inducing growth arrest (Krejci et al., J. Cell Sci., 118(21): 5089-5100 (2005)). To determine the dose of CNP variant and frequency of dosing that stimulate sufficient growth of bone cells, a dose response study was performed using the RCS cell assay as described in Example 6.

RCS cells were seeded at 10 x 10^3 cells per well in 24-well plates, grown for 24 hr, treated for 72 hr, and then counted. RCS cells were continuously exposed to FGF-2 (5 ng/mL) to simulate a constitutively active FGFR-3, which induced cell growth arrest (see bar #5 in **Figure 25**). Wild-type CNP22 (0.2 uM) was cultured continuously (72 hr), 1 hr daily or 2 hr daily. All stimulants were changed daily. Continuous exposure of RCS cells to 0.2 uM CNP22 in the presence of 5.0 ng/mL FGF-2 partially reversed FGF2-induced growth

arrest, leading to the growth of approximately 200 x 10^3 cells per well (bar #6 in Figure 25), compared to approximately 100 x 10^3 cells per well in the absence of CNP22 (bar #5 in Figure 25).

Both 1 hr exposure to CNP22 (0.2 uM) once a day and 2 hr exposure to CNP22 (0.2 uM) once a day achieved about 84% of the effect of continuous CNP22 (0.2 uM) exposure on chondrocyte growth (bars #7 and 8 in Figure 25). These results demonstrate that continuous exposure of growth-arrested chondrocytes to CNP22 is not required for reversal of cell growth arrest. Additionally, dose response studies demonstrate that lower doses of CNP22 are capable of reversing growth arrest (**Figure 26A**).

Furthermore, histological and cell morphological analysis of the extracellular matrix showed that CNP22 treatment antagonized FGF2-mediated loss of chondrosarcoma extracellular matrix and increased matrix synthesis. Exposure to FGF-2 decreased matrix synthesis and increased degradation, while addition of CNP22 to the FGF-2 cell culture increased matrix synthesis and partially inhibited FGF-2, as assessed by ^{35}S-sulfate and ^{3}H-Pro incorporation into, or decrease from, matrix (**Figures 27A-D**). Analysis of aggrecan and fibronectin production (mRNA and protein) in RCS cells cultured with FGF-2 and CNP22 shows that FGF-2 decreased aggrecan level and increased fibronectin level, which was inhibited by addition of CNP22 (**Figures 28A-C**). FGF-2 induces and activates matrix-processing molecules predominantly via Erk, and addition of CNP22 shows some effect on this activation.

Additional high-throughput assays for measuring growth arrest, such as crystal violet staining, are useful for measuring the effects of CNP22 and variants thereof on RCS cells.

Similar dose response studies can be conducted with the CNP variants described herein to determine their effective dose for reversing FGF2-induced growth arrest of RCS cells.

Example 8A

Ex Vivo Stimulation of Growth of Tibia and Femur from Mice with Mild Achondroplasia

Mouse tibial organ culture model has been used to demonstrate the efficacy of wild-type CNP22 in stimulating longitudinal bone growth. Treatment of wild-type tibiae with CNP22 at 10^{-8}, 10^{-7} or 10^{-6} M for 6 days increased longitudinal growth by 31%, 40% and

42%, respectively. Histological evaluation also showed expansion of the hypertrophic zone, e.g., an increase in the number and size of hypertrophic chondrocytes in the growth plate (Agoston et al., BMC Dev. Biol. 7:18 (2007)). Similar findings were observed in tibiae isolated from $FGFR3^{Ach}$ mice (Yasoda et al., Nat. Med. 10: 80-86 (2004)).

To determine the efficacy of CNP variants in stimulating longitudinal bone growth, CNP variants were tested in a mouse organ culture model of endochondral bone growth in wild-type mice and transgenic mice having a G380R mutation in the human FGFR-3 gene ($FGFR3^{wt/Ach}$ heterozygote) which represent a mouse model of mild achondroplasia. In brief, the pharmacological activity of wild-type CNP22 and CNP variants was compared in an organ culture model of embryonic or neonatal mouse tibiae, isolated from wild-type and $FGFR3^{wt/Ach}$ littermates. Overall bone growth and histological changes within the growth plate were assessed. Conditioned culture medium is also assessed for biomarkers of intracellular signaling (cGMP), cartilage metabolism (type II collagen, other collagens, aggrecan chondroitin sulfate), bone metabolism (bone alkaline phosphatase, osteocalcin, type 1 collagen [C-telopeptide, N-telopeptide]), and inflammation (interleukin-1, interleukin 6, interleukin-11).

Effective CNP variants are identified by their ability, e.g., to stimulate production of cGMP, and bone growth as measured by increased longitudinal bone length and expansion of the cells in the hypertrophic zone of the growth plate.

Measurement of Bone Growth

The efficacy of wtCNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) in stimulating longitudinal femoral growth was evaluated in the mouse organ culture model. For these experiments femora were isolated from 2-3 day old wild-type mice and cultured in alphaMEM supplemented with 0.2% BSA, 0.5 mM L-glutamine, 40 units penicillin/mL and 40 ug streptomycin/mL, for 8 days in the presence of vehicle, CNP22 or CNP variants. The treatment commenced at day 0 and was repeated every two days thereafter, as the medium was changed. Bones were measured prior to treatment and every two days thereafter, using a dissection microscope fitted with a 1 cm eye-piece reticule. Conditioned medium was used for biomarker analysis. At day 8 bones were fixed in 4% paraformaldehyde for 24 hr, decalcified in 5% formic acid for 24 hrs, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and stained with Alcian Blue for 30 min (pH 2.5; MasterTech).

Alcian Blue stains cartilage blue. Stained sections were visualized and photographed by brightfield microscopy. The thickness of the hypertrophic region of the growth plate cartilage was determined by image analysis.

Figure 29 illustrates the effect of wtCNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) on longitudinal growth of 3-day old wild-type mouse femurs treated with the CNP peptides every two days. The results were normalized to measurements prior to treatment (day 0). The studies were performed in triplicate (vehicle) or quadruplicate (CNP peptides). As shown in Figure 29, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), as well as CNP22, were effective in stimulating longitudinal femoral growth, with the N-terminal PEGylated CNP variant being the most effective.

The growth of wild-type and $FGFR3^{ach}$ mouse femur and tibia in response to CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) was also assessed. Culture of either wild-type or achondroplastic ($FGFR3^{ach}$) mouse tibia showed that CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) both increased the longitudinal growth of the tibia compared to vehicle or CNP22 (**Figures 30** and **31**). CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) also stimulated the growth of wild-type mouse femur (**Figure 32**). Moreover, each of CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 increased the longitudinal growth of $FGFR3^{ach}$ mouse femur compared to vehicle (**Figure 33**).

Furthermore, e*x vivo* distribution of CNP37 in the growth plate of $FGFR3^{ach}$ mouse tibia was evaluated. Bone samples were prepared as described above. Paraffin sections were cut and heat fixed for 1 hour at 60 °C. Antigen retrieval with 1% hyaluronidase at 37 °C (30 minutes) was followed by a 1 hour serum block (10% Normal Goat Serum). CNP22 antibody (1:500 dilution; Peninsula Laboratories Inc., San Carlos, California) was applied overnight at 4 °C. For immunodetection, Vectastain ELITE ABC kit (Vector, Burlingame, California) was used according to the manufacturer's recommendations. Specific bound peroxidase was visualized by incubation with DAB substrate kit (Vector) and the reaction was developed for 3 minutes. Slides were then dehydrated and mounted and photographed using a brightfield microscope. Staining for CNP in the growth plate of $FGFR3^{ach}$ mouse tibia showed that CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes (**Figure 34**), indicating that CNP37 was delivered to the chondrocytes.

In addition to the distribution of CNP variants in the bone growth plate, the *ex vivo* effects of CNP37, CNP22 and vehicle on cells in the FGFR3^{ach} and wild-type growth plate, e.g., hypertrophic cell size and cellularity of proliferating zone, were also evaluated. Bone samples were prepared, including Alcian Blue staining, as described above. Images of the entire proximal growth plate were taken at 4x magnitude. The growth plate is divided into three zones, starting from the epiphyseal side of cartilage: the resting zone (individual small chondrocytes), proliferating zone (columns of stacked chondrocytes parallel to the long axis of the bone), and hypertrophic zone (large chondrocytes and thin septa between the chondrocytes). In these regions, measurements were made by ImageJ software, including the number of proliferating chondrocytes per column and the density of hypertrophic chondrocytes. A test square (4x4 mm^2) at five different regions of the hypertrophic zone was used to determine the density of hypertrophic chondrocytes. The cell size of hypertrophic chondrocytes was calculated by 1 over determined cell density. Cellularity of proliferating columns was increased by CNP37 and CNP22 in both wild-type and FGFR3^{ach} mice (**Figures 35B** and **C**). Chondrocyte hypertrophy in FGFR3^{ach} mice was also increased as a result of culture with CNP22 or CNP37 (**Figures 36B** and **C**).

Ex vivo studies of cultures of mouse bones indicated that CNP37 was delivered to the growth plate and was able to increase chondrocyte cellularity and hypertrophy, which are associated with growth plate expansion and longitudinal bone growth. To assess the biodistribution of CNP37 in the bone growth plate *in vivo* and the *in vivo* effects of CNP37 on the growth plate (including total growth plate thickness, hypertrophic zone thickness, and cellularity of proliferating zone), bone samples were obtained from FGFR3^{ach} mice treated with vehicle or CNP37 as described above. For biodistribution and *in vivo* effects studies, tibias were fixed and stored in 70% ethanol. For immunohistochemistry, the samples were decalcified in 5% formic acid for 2 days, dehydrated and embedded in paraffin. Bones were sectioned at 5 um (microns), which were then deparaffinized, rehydrated, and used for CNP immunohistochemistry as described above. For cellular image analysis, bones were sectioned at 5 μm (microns), and then deparaffinized, rehydrated, and stained with Alcian Blue for 30 minutes (pH 2.5; MasterTech) and Hematoxylin & Eosin for 30 seconds. Stained sections were visualized and photographed by brightfield microscopy. Thicknesses of the growth plate and the proliferating and hypertrophic zones were measured using ImageJ software.

In vivo biodistribution studies demonstrated that, similar to the *ex vivo* studies, CNP immunoreactivity was increased in the regions of articular and hypertrophic chondrocytes in the tibia growth plate of FGFR3^{ach} mice treated with CNP37, indicating that CNP37 was delivered *in vivo* to the growth plate of FGFR3^{ach} mouse tibia (**Figure 37**). Furthermore, CNP37 treatment significantly increased the total growth plate thickness, proliferating zone thickness and hypertrophic zone thickness of FGFR3^{ach} mouse tibia *in vivo* (**Figures 38A-C**).

These results demonstrate that CNP variants of the disclosure penetrate into the growth plate of wild-type and achondroplastic animals, increase the number and size of chondrocytes, increase the thickness of the proliferating zone and the hypertrophic zone of the growth plate, and increase longitudinal bone growth in treated wild-type and achondroplasic animals. Therefore, the CNP variants are useful for stimulating bone growth in achondroplastic subjects.

Measurement of Biomarkers

In addition to measurement of bone growth in response to CNP variants, assay of the levels of biomarkers for cartilage and bone formation and growth induced in response to CNP variants is useful for evaluating the effect of CNP variants on bone growth.

Femurs and tibias were isolated from wild-type and FGFR3^{ach} mice as described above. Bones were cultured with CNP22 or a variant thereof for eight days with the replacement of media every two days. On the eighth day, media was collected and analyzed for the biomarkers cGMP (cyclic guanosine 3', 5' cyclic monophosphate) and fragments of cleaved collagen type II, a cartilage-specific marker for cartilage turnover. Both markers were measured using commercially available enzyme-linked immunosorbent assays (ELISA) for cGMP (Cayman Chemical Co., Ann Arbor, Michigan) and cleaved collagen type II (Cartilaps) (Immunodiagnostic Systems, Fountain Hills, Arizona), following the manufacturer's protocol.

Levels of cGMP and collagen type II fragments were measured from cell culture extracts after exposure to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). **Figures 39-42** show a large increase ($p < 0.01$) in the levels of cGMP in the media after exposure of ex-planted wild-type and FGFR3^{ach} mouse femurs and tibias to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). In addition, exposure of wild-type and FGFR3^{ach} mouse femurs to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) increased the levels of cleaved collagen type II, with the

treated FGFR3^{ach} mouse femurs showing significantly increased ($p < 0.05$) levels of collagen type II fragments (**Figure 43**). Elevated levels of collagen type II fragments indicate a turnover of the cartilage matrix, and cartilage turnover typically precedes new bone formation in growing bones.

Example 8B

Ex Vivo Stimulation of Growth of Femur from Mice with Severe Achondroplasia

The effect of a CNP variant on the growth of bones from mice with severe achondroplasia was evaluated *ex vivo*. Used in the study were transgenic mice expressing a human FGFR-3 gene having a Y367C mutation (FGFR3^{Y367C}) [S. Pannier et al., Biochim. Biophys. Acta, 1792(2): 140-147 (2009)], which represent a mouse model of severe achondroplasia. Femurs were isolated at embryonic day 16.5 and cultured for 6 days in the presence of 1 uM Pro-Gly-CNP37. Bone lengths were measured at Day 1 and Day 7. The bones were then paraffin-embedded, sectioned and stained with hematoxylin and eosin to assess histological changes and cellular morphology. Treatment of bone explants isolated from FGFR3^{Y367C} mice with Pro-Gly-CNP37 ("ProCNP38") resulted in increase in bone growth and expansion in the growth plate (**Figure 44**). Femurs from FGFR3^{Y367C} mice treated with vehicle for 6 days showed an 18% deficiency in growth length-wise compared to wild-type femurs treated with vehicle. Treatment of femurs from FGFR3^{Y367C} mice with 1 uM Pro-Gly-CNP37 for 6 days reduced the growth deficiency to just 11%, i.e., reduced the growth defect by around 40%.

Example 9

Serum/Plasma Stability of CNP Variants *In Vitro*

In preparation for pharmacokinetics (PK) studies, the stability of CNP variants in serum and/or plasma is evaluated.

Briefly, the analyte is isolated by the removal of serum or plasma proteins by either a 2% trichloroacetic acid precipitation or a 1:3 serum:acetonitrile precipitation. The precipitation mixture is vortexed at 14,000 rpm for five minutes, and a portion of the supernatant is removed and diluted with water prior to transfer to a silanized autosampler vial for analysis. Serum extracts are then analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) with electrospray ionization mass spectrometry (ESI-MS). A

single mass (m/z), shown to be specific for the CNP variant, is monitored for quantitation purposes.

Initially, analytical stability and recovery is determined. Analytical (RP-HPLC and ESI-MS) parameters are optimized through the analysis of matrix standards (serum extracts fortified with analyte post-precipitation). After optimization, analytical recovery is determined by spiking serum samples at known concentrations and comparison of the analyte response to that of matrix standards prepared at similar concentrations. Analyte stability in serum extracts is also determined to assure no significant losses occur after serum precipitation and prior to actual analysis. To test the effect of freezing on serum stability, a two-cycle freeze/thaw study is also performed. In this study a serum sample is spiked with CNP variant and analyzed prior to freezing overnight at -20 °C. The sample is then thawed at room temperature and re-analyzed. The process is repeated for a second freeze/thaw cycle.

Serum stability of CNP variant is determined by spiking of serum/plasma samples with CNP variant at a concentration of 10 ug/mL. The sample is placed in a 37 °C water bath for a period of three hours. At 30 minute intervals duplicate aliquots of serum are removed and analyzed. If rapid losses of analyte are evident (> 50% in 30 minutes), the study may be repeated with 10 minute timepoints.

In an exemplary method for determining the stability of CNP variants in murine plasma, a mixture of CNP variant (10 uL of a stock solution of about 2.5-5.0 mg/mL), heparinized murine plasma (50 uL, Bioreclamation, CD-1 Lith Hep 62231), and 5 M NaCl (10 uL) is incubated at 37 °C and 5% CO_2 for 0-5 hr, and then quenched with 10x protease inhibitor cocktail (15 uL, Sigma P2714). For extraction, 150 uL of MeOH/0.1% FA is added to 85 uL of the reaction mixture, and the resulting mixture is vortexed for 1 min and then centrifuged at 15 °C for 15 min. 75 uL of the supernatant is added to 300 uL of aqueous 0.1% FA. A small portion of the resulting mixture is subjected to analysis by LC/MS.

Example 10

Pharmacokinetics and cGMP Production in Rats and Mice

Studies were conducted in normal rats to evaluate the pharmacokinetics (PK) profile of CNP22 and certain CNP variants and the time courses of plasma cGMP concentration after single intravenous (i.v.) or subcutaneous (s.c.) administration of the CNP peptides. Plasma CNP immunoreactivity was determined by using a competitive radioimmunoassay (RIA)

with an anti-CNP rabbit polyclonal antibody. Plasma cGMP concentration was determined by RIA using a commercially available kit (YAMASA cyclic GMP Assay kit, YAMASA Corporation).

Normal male rats, 7-8 weeks of age, were used. Recombinant wild-type CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) were evaluated. A dosage of 20 nmol/kg of each CNP peptide as a solution in 5% mannitol was intravenously injected once into the tail, or a dosage of 50 nmol/kg of each CNP peptide as a solution in 0.03 mol/L acetic acid buffer solution, pH 4.0, containing 1%(w/v) benzyl alcohol and 10% (w/v) sucrose, was subcutaneously injected once into the back.

Plasma CNP immunoreactivity was determined by the competitive RIA using anti-CNP rabbit polyclonal antibody. Standard and QC samples were prepared. Fifty uL of the standard, QC and assay samples were added, respectively, to test tubes containing 50 uL of RIA buffer. Diluted anti-CNP rabbit polyclonal antibody (100 uL) was added to the tubes. All tubes were kept at 4 °C overnight. ^{125}I-[Tyr^{0}]-CNP22 solution (100 uL) and rabbit IgG solution (100 uL) were added and left at approximately 4 °C overnight. One milliliter of anti-rabbit IgG goat serum containing 10% polyethylene glycol was added, vortexed and left at approximately 4 °C for at least 1 hour, and then the insoluble fraction was precipitated by centrifugation. After aspiration of the supernatant, the amount of radiation (gamma line) in the sediment was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma cGMP concentrations in the sample at 5, 30, 60 and 90 minutes after i.v. dosing, or at 5, 30, 60, 120 and 180 minutes after s.c. dosing, were determined by the competitive RIA using anti-cGMP monoclonal antibody. Standard samples were prepared. 100 uL of the assay samples (standard solutions for the calibration curve or the diluted plasma samples for cGMP determination) were transferred to test tubes. Then 100 uL of anti-cGMP monoclonal antibody solution and 100 uL of ^{125}I-labeled succinyl cGMP tyrosine methyl ester solution were added to the tubes, respectively. All tubes were kept at 4 °C overnight. After the addition of 500 uL dextran charcoal solution, the tubes were vortexed and then placed on ice for 10 minutes. The reaction mixture was centrifuged and 500 uL of the supernatant was transferred from each sample to a new test tube. The amount of radiation (gamma line) in the supernatant was measured by a gamma-counter. Each sample was measured in duplicate, and the mean was adopted as the value determined.

Plasma CNP immunoreactivity was employed for pharmacokinetics (PK) analysis. PK analysis was performed using WINNONLIN® Professional (Pharsight Corporation). The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) after i.v. administration were calculated using PK parameters such as concentration at 0 hour (C_0: extrapolation, pmol/mL), total body clearance (CL_{tot}: mL/min/kg), distribution volume at steady state (V_{dss}: mL/kg), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min). The PK profiles of the CNP peptides after s.c. administration were calculated using PK parameters such as maximum plasma concentration (C_{max}: pmol/mL), time to reach C_{max} (T_{max}: min), area under the plasma concentration-time curve (AUC: pmol·min/mL), mean residence time (MRT: min), and half-life ($T_{1/2}$: min).

In plasma spike recovery experiments, the RIA detected CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) similarly (data not shown).

Procedures similar to those described above are employed to study in mice the PK profiles of CNP22 and variants thereof and their ability to stimulate cGMP production.

The PK profiles of CNP22, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) after i.v. administration in three rats are illustrated in **Figure 45**. As shown by Figure 45, CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life and a much greater bioavailability than CNP22. The half-life, $T_{1/2}$ (min), was 1.42 (± 0.45) for CNP22, 22.3 (± 1.5) for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), and 49.5 (± 28.0) for CNP37. The area under the curve, AUC (pmol·min/mL), was 320 (± 54) for CNP22, 1559 (± 568) for CNP37, and 2084 (± 424) for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36).

The PK profiles of the three CNP peptides after s.c. administration in three rats are depicted in **Figure 46**. Compared to CNP22, PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a much longer half-life (78.1 min (± 16.4) vs. 10.0 (± 5.0)) and a much greater bioavailability (60% (± 6%) vs. 19% (± 9%)).

The time courses of plasma cGMP concentrations after i.v. administration of the three CNP peptides in three rats are displayed in **Figure 47**. Figure 47 clearly demonstrates that i.v. administration of CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) resulted in much higher plasma levels of cGMP at 30, 60 and 90 minutes than i.v. administration of CNP22.

The time profiles of plasma cGMP concentrations after s.c. administration of the three CNP peptides in three rats are shown in **Figure 48**. Subcutaneous administration of PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) and CNP37 also resulted in substantially higher plasma concentrations of cGMP than s.c. administration of CNP22, with the difference relative to CNP22 increasing over time for PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36), but decreasing over time for CNP37.

The rat studies indicate that compared to wtCNP22, the CNP variants CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) had a substantially longer half-life *in vivo*, had a substantially greater bioavailability *in vivo*, and stimulated substantially higher levels of cGMP production *in vivo* for an extended period of time. The resistance of CNP37 and PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) to NEP degradation correlates to a longer plasma half-life *in vivo*, which in turn correlates to prolonged NPR-B/cGMP signaling *in vivo*. These results show that compared to CNP22, the CNP variants of the disclosure, administered by i.v. or s.c. injection (e.g., once daily), can be more effective in treating CNP-responsive conditions or disorders, such as bone-related disorders and vascular smooth muscle disorders.

Example 11

Pharmacokinetics Study in Mice

To determine CNP variants having increased NEP resistance, for efficacy study in FGFR3^{ach} mice (see Example 13), a pharmacokinetics (PK) study is carried out that compares the pharmacokinetics properties of CNP variants to wild-type CNP22. The FGFR3^{ach} mouse is a mutant mouse model of mild achondroplasia, containing a single transgene on a background of FVB mice.

Wild-type CNP22 or variant thereof is administered as a single intravenous (i.v.) dose in 6-week old wild-type FVB mice. Exemplary PK studies were conducted using wtCNP22. Six-week old FVB/N mice were intravenously administered wtCNP22 in a single dose at 100 nmol/kg. Mean plasma levels of CNP22 were calculated, and the estimated half-life of CNP22 was determined to be from 0.76 min to 1.03 min.

CNP variants displaying greater resistance to NEP degradation are expected to exhibit increased serum concentrations over time and a longer half-life *in vivo*.

Example 12

Efficacy of CNP Variants in Wild-Type Mice

The *in vivo* effects of CNP variants on bone growth were assessed in wild-type mice. Three week old FVB wild-type male mice received daily subcutaneous (s.c.) injection of either vehicle, G-CNP37 (200 nmol/kg) or PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) (200 nmol/kg) for 5 weeks. Body weight was measured at least once weekly. Tail length was measured at least once weekly using digital caliper readings, and body length (naso-anal length), bone length (tibia, femur, ulna and humerus), skull length (anterior to posterior cranial segment) and lumbar vertebrae 5 (LV5) length were measured after 5 weeks of treatment using a caliper. X-rays were taken at baseline and after 5 weeks of treatment.

Five weeks of treatment of wild-type mice with G-CNP37 resulted in significant body weight gain, with increased body weight being observed beginning at Day 9 ($p < 0.05$) (**Figure 49**). Treatment with G-CNP37 also resulted in significantly increased tail length, beginning at the second week after treatment ($p < 0.01$) (**Figure 50**).

Table 10 shows the percentage change in tail length, body (naso-anal) length, skull (anterior to posterior cranial segment) length, bone (femur, tibia, humerus and ulna) lengths, and lumbar vertebrae 5 (LV5) length in wild-type mice injected s.c. once daily with 200 nmol/kg of either G-CNP37 or PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

Table 10

	Tail	Naso-Anal	Anterior to Posterior (Skull)	Femur	Tibia	Humerus	Ulna	LV5
G-CNP37	119%**	114%**	104%*	109%**	105%**	104%**	101%	108%*
CNP27-PEO12	101%	104%**	101%	102%*	100%	102%*	96%*	103%

**p<0.01, *p<0.05

Treatment with G-CNP37 resulted in significantly increased tail length, body (naso-anal) length, skull length, proximal bone (femur and humerus) length, distal bone (tibia) length, and vertebral (lumbar vertebrae 5) length compared to treatment with vehicle.

Lower doses of Pro-Gly-CNP37 dosed daily s.c. at 5 nmol/kg, 20 nmol/kg or 70 nmol/kg for five weeks resulted in a dose-dependent increase in tail length, body (naso-anal) length, and bone lengths compared to vehicle. **Table 11** shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice injected s.c. once daily with 5 nmol/kg, 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37 for 5 weeks, relative to a value of 100% for wild-type mice treated with vehicle only.

Table 11

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
5 nmol/kg	107.6%**	102.7%**	103.3%**	101.7%**	101.1%	100.9%*
20 nmol/kg	107.8%**	107.6%**	107.1%**	103.4%**	102.3%**	102.5%**
70 nmol/kg	113.6%**	112.5%**	109.3%**	105.9%**	103.6%**	104.1%**

**p<0.01, *p<0.05

In another study, various dosing regimens of Pro-Gly-CNP37 were administered for nine weeks followed by one week of recovery. Wild-type FVB mice were dosed s.c. with:

(1) vehicle daily for nine weeks, followed by one week of recovery;

(2) 20 nmol/kg Pro-Gly-CNP37 daily for one week, followed by three doses per week for eight weeks and one week of recovery;

(3) 20 nmol/kg Pro-Gly-CNP37 on alternating weeks; or

(4) 5 nmol/kg Pro-Gly-CNP37 daily for nine weeks followed by one week of recovery.

Increased growth in tail length, body length, and bone lengths was observed in all treatment groups (Groups 2, 3 and 4) at the end of the study. **Table 12** shows the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in wild-type mice administered Pro-Gly-CNP37 ("Pro-CNP38") under the dose regimens described above, relative to a value of 100% for wt mice treated with vehicle only.

Table 12

	Total Pro-CNP38 received (nmol/kg)	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
Group 2	620	104.8%**	106.0%**	105.3%**	100.8%	102.5%	101.7%
Group 3	700	106.7%**	106.5%**	104.1%**	102.5%**	102.6%*	102.8%*
Group 4	350	104.9%**	105.1%**	107.3%**	102.7%**	102.3%**	103.6%**

**p<0.01, *p<0.05

While all dose regimens of Pro-Gly-CNP37 increased axial and appendicular growth parameters measured, daily dosing of Pro-Gly-CNP37 promoted appendicular growth (femur, tibia, humerus, and ulna) at a lower total dose level (Group 4) compared to regimens with less frequent dosing (Groups 2 and 3).

Example 13

Efficacy in Mouse Model of Mild Achondroplasia

The efficacy of CNP variants in enhancing growth and correcting achondroplasia was tested in a mouse model of mild achondroplasia, using a strain of transgenic mice expressing a human FGFR-3 gene having a G380R mutation ($FGFR3^{ach}$) (Wang et al., Proc. Natl Acad. Sci. U S A, 96(8): 4455–4460 (1999); Naski et al., Development USA, 125: 4977-4988 (1998); U.S. Patent Nos. 6,265,632 and 6,136,040).

At 3 weeks of age, $FGFR3^{ach}$ mice and their wild-type littermates were anesthetized to have lateral whole-body X-ray images taken by Faxitron, and randomized by body weight into the following treatment groups (n=8/group): (1) wild-type/vehicle, (2) $FGFR3^{ach}$/vehicle, (3) $FGFR3^{ach}$/CNP37, and (4) $FGFR3^{ach}$/PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36). The mice received once daily subcutaneous (s.c.) administration of designated test article (vehicle or 200 nmol/kg CNP variant) for 5 weeks. Satellite groups (n=3) were used to confirm exposure of each test article following a single subcutaneous administration on Day 1. Wild-type male FVB mice that received daily s.c. injection of vehicle for 5 weeks were used as a control for normal growth.

X-ray measurements at baseline and at the end of the study were performed to determine change in the head length, area of the skull, and the external auditory meatus (EAM, the ear canal running from the outer ear to the middle ear). Body weight and tail length were measured at least once weekly, using a digital caliper to measure tail length, and body (naso-anal) length was measured after 5 weeks of treatment. Bone (tibia, femur, ulna and humerus) lengths were measured using a digital caliper at necropsy.

On Day 37, all mice were sacrificed by terminal anesthesia and whole-animal photographs and X-ray images by Faxitron were taken. Left and right tibia, femur, humerus, and ulna were collected and measured using a digital caliper. The left portions of each bone were processed for histology, and the right portions were snap-frozen for archival. Samples

obtained from the bones were used to evaluate the effects of CNP variants on endochondral bone growth.

Treatment of FGFR3^{ach} mice with CNP37 by once daily s.c. injection for 5 weeks resulted in significantly increased body length (**Figure 51**), tail length (**Figure 52**), distal bone (ulna and tibia) lengths (**Figures 53A** and **B**), and proximal bone (humerus and femur) lengths (**Figures 54A** and **B**). Moreover, treatment with CNP37 increased the head length (**Figure 56**), area of the external auditory meatus (**Figure 57**), and spine length (**Figure 58**) via extension of the vertebral bodies. Additionally, treatment with CNP37 corrected rhizomelia (disproportion of the length of the proximal limbs) of FGFR3^{ach} mice, i.e., restored proportional growth of proximal bones, as assessed by the femur:tibia ratio (**Figure 55**). **Table 13** summarizes the percentage change in tail length, body (naso-anal) length, and bone (femur, tibia, humerus and ulna) lengths in FGFR3^{ach} mice injected s.c. once daily with 200 nmol/kg of either CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) for 5 weeks, relative to a value of 100% for FGFR3^{ach} mice treated with vehicle only.

Table 13

	Tail	Naso-Anal	Femur	Tibia	Humerus	Ulna
CNP37	115%**	109%**	112%**	107%**	105%*	106%**
CNP27-PEO24	100%	99%	100%	102%	100%	101%

**p<0.01, *p<0.05

The results of these studies show that CNP37 can stimulate spinal and long bone growth, help to correct rhizomelia by preferentially increasing the length of the femur over the tibia, and help to restore craniofacial proportions in FGFR3^{ach} mice. These results indicate that CNP37 and potentially other CNP variants may be effective in correcting the symptoms of achondroplasia and treating subjects having defects in bone growth or in need of increased bone growth.

Example 14

Measurement of Biomarkers and Evaluation of Immunogenicity in Wild-Type and Achondroplastic Mice

Measurement of biomarkers after CNP administration

The levels of bone growth biomarkers were measured in wild-type and achondroplastic ($FGFR3^{ach}$) mice.

Transgenic FVB $FGFR3^{ach}$ mice (a mouse model of mild achondroplasia) were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36) at 200 nmol/kg for each CNP compound, as described above. Plasma and serum were collected during the study. Harvested plasma was stored with 10x protease inhibitor at -80 °C until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. Fragments of cleaved collagen type II (cartilage-associated biomarkers), osteocalcin (bone-associated biomarker), and IgG (relating to immunogenicity) were measured from terminal bleed serum at the end of the study (Day 37). cGMP was measured using a commercially available ELISA kit (Cayman Chemical Co., Cat. No. 581021.1), cleaved collagen type II (Cartilaps) was measured using a commercially available kit from Immunodiagnostic Systems (Cat. No. 3CAL4000), and osteocalcin was measured using a commercially available kit from Biomedical Technologies Inc. (Stoughton, Massachusetts).

Figure 59 shows an increase in cGMP plasma levels in $FGFR3^{ach}$ mice treated with CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) compared to vehicle. **Figures 60** and **61** show that administration of CNP37 resulted in the greatest elevation of serum levels of cleaved collagen type II and osteocalcin. These results indicate that administration of the CNP peptides, in particular CNP37, to $FGFR3^{ach}$ mice led to increased levels of bone growth markers, suggesting increased bone formation and growth in CNP peptide-treated $FGFR3^{ach}$ mice.

For evaluation of biomarkers in wild-type mice, wild-type FVB mice were treated daily for 5 weeks by subcutaneous injection with either vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0), 200 nmol/kg G-CNP37, 200 nmol/kg PEO12-GANRR-CNP22(K4R) ("CNP27-PEO12") (SEQ ID NO: 36), or 20 nmol/kg or 70 nmol/kg Pro-Gly-CNP37. Plasma and serum were collected during the study. Harvested plasma was stored with 10x protease inhibitor at -80 °C until analysis. cGMP levels were measured 15 minutes post-injection on Day 36 from K2-ETDA plasma collection tubes. The levels of cleaved collagen type II, bone-specific alkaline phosphatase, and IgG (relating to

immunogenicity) were measured from terminal bleed serum at the end of the study (Day 37). cGMP and cleaved collagen type II (Cartilaps) were measured as described above. Bone-specific alkaline phosphatase was measured using a commercially available kit (Cusabio, Cat. No. CSB-E11914m).

Administration of G-CNP37 significantly increased ($p < 0.05$) the level of cGMP (**Figure 62**) and particularly the level of fragments of cleaved collagen type II (**Figure 63**) in wild-type mice compared to vehicle. The significantly higher level of collagen type II fragments resulting from administration of G-CNP37 indicates turnover of the cartilage matrix, suggesting that G-CNP37 stimulated new bone formation in growing bones in wild-type mice.

Both doses of Pro-Gly-CNP37 ("Pro-CNP38"), 20 and 70 nmol/kg, significantly increased ($p < 0.05$) plasma cGMP level 15 minutes after administration compared to wild-type mice treated with vehicle (**Figure 64**). Administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 also significantly increased ($p < 0.05$) the level of cleaved collagen type II compared to vehicle-treated mice (**Figure 65**), suggesting that the higher dose of Pro-Gly-CNP37 stimulated cartilage matrix turnover prior to new bone formation in wild-type mice. Furthermore, administration of the higher dose (70 nmol/kg) of Pro-Gly-CNP37 increased ($p < 0.05$) the level of bone-specific alkaline phosphatase compared to vehicle-treated mice (**Figure 66**), suggesting that the higher dose of Pro-Gly-CNP37 increased bone remodeling in wild-type mice.

Evaluation of immunogenicity of CNP variants

Because the CNP variants are peptide derivatives, it is possible that administration of the peptides may lead to an immunogenic response *in vivo*. To assess whether an immune response occurred after successive administrations of CNP variant, measurement of serum antibody levels was performed.

An IgG assay was performed to assess whether an IgG immune response was triggered by 5 week exposure of achondroplastic $FGFR3^{ach}$ mice to CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) ("CNP27-PEO24") (SEQ ID NO: 36). IgG is the most predominant immunoglobulin in mouse and human serum and is produced as part of the secondary immune response to an antigen. The IgG response to administration of the CNP peptides was determined as follows. 96-well plates were coated with 100 ng/mL CNP22, CNP37 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) in BupH PBS buffer

(Pierce/Thermo, Cat. No. 28372, Rockford, Illinois). After overnight incubation, plates were blocked with Casein-PBS blocking buffer (Pierce/Thermo, Cat. No. 37528) for two hours at room temperature with shaking at 300 rpm. After washing with wash buffer (1x PBS with 0.05% Tween), diluted serum samples from a terminal bleed (diluted 1:25) were added to the plate. Positive and negative controls were also loaded onto the plate. Positive control was 1:25 diluted serum (pooled from 6 individual FVB mice) with anti-CNP22 antibody added (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) at 1:1000 dilution. Negative control was the diluted pooled serum. After two hours of incubation, the plates were washed and secondary antibody diluted in blocking buffer was added to the wells. For the mouse serum samples, anti-mouse IgG Fcγ (peroxidase-conjugated affini-pure goat anti-mouse IgG, Fcγ fragment, Cat. No. 115-035-071, Jackson Immunoresearch, West Grove, Pennsylvania) was added at a 1:10,000 dilution. For the positive and negative controls, anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Cat. No. SC-2004, Santa Cruz, California) was added to blocking buffer. After two hours of incubation at room temperature with shaking at 300 rpm, plates were washed with wash buffer. 100 uL of TMB (One-step TMB, Pierce/Thermo, Cat. No. 34022) was added to all wells. Plates were incubated at room temperature for 15 minutes with shaking at 300 rpm. Colorimetric reactions were stopped by the addition of 100 uL of 2 N H_2SO_4. Plates were read at 450 nm (Spectramax, Molecular Devices, Sunnyvale, California) and data was analyzed using SoftMax Pro software (Molecular Devices).

Serum samples from $FGFR3^{ach}$ mice treated with CNP22 or PEO24-GANRR-CNP22(K4R) (SEQ ID NO: 36) indicated no positive IgG immune response in any of the mice. Only one out of nine CNP37-treated $FGFR3^{ach}$ mice showed a slightly positive IgG response.

The immunogenic response of wild-type mice administered G-CNP37, PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37, as measured by an increase in serum IgG level, was also assessed by examining terminal bleed serum samples as described above. None of the wild-type mice treated with PEO12-GANRR-CNP22(K4R) (SEQ ID NO: 36) or Pro-Gly-CNP37 (20 or 70 nmol/kg) showed a positive IgG immune response, and only one out of six wild-type mice administered G-CNP37 showed a slightly positive IgG response.

Measurement of biomarkers under different CNP dosing regimens

Wild-type FVB mice were treated daily for 9 weeks by subcutaneous (s.c.) injection of vehicle (30 mM acetic acid/acetate buffer, 1% benzyl alcohol, 10% sucrose, pH 4.0) or Pro-Gly-CNP37 ("Pro-CNP38") under different dosing regimens. Group 1 included vehicle-treated mice injected s.c. daily for 9 weeks followed by 1 week of no treatment. Group 2 included mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily for 1 week, followed by three doses per week for 8 weeks, followed by 1 week of no treatment. Group 3 contained mice treated with Pro-Gly-CNP37 (20 nmol/kg) once daily in alternate weeks (weeks 1, 3, 5, 7 and 9), with 1 week of no treatment following each week of treatment. Group 4 contained mice treated with Pro-Gly-CNP37 (5 nmol/kg) once daily for 9 weeks followed by 1 week of no treatment. Finally, Group 5 included mice treated with Pro-Gly-CNP37 (5 nmol/kg) once daily for 5 weeks without any week of no treatment. Terminal bleed serum was collected from the mice, and cleaved collagen type II, total alkaline phosphatase and total antibody levels were measured therefrom. Cleaved collagen type II levels were measured as described above. Total alkaline phosphatase levels, predominantly from the liver and bones, were measured with the assistance of a veterinary diagnostic laboratory testing facility (Antech).

A total antibody assay was developed to evaluate potential immune response. The platform used for the total antibody assay was an electrochemiluminescent assay (ECLA). The ECLA platform utilizes a biotin-labeled drug (here, biotin-Pro-Gly-CNP37) and a ruthenium-labeled drug (here, Ru-Pro-Gly-CNP37). The biotin-labeled drug binds to a streptavidin-coated plate that contains electrodes, and the ruthenium-labeled drug functions as the detection component of the assay, as ruthenium can be electrochemically stimulated. A drug-specific antibody (here, a CNP-specific antibody) binds to the biotin-labeled drug and the ruthenium-labeled drug and "bridges" the two labeled drugs. Among the advantages of the ECLA platform, any isotype of antibody (IgG, IgM, etc.) can be detected, and the ECLA assay is species-independent.

The total antibody assay was performed as follows. Pro-Gly-CNP37 was labeled with biotin in a 4:1 challenge ratio, and Pro-Gly-CNP37 was also labeled separately with ruthenium in a 10:1 challenge ratio. Both separate labeling reactions were quenched by the addition of glycine, and the samples from both reactions were buffer-exchanged to PBS. Low and high QCs were prepared by using commercially available anti-CNP22 antibody (Bachem rabbit anti-CNP22 IgG, Cat. No. T-4222) added at low and high concentrations to 5% diluted FVB mouse serum. Mouse serum samples were diluted to 5% using assay diluent (5% BSA in PBS, MSD Cat. No. R93AA-1). A working solution was prepared by adding

biotin-labeled Pro-Gly-CNP37 to assay diluent, then adding ruthenium-labeled Pro-Gly-CNP37 to assay diluent, and then combining the two solutions together. Twenty-five uL of low and high QCs were loaded onto the plate as controls. Then 25 uL of sample was added to a non-binding 96-well plate, followed by the addition of 50 uL of working solution to all wells. Samples and QCs were incubated with the working solution at room temperature for 2 hours with shaking at 350 rpm. In the meantime, an MSD streptavidin plate (MSD Cat. No. L13SA-1) was blocked with blocking buffer (MSD Cat. No. R93AA-1) at room temperature for 2 hours with shaking at 350 rpm. At the end of the two hour incubation, the MSD streptavidin plate was washed, and then 50 uL of sample or QC was transferred to the MSD plate. The plate was then incubated at room temperature for 1 hour with shaking at 350 rpm. At the end of the 1 hour incubation, the MSD plate was washed and 150 uL of 2x read buffer (MSD Cat. No. R92TC-2) was added to the plate. The plate was read using an MSD PR400 machine.

All five groups of mice exhibited substantially comparable levels of cleaved collagen type II (**Figure 67**). Treatment of the mice in Group 5 with 5 nmol/kg Pro-Gly-CNP37 once daily for 5 weeks significantly increased ($p < 0.001$) total alkaline phosphatase level (**Figure 68**), which is indicative of bone remodeling. Serum samples from each of the four groups of mice treated with Pro-Gly-CNP37 did not show a positive antibody response in the total antibody assay.

Not to be bound by any theory, there are possible explanations as to why the four groups of CNP-treated mice did not exhibit statistically significant difference in cleaved collagen type II level compared to vehicle-treated mice, and why only the CNP-treated mice in Group 5 exhibited a statistically significant increase in total alkaline phosphatase level compared to vehicle-treated mice. It is possible that because the vehicle-treated mice in the study were also growing, cleaved collagen type II and alkaline phosphatase, which are biomarkers of growth, were also produced in the vehicle-treated mice. In addition, there was a one week period of no treatment for the mice in each of Groups 1 to 4, which possibly diluted out any changes in the cleaved collagen type II and total alkaline phosphatase levels between the CNP-treated mice in Groups 1-3 and the vehicle-treated mice. There was no period of no treatment for the CNP-treated mice in Group 5, and those mice exhibited significantly ($p < 0.001$) greater total alkaline phosphatase level compared to vehicle-treated mice.

Example 15

Dose Responses of CNP Variants in Mice

The effects of varying doses of CNP variants were assessed in wild type FVB mice.

For the dose study, in two separate studies (S1 and S2), groups of 10 mice were administered Pro-Gly-wtCNP37 ("Pro-CNP38") at 20 and 70 nmol/kg subcutaneously once daily for 36 injections. Tail length and body weight were measured over the course of treatment. Animals were sacrificed at the end of the experiment and bone length assessed.

Tail length of vehicle treated animals was approximately 8 cm at day 36, whereas animals treated with 20 nmol/kg Pro-CNP38 exhibited tail length of approximately 8.75 cm and 70 nmol/kg Pro-CNP38 increased tail length to approximately 9.5 cm. Administration of Pro-CNP38 in either dose induces a significant ($p<.05$) relative increase in total body length compared to control treated animals, demonstrating approximately a 130% increase in growth velocity at 20 nmol/kg and approximately a 160% increase in growth velocity at 70 nmol/kg Pro-CNP38 (**Figure 69**).

Treatment with Pro-CNP 38 also significantly increased bone length in most of the long bones assessed as well as total naso-anal (body length) of the animal. **Table 14** is representative of the relative % increase in bone length in treated animals in comparison to vehicle treated animals.

Table 14

Pro-CNP38	Tail	Naso-Anal	LV4-6	Femur	Tibia	Humerus	Ulna
20 nmol/kg	8%*	7%*	10%*	7%*	4%*	3%*	3%
70 nmol/kg	16%*	13%*	13%*	10%*	7%*	4%*	5%*

Relative %increase, * $p<0.05$ ANNOVA (Dunnett's) v vehicle

Bone mineral density (BMD) and bone mineral content (BMC) were also evaluated after administration of Pro-CNP38 at different doses. Results (**Figure 70**) showed that administration of Pro-CNP38 at 70 nmol/kg significantly decreased bone mineral density (**Figure 70A**) and increased bone mineral content (**Figure 70B**), suggesting there is a delay in bone mineralization in treated animals, but the mineralization process itself is not adversely affected by treatment with CNP.

There were no significant changes in organ weight between Pro-CNP38 or vehicle treated animals.

Bioanalytical studies of dose response in mice

Bioanalytical studies were carried out to measure markers of CNP activity after in vivo administration of varying doses of Pro-CNP38. Plasma cGMP levels, serum levels of collagen type II and serum levels of alkaline phosphatase from in vivo samples were analyzed. Wild type mice were administered 20 and 70 nmol/kg Gly-wtCNP37 ("CNP38"), 20 and 70 nmol/kg Pro-Gly-wtCNP37 ("Pro-CNP38") and 70 and 200 nmol/kg of GHKSEVAHRFK-wtCNP27 ("HSA-CNP27") (SEQ ID NO: 144) subcutaneously once daily for 36 days. Plasma was collected 15 minutes after the last injection on day 36 and mice were sacrified 24 hr later. At sacrifice, terminal bleed serum was collected and used for biomarker analysis as described previously.

Figure 71 shows that 20 nmol/kg CNP38 and 70 nmol/kg HSA-CNP27 signficantly increased plasma cGMP ($p<0.01$), raising plasma cGMP levels to approximately 300 pmol and 400 pmol, respectively. Adminsitration of 70 nmol/kg CNP38 increased cGMP to approximately 500 pmol ($p<0.01$), while administration of 70 nmol/kg Pro-CNP38 increased cGMP to approximately 575 pmol ($p<0.001$). Administration of 200 nmol/kg HSA-CNP27 increased cGMP to approximately 675 pmol ($p<0.001$).

CNP variants also significantly increased serum levels of cleaved collagen type II (**Figure 72**). CNP38 at 20 nmol/kg increased collagen to approximately 9 pg/ml ($p<0.05$), CNP38 at 70 nmol/kg increased collagen to approximately 8 pg/ml ($p<0.05$), Pro-CNP38 at 20 nmol/kg increased collagen to approximately 12 pg/ml ($p<0.05$), Pro-CNP38 at 70 nmol/kg increased collagen to approximately 16 pg/ml ($p<0.05$), HSA-CNP27 at 70 nmol/kg increased collagen to approximately 10 pg/ml, and HSA-CNP27 at 200 nmol/kg increased collagen to approximately 10 pg/ml ($p<0.05$).

Serum alkaline phosphatase (AP) levels also increased after administration of CNP variants (**Figure 73**). CNP38 at 20 nmol/kg increased AP to approximately 130 IU/L, CNP38 at 70 nmol/kg increased AP to approximately 160 IU/L ($p<0.001$), Pro-CNP38 at 20 nmol/kg increased AP to approximately 155 IU/L ($p<0.001$), Pro-CNP38 at 70 nmol/kg increased AP to approximately 180 IU/L ($p<0.001$), HSA-CNP27 at 70 nmol/kg increased AP to approximately 120 IU/L, and HSA-CNP27 at 200 nmol/kg increased AP to

approximately 140 IU/L ($p<0.01$). **Table 15** illustrates the percent of total AP that is bone specific.

Table 15

	Total AP IU/L	Bone –specific AP IU/L	% of Total Alk Phos
Vehicle	109.8	24.7	22.5
CNP38 (20 nmol/kg)	135.9	47.9	35.2
CNP38 (70 nmol/kg)	166.3	63.5	38.2
Pro-CNP38 (20 nmol/kg)	159.9	65.9	41.2
Pro-CNP38 (70 nmol/kg)	183.6	78.4	42.7
HSA-CNP27 (20 nmol/kg)	121.4	51.7	42.6
HSA-CNP27 (70 nmol/kg)	136.1	76.5	56.2

Analysis of anti-CNP antibodies showed that only HSA-CNP27 elicited an IgG antibody response in mice.

The results above illustrate that administration of CNP variants increases the concentration of collagen type II and alkaline phosphatase in serum, indicating that CNP increases factors relevant for increased bone growth, and suggest that administration of CNP variants at doses as low as 20 nmol/kg are effective in increasing bone growth in vivo.

cGMP response after different dose regimens

Bioanalytical analysis was also assessed at different times after administration of Pro-Gly-wtCNP37 ("Pro-CNP38") to wild type CD-1 mice, 8 – 10 weeks old, (n=3 per treatment group). Pro-CNP38 was given in a single subcutaneous dose of 200 nmol/kg and levels of cGMP measured at 15 minutes, 3 hours, 1 day, 2 days and 3 days post injection in the plasma, epiphysis, cortical bone (marrow removed), lung and brain. Blood was collected on K2EDTA. Tibial and femoral epiphysis and cortical bone, ear pinna, brains, kidneys and lungs were harvested, placed in boiling water for 5 minutes, then frozen to -70°C. Both plasma and tissue were assayed for cGMP (Cayman Chemical cyclic GMP ELISA kit).

Results showed that cGMP levels increased at 15 minutes post injection in plasma (approximately 1300 pmol/ml) and epiphysis (approximately 2.5 pmol/ml/mg). By 3 hours,

plasma levels had decreased approximately to control levels, while levels in the epiphysis were apprimately 3 fold lower than cGMP levels at 15 minutes post injection, but higher than control levels. Levels of cortical bone increased to approximately 0.5 pmol/ml/mg at 15 minutes and remained at this level at 3 hours. Levels of cGMP at all timepoints were back to control levels by 1 day after injection. Little to no cGMP was detected in lung or brain at any timepoint.

cGMP levels were also measured in mice administered multiple injections of Pro-CNP38. Groups of mice (n=3) were given Pro-CNP38 as follows: 20 nmol/kg single dose, subcutaneously; 200 nmol/kg single dose, subcutaneously; 20 nmol/kg subcutaneously on days 0 and 1; 200 nmol/kg subcutaneously on days 0 and 1; 20 nmol/kg subcutaneously on days 0 and 3; 200 nmol/kg subcutaneously on days 0 and 3. Mice were sacrificed 15 minutes after the final dose of Pro-CNP38 and plasma levels of cGMP analyzed. There does not appear to be a modulation of the plasma cGMP signal due to the different dose regimens. cGMP responses in the cartilage are also investigated.

Evaluation of potential desensitization of NPR-B receptor

Histological analysis shows that CNP, when administered daily at 200 nmol/kg, accumulates in the growth plate of animals, based on increased CNP immunoreactivity. It is possible that this accumulation, or daily stimulation of the CNP receptor, in the growth plate could desensitize the CNP receptor.

To determine if multiple dosing desensitizes the NPR-B receptor *in vitro*, normal human articular chondrocytes were cultured with Pro-Gly-wtCNP37 ("Pro-CNP38") for varying times and cGMP secretion measured.

Primary normal human chondrocytes isolated from articular cartilage were cultured as recommended by the supplier (Lonza). At 60-80 % confluence the chondrocytes were treated with 1 uM Pro-CNP38 twice, with increasing amounts of time between treatments (first treatment at time 0, then at 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, 6 hrs after the initial treatment). In the following experiment the treatments were either applied twice, with increasing amounts of time between treatment (first treatment at time 0, then at 6, 16, 24, 48 hrs after initial treatment) or only once, in parallel to the second treatment (only at 6, 16, 24 and 48 hrs-naïve responses). Treatments were either applied for 15 min only (acute treatment), or for the duration of the experiment (chronic treatment when CNP was left in the

medium). Cell lysates and conditioned medium were collected and analyzed for total cGMP secretion (Molecular Devices ELISA).

In a short term experiment, cells were stimulated with Gly-wtCNP37 ("CNP38") 1 uM two times for 15 minutes (acute treatment), or two times with CNP38 throughout culture (chronic treatment). The periods between treatments were 15 min, 30 min, 60 min, 2 hrs, 3 hrs, 4 hrs, and 6 hrs. Peak cGMP stimulation was obtained by treating cells once only, at the last time point of the experiment, (6 hours; > 0.1 pmol/well in the acute experiment and 0.2 pmol/well in the chronic experiment). In the acute experiment, when the cells are treated twice, the response decreases to 0.1 pmol/well if the cells are treated at time 0 and then again at 15 minutes. In the acute experiment, when the cells are treated twice, the response decreases to approximately 0.5 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, 2, 3, and 4 hours. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.16 pmol/well if the cells are treated at time 0 and at 15 minutes. In the chronic experiment, when the cells are treated twice, the response decreases to approximately 0.6 pmol/well if the cells are treated at time 0 and again at 30 min, 60 min, or 2 hours. In the chronic experiment, when the cells are treated twice, the response decreases to < 0.5 pmol/well if the cells are treated at time 0 and again at 2, 4 or 6 hours.

Long term studies were also carried out. For acute treatment, 1 uM CNP38 was added to cell culture as described above. For chronic treatment, 1 uM CNP38 was added to cell culture throughout the experiment duration as described above. Results indicate that the NPR-B receptor can desensitize after repeated, daily CNP administration *in vitro*, and 48 hours between doses is sufficient to recover to 60% of the maximial NPR-B response to CNP38, if the CNP is removed after the first dose, and to <40% if the CNP is incubated throughout the experiment (**Figure 74**).

To evaluate whether treatment with a CNP variant desensitizes the NPR-B receptor *in vivo*, experiments were conducted with wild-type mice. CD-1 male mice 8-10 weeks of age were injected subcutaneously with vehicle control or Pro-Gly-wtCNP37 ("Pro-CNP38") at 200 nmol/kg on the appropriate days. The mice were either injected with Pro-CNP38 daily for up to 8 days, or were injected with Pro-CNP38 on the first day, the first and second days, or the first and third days of the study. Fifteen minutes following the final injection, the mice (n = 3 per treatment group) were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was flushed from the body with PBS via an aortic

cannula. The kidneys and right tibias, right femurs and left femurs were collected, boiled in water for 5 minutes and/or snap frozen in liquid nitrogen and stored on dry ice or in a -70 °C freezer until cGMP assay. For estimation of cGMP production in cartilage, distal femurs and/or proximal tibias were dissected, weighed and pulverized using Covaris Cryoprep CP02. Powdered samples were homogenized using a Covaris E210 sonicator in 5% perchloric acid, and neutralized in 60% KOH. Samples were then centrifuged at 10,000 rpm at 4 °C for 5 minutes, and the supernatant was used for the cGMP assay (Cyclic GMP Enzyme Immunoassay Kit, Cayman, Michigan). Additionally, left tibias were collected, fixed in 10% normal buffered formalin, and saved for further immunohistochemical analysis.

Figure 75A shows that repeated daily treatment of wild-type mice with 200 nmol/kg of Pro-CNP38 for 1, 4, 6, 7 and 8 days did not result in desensitization of the cGMP response. To the contrary, potentiation of the cGMP response was observed after 4 days of daily treatment. Further daily treatment resulted in a plateau of the cGMP reponse, up to 8 days. The results indicate that daily treatment of wild-type mice with 200 nmol/kg of Pro-CNP38 for up to 8 days do not desensitize the cGMP response.

The kinetics of the cGMP response in distal femoral cartilage after treatment of the wild-type mice with Pro-CNP38 was also investigated. Treatment of the mice once a day for two days potentiated the cGMP response to Pro-CNP38, compared with the cGMP response to a single treatment (**Figure 75B**). When the mice were treated on the first and third days, but not on the first and second days, the cGMP response after the second treatment (on the third day) was substantially similar to the cGMP response observed after a single treatment on day one (Figure 75B). The results suggest that dosing on consecutive days is beneficial for potentiating the cGMP response to Pro-CNP38 in this mouse study.

Activation of NPR-B in different tissues

To evaluate potential activation of NPR-B in different tissues by a CNP variant, wild-type male CD-1 mice were injected with 200 nmol/kg of Gly-CNP37, and the cGMP response was measured in certain tissues at different time points. Two mice were used for each treatment group. Each mouse received one subcutaneous injection of Gly-CNP37 or vehicle control. Fifteen, 30 or 60 minutes or 3 hours following the injection, the mice were deeply anesthetized and ex-sanguinated via thoracotomy and aortic cannulation. Circulating blood was removed by a PBS flush with an aortic cannula. Heart, liver, lung, kidney, ear pinna, aorta and brain were collected. All tissues were boiled in water for 5 minutes, finely

dissected (including flushing of marrow from femoral cortices with PBS), weighed, cooled in liquid nitrogen, and pulverized in a BioPulverizer. The resulting powdered samples were homogenized with a Polytron in 6% pre-chilled perchloric acid and neutralized with 60% KOH. Samples were then centrifuged at 10,000 rpm and 4 °C for 5 minutes, and the supernatant was used for the assay of cGMP (Cyclic GMP Enzyme Immunoassay Kit, Cayman Chemical Company, Ann Arbor, Michigan). The results were normalized for tissue weight.

Secretion of cGMP in response to treatment with Gly-CNP37 ("CNP" in Figure 76) was detectable in distal femurs (cartilage and bone), femoral cortices (bone), ear pinna (cartilage), and kidney (**Figures 76A-D**). Maximal cGMP responses in those tissues were observed 15 min after treatment. Liver, heart, lung and brain tissues did not exhibit appreciable cGMP secretion in response to Gly-CNP37 relative to vehicle control at the studied time points (**Figures 76E-H**). The results indicate that treatment with 200 nmol/kg of Gly-CNP37 stimulated cGMP secretion in cartilage, bone and renal tissues.

Example 16

Dose Responses of CNP Variants in Monkeys

The effects of the CNP variant Pro-Gly-CNP37 on bone growth and the levels of bone growth-related biomarkers are evaluated in cynomolgus monkeys. Eight normal juvenile cynomolgus monkeys (about 2.5 years of age at the start of the on-going study) are subcutaneously injected daily with 10 or 36 μg/kg/day of Pro-Gly-CNP37 (n = 4 per dose group). Four such monkeys are administered vehicle as control. The total length of treatment is 6 months. Various measures of growth plate expansion and bone growth are made by digital X-ray and magnetic resonance imaging, and by measurement of limb and body lengths externally. Blood and urine samples are collected periodically for clinical pathology and measurement of levels of Pro-Gly-CNP37 and biomarkers. Following termination of the study, gross pathology is performed and tissue samples are evaluated histologically for assessment of efficacy and safety.

Data obtained thus far in the on-going study show that both doses of Pro-Gly-CNP37 have increased growth plate width by digital X-ray (**Figure 77**), increased right and left tibia lengths by digital X-ray (**Figures 78A** and **B**), increased leg length by external measurement (**Figure 79**), increased arm length by external measurement (**Figure 80**), increased body length by external measurement (**Figure 81**), and increased the serum level of alkaline

phosphatase, a biomarker for bone formation (**Figure 82**). The data demonstrate that Pro-Gly-CNP37 can stimulate bone growth in normal juvenile cynomolgus monkeys at hemodynamically acceptable doses.

Example 17

Effects of CNP Variants on Cardiovascular System in Mice

Natriuretic peptides such as CNP have been reported to affect the cardiovascular system. Wang et al. (Eur J Heart Fail. 9:548-57. 2007) describe that CNP has been shown to have a cardioprotective effect in preventing myocardial ischaemia/reperfusion injury and improving cardiac remodelling after myocardial infarction in rats. Wang demonstrated that mice overexpressing CNP have reduced incidence of cardiac hypertrophy caused by myocardial infarction. Additionally, CNP has been shown to cause endothelium-independent vasodilation (M. Honing *et al.*, Hypertension, 37:1179–1183 (2001)) and therefore may transiently decrease blood pressure *in vivo*.

To assess the effects of CNP variants on the cardiovascular system, the blood pressure and heart rate in anesthetized wild-type FVB mice is studied following subcutaneous injection of the variants.

After a pilot study to define a broad dose range of cardiovascular activity, a dose-response study is conducted to examine the effects of three different dose levels of each CNP variant. Three male FVB mice aged 8 weeks comprise each treatment group. Doses are administered subcutaneously to anesthetized mice, and systolic, diastolic and mean arterial pressure (MAP), as well as heart rate, are monitored via implanted intraarterial pressure transducers.

Example 18

Formulation of CNP Variants

CNP preformulation studies were carried out to assess the stability of CNP variant Gly-wtCNP37 ("CNP38") at different pH's (pH 3, 4, 5, 6, 7 and 8) and temperatures (5 °C, 25 °C, and 40 °C) over time. CNP38 exhibited greater stability at pH 4-6 than at the other pH's in the studies. CNP38 was stable at 5 °C at pH 4-6, with ≥ about 95% of CNP38 remaining after 15 weeks. When the temperature was raised to 25 °C, at pH 4 about 85% of CNP38 remained after 15 weeks, at pH 5 about 85% remained after 15 weeks, and at pH 6

about 80% remained after 15 weeks. When the temperature was raised to 40 °C, at pH 4 about 55-60% of CNP38 remained after 15 weeks, at pH 5 about 65% remained after 15 weeks, and at pH 6 about 40% remained after 15 weeks. **Figure 83** illustrates the observed plot of pseudo-first order degradation rate constant (K_{obs}) vs. pH from pH 3 to 8 and at 5 °C, 25 °C and 40 °C. The stability data for CNP38 in the preformulation studies suggests CNP formulations having a pH in the range from about 4 to about 6. An acidic pH (e.g., pH ≤ about 6) can promote the stability of a CNP variant by, e.g., minimizing or avoiding deamidation of asparagine and/or glutamine residue(s), isomerization of aspartic acid residue(s), or degradation of the CNP variant by other pathways.

CNP variants can be formulated in pharmaceutical carriers for administration to subjects affected by, e.g., bone growth conditions. In some embodiments, liquid formulations of CNP variants are formulated according to any combinations of the ingredients and their amounts or concentrations in Table 16.

Table 16

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent	NaCl	140 mM ± 20 mM
Isotonicity-adjusting agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.1% or 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5% - 100% (neat)[1]
Stabilizer	Methionine	0.01% - 0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1% - 1%
Stabilizer	Thioglycerol	0.1% - 1%
Anti-adsorbent	Polysorbate 20 Polysorbate 80 Benzyl alcohol	0.001% - 0.5% 0.001% - 0.5% 0.5% - 1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants. For lyophilized formulations, 4-6% or 6-20% mannitol or sucrose can be substituted for NaCl.

In certain embodiments, lyophilized formulations of CNP variants are prepared from formulations formulated according to any combinations of the ingredients and their amounts or concentrations in Table 17.

Table 17

Ingredient Class	Ingredient	Concentration Range
Active ingredient	CNP variant	10 mg/mL ± 9.9 mg/mL
Buffering agent	Acetic acid/acetate	10 mM ± 5 mM, or pH 5 ± 1
Buffering agent	Citric acid/citrate	10 mM ± 5 mM, or pH 5 ± 1
Isotonicity-adjusting agent/ bulking agent	Sorbitol	5% ± 3%
Isotonicity-adjusting agent/ bulking agent	Mannitol	5% ± 3%
Isotonicity-adjusting agent/ bulking agent	Sucrose	10% ± 5%
Preservative	m-Cresol	0.4% ± 0.2%
Preservative/anti-adsorbent	Benzyl alcohol	1.5% ± 0.5%
Stabilizer	Glycerin (glycerol)	5% - 100% (neat)[1]
Stabilizer	Methionine	0.01% - 0.2%
Stabilizer	Ascorbic acid/ ascorbate salt	0.1% - 1%
Stabilizer	Thioglycerol	0.1% - 1%
Anti-adsorbent	Polysorbate 20 Polysorbate 80 Benzyl alcohol	0.001% - 0.5% 0.001% - 0.5% 0.5% - 1.5%

[1]Glycerin is used to minimize or prevent water-driven hydrolysis, deamidation, isomerization or cleavage of CNP variants.

In certain embodiments, a formulation comprising a CNP variant has a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5. In some embodiments, for pH 4-5.5 a suitable buffering agent is acetic acid/acetate (e.g., sodium acetate), and for pH 5.5-6 a suitable buffering agent is citric acid/citrate. Citric acid/citrate

(e.g., sodium citrate) is also a suitable buffering agent in the range of pH 3-6 or pH 4-6. In certain embodiments, the buffering agent has a concentration in the formulation of about 2-50 mM, or about 2-40 mM, or about 2-30 mM, or about 5-30 mM, or about 2-20 mM, or about 5-20 mM, or about 5-15 mM.

To minimize or avoid deamidation of a CNP variant, the variant can be formulated in pharmaceutically acceptable organic cosolvents, such as glycerin, ethanol, and propylene glycol. Because deamidation occurs by hydrolysis, substitution of an organic cosolvent for water minimizes contact of the CNP variant with water. The concentration of one or more organic solvents in an organic-aqueous solvent system can be, e.g., from about 10% to about 99%, or about 100% if water is not used.

Also to minimize or avoid deamidation of a CNP variant, water can be removed from the formulation by lyophilization. In some embodiments, lyophilized formulations contain any combinations of the following components:

buffer: sodium acetate and acetic acid, or sodium citrate and citric acid;

isotonicity/bulking agent: mannitol (e.g., 3-10%, 2-8% or 4-6%);
sucrose (e.g., 6-20%, 5-15% or 8-12%);

antioxidants: methionine and/or ascorbic acid with molal ratio of each antioxidant to CNP variant from about 0.1:1 to about 1:1, or from about 0.5:1 to about 5:1, or from about 1:1 to about 15:1, or from about 1:1 to about 10:1, or from about 3:1 to about 10:1.

Deamidation can also be minimized or avoided by storing a CNP composition (e.g., a liquid formulation or a lyophilized formulation) at lower temperature, such as at about 5 °C, 0 °C, -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, or -100 °C.

To minimize or avoid oxidation of oxidizable residues (e.g., methionine) in a CNP variant, the variant can be formulated with one or more antioxidants. Exemplary antioxidants include, but are not limited to, methionine, ascorbic acid, and thioglycerol. Oxidation of, e.g., methionine residues can also be minimized or prevented by purging oxygen from a liquid medium (if a liquid formulation) with nitrogen or argon, and/or by purging oxygen from a container or packaging with nitrogen or argon.

In some embodiments, to minimize or prevent adsorption (e.g., adsorption of a CNP variant to plastic or glass), Polysorbate 20, Polysorbate 80 or benzyl alcohol, or a combination thereof, is added to a CNP formulation. In certain embodiments, each of the

anti-adsorbent(s) is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%. Exemplary range(s) of anti-adsorbent(s) in the formulation include without limitation from about 0.001% to about 0.5% of Polysorbate 20, from about 0.001% to about 0.5% of Polysorbate 80, and/or from about 0.5% to about 1.5% of benzyl alcohol.

In certain embodiments, a liquid CNP formulation comprises, or a lyophilized CNP formulation is prepared from a formulation that comprises, (1) an acetic acid/acetate (e.g., sodium acetate) buffer having a concentration of about 30 mM ± 5 or 10 mM buffering agent and a pH of about 4 ± 0.5 or 1, and (2) benzyl alcohol (e.g., as a preservative and/or anti-adsorbent) at a concentration of about 1% ± 0.5%, and optionally (3) sucrose at a concentration of about 10% ± 5%.

Example 19

Clinical Evaluation of CNP Variants

The following example provides guidance on the parameters to be used for the clinical evaluation of compositions comprising CNP22 or variants thereof in the therapeutic methods of the present disclosure. As discussed herein, CNP22 or variants thereof will be used in the treatment of disorders responsive to CNP, including disorders of the bone and vascular smooth muscle. Clinical trials will be conducted which will provide an assessment of doses of CNP22 or variants thereof for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 24 weeks to collect sufficient safety information on about 100 evaluable patients. The initial dose for the trials will vary from about 0.001 to about 1.0 mg/kg/week, or any of the doses described herein. In the event that the initial dose in this range does not produce a significant direct clinical benefit, the dose should be increased within this range or beyond this range as necessary, and maintained for an additional minimal period of, but not necessarily limited to, 24 weeks to establish safety and to evaluate efficacy further.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (including kidney and liver functions), urinalysis, and CBC with differential. In addition, other parameters relevant to clinical benefit will be monitored. The present example also includes the determination of pharmacokinetic parameters of CNP22 or

variants thereof, including absorption, distribution, metabolism, excretion, and half-life and bioavailability in the blood. It is anticipated that such analyses will help relate dose to clinical response.

Methods

Patients will undergo a baseline medical history and physical exam, and a standard set of clinical laboratory tests (including CBC, Panel 20, CH50, and UA). The patients will be followed closely with weekly visits to the clinic. The patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Diagnosis and Inclusion Criteria

The patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder. A specific example of a potentially CNP-responsive, bone-related disorder is achondroplasia, which may be confirmed by genetic testing and other evidence of an FGFR-3 mutation or dysfunction. The ideal age range of achondroplasia patients will be from infant (< 1 year of age) to pre-adolescent (< 13 years of age). A patient will be excluded from this study if the patient is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

Safety

Therapy with CNP22 or variants thereof will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

It has been shown that compared to wild-type CNP22, certain CNP variants of the disclosure are much more resistant to NEP degradation *in vitro*, have a much longer plasma half-life and bioavailability in rats, stimulate a much higher level of cGMP production in rats, and/or induce a significantly greater increase in long bone length and body length in achondroplastic mice. Furthermore, it has been shown that short duration dose regimen treatments with CNP22 are nearly as effective as continuous CNP22 treatment in reversing

FGF2-induced arrest of chondrocyte growth *in vitro*. These results, among others described herein, demonstrate the utility of CNP variants of the disclosure in treating CNP-responsive conditions or disorders such as, e.g., bone-related disorders and vascular smooth muscle disorders.

It is understood that every embodiment of the disclosure described herein may optionally be combined with any one or more of the other embodiments described herein. Every patent literature and every non-patent literature cited herein are incorporated herein by reference in their entirety.

Numerous modifications and variations to the disclosure, as set forth in the embodiments and illustrative examples described herein, are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the disclosure.

WHAT IS CLAIMED IS:

1. A variant of C-type natriuretic peptide (CNP) selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 152);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);

ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);

KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);

YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);

KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);

GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);

ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);

NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);

KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);

LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);

SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);

KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 177);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 145);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

2. A pharmaceutical composition comprising a CNP variant, and a pharmaceutically acceptable excipient, carrier or diluent.

3. The composition of claim 2, which is a lyophilized formulation.

4. The composition of claim 3, wherein the lyophilized formulation is prepared from a formulation that comprises a buffer having a pH from about 4 to about 6.

5. The composition of claim 4, wherein the buffer is a citric acid/citrate buffer or an acetic acid/acetate buffer.

6. The composition of claim 4 or 5, wherein the lyophilized formulation is prepared from a formulation that further comprises an isotonicity-adjusting agent and/or a bulking agent.

7. The composition of claim 6, wherein the isotonicity-adjusting agent and/or the bulking agent are selected from the group consisting of mannitol, sucrose, sorbitol, and combinations thereof.

8. The composition of any one of claims 4 to 7, wherein the lyophilized formulation is prepared from a formulation that further comprises an antioxidant.

9. The composition of claim 8, wherein the antioxidant is selected from the group consisting of methionine, ascorbic acid, salt forms of ascorbic acid, thioglycerol, and combinations thereof.

10. The composition of any one of claims 2 to 9, wherein the CNP variant is a CNP variant according to claim 1.

11. A CNP variant according to claim 1 or a pharmaceutical composition according to any one of claims 2 to 10 for use in the treatment of a bone-related disorder or skeletal dysplasia selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia.

12. The CNP variant or pharmaceutical composition of claim 11, wherein the bone-related disorder or skeletal dysplasia is achondroplasia.

13. A CNP variant according to claim 1 or a pharmaceutical composition according to any one of claims 2 to 10 for use in the treatment of a vascular smooth muscle disorder selected from the group consisting of hypertension, restenosis, arteriosclerosis, acute decompensated heart failure, congestive heart failure, cardiac edema, nephredema, hepatic edema, acute renal insufficiency, and chronic renal insufficiency.

14. A method for recombinant production of a CNP variant, comprising culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker.

15. The method of claim 14, wherein the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant polypeptide linked to the polynucleotide encoding the cleavable peptide or protein.

16. The method of claim 14 or 15, wherein expression of the polynucleotides encoding the fusion polypeptide is enhanced using an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible vector.

17. The method of claim 16, wherein the host cell is cultured for a time period at a temperature from about 20 °C to about 40 °C in the presence of about 0.4 mM to about 1.5 mM IPTG.

18. The method of any one of claims 15 to 17, wherein the vector is a plasmid.

19. The method of claim 18, wherein the plasmid is selected from the group consisting of pJexpress, pJexpress401, pJexpress404, pET-15b, pET-21a, pET-22b, pET-31b, pET-32a, pET-41a, pMAL, pMAL-c2X, pQE-30, pET-SUMO, and pTYB11.

20. The method of any one of claims 14 to 19, wherein the cleavable peptide or protein is selected from the group consisting of histidine tags, human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E), ketosteroid isomerase, maltose-binding protein, ß-galactosidase, glutathione-S-transferase, thioredoxin, chitin-binding domain, BMP-2, BMP-2 mutants, BMP-2(C/A), and mutants and fragments thereof.

21. The method of claim 20, wherein the cleavable peptide or protein is selected from the group consisting of human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), and TAF12(C/A & 10D/10E).

22. The method of any one of claims 14 to 21, wherein the cleavable peptide or protein is cleaved by a cleaving agent.

23. The method of claim 22, wherein the cleaving agent is selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.

24. The method of any one of claims 14 to 23, wherein the host cell is bacterial.

25. The method of claim 24, wherein the bacterial host cell is *E. coli*.

26. The method of claim 25, wherein the *E. coli* cell is selected from the group consisting of BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41(DE3), C43(DE3), Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3).

27. The method of claim 26, wherein the *E. coli* cell is BL21(DE3).

28. The method of any one of claims 14 to 27, wherein the fusion polypeptide is expressed as a soluble protein or as an inclusion body.

29. The method of any one of claims 14 to 28, further comprising isolating the expressed fusion polypeptide from the host cell or culture medium.

30. The method of claim 29, further comprising contacting the isolated fusion polypeptide with a cleaving agent selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.

31. The method of claim 30, wherein the cleaving agent is formic acid.

32. The method of claim 30 or 31, wherein the contacting the isolated fusion polypeptide with a cleaving agent is conducted in the presence of about 1% to about 10% formic acid at a temperature from about 50 °C to about 70 °C.

33. The method of claim 32, wherein the contacting in the presence of formic acid is conducted for a time period from about 5 hours to about 36 hours.

34. The method of any one of claims 14 to 33, which produces a CNP variant selected from the group consisting of:
R-CNP22(K4R) (Analog AZ) (SEQ ID NO: 41);
ER-CNP22(K4R) (Analog BA) (SEQ ID NO: 39);
GANPR-CNP22(K4R) (Analog CI) (SEQ ID NO: 37);
GANQQ-CNP22(K4R) (Analog CH) (SEQ ID NO: 69);
AAWARLLQEHPNA-CNP22 (Analog CA) (SEQ ID NO: 61);
AAWARLLQEHPNAR-CNP22 (Analog CB)(SEQ ID NO: 62);
DLRVDTKSRAAWAR-CNP22 (Analog CC) (SEQ ID NO: 63);

GQPREPQVYTLPPS-CNP22 (Analog CF) (SEQ ID NO: 79);

GERAFKAWAVARLSQ-CNP22 (Analog CE) (SEQ ID NO: 81);

GHHSHEQHPHGANQQ-CNP22 (Analog CQ) (SEQ ID NO: 76);

GQEHPNARKYKGANPK-CNP22 (Analog CS) (SEQ ID NO: 71);

GQEHPNARKYKGANQK-CNP22 (Analog CT) (SEQ ID NO: 130);

GAHHPHEHDTHGANQQ-CNP22 (Analog CR) (SEQ ID NO: 77);

GQTHSSGTQSGANQQ-CNP22(K4R) (Analog CN) (SEQ ID NO: 87);

SPKMVQGSG-CNP17-KVLRRH (Analog CD) (SEQ ID NO: 68);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 145);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188); and

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193).

35. The method of any one of claims 14 to 34, which does not produce CNP-22 or CNP-53.

36. A CNP variant produced according to the method of any one of claims 14 to 33, wherein the CNP variant is selected from the group consisting of:

R-CNP22(K4R) (Analog AZ) (SEQ ID NO: 41);

ER-CNP22(K4R) (Analog BA) (SEQ ID NO: 39);

GANPR-CNP22(K4R) (Analog CI) (SEQ ID NO: 37);

GANQQ-CNP22(K4R) (Analog CH) (SEQ ID NO: 69);

AAWARLLQEHPNA-CNP22 (Analog CA) (SEQ ID NO: 61);

AAWARLLQEHPNAR-CNP22 (Analog CB)(SEQ ID NO: 62);

DLRVDTKSRAAWAR-CNP22 (Analog CC) (SEQ ID NO: 63);

GQPREPQVYTLPPS-CNP22 (Analog CF) (SEQ ID NO: 79);

GERAFKAWAVARLSQ-CNP22 (Analog CE) (SEQ ID NO: 81);

GHHSHEQHPHGANQQ-CNP22 (Analog CQ) (SEQ ID NO: 76);

GQEHPNARKYKGANPK-CNP22 (Analog CS) (SEQ ID NO: 71);

GQEHPNARKYKGANQK-CNP22 (Analog CT) (SEQ ID NO: 130);

GAHHPHEHDTHGANQQ-CNP22 (Analog CR) (SEQ ID NO: 77);

GQTHSSGTQSGANQQ-CNP22(K4R) (Analog CN) (SEQ ID NO: 87);

SPKMVQGSG-CNP17-KVLRRH (Analog CD) (SEQ ID NO: 68);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)];

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)];

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)];

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 182);

PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);

MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 192);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 145);

MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188); and

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193).

37. A host cell comprising an expression vector, the vector comprising a first polynucleotide encoding a CNP variant polypeptide linked to a second polynucleotide encoding a cleavable peptide or protein.

38. The host cell of claim 37, wherein the cleavable peptide or protein is selected from the group consisting of histidine tags, human transcription factor TAF12, TAF12 fragments, TAF12 histone fold domain, mutants of TAF12 and fragments thereof, TAF12(C/A), TAF12(D/E), TAF12(4D/4E), TAF12(6D/6E), TAF12(10D/10E), TAF12(C/A & D/E), TAF12(C/A & 4D/4E), TAF12(C/A & 6D/6E), TAF12(C/A & 10D/10E), ketosteroid isomerase, maltose-binding protein, ß-galactosidase, glutathione-S-transferase, thioredoxin, chitin-binding domain, BMP-2, BMP-2 mutants, BMP-2(C/A), and mutants and fragments thereof.

39. The host cell of claim 37 or 38, wherein the host cell is bacterial.

40. The host cell of claim 39, wherein the bacterial host cell is *E. coli*.

41. The host cell of claim 40, wherein the *E. coli* cell is selected from the group consisting of BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pGro7, ArcticExpress(DE3), C41(DE3), C43(DE3), Origami B(DE3), Origami B(DE3)pLysS, KRX, and Tuner(DE3).

42. The host cell of claim 41, wherein the *E. coli* cell is BL21(DE3).

43. The host cell of any one of claims 37 to 42, wherein the vector is a plasmid.

44. The host cell of claim 43, wherein the plasmid is selected from the group consisting of pJexpress, pJexpress401, pJexpress404, pET-15b, pET-21a, pET-22b, pET-31b, pET-32a, pET-41a, pMAL, pMAL-c2X, pQE-30, pET-SUMO, and pTYB11.

45. The host cell of any one of claims 37 to 44, wherein the host cell is transformed with the vector prior to cell culture.

46. The host cell of any one of claims 37 to 45, wherein the host cell is cultured in a medium under conditions suitable for expression of a fusion polypeptide encoded by the polynucleotides, wherein the fusion polypeptide comprises the CNP variant polypeptide directly linked to the cleavable peptide or protein or indirectly linked thereto via a linker.

47. The host cell of claim 46, wherein the fusion polypeptide is expressed as a soluble protein or as an inclusion body.

48. The host cell of claim 46 or 47, wherein the expressed fusion polypeptide is isolated from the host cell or culture medium.

49. The host cell of claim 48, wherein the isolated fusion polypeptide is contacted with a cleaving agent selected from the group consisting of formic acid, cyanogen bromide (CNBr), hydroxylamine, protein self cleavage, Factor Xa, enterokinase, ProTEV, and SUMO protease.

50. The host cell of any one of claims 37 to 49, wherein the CNP variant polypeptide is not CNP-22 or CNP-53.

51. A method of assessing the effect of a CNP peptide or variant on the level of at least one bone- or cartilage-associated biomarker in a subject, comprising assaying the level of at least one bone- or cartilage-associated biomarker in a biological sample from a subject that has been administered a CNP peptide or variant.

52. The method of claim 51, further comprising administering the CNP peptide or variant to the subject before assaying the level of the at least one bone- or cartilage-associated biomarker.

53. A method of treating a CNP-responsive condition or disorder, comprising

administering a CNP peptide or variant to a subject, and

monitoring the level of at least one bone- or cartilage-associated biomarker in the subject,

wherein an increase in the level of the at least one bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject or the condition or disorder.

54. The method of claim 53, further comprising adjusting the amount or frequency of administration of the CNP peptide or variant, wherein

i) the amount or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a target level; or

ii) the amount or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level.

55. The method of any one of claims 51 to 54, wherein the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

56. The method of any one of claims 51 to 55, wherein the CNP peptide or variant is selected from the group consisting of:
R-CNP22(K4R) (Analog AZ) (SEQ ID NO: 41);
AAWARLLQEHPNA-CNP22 (Analog CA) (SEQ ID NO: 61);
AAWARLLQEHPNAR-CNP22 (Analog CB) (SEQ ID NO: 62);
DLRVDTKSRAAWAR-CNP22 (Analog CC) (SEQ ID NO: 63);
GQPREPQVYTLPPS-CNP22 (Analog CF) (SEQ ID NO: 79);
GERAFKAWAVARLSQ-CNP22 (Analog CE) (SEQ ID NO: 81);
GHHSHEQHPHGANQQ-CNP22 (Analog CQ) (SEQ ID NO: 76);
GQEHPNARKYKGANPK-CNP22 (Analog CS) (SEQ ID NO: 71);
GQEHPNARKYKGANQK-CNP22 (Analog CT) (SEQ ID NO: 130);
GAHHPHEHDTHGANQQ-CNP22 (Analog CR) (SEQ ID NO: 77);
GQTHSSGTQSGANQQ-CNP22(K4R) (Analog CN) (SEQ ID NO: 87);
SPKMVQGSG-CNP17-KVLRRH (Analog CD) (SEQ ID NO: 68);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 179);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 185);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 190);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 180);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-53) (SEQ ID NO: 4);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 146);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 147);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 148);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 149);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 150);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 151);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 153);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 153);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 154);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 155);

WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 156);

ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 157);

RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 158);

LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 159);

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 160);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 60);

EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 161);

HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 162);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 163);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 164);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 165);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 166);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 167);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 168);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 169);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 170);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 171);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 172);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 173);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 174);
GLSKGCFGLKLDRIGSMSGLGC (CNP-22) (SEQ ID NO: 1);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 175);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 176);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 177);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 178);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N)] (SEQ ID NO: 181);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 186);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 191);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 75);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 181);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 145);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 191);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 144);

GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 183);

PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 188);

MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 193);

GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 36);

GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEG1K-CNP22 (SEQ ID NO: 1);

PEO12-CNP22 (SEQ ID NO: 1);

PEO24-CNP22 (SEQ ID NO: 1);

PEO12-R-CNP22(K4R) (SEQ ID NO: 41);

PEO24-R-CNP22(K4R) (SEQ ID NO: 41);

PEO12-ER-CNP22(K4R) (SEQ ID NO: 39);

PEO24-ER-CNP22(K4R) (SEQ ID NO: 39);

PEO12-GANPR-CNP22(K4R) (SEQ ID NO: 37);

PEO24-GANPR-CNP22(K4R) (SEQ ID NO: 37);

PEO12-GANQQ-CNP22(K4R) (SEQ ID NO: 69);

PEO24-GANQQ-CNP22(K4R) (SEQ ID NO: 69);

PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 36);

PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189);

PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194);

PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 36);

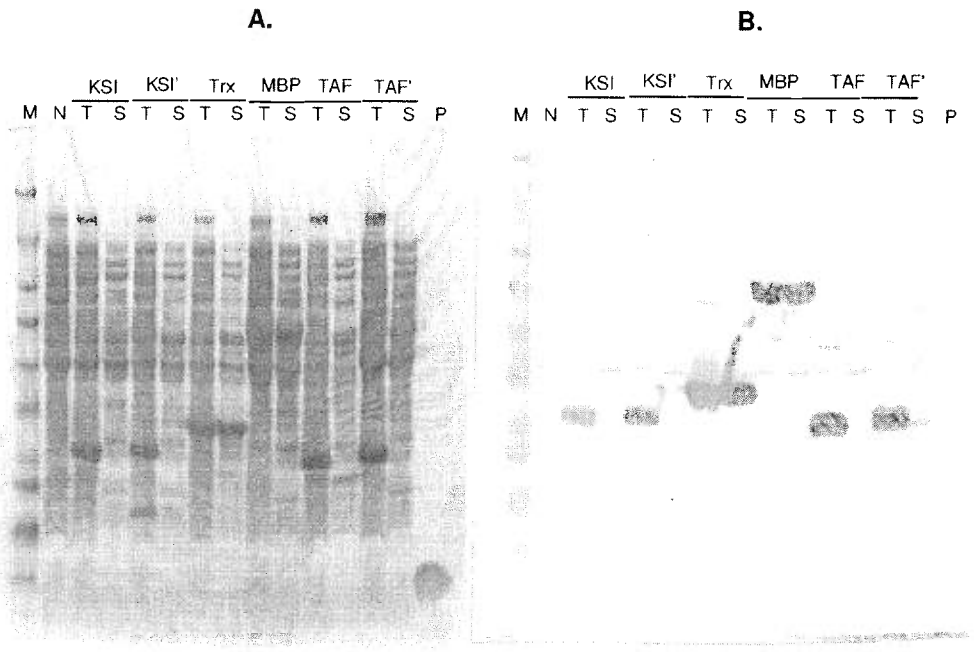
PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 184);

PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 189); and

PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 194).

57. The method of claim 56, wherein the CNP peptide or variant is not CNP-22 or CNP-53.

FIGURE 1



Coomassie blue stain

Western blot with anti-CNP antibody

FIGURE 2

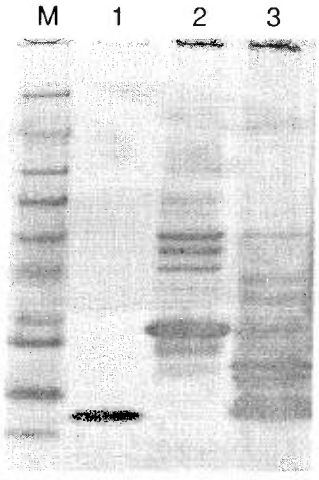


FIGURE 3

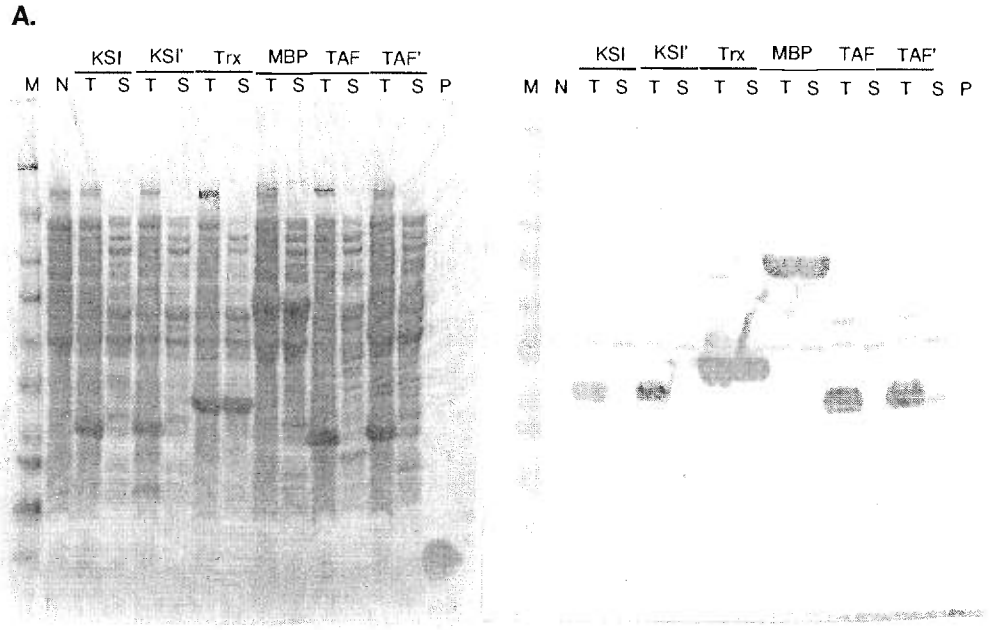

A.
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
KSI KSI' Trx MBP TAF TAF'
M N T S T S T S T S T S T S P
Coomassie blue stain
Western blot with anti-CNP antibody
B.
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
BMP-Pro-CNP37 TAF-Pro-CNP37
M C Tu T S P Tu T S P
Coomassie blue stain
Western blot with anti-CNP antibody

FIGURE 3 continued

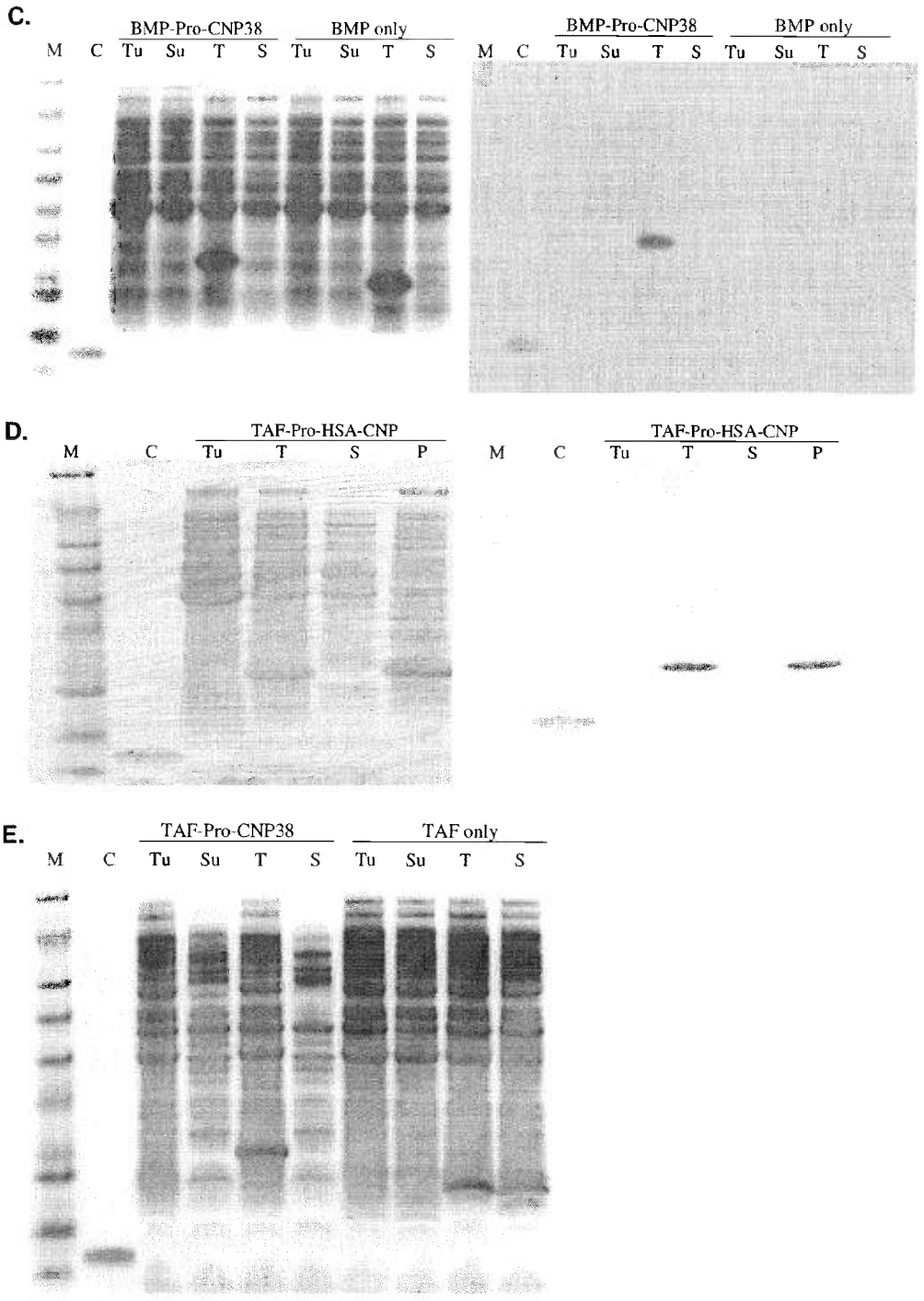


FIGURE 4

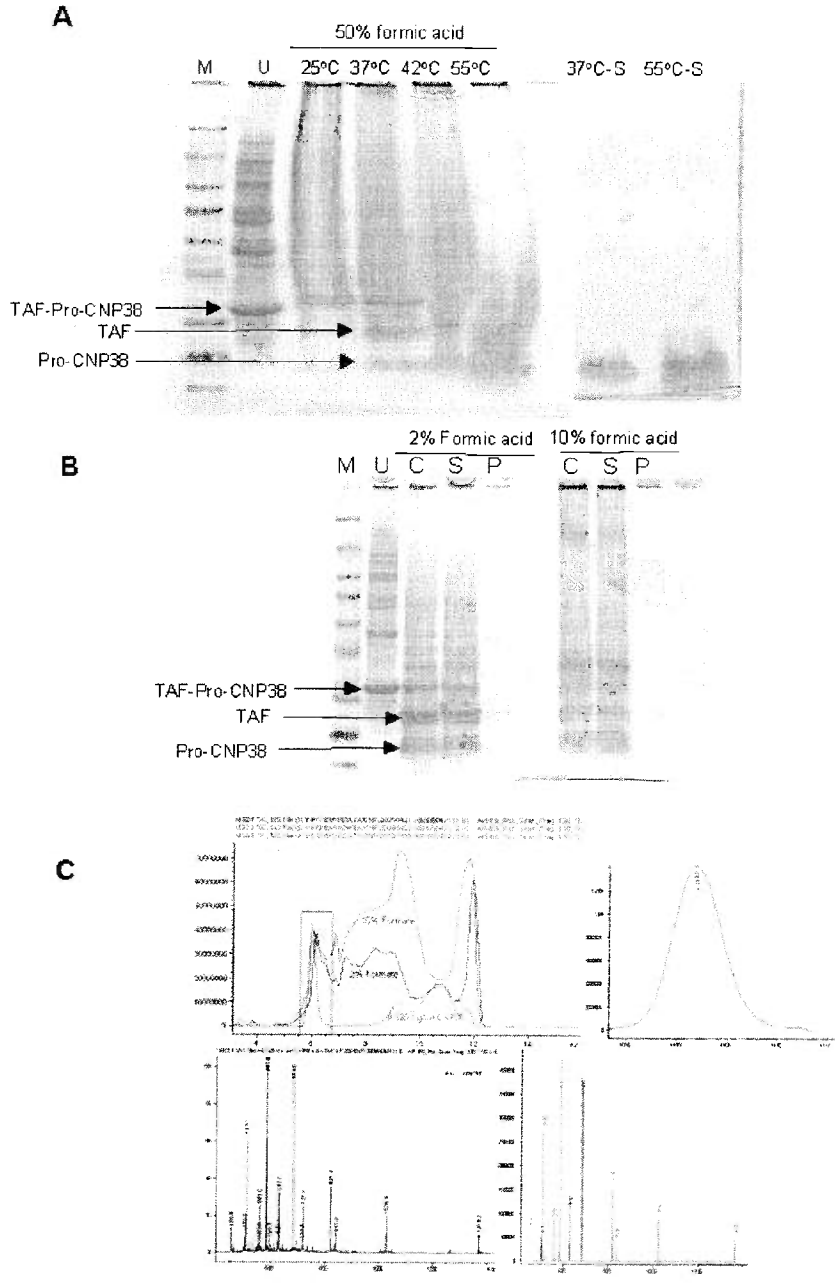

A
50% formic acid
M
U
25°C
37°C
42°C
55°C
37°C-S
55°C-S
TAF-Pro-CNP38
TAF
Pro-CNP38
B
2% Formic acid
10% formic acid
M
U
C
S
P
C
S
P
TAF-Pro-CNP38
TAF
Pro-CNP38
C

FIGURE 5

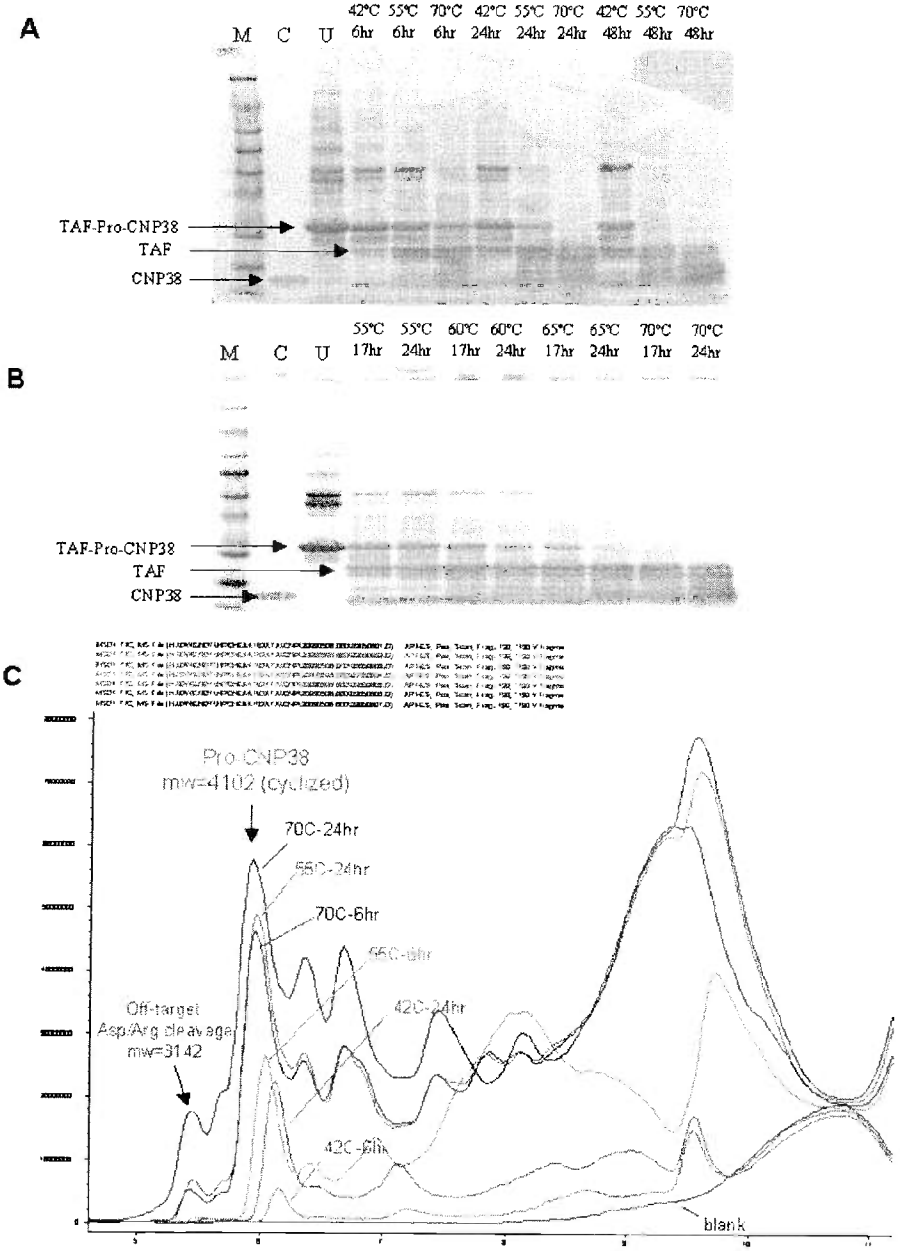

A
M C U
42°C 6hr
55°C 6hr
70°C 6hr
42°C 24hr
55°C 24hr
70°C 24hr
42°C 48hr
55°C 48hr
70°C 48hr
TAF-Pro-CNP38
TAF
CNP38
B
M C U
55°C 17hr
55°C 24hr
60°C 17hr
60°C 24hr
65°C 17hr
65°C 24hr
70°C 17hr
70°C 24hr
TAF-Pro-CNP38
TAF
CNP38
C
Pro-CNP38
mw=4102 (cyclized)
70C-24hr
55C-24hr
70C-6hr
55C-6hr
42C-24hr
Off-target
Asp/Arg cleavage
mw=3142
42C-6hr
blank

FIGURE 6

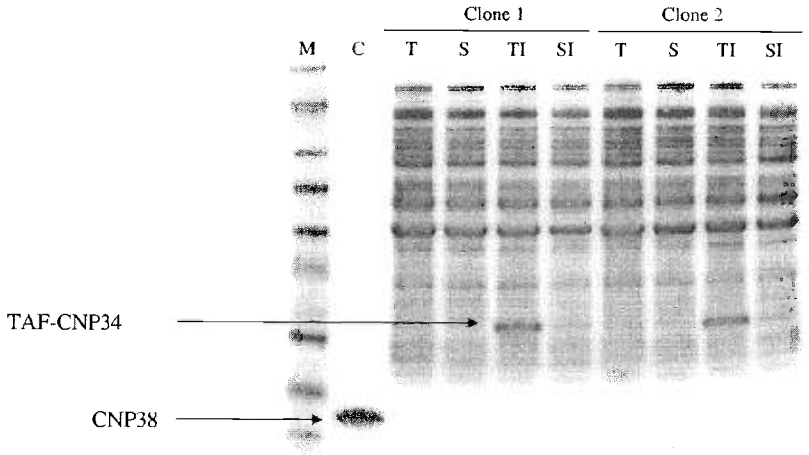


FIGURE 7

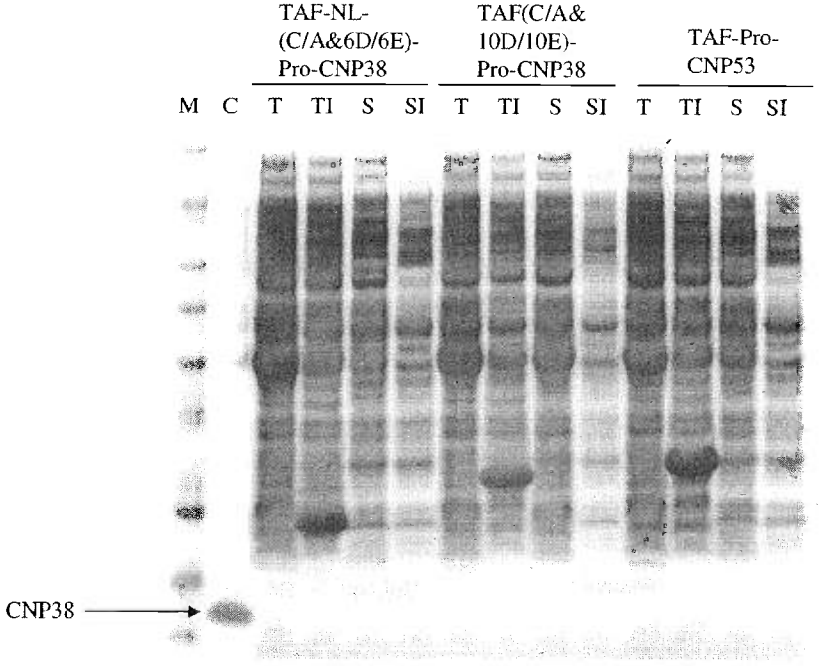


FIGURE 8

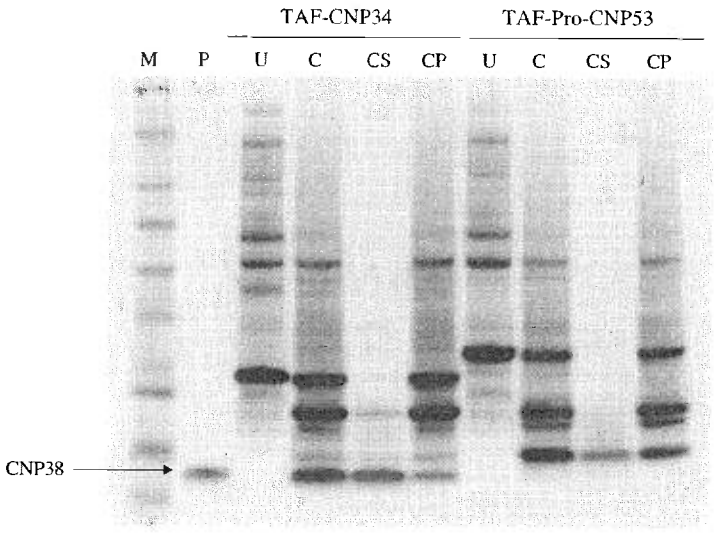


FIGURE 9

Calc. CNP34 monoisotopic mass = 3553.88

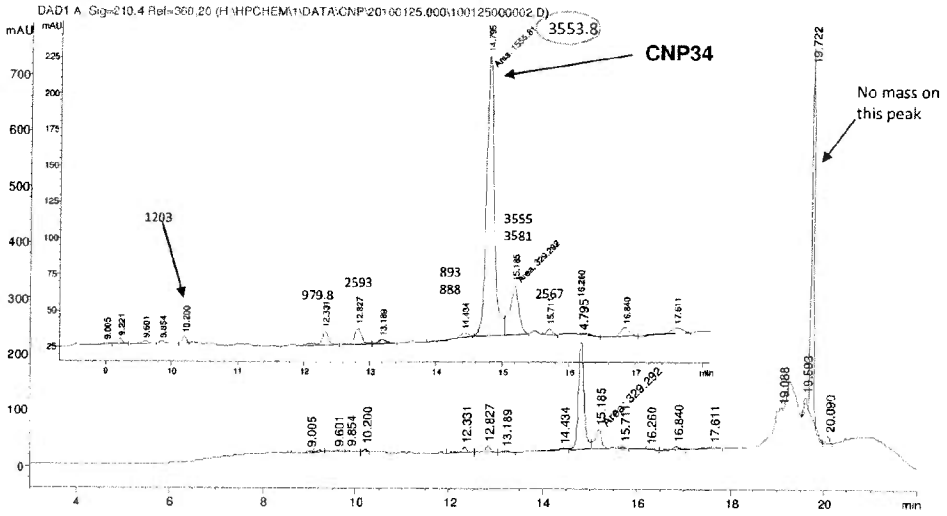


FIGURE 10

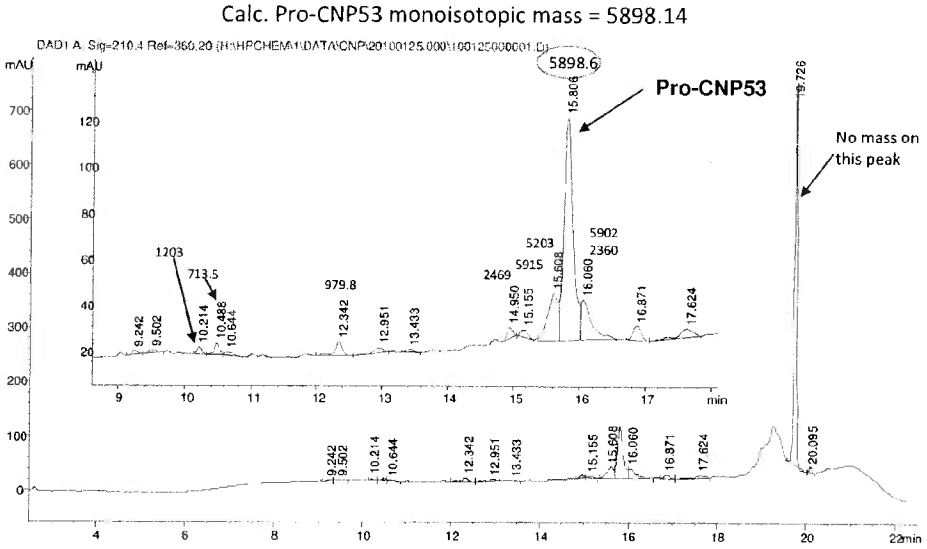


FIGURE 11

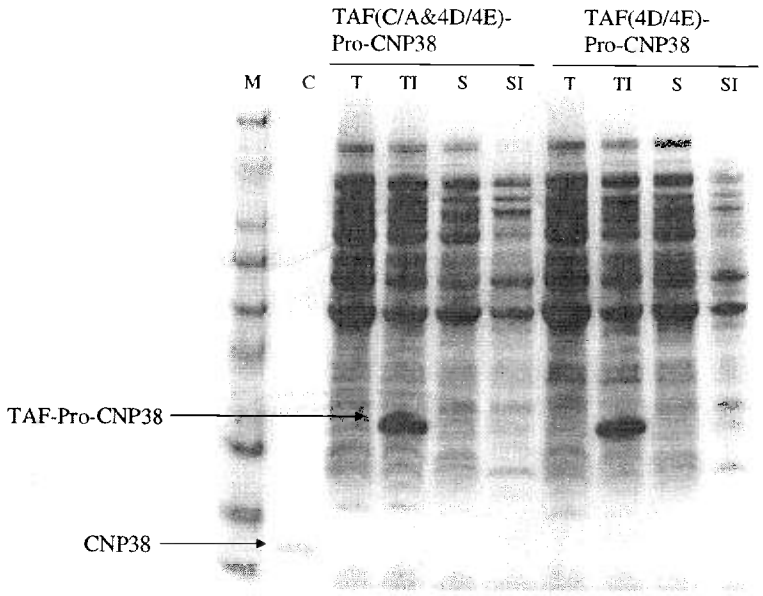


FIGURE 12

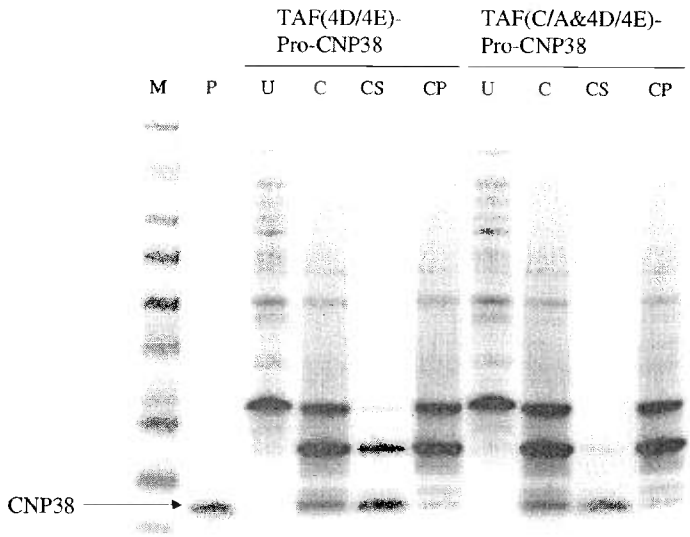


FIGURE 13

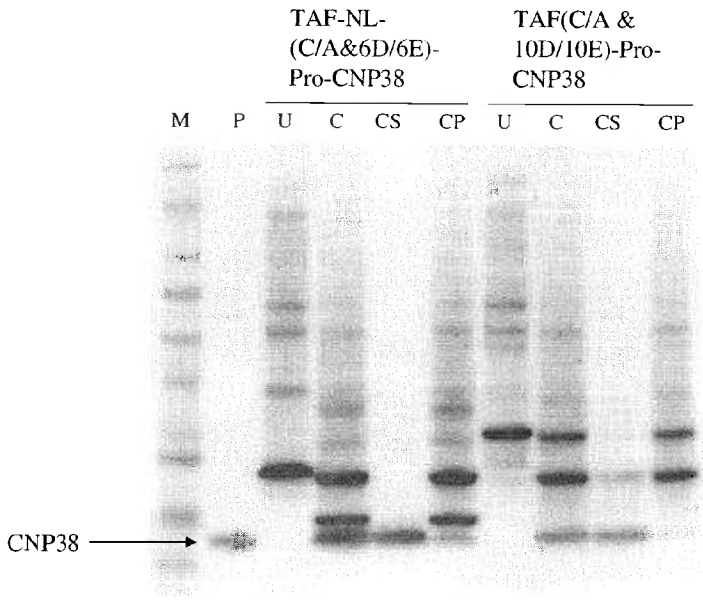


FIGURE 14

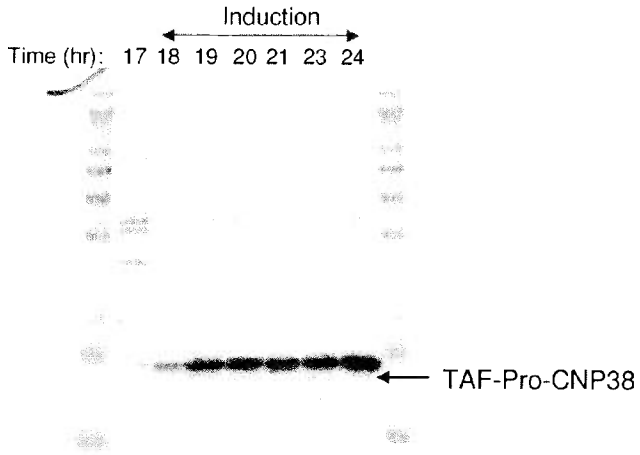


FIGURE 15

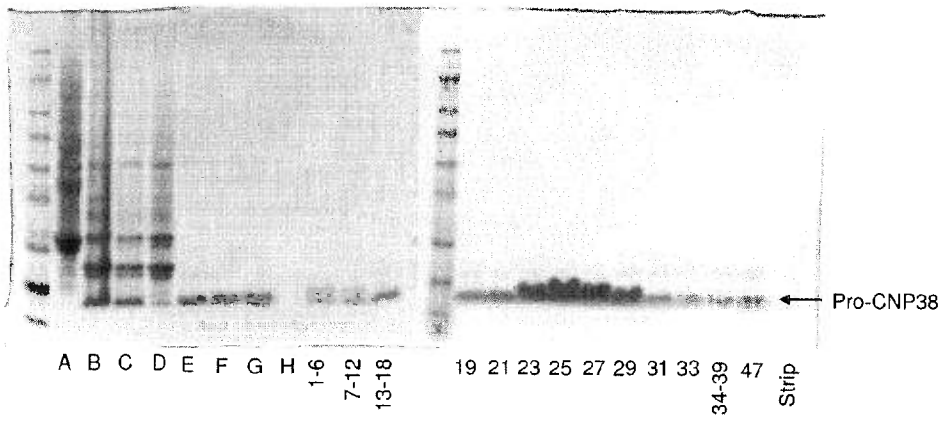


FIGURE 16

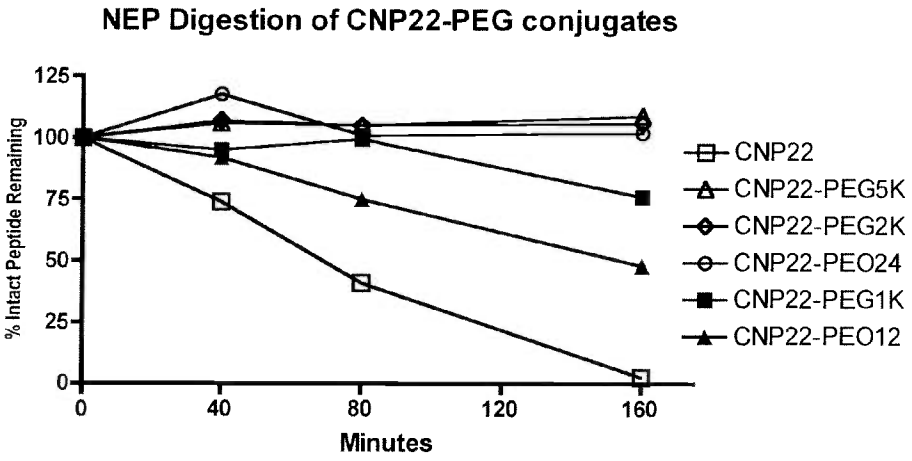


FIGURE 17

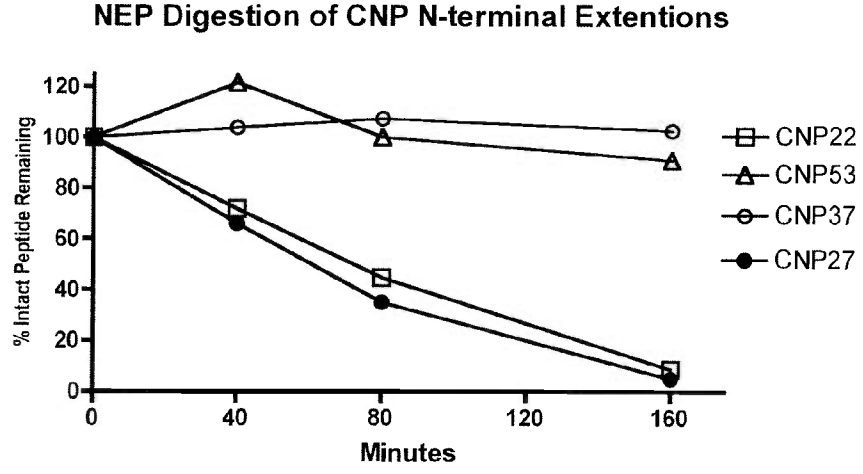


FIGURE 18

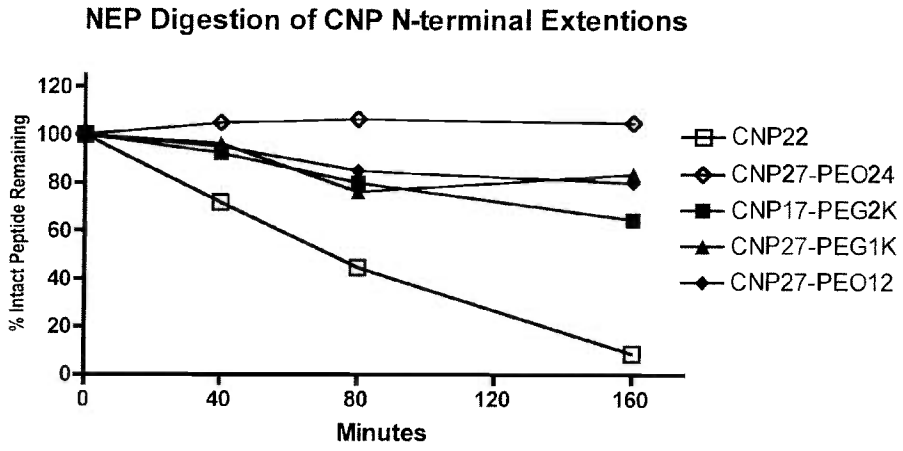


FIGURE 19

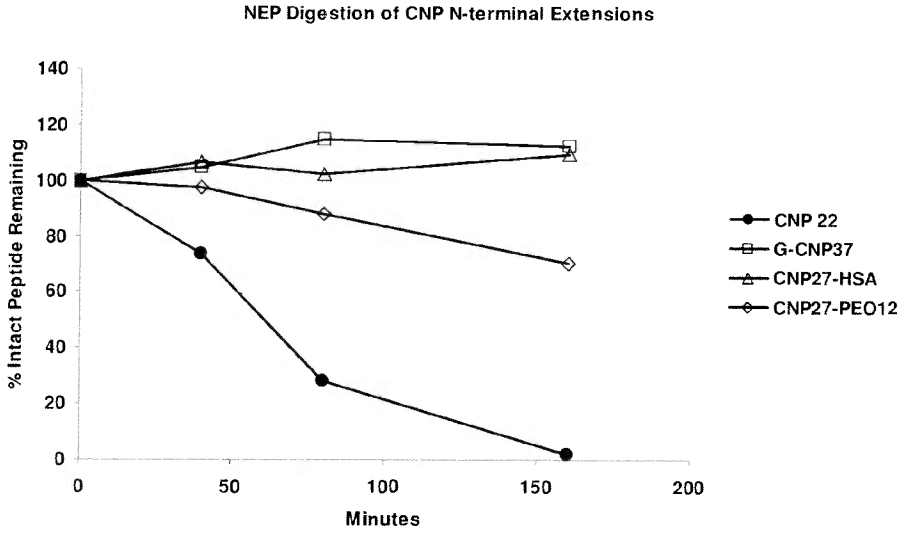


FIGURE 20

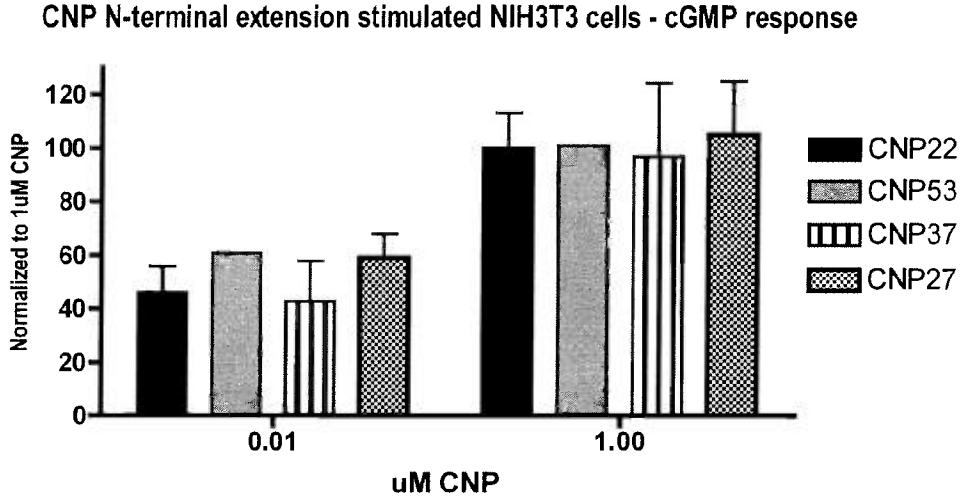


FIGURE 21

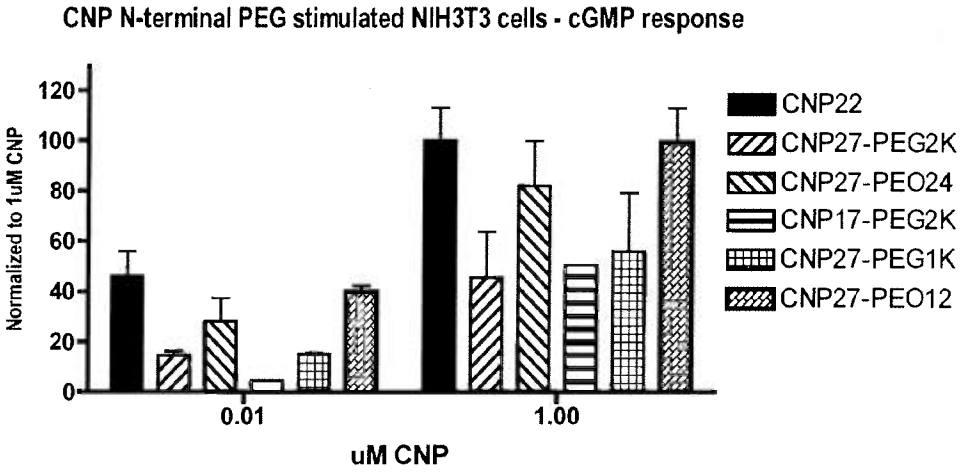


FIGURE 22

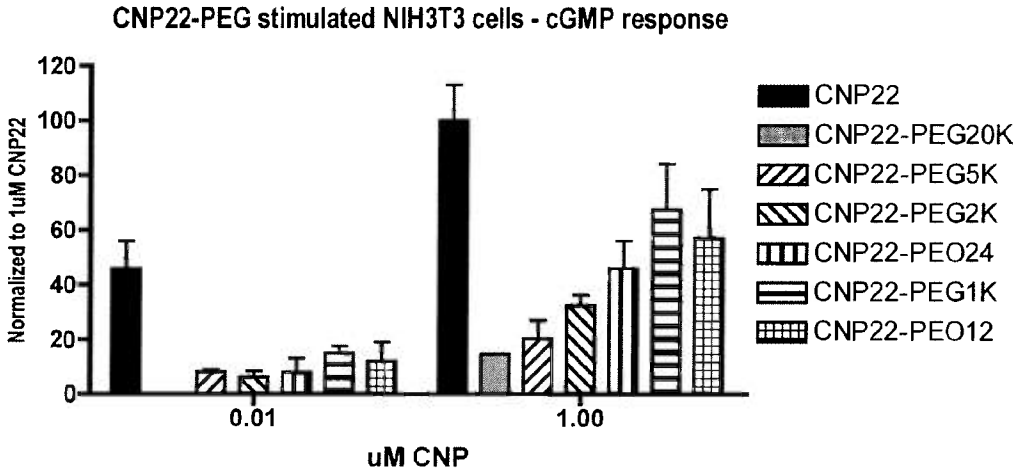


FIGURE 23

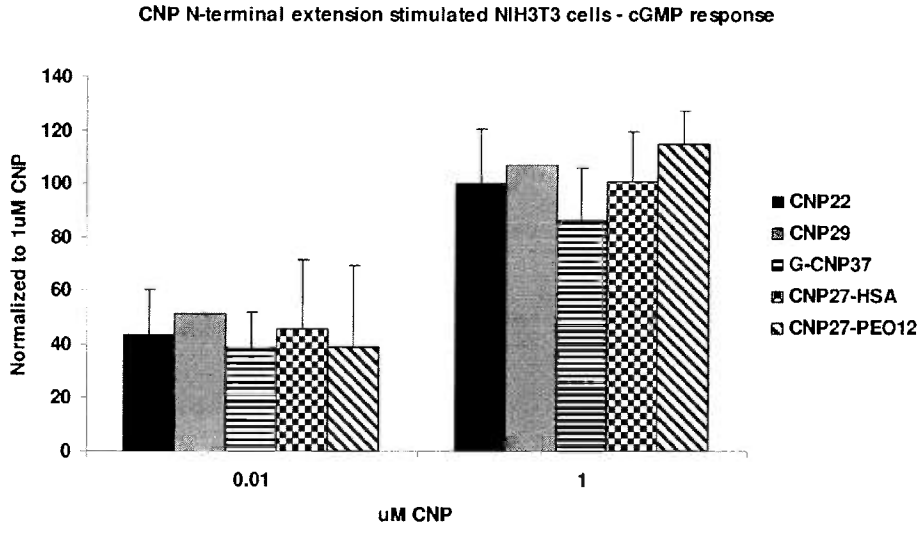
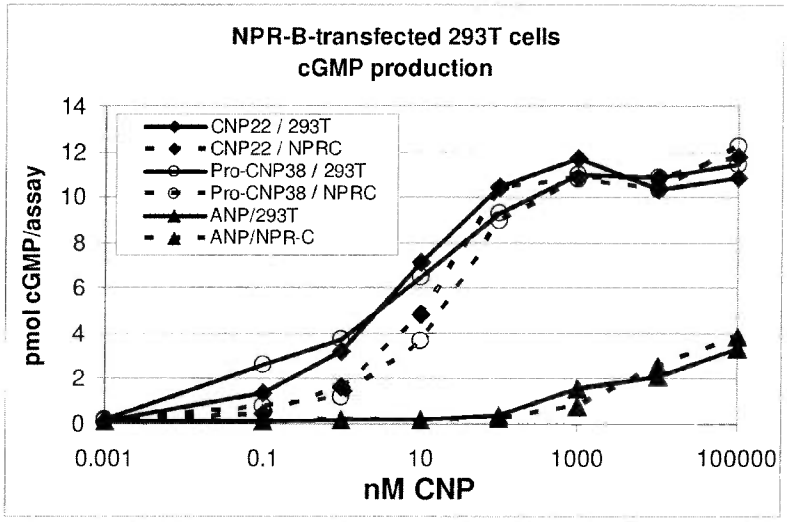


FIGURE 24

A.


NPR-B-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

B.

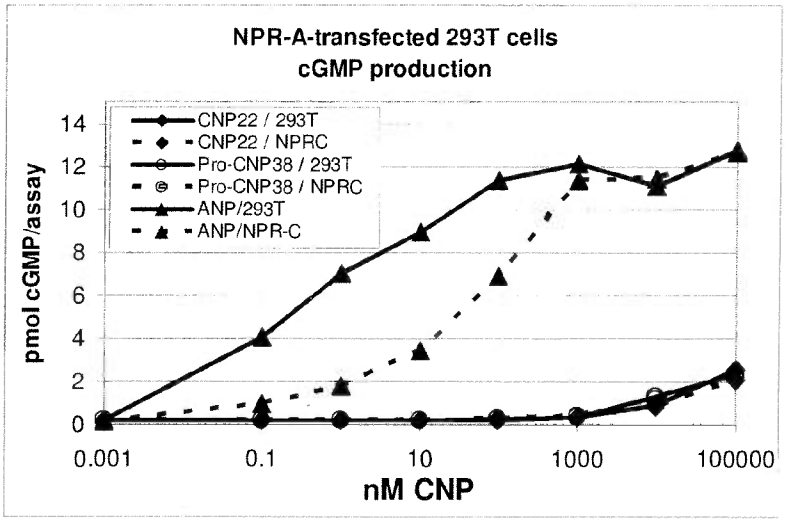
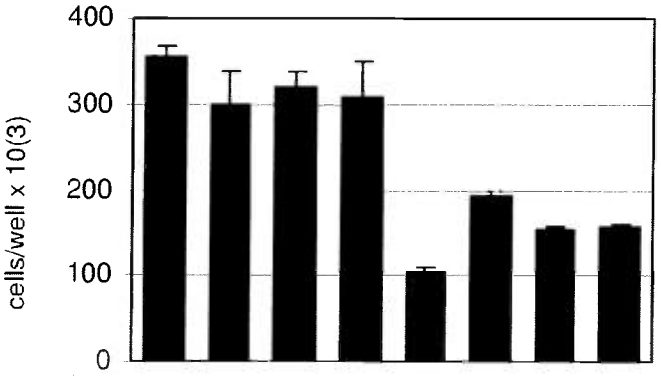

NPR-A-transfected 293T cells
cGMP production
pmol cGMP/assay
nM CNP
CNP22 / 293T
CNP22 / NPRC
Pro-CNP38 / 293T
Pro-CNP38 / NPRC
ANP/293T
ANP/NPR-C
0
2
4
6
8
10
12
14
0.001
0.1
10
1000
100000

FIGURE 25



Bars from left to right:

1	control (no CNP22, no FGF2)
2	CNP22 (0.2 uM) continuous
3	CNP22 (0.2 uM) 1 hr once daily
4	CNP22 (0.2 uM) 2 hr once daily
5	FGF2 (5 ng/mL) continuous
6	FGF2 + CNP22 (0.2 uM) continuous
7	FGF2 + CNP22 (0.2 uM) 1 hr daily
8	FGF2 + CNP22 (0.2 uM) 2 hr daily

FIGURE 26

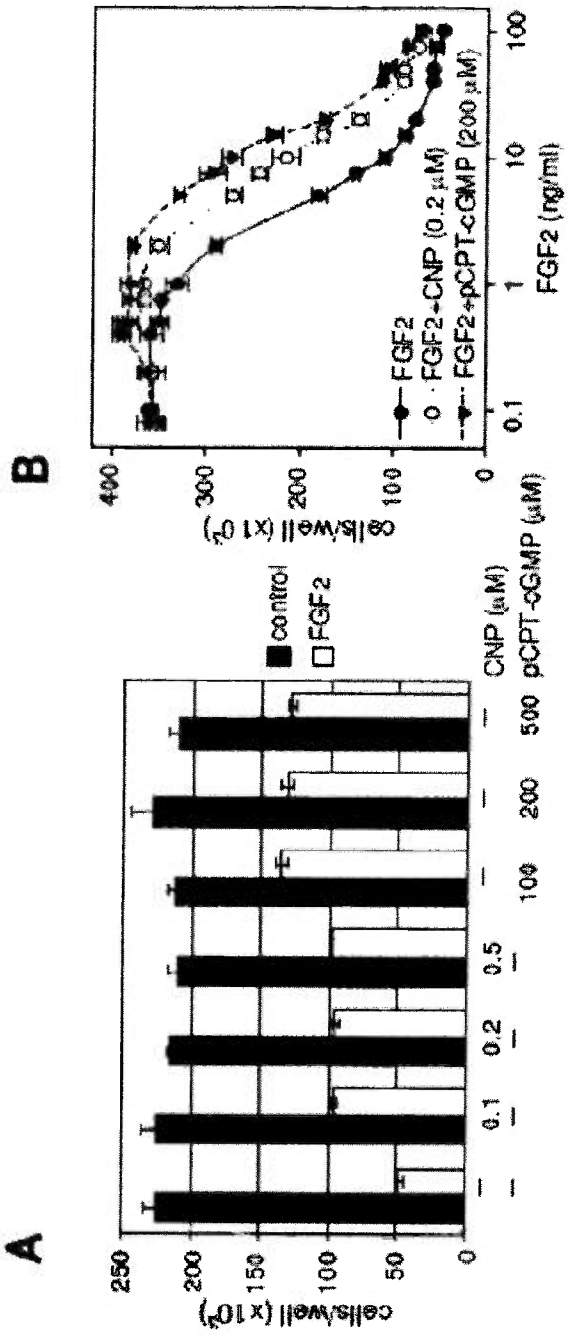

A
B
cells/well (x 10⁵)
control
FGF2
CNP (μM)
pCPT-cGMP (μM)
FGF2
FGF2+CNP (0.2 μM)
FGF2+pCPT-cGMP (200 μM)
FGF2 (ng/ml)

FIGURE 27

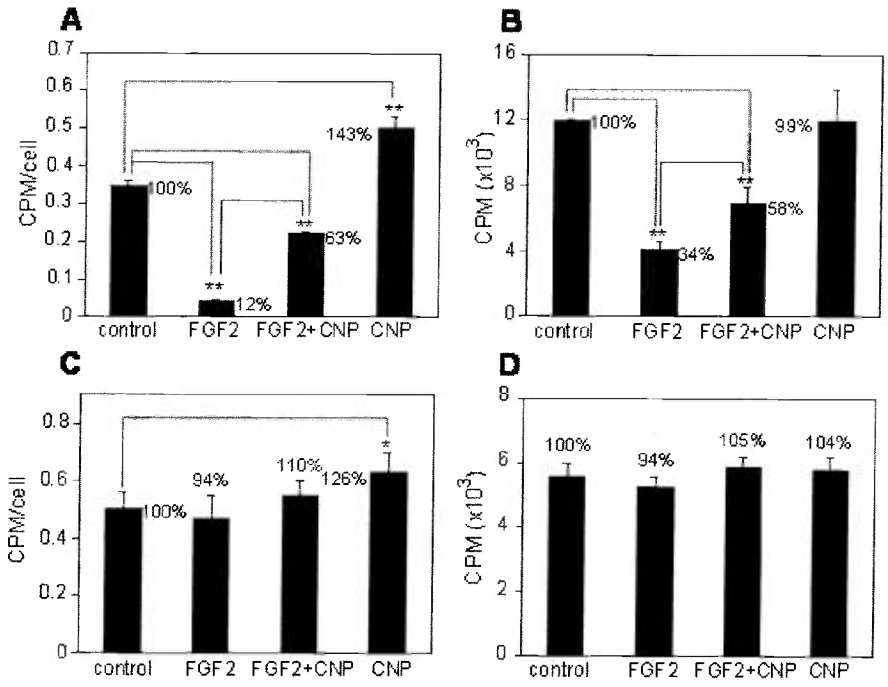

A
CPM/cell
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100%
12%
63%
143%
**
control
FGF2
FGF2+CNP
CNP
B
CPM (x10^3)
16
12
8
4
0
100%
34%
58%
99%
**
control
FGF2
FGF2+CNP
CNP
C
CPM/cell
0.8
0.6
0.4
0.2
0
100%
94%
110%
126%
*
control
FGF2
FGF2+CNP
CNP
D
CPM (x10^3)
8
6
4
2
0
100%
94%
105%
104%
control
FGF2
FGF2+CNP
CNP

FIGURE 28

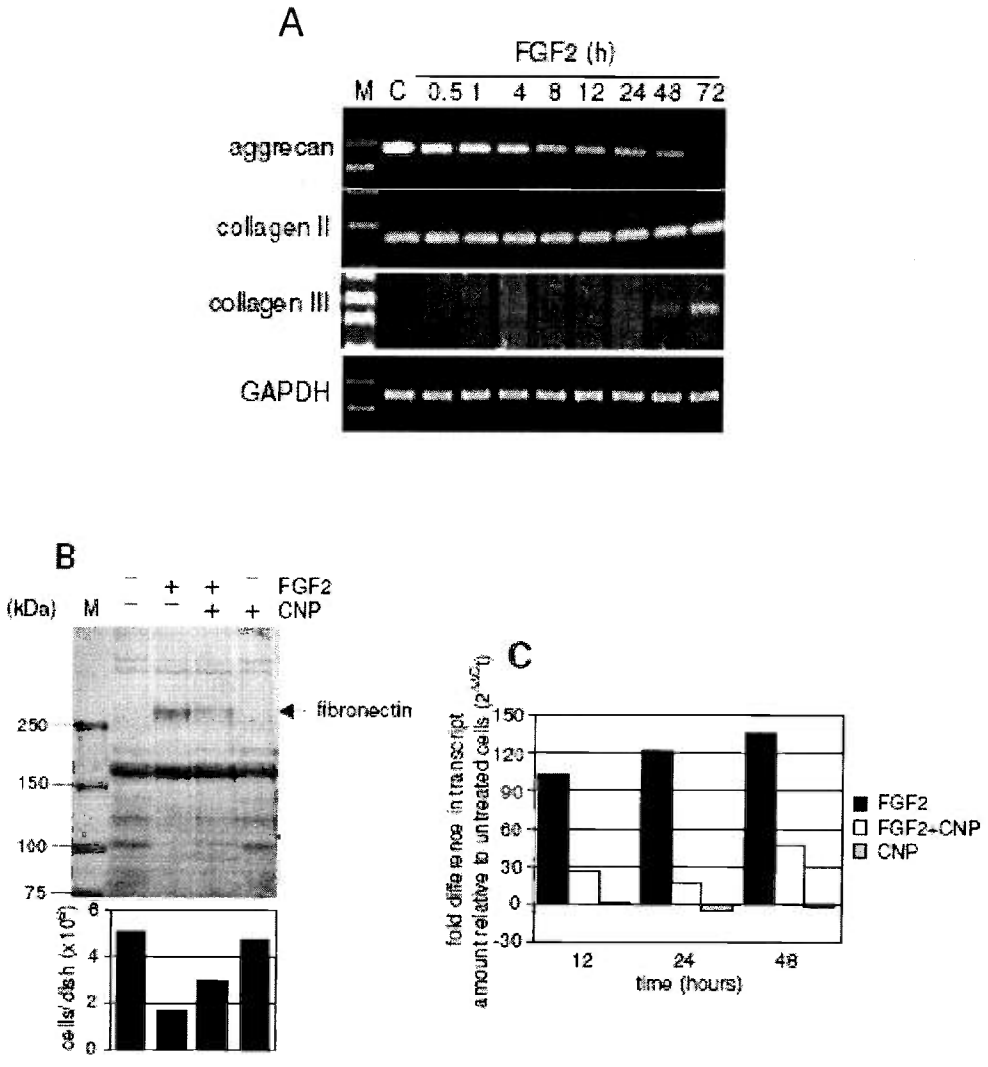

A
FGF2 (h)
M C 0.5 1 4 8 12 24 48 72
aggrecan
collagen II
collagen III
GAPDH
B
FGF2
CNP
(kDa) M
250
150
100
75
fibronectin
cells/ dish (x 10⁵)
C
fold difference in transcript amount relative to untreated cells
time (hours)
FGF2
FGF2+CNP
CNP

FIGURE 29

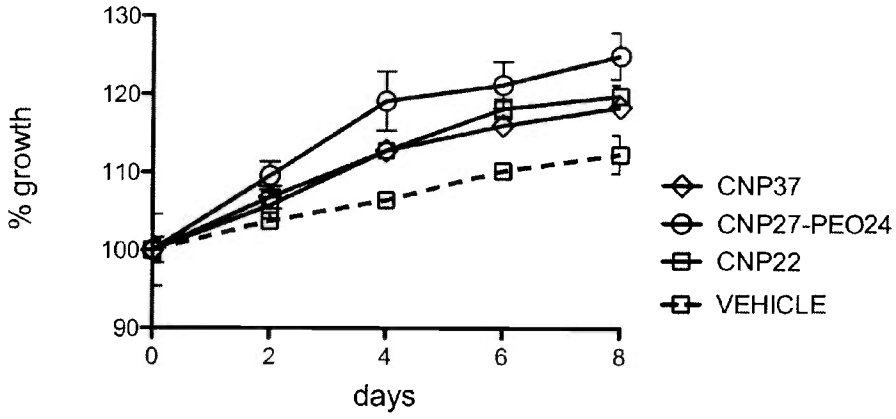


FIGURE 30

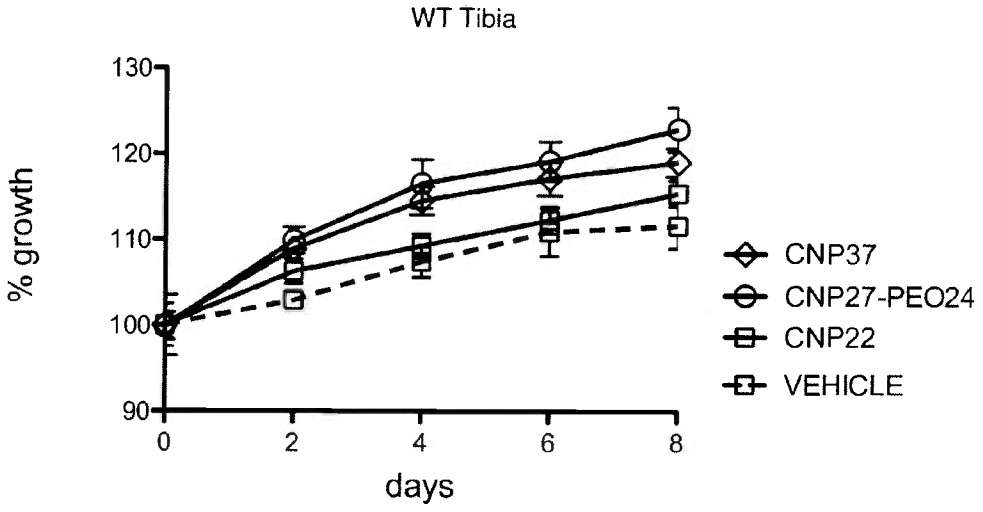


FIGURE 31

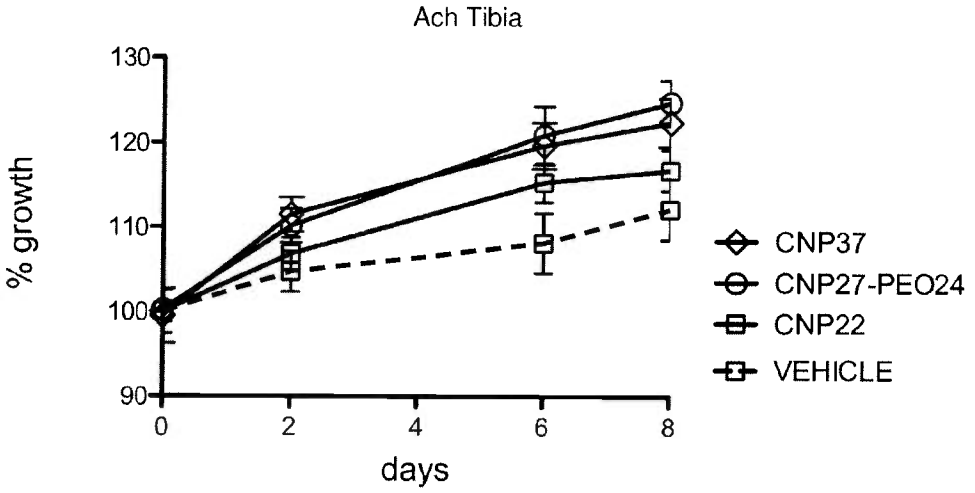


FIGURE 32

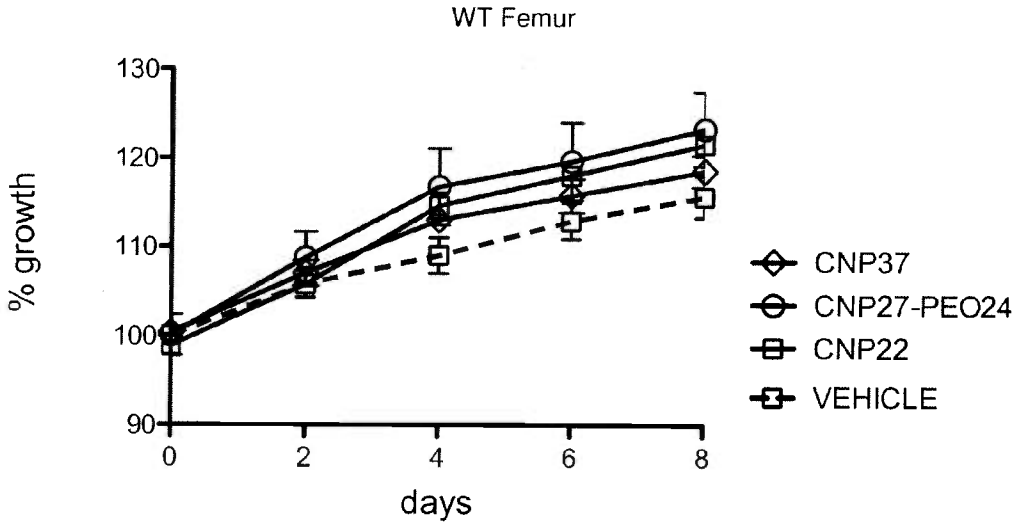


FIGURE 33

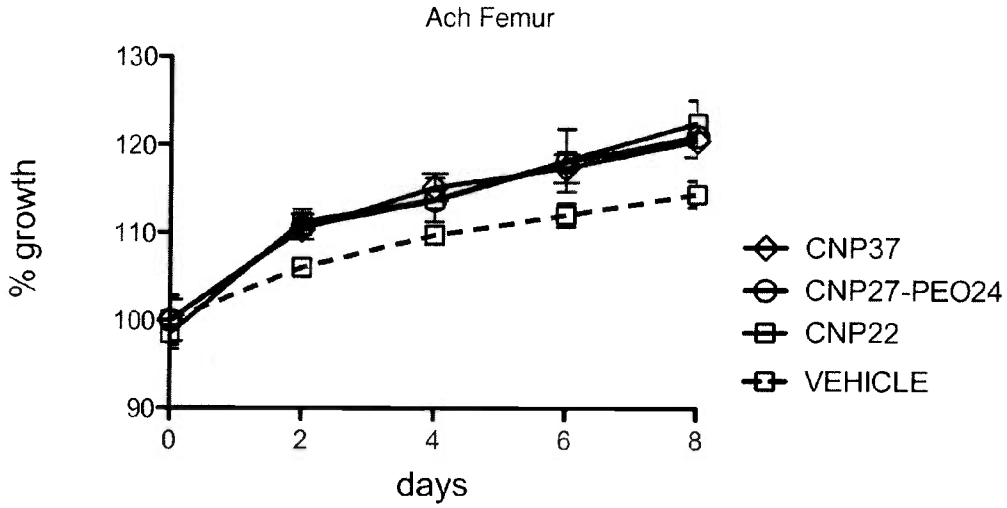

Ach Femur
130
120
110
100
90
% growth
0
2
4
6
8
days
CNP37
CNP27-PEO24
CNP22
VEHICLE

FIGURE 34

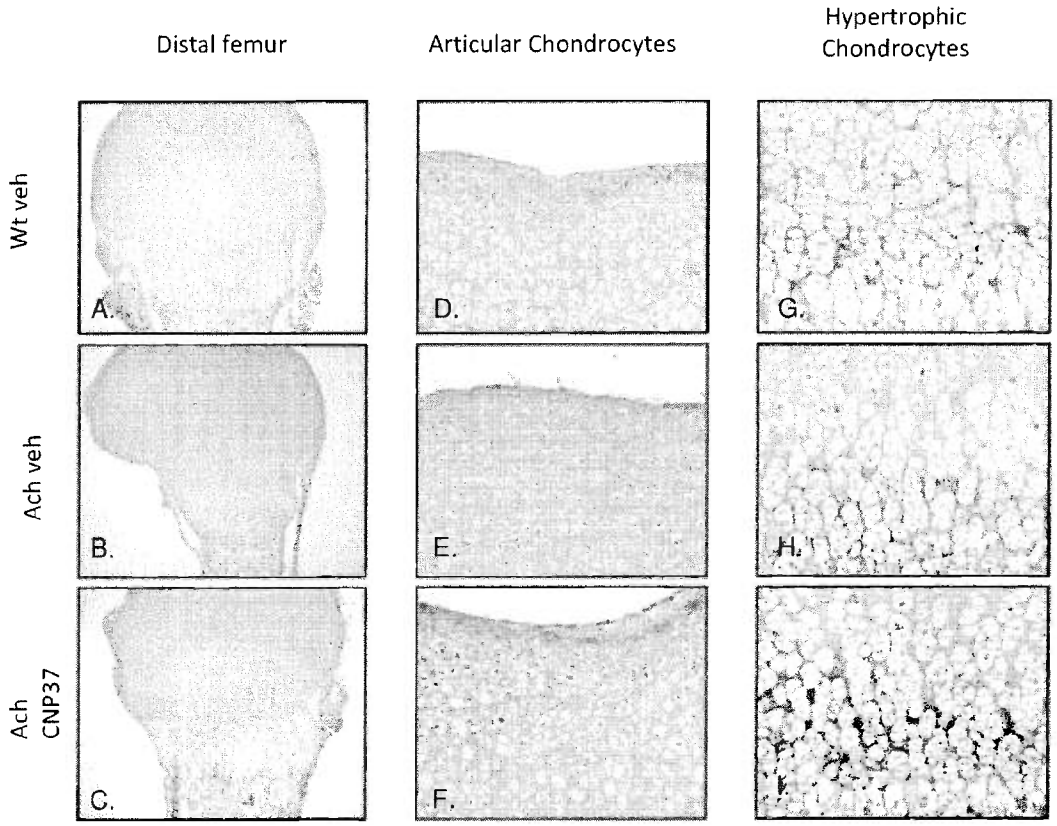

Distal femur
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.

FIGURE 35

Cellularity of Proliferating Columns

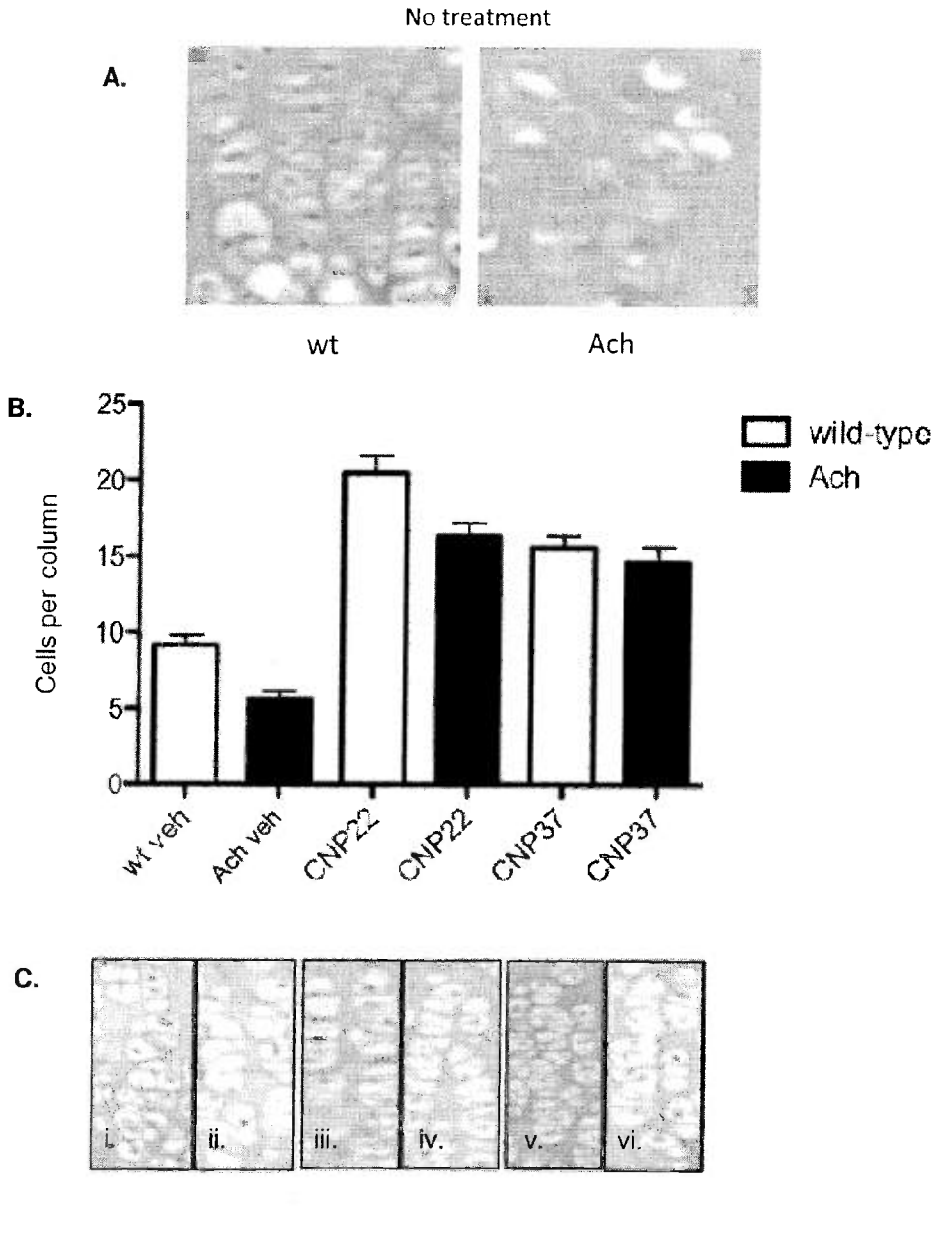


FIGURE 36

Chondrocyte Hypertrophy

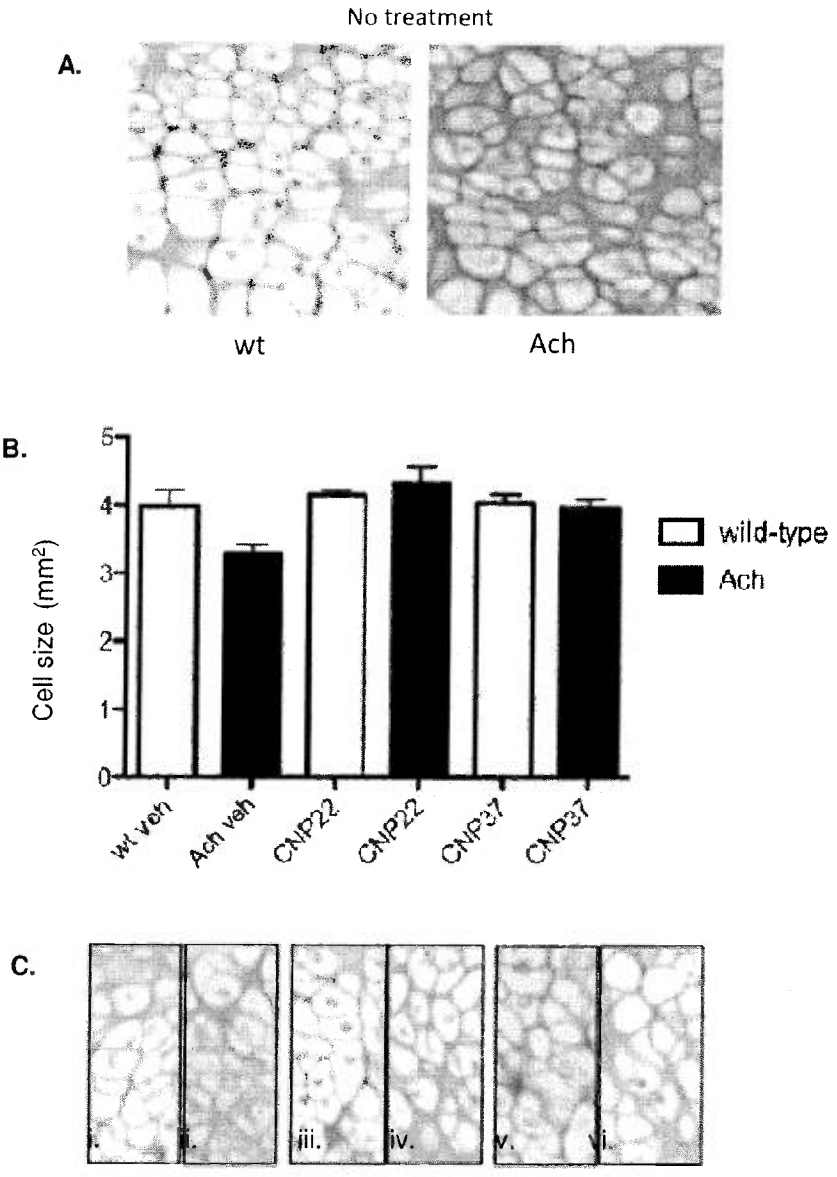


FIGURE 37

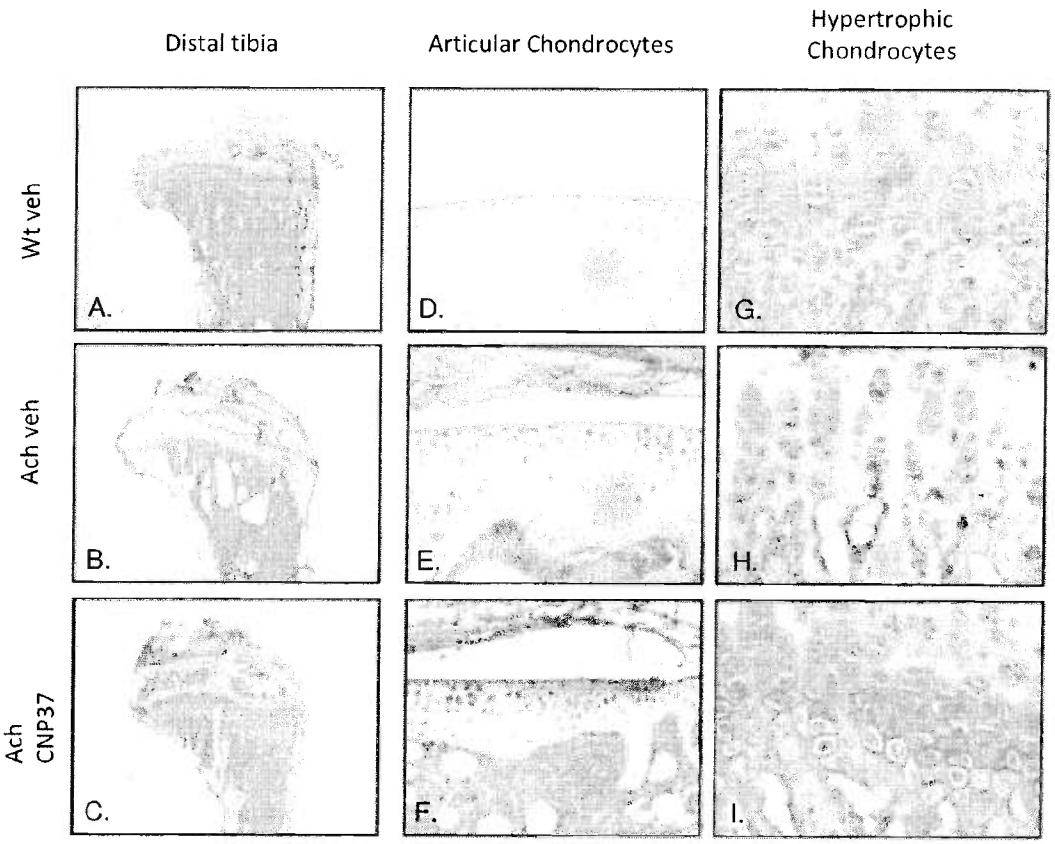
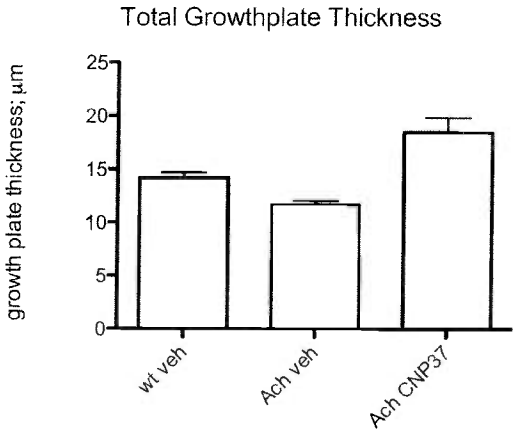
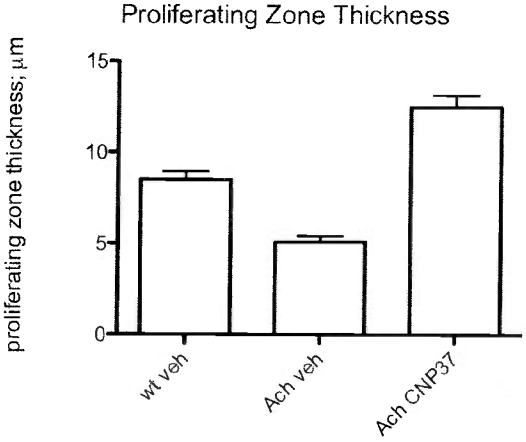

Distal tibia
Articular Chondrocytes
Hypertrophic Chondrocytes
Wt veh
Ach veh
Ach CNP37
A.
B.
C.
D.
E.
F.
G.
H.
I.

FIGURE 38

A.



B.



C.

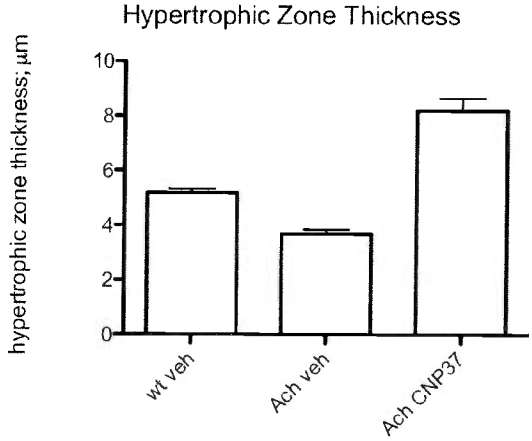


FIGURE 39

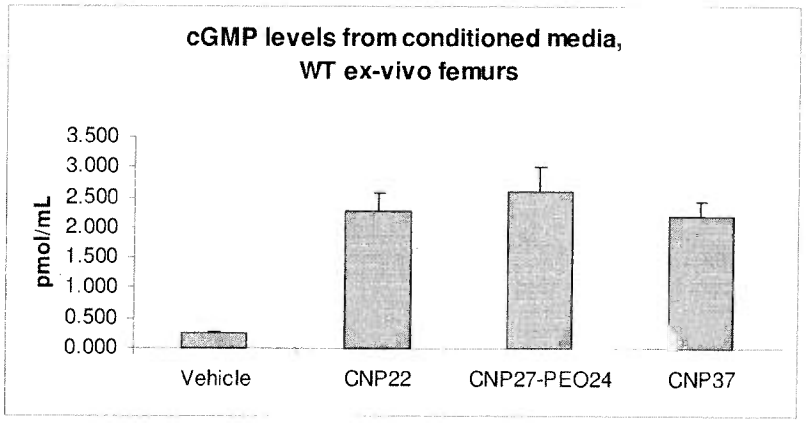


FIGURE 40

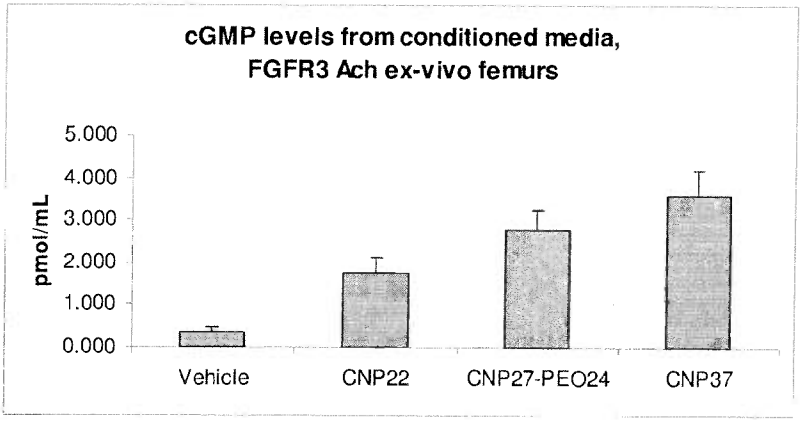


FIGURE 41

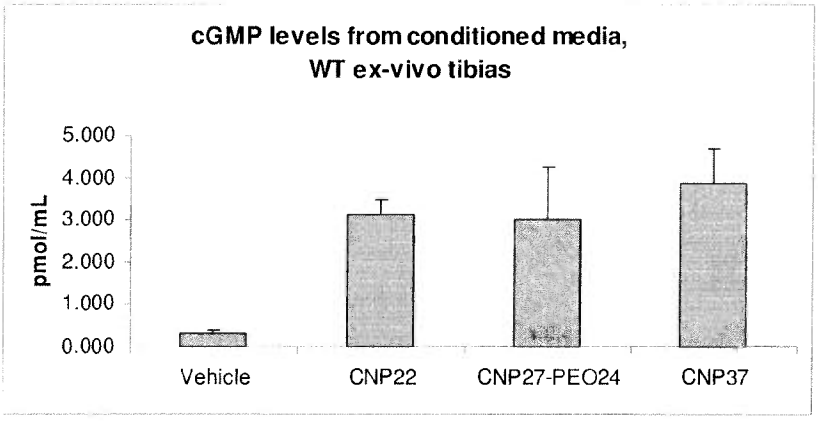


FIGURE 42

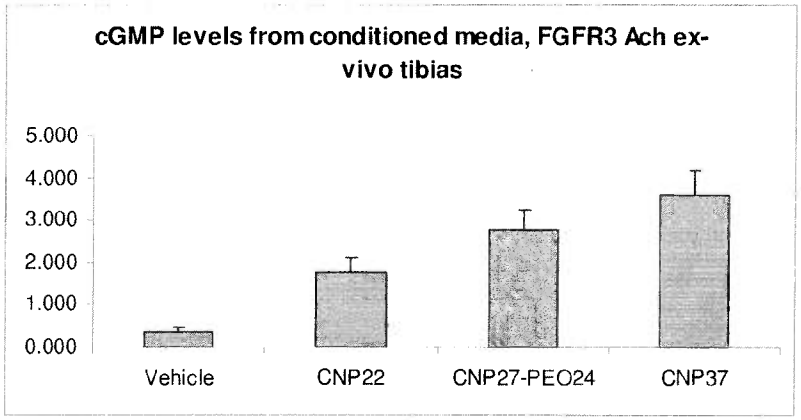


FIGURE 43

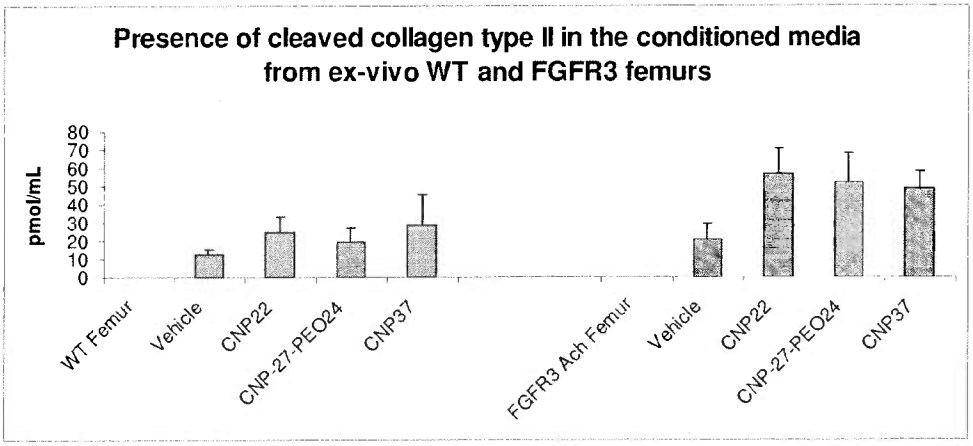


FIGURE 44

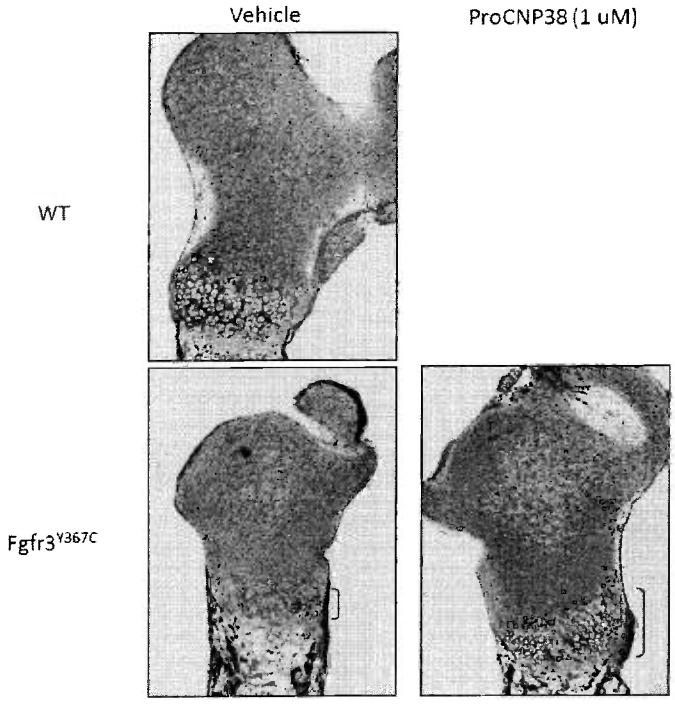


FIGURE 45

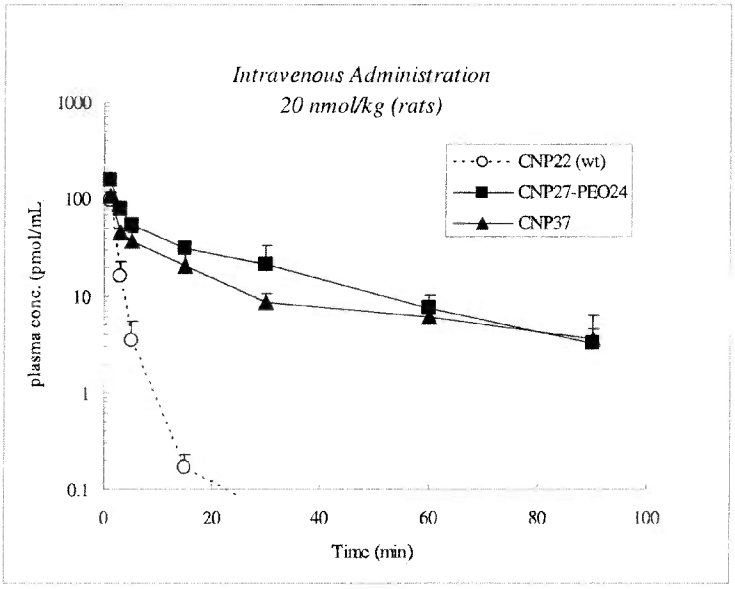


FIGURE 46

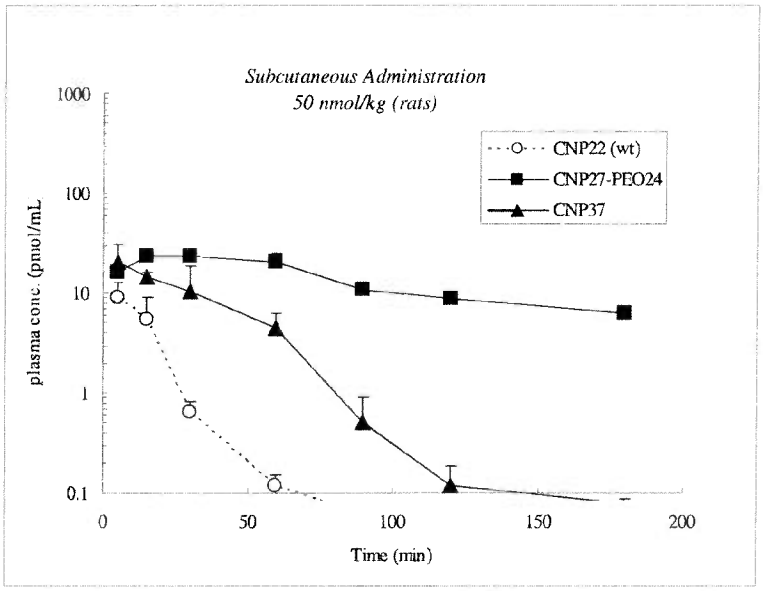


FIGURE 47

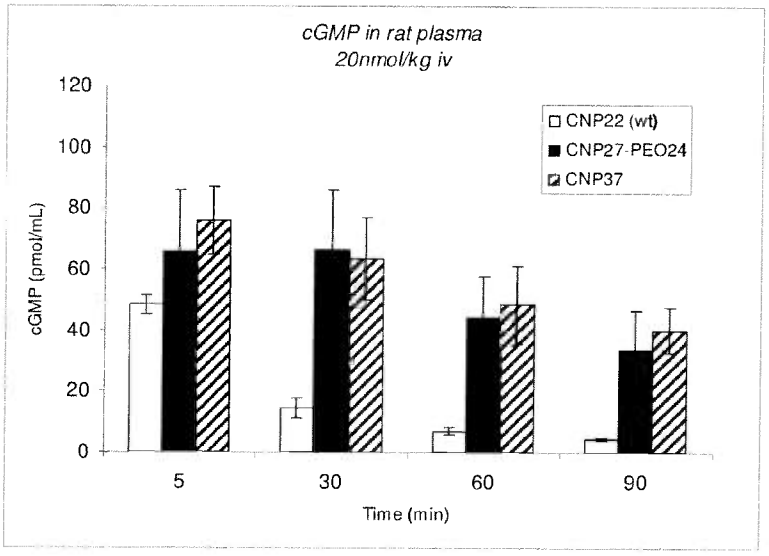


FIGURE 48

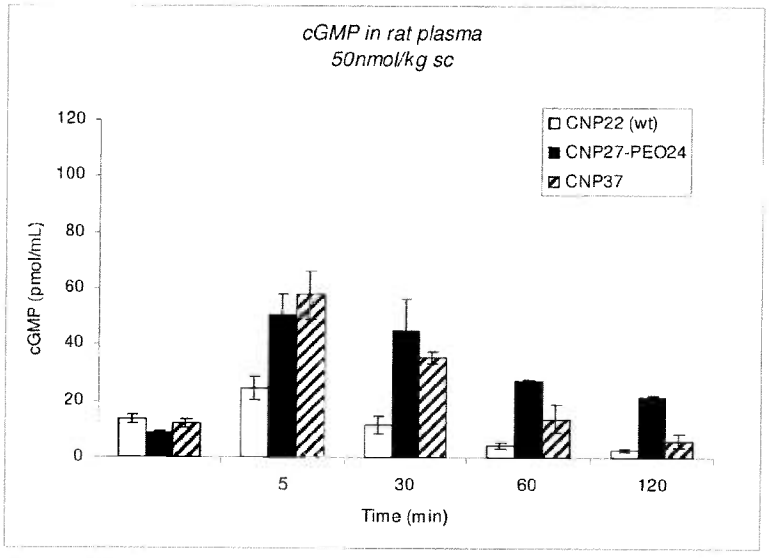


FIGURE 49

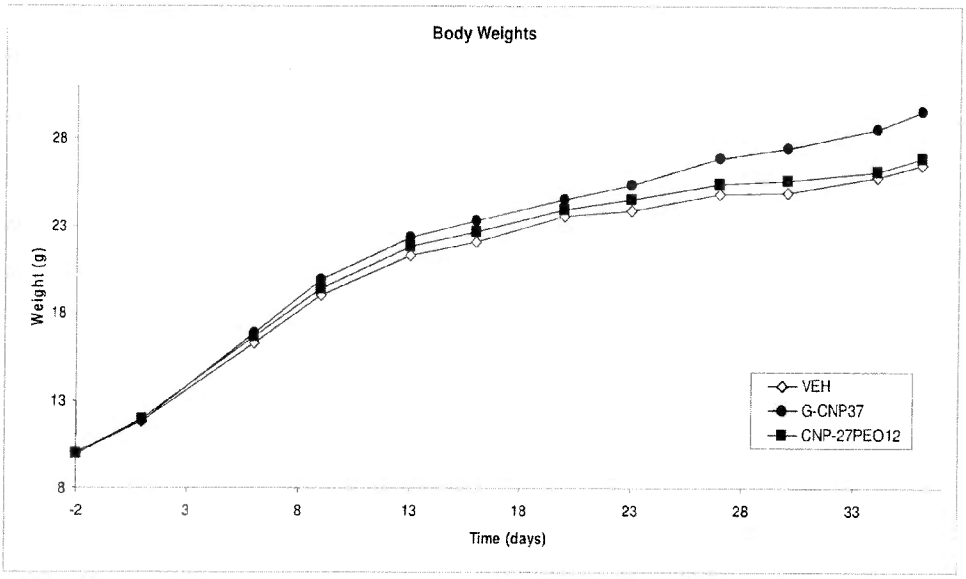


FIGURE 50

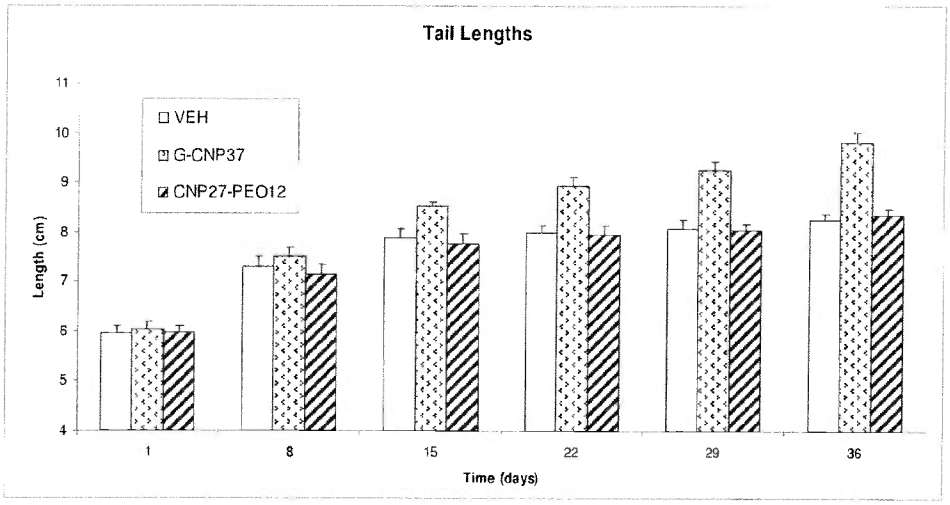


FIGURE 51

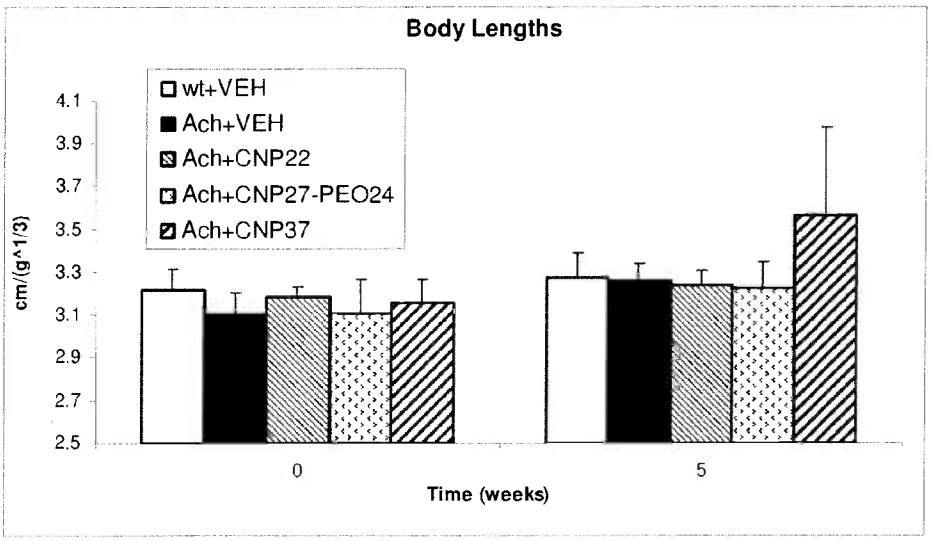


FIGURE 52

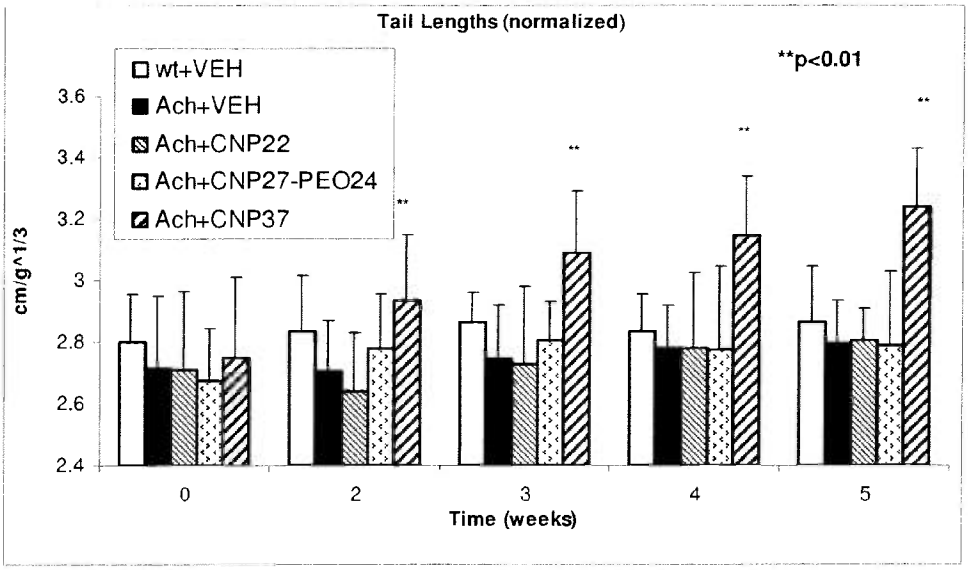
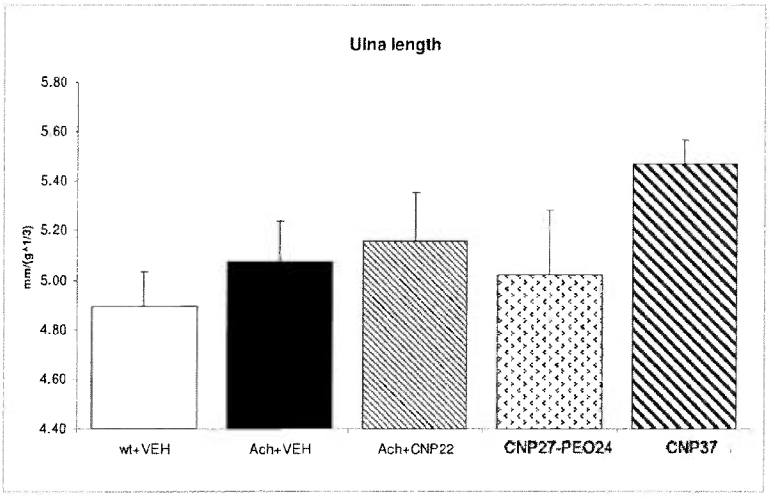


FIGURE 53

A.


Ulna length
5.80
5.60
5.40
5.20
5.00
4.80
4.60
4.40
mm/(g^1/3)
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

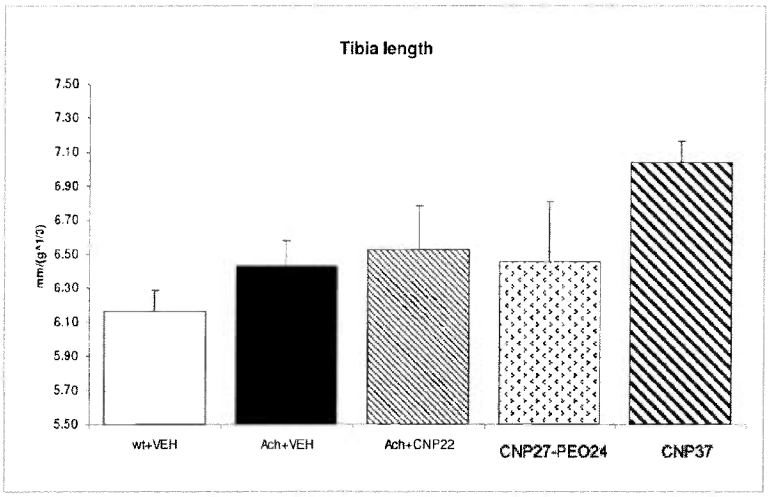
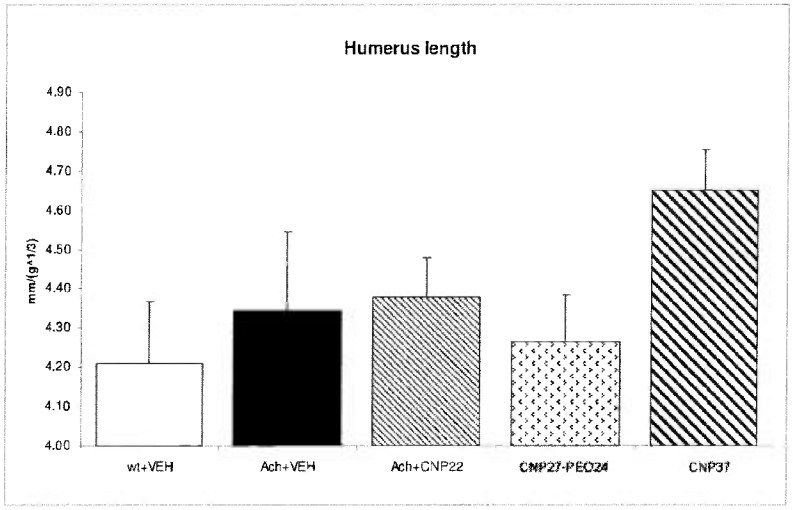

Tibia length
7.50
7.30
7.10
6.90
6.70
6.50
6.30
6.10
5.90
5.70
5.50
mm/(g^1/3)
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 54

A.


Humerus length
mm/(g^1/3)
4.90
4.80
4.70
4.60
4.50
4.40
4.30
4.20
4.10
4.00
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

B.

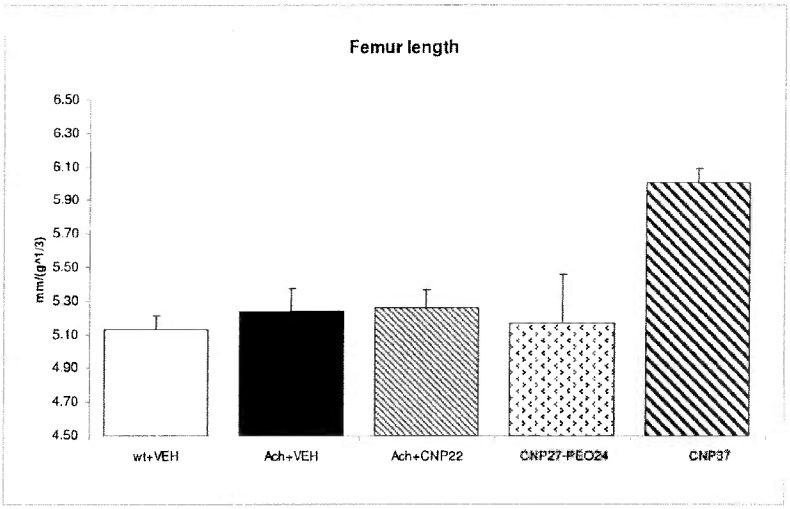

Femur length
mm/(g^1/3)
6.50
6.30
6.10
5.90
5.70
5.50
5.30
5.10
4.90
4.70
4.50
wt+VEH
Ach+VEH
Ach+CNP22
CNP27-PEO24
CNP37

FIGURE 55

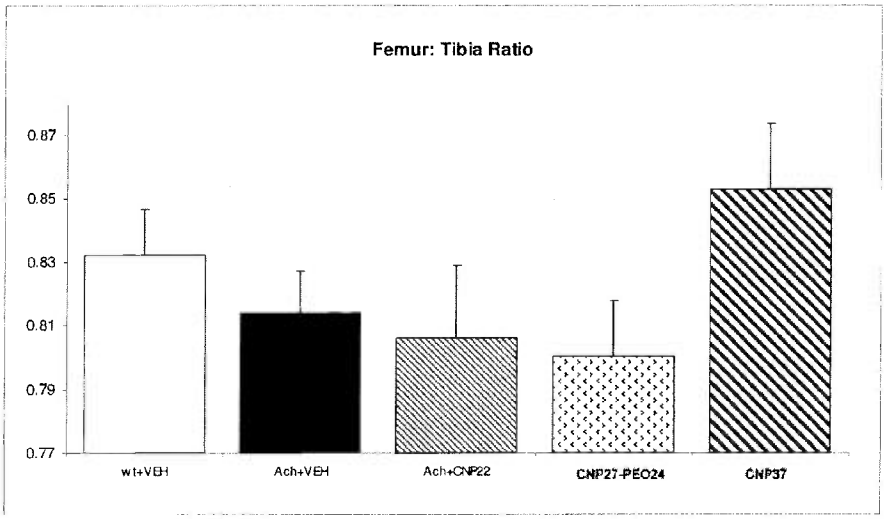


FIGURE 56

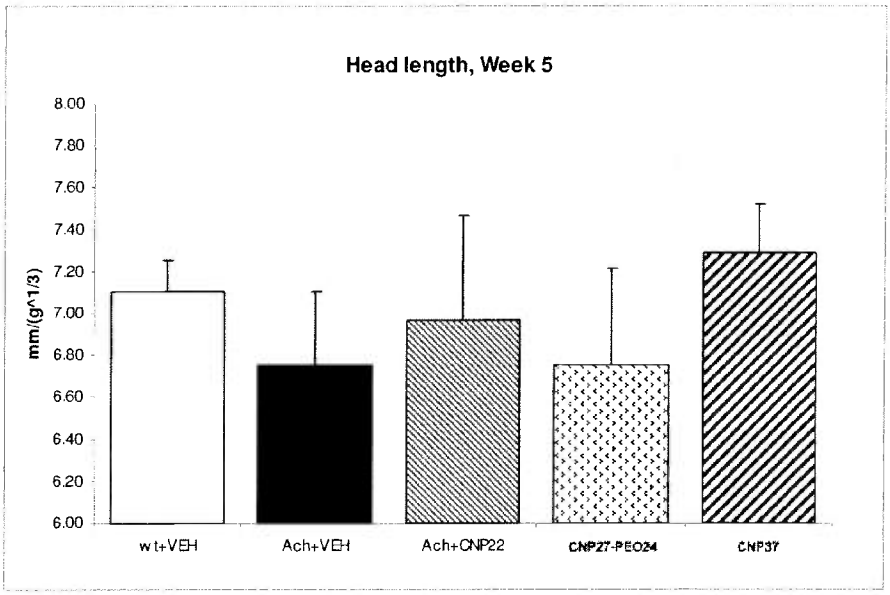


FIGURE 57

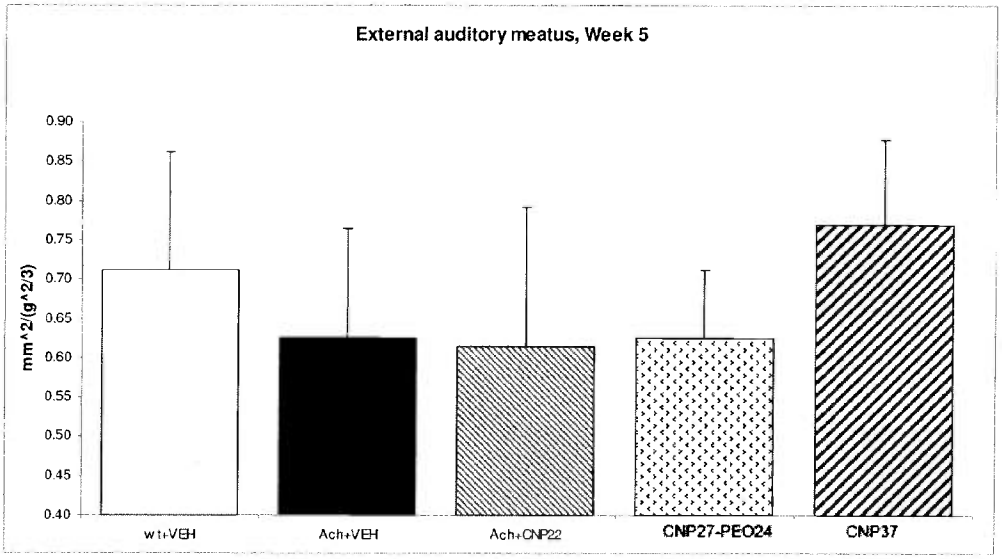


FIGURE 58

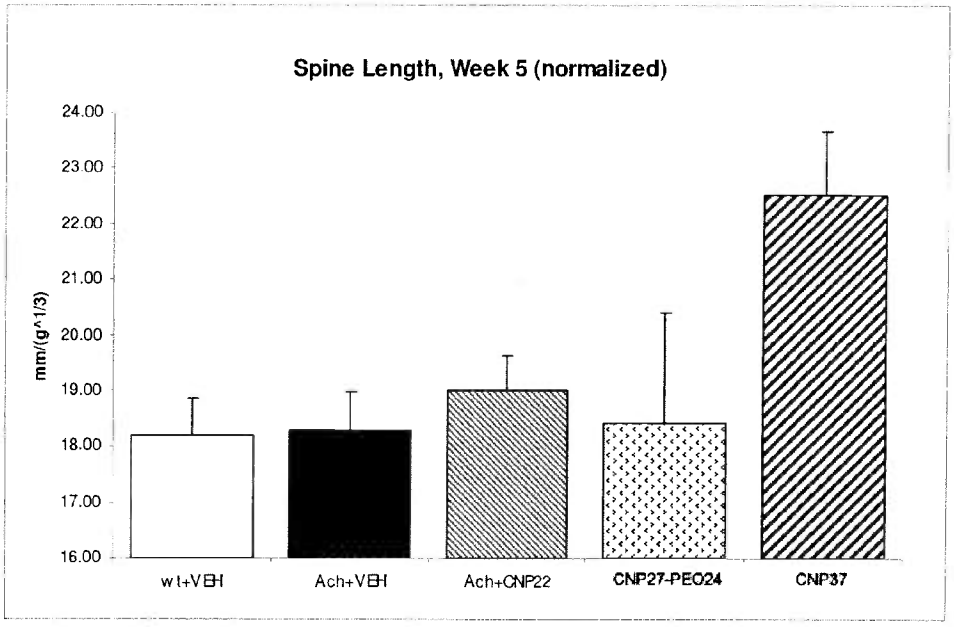


FIGURE 59

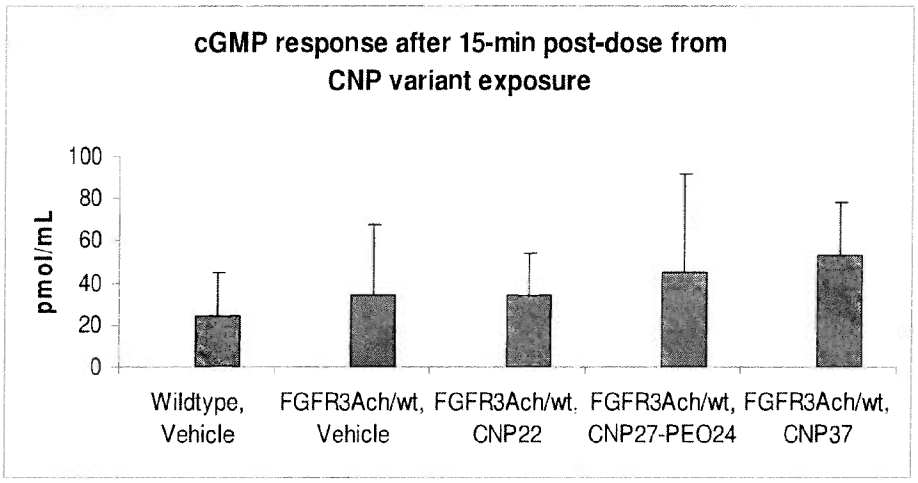


FIGURE 60

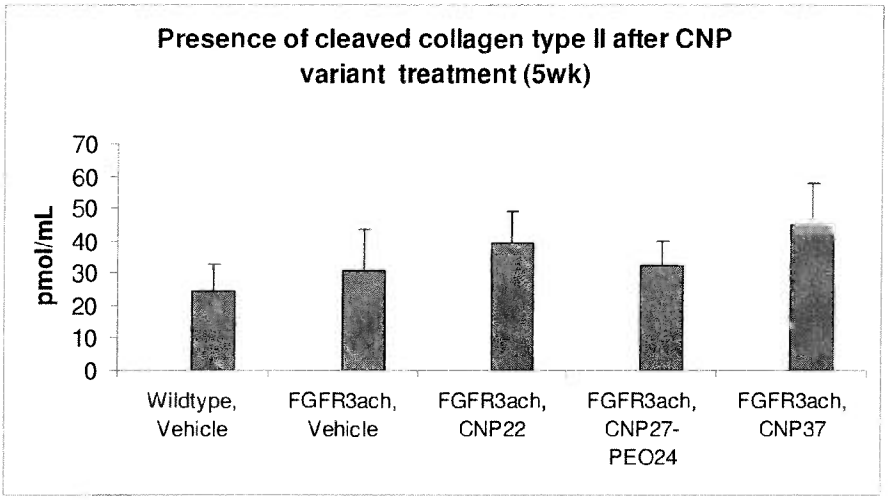


FIGURE 61

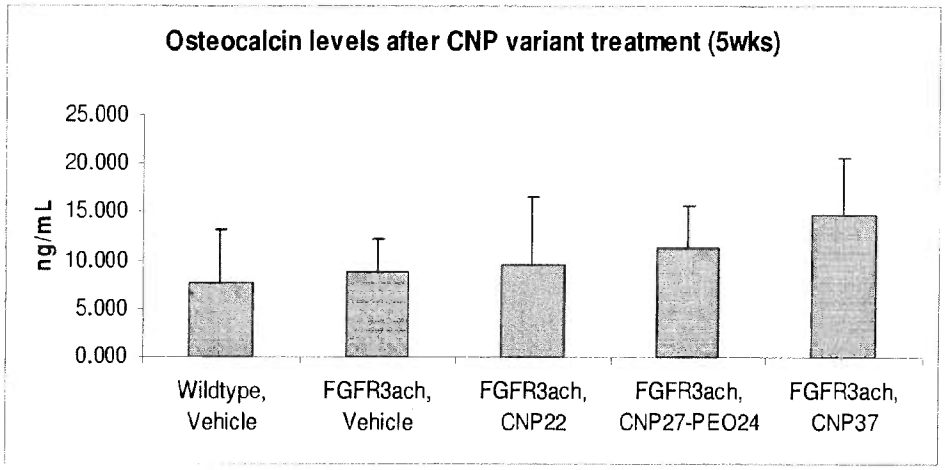


FIGURE 62

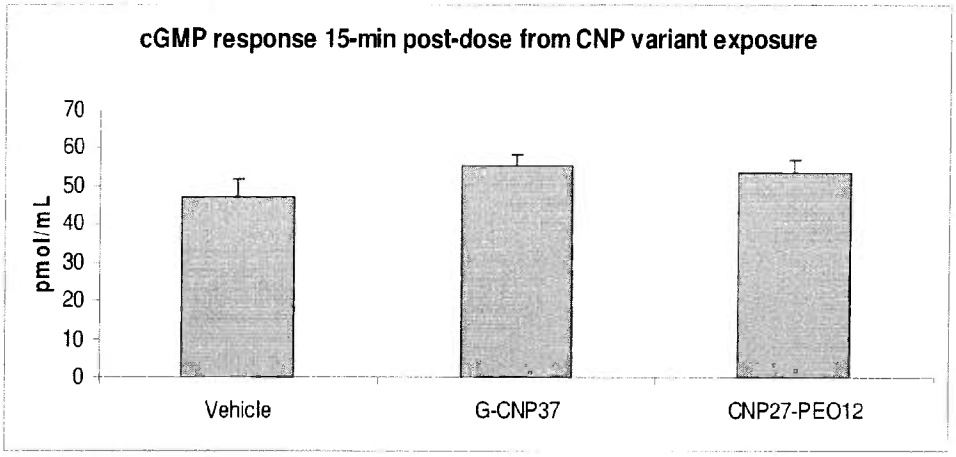


FIGURE 63

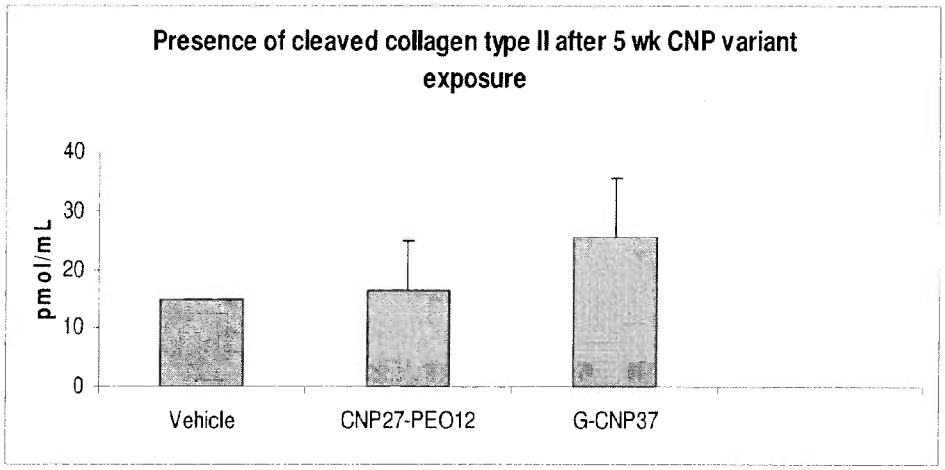
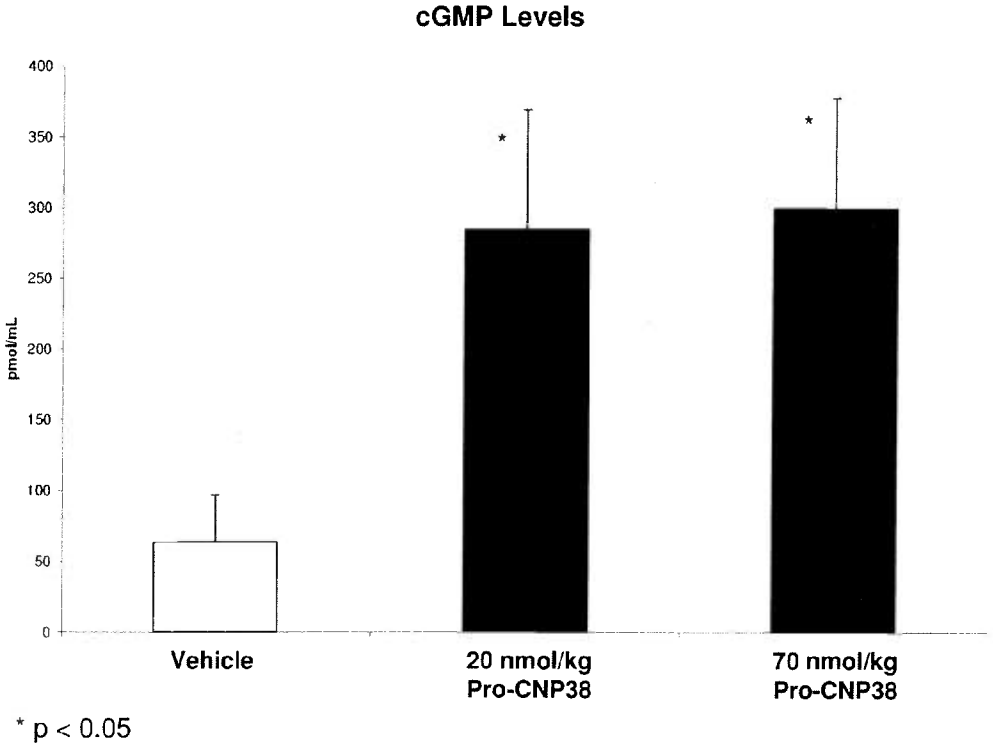


FIGURE 64



* $p < 0.05$

FIGURE 65

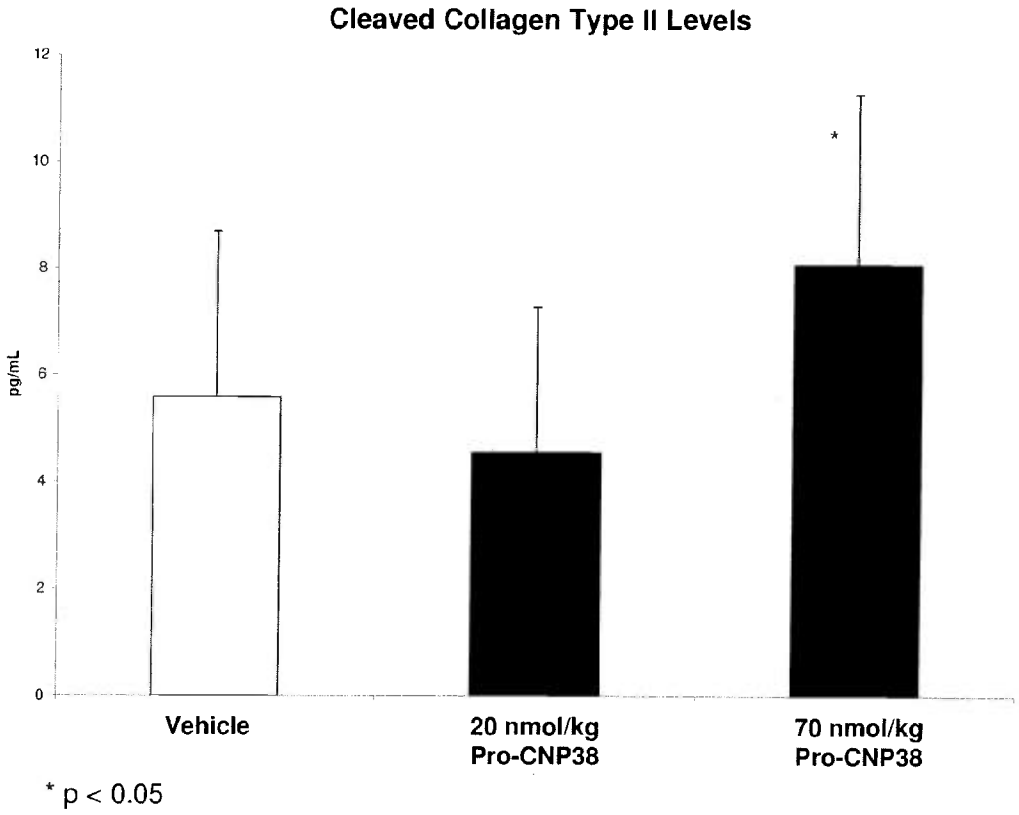

Cleaved Collagen Type II Levels
12
10
8
6
4
2
0
pg/mL
*
Vehicle
20 nmol/kg
Pro-CNP38
70 nmol/kg
Pro-CNP38
* p < 0.05

FIGURE 66

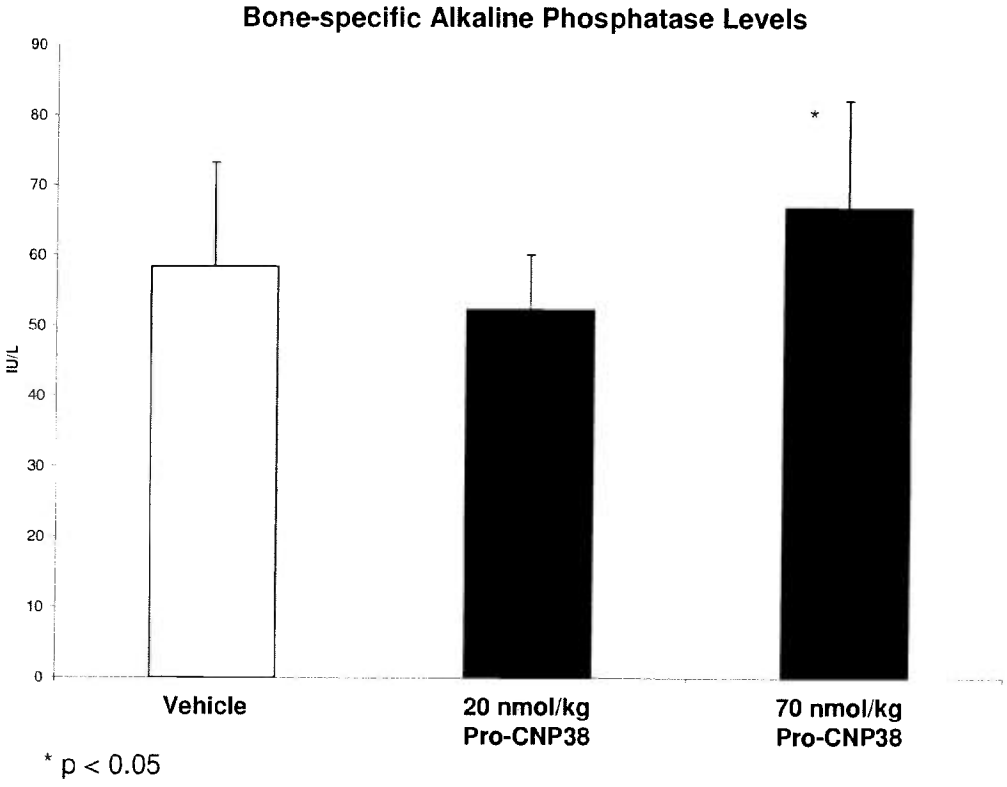

Bone-specific Alkaline Phosphatase Levels
90
80
70
60
50
40
30
20
10
0
IU/L
*
Vehicle
20 nmol/kg
Pro-CNP38
70 nmol/kg
Pro-CNP38
* p < 0.05

FIGURE 67

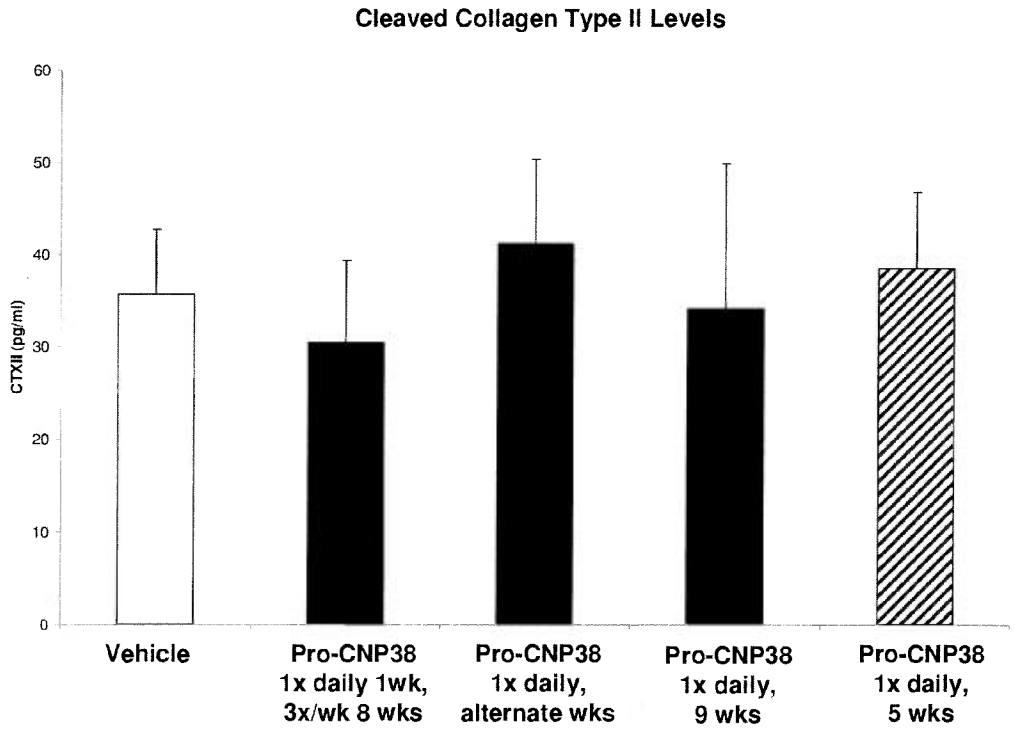

Cleaved Collagen Type II Levels
CTXII (pg/ml)
60
50
40
30
20
10
0
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks

FIGURE 68

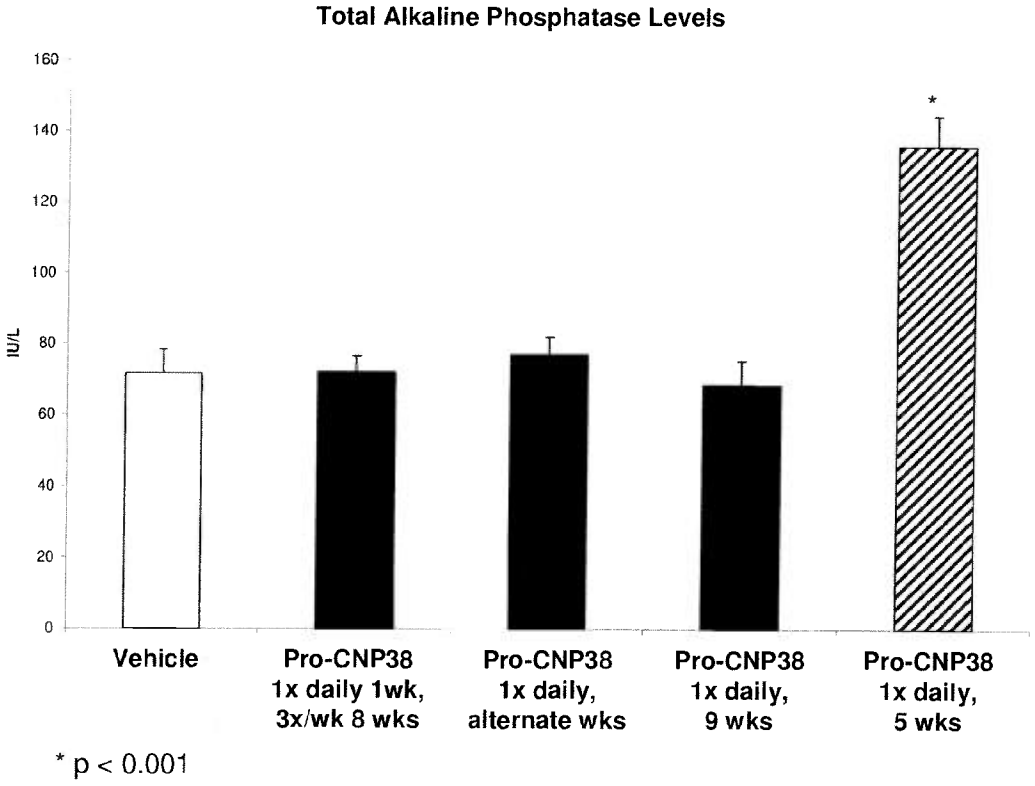

Total Alkaline Phosphatase Levels
160
140
120
100
80
60
40
20
0
IU/L
*
Vehicle
Pro-CNP38 1x daily 1wk, 3x/wk 8 wks
Pro-CNP38 1x daily, alternate wks
Pro-CNP38 1x daily, 9 wks
Pro-CNP38 1x daily, 5 wks
* p < 0.001

FIGURE 69

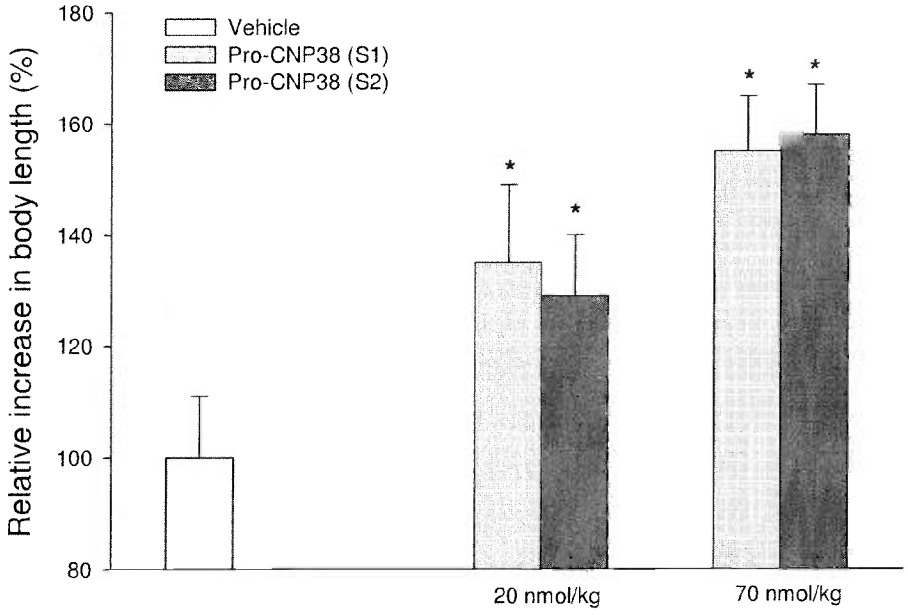
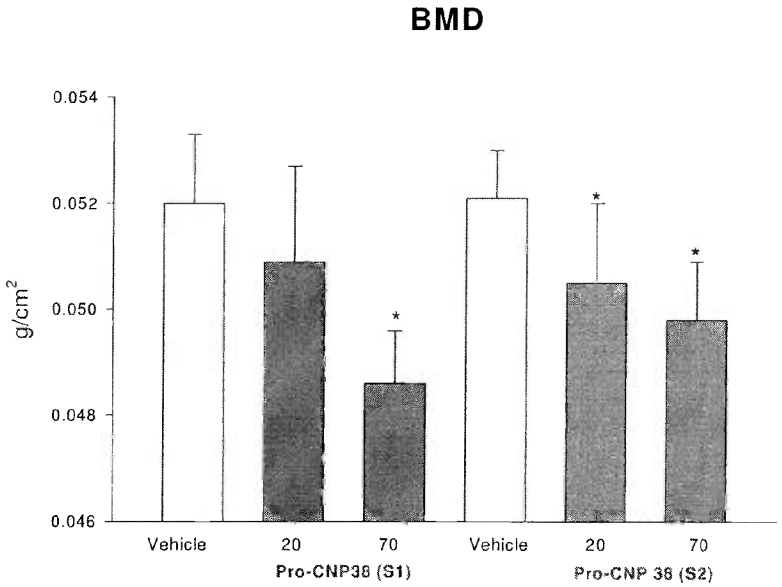

Relative increase in body length (%)
180
160
140
120
100
80
Vehicle
Pro-CNP38 (S1)
Pro-CNP38 (S2)
*
*
*
*
20 nmol/kg
70 nmol/kg

FIGURE 70

A.


BMD
0.054
0.052
0.050
0.048
0.046
g/cm2
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

B.

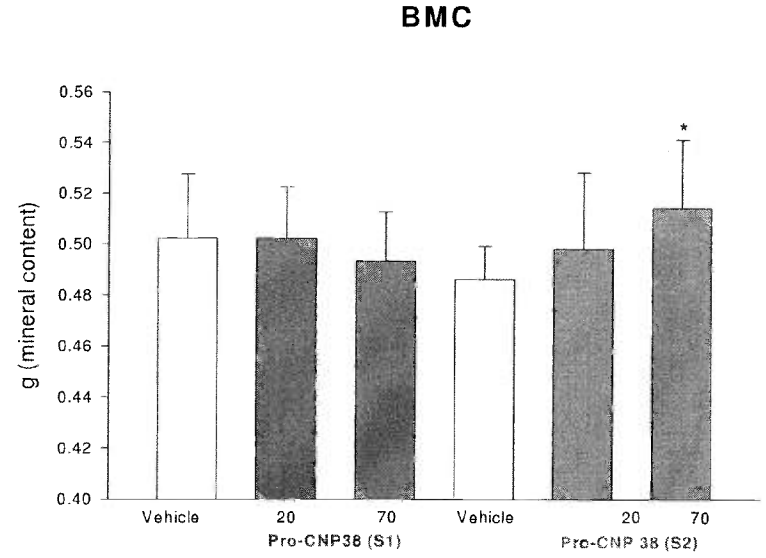

BMC
0.56
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
g (mineral content)
Vehicle
20
70
Vehicle
20
70
Pro-CNP38 (S1)
Pro-CNP 38 (S2)

FIGURE 71

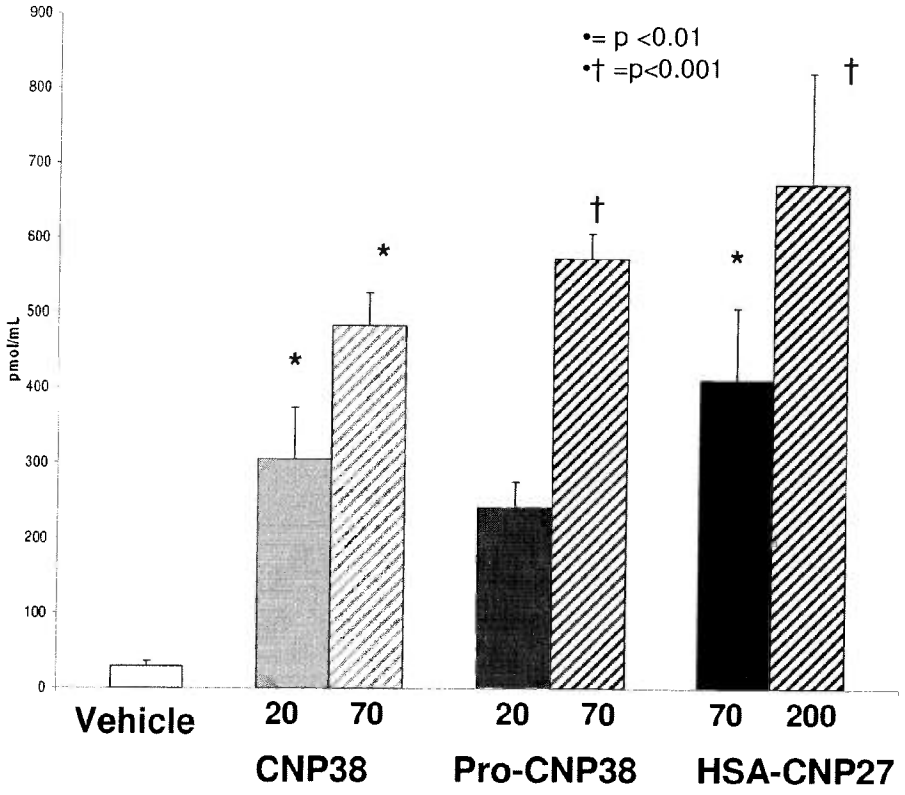

900
800
700
600
500
400
300
200
100
0
pmol/mL
•= p <0.01
•† =p<0.001
*
*
*
†
†
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 72

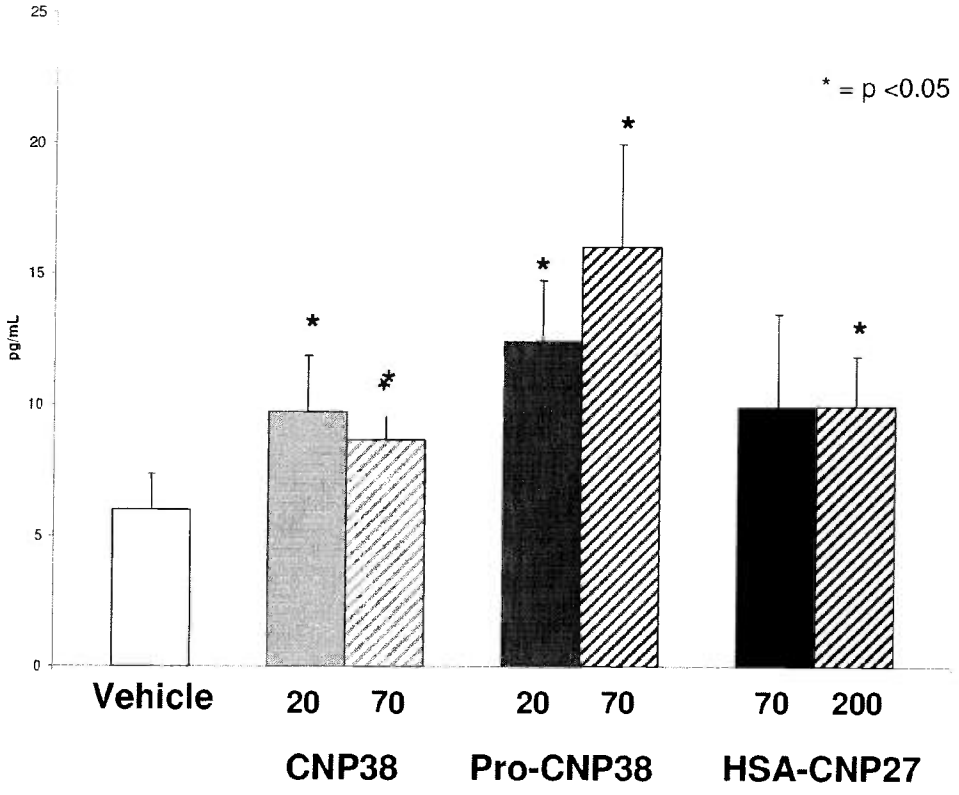

25
20
15
10
5
0
pg/mL
* = p <0.05
*
*
*
*
*
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 73

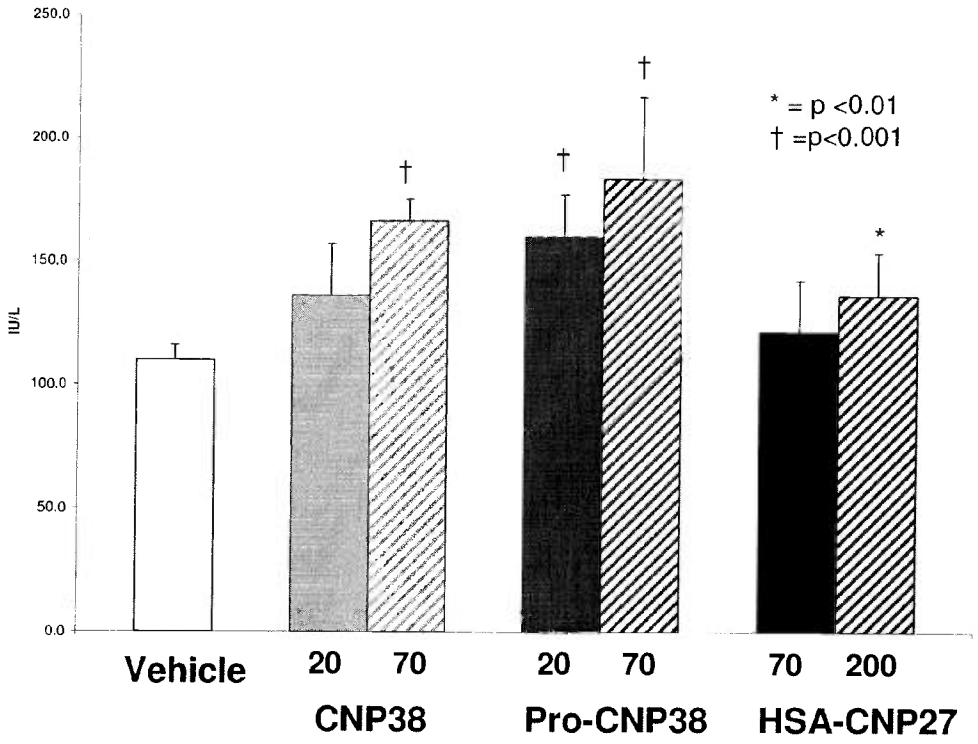
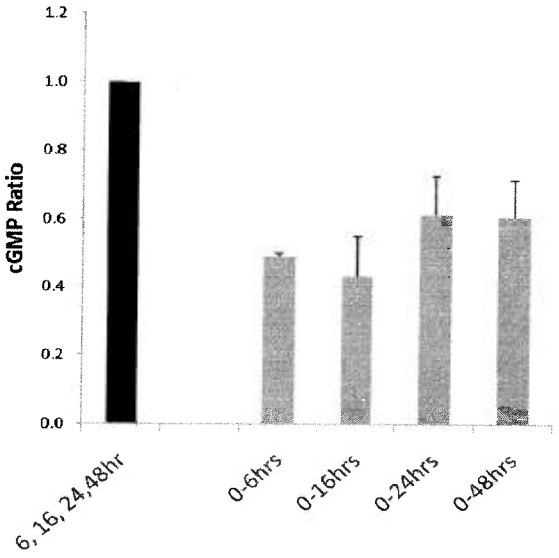

250.0
200.0
150.0
100.0
50.0
0.0
IU/L
* = p <0.01
† =p<0.001
†
†
†
*
Vehicle
20
70
20
70
70
200
CNP38
Pro-CNP38
HSA-CNP27

FIGURE 74

A.


cGMP Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6, 16, 24,48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

B.

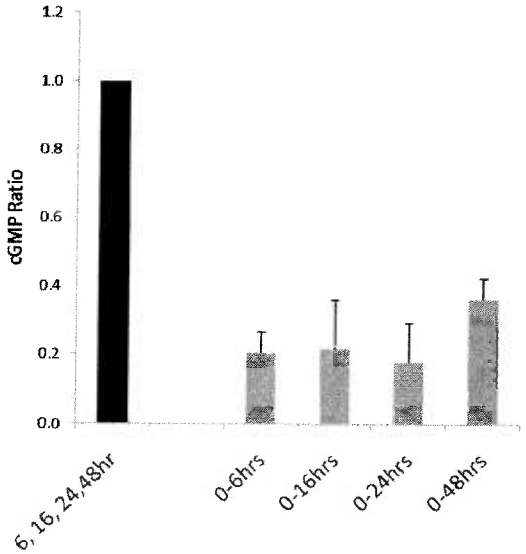
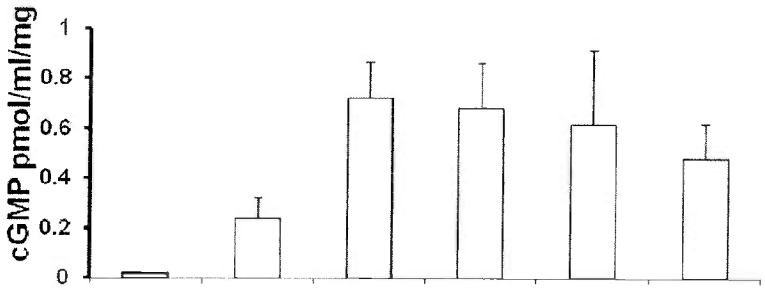

cGMP Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6, 16, 24,48hr
0-6hrs
0-16hrs
0-24hrs
0-48hrs

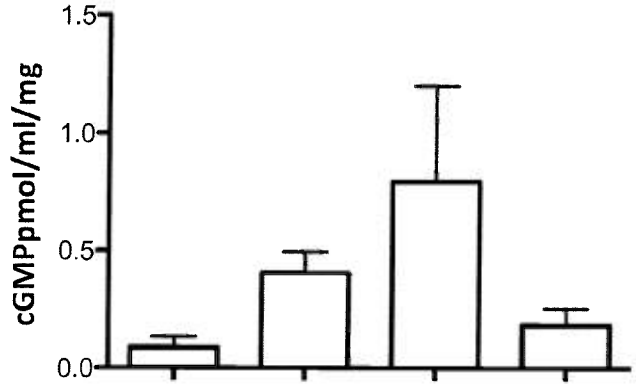
FIGURE 75

A:



# of daily treatments:	1	1	4	6	7	8
ProCNP38 200nmol/kg:	-	+	+	+	+	+

B:



Single treatment on days:	1	1	1,2	1,3
ProCNP38 200nmol/kg:	-	+	+	+

FIGURE 76

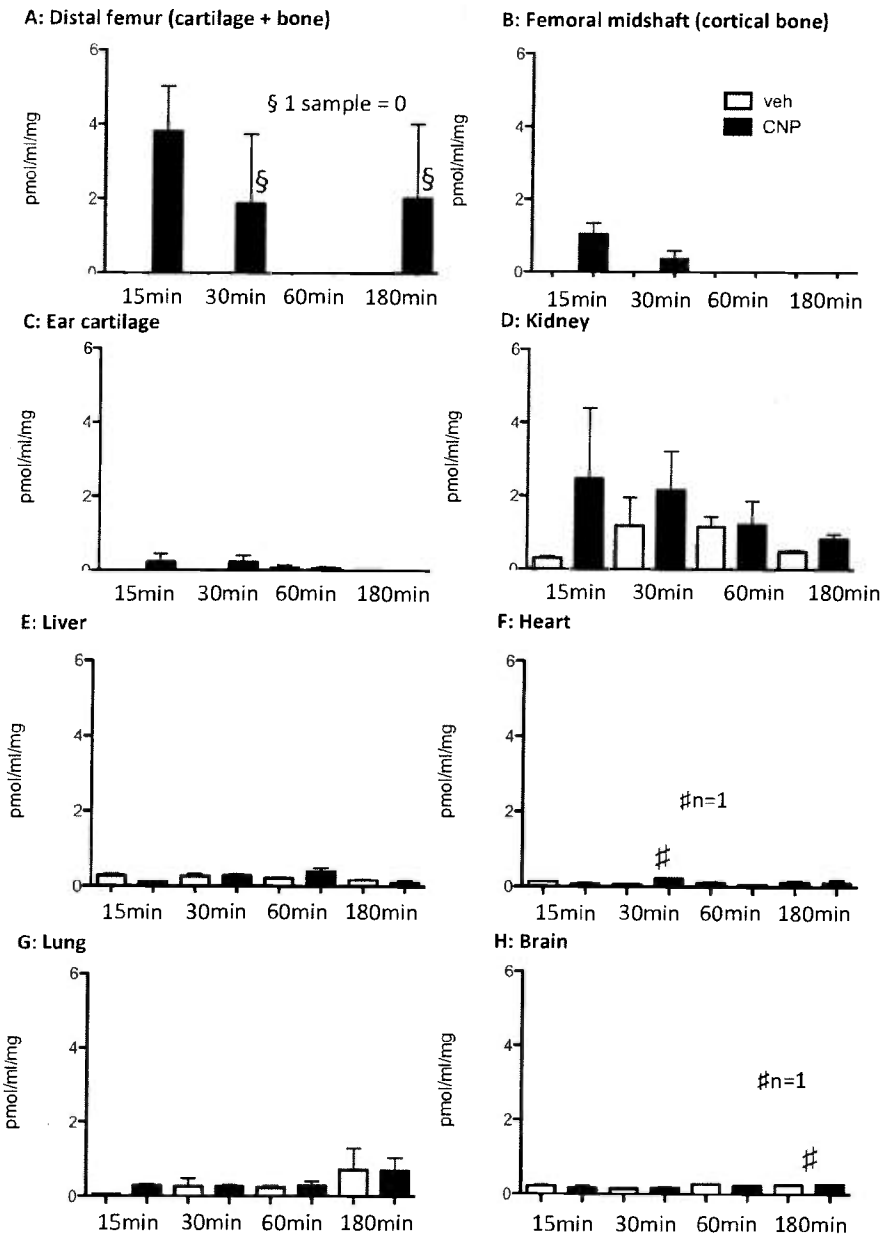

A: Distal femur (cartilage + bone)
§ 1 sample = 0
pmol/ml/mg
15min 30min 60min 180min
B: Femoral midshaft (cortical bone)
veh
CNP
C: Ear cartilage
D: Kidney
E: Liver
F: Heart
#n=1
G: Lung
H: Brain
#n=1

FIGURE 77

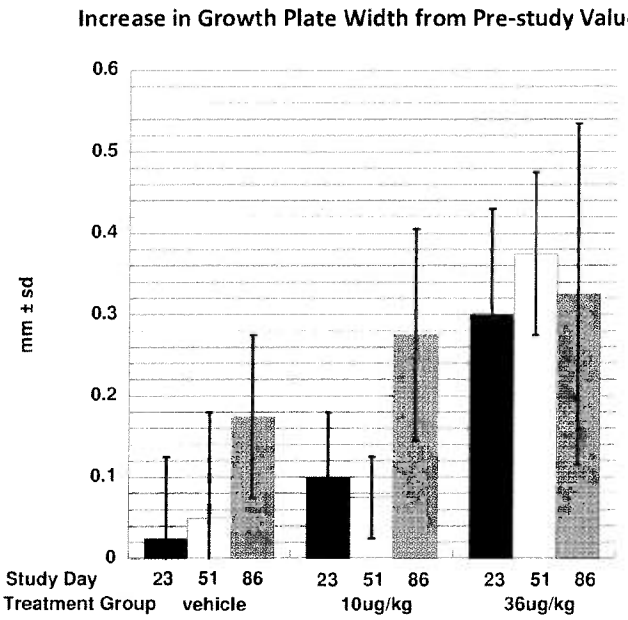


FIGURE 79

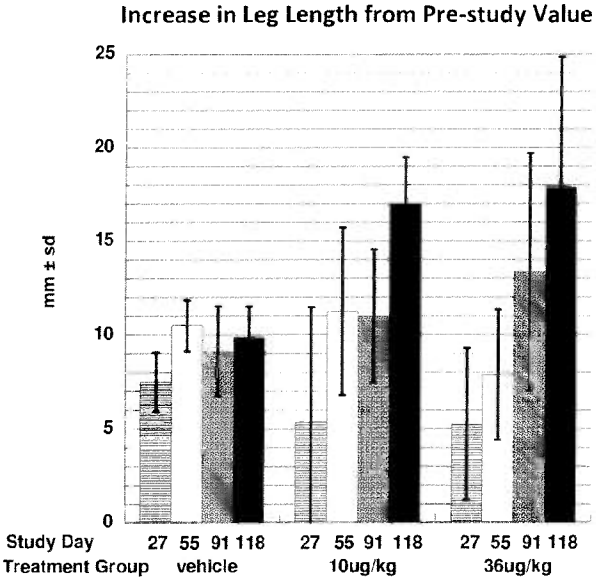
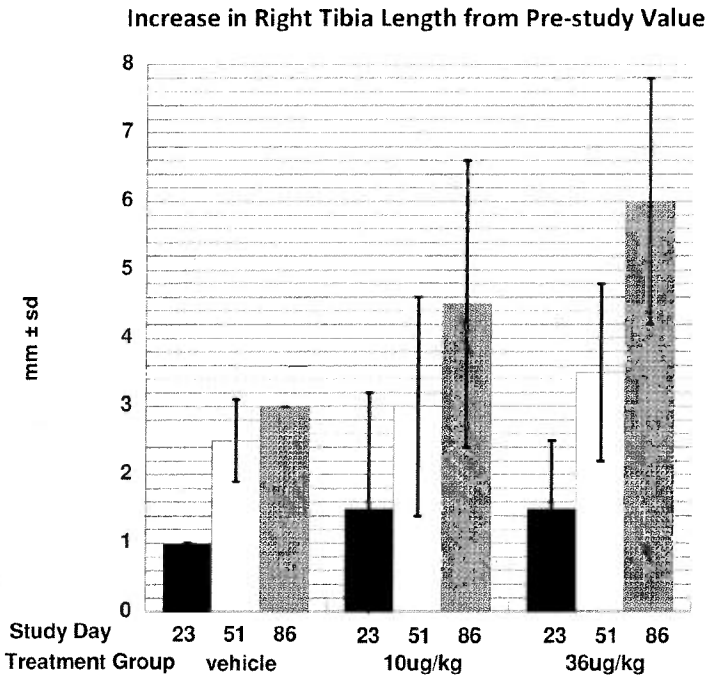


FIGURE 78

A.


Increase in Right Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

B.

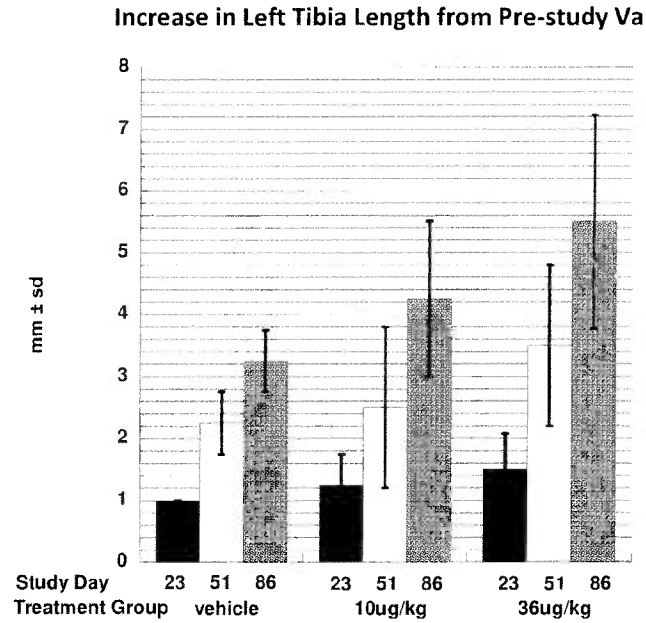

Increase in Left Tibia Length from Pre-study Value
mm ± sd
0
1
2
3
4
5
6
7
8
Study Day
23 51 86 23 51 86 23 51 86
Treatment Group
vehicle
10ug/kg
36ug/kg

FIGURE 80

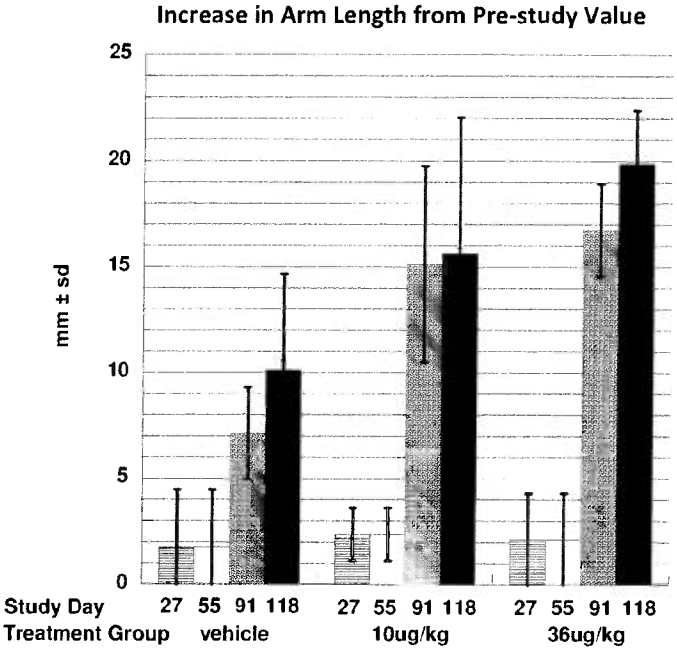


FIGURE 81

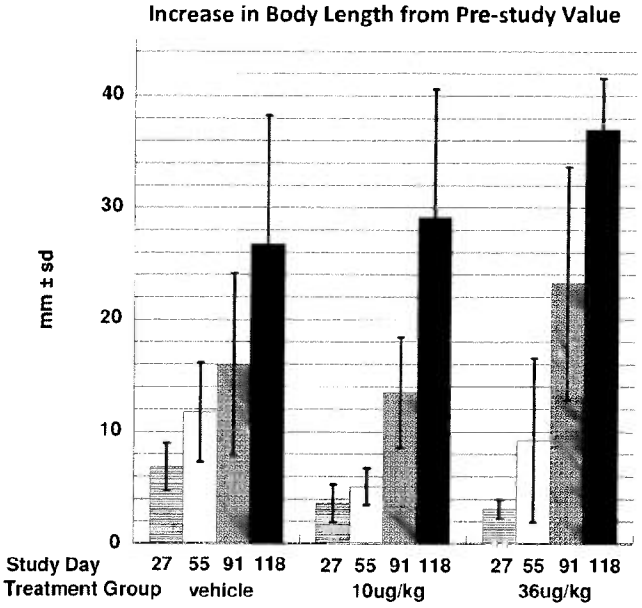


FIGURE 82

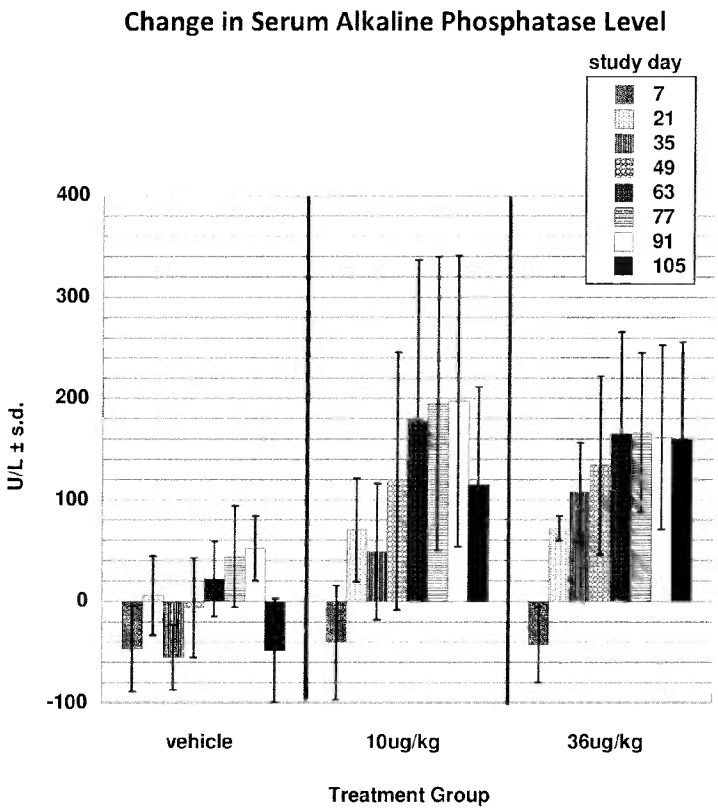
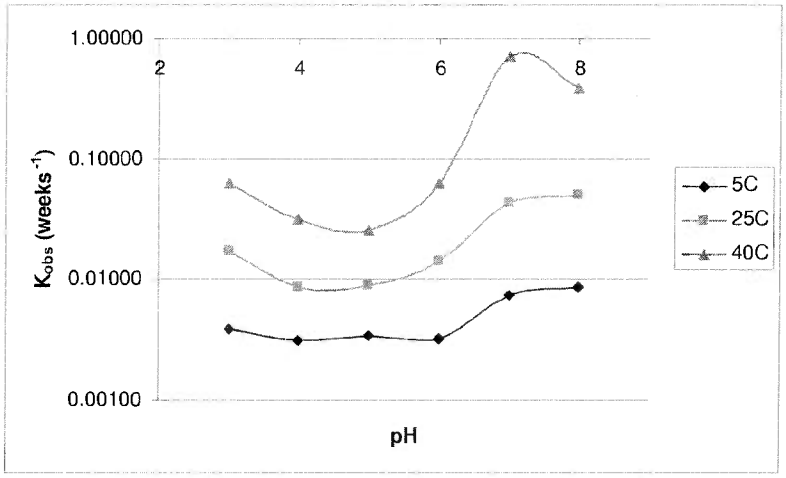


FIGURE 83



Publication Number: WO 2021/030411 A1
Publication Date: Feb 18, 2021

Abstract

The present disclosure, relates, in general, to hydrophobic salts of hydrophilic peptides that form low solubility materials in aqueous solutions and are capable of extended or sustained release of the peptide component when administered to a subject. Hydrophobic salts of C-type natriuretic peptides and uses thereof are also disclosed.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 February 2021 (18.02.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/030411 A1

(51) International Patent Classification:
A61K 47/52 (2017.01) *A61P 19/00* (2006.01)
A61K 47/54 (2017.01)

(21) International Application Number:
PCT/US2020/045885

(22) International Filing Date:
12 August 2020 (12.08.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/885,705	12 August 2019 (12.08.2019)	US
62/935,052	13 November 2019 (13.11.2019)	US
62/963,354	20 January 2020 (20.01.2020)	US
62/964,848	23 January 2020 (23.01.2020)	US
63/038,652	12 June 2020 (12.06.2020)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: HYDROPHOBIC PEPTIDE SALTS FOR EXTENDED RELEASE COMPOSITIONS

(57) Abstract: The present disclosure, relates, in general, to hydrophobic salts of hydrophilic peptides that form low solubility materials in aqueous solutions and are capable of extended or sustained release of the peptide component when administered to a subject. Hydrophobic salts of C-type natriuretic peptides and uses thereof are also disclosed.

WO 2021/030411 A1

HYDROPHOBIC PEPTIDE SALTS FOR EXTENDED RELEASE COMPOSITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. Provisional Patent Application No. 62/885,705, filed August 12, 2019, U.S. Provisional Patent Application No. 62/935,052, filed November 13, 2019, U.S. Provisional Patent Application No. 62/963,354, filed January 20, 2020, U.S. Provisional Patent Application No. 62/964,848, filed January 23, 2020, and U.S. Provisional Patent Application No. 63/038,652, filed June 12, 2020, herein incorporated by reference in their entireties.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification as a text file. The name of the text file containing the Sequence Listing is " 54627_Seqlisting.txt", which was created on August 6, 2020 and is 54,454 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

FIELD OF THE DISCLOSURE

[0003] The present disclosure, relates, in general, to hydrophobic salts of hydrophilic peptides that form low solubility materials in aqueous solutions and are capable of extended or sustained release of the peptide component when administered to a subject.

BACKGROUND

[0004] Sustained delivery therapeutics are desirable, for example, to reduce the number of doses, or reduce the amount of drug a subject may receive in order to achieve a therapeutic benefit. However, certain types of active ingredients in drugs are difficult to formulate into sustained release compositions (e.g., enterically coated, dose responsive capsules or tablets, or microspheres, such as liposomes or nanoparticles), that may deliver the therapeutic at certain sites in vivo or have certain slow release properties that allow drug to escape from the particle slowly over time.

SUMMARY

[0005] The present disclosure relates to compositions comprising salts of electrostatically charged peptides that have low solubility in solution such that the salts form solids or semi-solids in aqueous medium. It is shown here that such salts have slower dissolution rates than

the non-salt form of the peptide in aqueous solution, and can be used for extended release therapeutics without having to be reformulated in a typical extended release format.

[0006] Provided herein is a composition comprising a hydrophobic salt of an electrostatically charged peptide, the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion. In various embodiments, the salt is a hydrophobic peptide salt.

[0007] In various embodiments, the % peptide in the peptide salt by weight is at least about 10% peptide by weight in the peptide salt. In various embodiments, the peptide in the peptide salt by weight is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70% or more. In various embodiments, the % of active peptide in the peptide salt by weight is at least about 5% by weight. In various embodiments, % of active peptide in the peptide salt by weight is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35% or more. In various embodiments, the % active peptide as compared to the total peptide present in the salt is at least about 50%, 60%, 70%. 80% or more. In various embodiments, the hydrophobic peptide salts have slow dissolution, and are not immediately soluble at 1 mg/mL in 1x phosphate buffered saline (PBS).

[0008] In various embodiments, the hydrophobic counterion is complexed to the electrostatically charged peptide via a non-covalent bond.

[0009] In various embodiments, the hydrophobic peptide salt further comprises a polyvalent cation complexed to the peptide-hydrophobic counterion complex. In various embodiments, the electrostatically charged peptide, hydrophobic counterion, and polyvalent cation are complexed via non-covalent bonds. In various embodiments, the polyvalent cation complexed to the peptide-hydrophobic counterion complex is a metal cation.

[0010] In various embodiments, the peptide salt comprising the hydrophobic counterion has a cLogP of about 0 to about 10, or the conjugate acid of the hydrophobic counterion has a pKa of -2 to 5, or both. In various embodiments, the peptide salt comprising the hydrophobic counterion has a cLogP of about 2 to about 9, or the conjugate acid of the hydrophobic counterion has a pKa less than about 5, or both. In various embodiments, the peptide salt comprising the hydrophobic counterion has a cLogP of about 2 to about 9, and the conjugate acid of the hydrophobic counterion has a pKa less than about 5. In various embodiments, the peptide salt comprising the hydrophobic counterion has a cLogP of about 2 to about 9, and the conjugate acid of the hydrophobic counterion has a pKa of about 0 to about 5.

[0011] In various embodiments, the hydrophobic counterion is selected from the group consisting of a deprotonated fatty acid, a deprotonated bile acid, naphthoate and derivatives

thereof, nicotinate and derivatives thereof, an alkyl sulfonate, a dialkyl sulfosuccinate, a phospholipid, an alkyl sulfonate, an aryl sulfonate, an alkylbenzene sulfonate, an alkyl sulfate, an aryl sulfate, a dextran sulfate, an alkylbenzene sulfate, an ionic surfactant, and a combination of any of the foregoing. In various embodiments, the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristate, palmitate, stearate, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidate, decanoate, 2-naphthalenesulfonate, 1-heptanesulfonate, 1-octanesulfonate monohydrate, 1-decanesulfonate, dodecyl sulfate, dextran sulfate, and dodecyl benzenesulfonate. In various embodiments, the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate. In various embodiments, the counterion is selected from the group consisting of oleate, pamoate, deoxycholate, decanoate and docusate.

[0012] In various embodiments, the polyvalent cation has a charge of +2, +3 or +4, or higher. In various embodiments, the polyvalent cation has a charge of +2, +3 or +4. In certain embodiments, the polyvalent cation has a charge of +2. In certain embodiments, the polyvalent cation has a charge of +3. In certain embodiments, the polyvalent cation has a charge of +4. In various embodiments, the cation is a metal cation. In various embodiments, the cation comprises a metal selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), iron (Fe), manganese (Mn), cobalt (Co), nickel (Ni), titanium (Ti), vanadium (V), platinum (Pt), copper (Cu) and gold (Au). In various embodiments, the cation comprises zinc or calcium. In various embodiments, the cation is Mg^{2+}, Zn^{2+} or Ca^{2+}. In various embodiments, the cation is Zn^{2+} or Ca^{2+}. In certain embodiments, the cation is Zn^{2+}. In certain embodiments, the cation is Ca^{2+}.

[0013] In various embodiments, the peptide salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form. In various embodiments, the peptide salt is in solid form. In various embodiments, the peptide salt is in an amorphous form. In various embodiments, the peptide salt is in gel form. In various embodiments, the peptide salt is suspended in or linked to a gel.

[0014] In various embodiments, the electrostatically charged peptide is a C-type natriuretic peptide (CNP). In various embodiments, the CNP is a CNP variant. CNP and CNP variants contemplated herein are described more fully in the Detailed Description. In various

embodiments, the CNP is complexed to the hydrophobic counterion to form a hydrophobic CNP salt complex. In various embodiments, the hydrophobic CNP salt further comprises a polyvalent cation complexed to the CNP-hydrophobic counterion complex, forming a CNP-cation-hydrophobic counterion salt complex. In various embodiments, the polyvalent cation is a metal cation.

[0015] In various embodiments, the CNP is selected from the group consisting of: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1) LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4) and salts thereof. In various embodiments, the CNP salt useful for forming the hydrophobic CNP salt herein, is CNP-acetate.

[0016] In various embodiments, the CNP is selected from the group consisting of PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5); PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1); PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6); PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6); PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5); and PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 7).

[0017] In various embodiments, the CNP variant peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the acetyl group is on an amino acid side chain within the peptide sequence. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus.

[0018] In various embodiments, the CNP variant is selected from the group consisting of

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8);

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9);

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10);

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11);

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12);

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 13); and

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 14).

[0019] In various embodiments, the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate. In various embodiments, if a polyvalent cation is present, the polyvalent cation comprises zinc or calcium. In various embodiments, if a polyvalent cation is present, the polyvalent cation comprises magnesium, zinc or calcium. In various embodiments, the cation is Mg^{2+}, Zn^{2+} or Ca^{2+}. In various embodiments, the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the polyvalent cation is Zn^{2+}. In various embodiments, the polyvalent cation is Ca^{2+}.

[0020] In various embodiments, the composition further comprises an excipient, diluent or carrier. In various embodiments, the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier. Also provided is a sterile pharmaceutical composition comprising the hydrophobic salt composition described herein.

[0021] Further contemplated by the disclosure is a modified release composition comprising a hydrophobic peptide salt described herein. In various embodiments, the modified release composition is an extended release composition, sustained release composition or delayed release composition.

[0022] In various embodiments, the extended release composition comprises a hydrophobic peptide salt, wherein the peptide salt solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle is resuspended in an aqueous solution or in oil. In various embodiments, the aqueous solution is water, saline, or buffer.

[0023] In various embodiments, the oil comprises a triglyceride or a fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. In various embodiments, the fatty acid is a short chain, medium chain or long chain fatty acid. In various embodiments, when the fatty acid is in a triglyceride, the fatty acid is saturated or unsaturated, and can be a medium chain or long chain fatty acid.

[0024] In various embodiments, the fatty acid is a C-6 to C-20 fatty acid. In various embodiments, the fatty acid is a C-6, C-8, C-10, C-12, C-14, C-16, C-18, or C-20 fatty acid. In various embodiments, the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid.

[0025] In various embodiments, for the extended release composition, (i) less than about 20% of peptide is released by day 1; and (ii) about 90% of peptide is released weekly, or about

90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6.

[0026] In various embodiments, less than about 20% of peptide is released by day 1 at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50%, or about 60% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50%, or about 60% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6.

[0027] In various embodiments, (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60% about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6; or alternatively ii) about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released bi-weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released every three weeks; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released monthly, at pH 7 to 7.6

[0028] In various embodiments, about 90% of peptide is released weekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released bi-weekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that the release can be at a pH between pH 7.0 to 7.6, between pH 7.1 to 7.5, between pH 7.2 to 7.4, between pH 7.2 to 7.6, or between pH 7.0 to 7.4.

[0029] In various embodiments, the extended release composition comprises an excipient, diluent or carrier. In various embodiments, the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier. In various embodiments, provided is a sterile pharmaceutical composition comprising the extended release composition.

[0030] Also provided herein is a method of making a hydrophobic peptide salt composition as described herein, such as a hydrophobic CNP salt. Ionic surfactants are good candidates as counterions since the polar headgroup is perpetually charged irrespective of complexation pH. Modulation of pH at which complexation occurs results in different amounts of charge on the peptide and this allows for the control of peptide:surfactant complexation stoichiometry and possibly control over resulting precipitate size. Metal cations can be used as bridges for anionic side chains of peptide amino acids to bind to anionic hydrophobic counterions. The order and rate of cation and counterion addition to the peptide is important to minimize precipitation of metal cation with anionic hydrophobic counterions.

[0031] In various embodiments, the disclosure contemplates a method of making a composition comprising a salt of an electrostatically charged peptide comprising, a) contacting an electrostatically charged peptide in an aqueous solution with a hydrophobic counterion in solution; b) mixing the electrostatically charged peptide solution with the hydrophobic counterion solution in a manner sufficient for the peptide and counterion to form a complex, wherein the formation of the peptide-counterion complex results in formation of a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form comprising the hydrophobic peptide salt. In various embodiments, the peptide salt is a hydrophobic CNP salt.

[0032] In various embodiments, the method optionally comprises before step (b), contacting the electrostatically charged peptide in solution with a polyvalent cation in aqueous solution, forming a peptide-cation complex. In various embodiments, the polyvalent cation is a metal cation.

[0033] In various embodiments, the mixing is carried out by dropwise addition of the hydrophobic counterion solution to the electrostatically charged peptide solution. In various embodiments, the solutions are mixed by vortexing after addition of each drop of hydrophobic counterion solution, or other means for mixing known in the art.

[0034] In various embodiments, the method further comprises step (c) washing the peptide salt in buffer or water. In various embodiments, the washing is carried out in an aqueous solution, e.g., buffer or water.

[0035] In various embodiments, the method further comprises step (d) obtaining the peptide salt by centrifugation to form a peptide salt pellet. In various embodiments, if the salt is in gel form, the salt is obtained by centrifugation or by decanting the liquid phase followed by lyophiliation or other drying methods

[0036] In various embodiments, the method further comprises step (e) removing water from the peptide salt pellet. It is contemplated that water or another aqueous solution can be removed from the pellet by lyophilization or drying using techniques known in the art.

[0037] In various embodiments, the method further comprises resuspending the pellet in an aqueous solution or oil. In various embodiments, the aqueous solution is water, saline, or buffer. In various embodiments, the oil comprises a triglyceride or a fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. In various embodiments, the fatty acid in the triglyceride is saturated or unsaturated, or combinations thereof.

[0038] The fatty acid may be the oil itself or in a triglyceride. In various embodiments, the fatty acid is a short chain, medium chain or long chain fatty acid. In various embodiments, when the fatty acid is in a triglyceride, the fatty acid is saturated or unsaturated, and can be a medium chain or long chain fatty acid. In various embodiments, the fatty acid is a C-6 to C-20 fatty acid. In various embodiments, the fatty acid is a C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acid. In various embodiments, the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid.

[0039] In various embodiments, the synthetic method contemplates using a peptide: hydrophobic counterion ratio of at least one molar equivalent of hydrophobic counterion to the total number of positively charged amino acids in the peptide. In various embodiments the peptide: hydrophobic counterion ratio used in the synthetic method is between 1:1 to 1:20, or from 1:1 to 1:50. The peptide: counterion ratio used in the synthetic method can be between 1:2 to 1:15, 1:2 to 1:10, 1:2 to 1:8, 1:3 to 1:10, or 1:4 to 1:10. In various embodiments, the peptide: counterion ratio is 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19 or 1:20. In various embodiments, the peptide: hydrophobic counterion ratio used in the synthetic method is at least two molar equivalents of hydrophobic counterion to total number of positively charged amino acids in the peptide. In various embodiments, the peptide: hydrophobic counterion ratio used in the synthetic method is at least

three molar equivalents of hydrophobic counterion to total number of positively charged amino acids in the peptide.

[0040] In various embodiments, the synthetic method contemplates using a peptide: cation ratio of at least one molar equivalents of cation to total number of negatively charge amino acids in the peptide. In various embodiments, the peptide: cation ratio used in the synthetic method is between 1:1 to 1:10. The peptide: cation ratio used in the synthetic method can be 1:2 to 1:10, 1:3 to 1:10, 1:1 to 1:5, 1:2 to 1:5, of 1:2 to 1:8. In various embodiments, the peptide: cation ratio used in the synthetic method is 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, or 1:10. In various embodiments, the peptide: cation ratio used in the synthetic method at least two molar equivalents of cation to total number of negatively charged amino acids in the peptide. In various embodiments, the peptide: cation ratio used in the synthetic method is at least three molar equivalents of cation to total number of negatively charged amino acids in the peptide. Further contemplated are combinations of the peptide: cation and peptide: hydrophobic counterion ratios described above.

[0041] An exemplary ratio is one counterion per positive charge on a peptide. For the polyvalent cation, an exemplary ratio is approximately 1 metal cation per negative charge site in the peptide, and 2 polyvalent cations per negative charge site, or a 2X molar excess. For example, one or two polyvalent cations such as Zn^{2+} or Ca^{2+} can be used in combination with 6-8 counterions, or more if a hydrophobic interaction is involved.

[0042] In various embodiments, the hydrophobic counterion is complexed via a non-covalent bond.

[0043] In various embodiments, if the salt complex further comprises a polyvalent cation complexed to the peptide-counterion complex, the cation is complexed via a non-covalent bond. In various embodiments, the electrostatically charged peptide, hydrophobic counterion, and cation are complexed via a non-covalent bonds.

[0044] In various embodiments, the CNP is selected from the group consisting of: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1).

[0045] Also provided is a method of treating a bone-related disorder or skeletal dysplasia in a subject in need thereof comprising administering to the subject a composition comprising a hydrophobic salt of C-type natriuretic peptide (CNP), including compositions and extended release compositions as described herein.

[0046] In various embodiments, the bone-related disorder or skeletal dysplasia is selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature.

[0047] In various embodiments the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

[0048] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome).

[0049] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome), or insulin growth factor 1 receptor (IGF1R).

[0050] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3.

[0051] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0052] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from a RASopathy. In various embodiments, the RASopathy is Noonan

syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, or LEOPARD syndrome.

[0053] In one embodiment, the RASopathy is hereditary gingival fibromatosis type 1.

[0054] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R).

[0055] In various embodiments, the short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0056] In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and having at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. In various embodiments, the short stature is associated with one or more mutations in a gene associated with short stature, such as, collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R), or combinations thereof. In various embodiments, the short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0057] In various embodiments, the short stature is a result of mutations in multiple genes as determined by polygenic risk score (PRS). In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in FGFR3 and a low PRS. In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in IGF1R and a low PRS. In various embodiments, the subject has a mutation in NPPC and a low PRS. In various embodiments, the subject has a mutation in SHOX and a low PRS. In various embodiments, the subject has one or more mutation in one or more of FGFR3, IGF1R, NPPC, NPR2 and SHOX, and a low PRS. In various embodiments, the PRS is 1 or 2. In various embodiments, the PRS is 1. In various embodiments, the PRS is 2.

[0058] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the acetyl group is on an amino acid side chain within the peptide sequence. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker. In various embodiments, the variant is a hydrophobic salt of an electrostatically charged CNP peptide, the salt comprising the electrostatically charged CNP peptide complexed with a hydrophobic counterion.

[0059] The disclosure also contemplates a method of elongating a bone or increasing long bone growth in a subject in need thereof, comprising administering to the subject a sustained release composition comprising a salt of C-type natriuretic peptide (CNP), and wherein the administering elongates a bone or increases long bone growth, including compositions and extended release compositions as described herein.

[0060] In various embodiments, the CNP is a CNP variant. In various embodiments, the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4); and,
pharmaceutical salts thereof. In various embodiments, the CNP is a CNP-acetate.

[0061] In various embodiments, the CNP is selected from the group consisting of
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5); and
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 7).

[0062] In various embodiments, the CNP variant further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various

embodiments, the acetyl group is on a side group of an amino acid of the peptide. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus.

[0063] In various embodiments, the variant is selected from the group consisting of

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8);

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2; (SEQ ID NO: 9)

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10);

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11);

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12);

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 13); and

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 14).

[0064] In various embodiments, the composition is administered subcutaneously, intradermally, intraarticularly, orally, or intramuscularly.

[0065] In various embodiments, the composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

[0066] In various embodiments, the composition is an extended release composition.

[0067] Also provided is a hydrophobic salt of C-type natriuretic peptide (CNP) comprising a CNP in complex with a hydrophobic counterion. In various embodiments, the hydrophobic CNP salt further comprises a cation in complex with the CNP and hydrophobic counterion. In various embodiments, the hydrophobic CNP salt is a purified salt. In various embodiments, the salt has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% purity, or more.

[0068] In various embodiments, the hydrophobic salt comprising a hydrophobic counterion selected from the group consisting of oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate. In various embodiments, hydrophobic salt comprises a cation of Zn^{2+} or Ca^{2+}.

[0069] In various embodiments, the hydrophobic salt is selected from the group consisting of CNP-oleate, CNP-pamoate, CNP-deoxycholate, CNP-decanoate and CNP-docusate. In various embodiments, he hydrophobic salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), CNP–Ca^{+2}(deoxycholate), CNP–Ca^{+2}(decanoate), CNP–Ca^{+2}(docusate),

CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), CNP–Zn^{+2}(deoxycholate), CNP–Zn^{+2}(decanoate), and CNP–Zn^{+2}(docusate).

[0070] In various embodiments, the hydrophobic salt comprises a CNP selected from the group consisting of: PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1), LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4), and salts thereof. In various embodiments, the hydrophobic salt comprises a CNP which is CNP-acetate.

[0071] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the acetyl group is on a side group of an amino acid of the peptide. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker. In various embodiments, the peptide comprises a hydrophobic salt of an electrostatically charged peptide, the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion.

[0072] The disclosure also provides a composition comprising a hydrophobic peptide salt, e.g., a hydrophobic CNP salt, as described herein for use in treating skeletal dysplasia or bone-related disorder as described herein. In certain embodiments, the disclosure provides use of a composition comprising a hydrophobic peptide salt, e.g., a hydrophobic CNP salt, as described herein in the preparation of a medicament for treating skeletal dysplasia or bone-related disorder as described herein. In various embodiments, the hydrophobic peptide salt is a hydrophobic CNP salt as described herein.

[0073] It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the invention and, as such, is meant to be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as “one embodiment”, “some embodiments”, “certain embodiments”, “further embodiment”, “specific exemplary embodiments”, and/or “another embodiment”, each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination.

Such features or combinations of features apply to any of the aspects of the invention. Where examples of values falling within ranges are disclosed, any of these examples are contemplated as possible endpoints of a range, any and all numeric values between such endpoints are contemplated, and any and all combinations of upper and lower endpoints are envisioned.

[0074] The headings herein are for the convenience of the reader and not intended to be limiting. Additional aspects, embodiments, and variations of the invention will be apparent from the Detailed Description and/or Drawing and/or claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0075] Figures 1A-1D show dissolution profiles of the different hydrophobic CNP salts in water at pH 6.5.

[0076] Figure 2 shows dissolution profiles of the different hydrophobic CNP salts in water at pH 6.5. The control is CNP-acetate.

[0077] Figure 3 shows the effects of a CNP variant (Pro-Gly-CNP37) on cells carrying either NPR2 homozygous or heterozygous mutations, as measured by cGMP stimulation.

[0078] Figure 4 shows the nucleotide and predicted protein sequence of the first exon in NPR2 mutant clones transfected into RCS cells.

[0079] Figure 5 shows exemplary NPR2 mutations analyzed for response to CNP

[0080] Figure 6 shows exemplary mutations associated with short stature in FGFR3, IGF1R, NPPC, NPR2 and SHOX.

[0081] Figures 7A-7F illustrate the combined effect of PRS and rare coding variants on Height. Fig. 7A. Effects on height as a quantitative trait, samples were divided in five groups based on their PRS, violin-plots with horizontal lines representing the 25%, 50% and 75% percentile of height. Samples were grouped by carrying status of missense, loss of function or None in any of the five core genes. Fig. 7B. Effect reflected on Odds ratios for "Idiopathic Short Stature" or ISS. Odds for ISS using PRS =3 as reference vs the other PRS groups. Fig. 7C. Odds for ISS using PRS =1 as reference vs having missense and/or loss of function variants in core genes. Fig. 7D. Odds for ISS using PRS =1 non-carriers as reference vs having missense and/or loss of function variants in core genes. Fig. 7E. Odds for ISS using PRS =2 non-carriers as reference vs having missense and/or loss of function variants in core genes. Fig. 7F. Odds

for ISS using PRS =3 non-carriers as reference vs having missense and/or loss of function variants in core genes.

[0082] Figure 8A shows the release profile of CNP Zn-pamoate salt. Data shown is an average of 3 wells. Figure 8B show the release profiles of different CNP peptide salts. Data shown is an average of 4 wells.

[0083] Figures 9A-9B illustrate the dissolution profiles of CNP pamoate salts as cumulative release profile (Figure 9A) or % released (Figure 9B). Data shown is an average of 3 wells for the 1xPBS data, and 2 wells for the 1x PBS+0.05% PS80 data.

[0084] Figures 10A to 10C show the dissolution profiles of the docusate salts either after lyophilization and storage (Figure 10A), or made fresh and then analyzed (Figures 10B-10C).

[0085] Figure 11 shows the release profile of CNP salt over 7 days in vivo.

DETAILED DESCRIPTION

[0086] The present disclosure relates to salts of hydrophilic peptides that are solid, semi-solid gel or other salt form that are capable of extended release of the peptide active ingredient when put in aqueous solutions. For example, it is shown herein, that unexpectedly, complexation of a hydrophilic C-type natriuretic peptide (CNP) with charged, hydrophobic counterions generates low solubility peptide salts under aqueous conditions. The present disclosure shows that the peptide-hydrophobic counterion salt complex itself, including salts comprising a peptide-counterion-cation complex, can be used in a modified or extended release composition without having to encapsulate the peptide complex into a liposome or microsphere/nanoparticle. Such compositions are useful for extended release applications, e.g., in treating skeletal dysplasias and bone growth disorders as described herein.

[0087] As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

[0088] The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood

that the term "about" or "approximately" applies to each one of the numerical values in that series.

[0089] The term "electrostatically charged peptide" as used herein refers to a peptide, e.g., a string of amino acids from 5 to 100 amino acids, that comprises charged amino acids. The peptide can have positively charged amino acids, negatively charged amino acids, or a mixture of both, such that the electrostatically charged peptide has an overall net charge and is capable of interacting with other charged moieties, e.g., a cation, anion or a counterion having charged species opposite to those in the peptide to which the counterion may bind. The electrostatically charged peptide can have a net positive or a net negative charge. When the peptide has a net positive charge, it can interact with a charged moiety that has one or more negative charges. When the peptide has a net negative charge, it can interact with a charged moiety that has one or more positive charges.

[0090] The term "hydrophobic counterion" as used herein refers to a group of electrostatically charged moieties that are hydrophobic in nature and are capable of interacting with to hydrophilic peptides. In various embodiments, the hydrophobic counterion is selected based on its cLogP value, the pKa value of its conjugate acid, or both. In various embodiments, a hydrophobic counterion has a cLogP of about 0 to about 10, or a pKa of its conjugate acid of about -2 to about 5, or both. In various embodiments, the hydrophobic counterion has a net negative charge and can interact with an electrostatically charged peptide having a net positive charge. In various embodiments, hydrophobic counterions include deprotonated fatty acids, deprotonated bile acids, naphthoate and derivatives thereof, nicotinate and derivatives thereof, alkyl sulfonates, dialkyl sulfosuccinates, phospholipids, alkyl sulfonates, aryl sulfonates, alkylbenzene sulfonates, alkyl sulfates, aryl sulfates, dextran sulfates, ionic surfactants, and alkylbenzene sulfates. In some embodiments, the hydrophobic counterion is a zwitterion (e.g., phosphatidylethanolamine). In various embodiments, hydrophobic counterions include, but are not limited to, palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristate, palmitate, stearate, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidate, decanoate, 2-naphthalenesulfonate, 1-heptanesulfonate, 1-octanesulfonate monohydrate, 1-decanesulfonate, dodecyl sulfate, dextran sulfate, and dodecyl benzenesulfonate. In some embodiments, the hydrophobic counterion has a net positive charge and can interact with an electrostatically charged peptide having a net negative charge.

[0091] The term "peptide salt" or "hydrophobic peptide salt", as used herein refers to a complex between an electrostatically charged peptide and a counterion, e.g., a hydrophobic counterion, such that the moieties are in a complex and form a salt. The peptide and counterion may be complexed non-covalently. In various embodiments, the peptide–counterion salt further comprises a polyvalent cation, such that the complex contains a peptide-cation-counterion in complex. Peptide salt or hydrophobic peptide salt refers to both a peptide-counterion complex and a peptide-cation-counterion complex.

[0092] In various embodiments, the peptide and cation are complexed non-covalently. In various embodiments, the peptide, cation, and hydrophobic counterion in the peptide salt are complexed via non-covalent bonds.

[0093] The term "C-type natriuretic peptide" or "CNP" refers to a small, single chain peptide having a 17-amino acid loop structure at the C-terminal end (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) and variants thereof. CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide, which is cleaved to generate pro-CNP, and an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22). "CNP salt" or "hydrophobic CNP salt" refers to salts as described herein comprising a counterion, such as a hydrophobic counterion, respectively, and optionally, further comprising a polyvalent cation, and comprising a CNP or CNP variant.

[0094] In various embodiments, a "CNP variant" is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. In various embodiments, a CNP variant peptide may comprise from about 1 to about 53, or 1 to 38, or 1 to 37, or 1 to 35, or 1 to 34, or 1 to 33, or 1 to 32, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, a CNP variant may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

[0095] Provided herein are modified release compositions comprising a hydrophobic peptide salt described herein. Modified-release compositions include those that deliver a drug with a delay after its administration (delayed-release dosage) or for a prolonged period of time (extended-release dosage). Various embodiments of a peptide salt provided herein include modified-release compositions, such as extended release, sustained or controlled release, and

delayed release. The term "extended release composition" refers to a composition formulated in a manner in order to make the active ingredient/drug available over an extended period of time following administration (US Pharmacopeia). Extended-release dosage include sustained-release (SR) or controlled-release (CR) forms. Sustained release maintains drug release over a sustained period but not necessarily at a constant rate, while CR maintains drug release over a sustained period at a nearly constant rate (Pharmaceutics: Drug Delivery and Targeting, Yvonne Perrie, Thomas Rades, Pharmaceutical Press, 2009). Delayed-release compositions or products are modified to delay release of the drug substance for some period of time after initial administration.

[0096] The term "effective amount" refers to a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

[0097] "Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

[0098] A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs or symptoms of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

[0099] A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or

physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

[0100] "Pharmaceutical composition" or "formulation" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of a hydrophobic peptide salt, e.g., a CNP salt, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

[0101] "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

[0102] A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

[0103] By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

[0104] "Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

[0105] As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender. In various embodiments, the subject is human. In various embodiments the subject is a child or adolescent. In various embodiments, the subject is an infant.

Electrostatically Charged Peptides and Peptide Salts

[0106] Peptide therapeutics are attractive biological therapeutic agents, but are often disadvantaged by low stability and short half-life in solution (Tang et al., Eur J Pharm Sci. 102:63-70, 2017). Attempts to improve efficacy of peptide therapeutics include attempts to encapsulate hydrophilic peptides into biodegradable particles such as liposomes or polymer particles. However, this has been difficult due to the cationic nature of these peptides and their ability to electrostatically interact with liposomes of negatively charged polymers (Griesser et al., Int J Pharmaceutics 520:267-274, 2017). Generation of hydrophobic ion pairs between hydrophilic peptides and hydrophobic moieties has been one means used to enable better encapsulation of hydrophilic polymers into microparticles or liposomes (Lu et al., Mol. Pharmaceutics 15:216-225, 2018). Hydrophobic ion pairs are formed when charged residues in the peptides interact with the oppositely charged ions in the hydrophobic moiety (Tang et al., *supra*). In certain instances, it can lead to precipitation of the hydrophobic ion pair out of solution making it easier to encapsulate into a liposome or polymeric nanoparticle (Griesser et al., *supra*)

[0107] It has been discovered herein that hydrophobic ion complexes between hydrophilic CNP peptides and hydrophobic counterions generate CNP peptide salts. Generation of

hydrophobic ion pairs between hydrophilic peptides and hydrophobic counterions can be enhanced by first contacting the hydrophilic peptide with a polyvalent cation (e.g., a metal cation) to enhance the interaction between the peptide and hydrophobic counterion. For example, the polyvalent cation can complex with the negatively-charged functional groups of the hydrophilic peptide, increasing the number of positive charges on the hydrophilic peptide available for complexation with the hydrophobic counterion, such as a hydrophobic anion. Thus, the polyvalent cation can bridge together the negative charges of the hydrophilic peptide and the negative charges of the hydrophobic counterion. Additionally, the present disclosure shows that the peptide-hydrophobic counterion salt complex or peptide-cation-hydrophobic counterion salt complex itself can be used in a modified or extended release composition without having to encapsulate the peptide complex into a liposome or microsphere/nanoparticle.

[0108] Electrostatically charged peptides can be a string of amino acids from 5 to 100 amino acids that comprise charged amino acids and have an overall net charge. The peptide can have positively charged amino acids, negatively charged amino acids, or a mixture of both, such that the electrostatically charged peptide is capable of interacting with other charged moieties, e.g., a cation, anion or a counterion or a combination thereof having charged species opposite to those in the peptide. In various embodiments, the electrostatically charged peptide has a net positive charge. An electrostatically charged peptide having a net positive charge can complex with a hydrophobic counterion having negative charges, such as a hydrophobic counterion having a net negative charge. In various embodiments, the electrostatically charged peptide has a net negative charge. An electrostatically charged peptide having a net negative charge can complex with a hydrophobic counterion having positive charges, such as a hydrophobic counterion having a net positive charge. In various embodiments, the electrostatically charged peptides have at least two amino acids having the same type of charge (e.g., two positively charged amino acids or two negatively charged amino acids).

[0109] Hydrophilic peptides refer to peptides that have high solubility in aqueous solution. Hydrophilic peptides contemplated herein include peptides between 5 and 100 amino acids, that have a net charge of between +3 to +15, or between +4 and +15, or between +3 to +12, or between +4 and +12, or +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, or +15 or any range between these numbers. In various embodiments, the hydrophilic peptide has solubility of greater than 10 mg/mL in aqueous solution, or greater than 5 mg/mL solubility. In various embodiments, hydrophilic peptide also refers to peptides that have high solubility in aqueous solution, e.g., having cLogP of less than 1.

[0110] *Hydrophobic counterions*

[0111] In order to generate a salt of an electrostatically charged peptide as described herein, the peptide is complexed with a counterion. In the case of hydrophilic peptides, the counterion is a hydrophobic counterion. In various embodiments, the hydrophobic counterion has a net negative charge and forms a salt with a hydrophilic peptide having a net positive charge.

[0112] It is contemplated that the counterion is complexed to the charged peptide via non-covalent bonding. The counterion may be non-covalently associated with the peptide via electrostatic interactions.

[0113] When a hydrophobic counterion is used, the hydrophobic counterion exhibits a cLogP of about 0 to about 10, or its conjugate acid exhibits a pKa from about -2 to about 5, or both. In various embodiments, the hydrophobic counterion has a cLogP of about 2 to about 9, about 3 to 8, about 4 to 7, or about 5 to 9. In various embodiments, the cLogP is about 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In various embodiments, the conjugate acid of the hydrophobic counterion has a pKa of about -1 to 4, 0 to 3, 0 to 5, 1 to 4 or 2 to 5. In various embodiments, the conjugate acid of the hydrophobic counterion has pKa of about -2, -1, 0, 1, 2, 3, 4, or 5. Further contemplated is a hydrophobic counterion having a combination of any of these values and ranges in between the values. In various embodiments, the hydrophobic counterion has a cLogP of about 2 to about 9, or its conjugate acid has a pKa less than about 5, or both. In various embodiments, the hydrophobic counterion has a cLogP of about 2 to about 9, and its conjugate acid has a pKa less than about 5.

[0114] In various embodiments, the counterion is an anion. In various embodiments, the counterion is a zwitterion. In various embodiments, the counterion is an anionic or zwitterionic detergent. In various embodiments, the hydrophobic counterion is selected from the group consisting of a deprotonated fatty acid, a deprotonated bile acid, a naphthoate and derivatives thereof, a nicotinate and derivatives thereof, an alkyl sulfonate, a dialkyl sulfosuccinate, a phospholipid, an alkyl sulfonate, an aryl sulfonate, an alkylbenzene sulfonate, an alkyl sulfate, an aryl sulfate, a dextran sulfate, an alkylbenzene sulfate, and ionic surfactants. In various embodiments, the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate, sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, and sodium dodecyl benzenesulfonate. In

various embodiments, the hydrophobic counterion is oleate, pamoate, deoxycholate, decanoate or docusate.

[0115] In various embodiments, at least one hydrophobic counterion is complexed to the hydrophilic peptide (when no polyvalent cation is present) or to the complex comprising the hydrophilic peptide and polyvalent cation (when cation is present). In various embodiments, at least two hydrophobic counterions are complexed to the hydrophilic peptide (when no cation is present) or to the complex comprising the hydrophilic peptide and polyvalent cation (when cation is present). In various embodiments, at least three hydrophobic counterions are complexed to the hydrophilic peptide (when no cation is present) or to the complex comprising the hydrophilic peptide and polyvalent cation (when cation is present). In various embodiments, at least four hydrophobic counterions are complexed to the hydrophilic peptide (when no cation is present) or to the complex comprising the hydrophilic peptide and polyvalent cation (when cation is present). In various embodiments, each positive charge of the hydrophilic peptide is complexed to a hydrophobic counterion. For example, if a peptide has four positively charged amino acids, then it can complex to four hydrophobic counterions. Similarly, if the peptide has four positively charged amino acids and is complexed to two cations, for a total of six positive charges, then the peptide can complex to six hydrophobic counterions. In various embodiments, not all of the positive charges of the hydrophilic peptide (when no cation is present) or of the complex comprising the hydrophilic peptide and polyvalent cation (when cation is present) are complexed to a hydrophobic counterion. For example, if a peptide has four positively charged amino acids, then it can complex to three hydrophobic counterions, or two hydrophobic counterions, or to one hydrophobic counterion. Similarly, if the peptide has four positively charged amino acids and is complexed to two cations, for a total of six positive charges, then the peptide can complex to five hydrophobic counterions, or to four hydrophobic counterions, or to three hydrophobic counterions, or two hydrophobic counterions, or to one hydrophobic counterion.

[0116] *Polyvalent Cations*

[0117] In various embodiments, the peptide salt further comprises a polyvalent cation complexed to the peptide-counterion complex. It is contemplated that the cation is complexed to the charged peptide via a non-covalent bond. The cation may be non-covalently associated with the peptide via electrostatic interactions.

[0118] It is contemplated that the polyvalent cation has a charge of +2, +3 or +4 or higher. In embodiments, the cation has a charge of +2. In embodiments, the cation has a charge of +3.

In embodiments, the cation has a charge of +4. In various embodiments, the polyvalent cation is a metal cation. Metal cations include those of the Group II and Group III metals. Suitable polyvalent cations can comprise a metal selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), iron (Fe), manganese (Mn), cobalt (Co), nickel (Ni), titanium (Ti), vanadium (V), platinum (Pt), copper (Cu) and gold (Au). In various embodiments, the polyvalent cation comprises magnesium, zinc, or calcium. In various embodiments, the polyvalent cation comprises zinc or calcium. In various embodiments, the polyvalent cation comprises zinc. In various embodiments, the polyvalent cation comprises calcium. In various embodiments, the polyvalent cation is selected from the group consisting of Mg^{2+}, Zn^{2+}, and Ca^{2+}. In various embodiments, the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the polyvalent cation is Zn^{2+}. In various embodiments, the polyvalent cation is Ca^{2+}.

[0119] In various embodiments, at least one polyvalent cation is complexed to the hydrophilic peptide. In various embodiments, at least two cations are complexed to the hydrophilic peptide. In various embodiments, at least three cations are complexed to the hydrophilic peptide. In various embodiments, each negative charge of the hydrophilic peptide is complexed to a polyvalent cation. For example, if a peptide has four negatively charged amino acids, then it can complex to four polyvalent cations, or three polyvalent cations, or two polyvalent cations, or one polyvalent cation.

[0120] *Peptide Salts*

[0121] In various embodiments, the peptide salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form. In various embodiments, the peptide salt is in solid, semi-solid, or gel form. In various embodiments, the peptide salt is in solid or gel form. In various embodiments, the peptide salt is in solid form. In various embodiments, the peptide salt is in amorphous form. In various embodiments, the peptide salt is in gel form. In various embodiments, the peptide salt is suspended in or linked to a gel.

[0122] It is contemplated that the peptide salt is in particle form, and in particular, as solid particles. In various embodiments, the particle is between 1 and 10,000 micrometers (um), between 1 um to 2000 um, between 2 um to 1000 um, between 5 um to 500 um, between 10 um to 1000 um, between 50 um to 500 um, between 100 um to 800 um, between 200 um to 600 um, between 300 um to 500 um, between 100 um to 300 um, between 50 um to 100 um, or

between 10 um to 50 um. In various embodiments, the particle is a nanoparticle. In various embodiments, the nanoparticle is between 5 nanometers (nm) to 1000 nm, between 8 nm to 900 nm, between 10 nm to 800 nm, between 20 nm to 600 nm, between 50 nm to 500 nm, between 50 nm to 400 nm, between 20 to 300 nm, between 300 to 800 nm, between 200 to 600 nm, between 100 nm to 300 nm or between 50 nm to 200 nm.

[0123] In some embodiments, the hydrophilic peptide of the peptide salt is CNP or a CNP variant, as described herein, and the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate, sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, and sodium dodecyl benzenesulfonate. In some embodiments, the hydrophilic peptide salt is CNP or a CNP variant, as described herein, and the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, and decanoate. In various embodiments, the peptide salt is selected from the group consisting of CNP-oleate, CNP-pamoate, CNP-deoxycholate, and CNP-decanoate, In some embodiments, the hydrophilic peptide salt is CNP or a CNP variant, as described herein, and the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, decanoate and docusate. In various embodiments, the peptide salt is selected from the group consisting of CNP-oleate, CNP-pamoate, CNP-deoxycholate, CNP-decanoate and CNP-docusate. In various embodiments, the peptide salt is selected from the group consisting of CNP-oleate, CNP-pamoate, and CNP-docusate. In various embodiments, the peptide salt is selected from the group consisting of CNP-deoxycholate, CNP-decanoate and CNP-docusate. In various embodiments, the peptide salt is selected from the group consisting of CNP-oleate and CNP-pamoate. In various embodiments, the peptide salt is CNP-oleate. In various embodiments, the peptide salt is CNP-pamoate. In various embodiments, the peptide salt is CNP-docusate.

[0124] In various embodiments, the hydrophilic peptide of the peptide salt is Pro-Gly CNP37 (PG-CNP37), and the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate, sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, and sodium dodecyl benzenesulfonate. In

some embodiments, the hydrophilic peptide salt is PG-CNP37, as described herein, and the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, and decanoate. In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37-oleate, PG-CNP37-pamoate, PG-CNP37-deoxycholate, and PG-CNP37-decanoate, In some embodiments, the hydrophilic peptide salt is PG-CNP37, as described herein, and the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, decanoate and docusate. In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37-oleate, PG-CNP37-pamoate, PG-CNP37-deoxycholate, PG-CNP37-decanoate and PG-CNP37-docusate. In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37-oleate, PG-CNP37-pamoate, and PG-CNP37-docusate. In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37-deoxycholate, PG-CNP37-decanoate and PG-CNP37-docusate. In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37-oleate and PG-CNP37-pamoate. In various embodiments, the peptide salt is PG-CNP37-oleate. In various embodiments, the peptide salt is PG-CNP37-pamoate. In various embodiments, the peptide salt is PG-CNP37-docusate.

[0125] In various embodiments, the peptide-counterion salt further comprises a polyvalent cation. In some embodiments, the hydrophilic peptide of the peptide salt is CNP or a CNP variant, as described herein; the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate, sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, and sodium dodecyl benzenesulfonate; and the polyvalent cation comprises a metal selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), iron (Fe), manganese (Mn), cobalt (Co), nickel (Ni), titanium (Ti), vanadium (V), platinum (Pt), copper (Cu) and gold (Au). In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, and decanoate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), CNP–Ca^{+2}(deoxycholate), CNP–Ca^{+2}(decanoate), CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), CNP–Zn^{+2}(deoxycholate) and CNP–Zn^{+2}(decanoate). In various

embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is selected from the group consisting of oleate, pamoate, deoxycholate, decanoate and docusate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is selected from the group consisting of oleate, pamoate, and docusate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is selected from the group consisting of deoxycholate, decanoate and docusate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is selected from the group consisting of oleate and pamoate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is oleate; and the polyvalent cation is Zn^{2+} or Ca^{2+}. In various embodiments, the hydrophilic peptide salt is CNP, as described herein, the hydrophobic counterion is pamoate; and the polyvalent cation is Zn^{2+} or Ca^{2+}.

[0126] In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), CNP–Ca^{+2}(deoxycholate), CNP–Ca^{+2}(decanoate), CNP–Ca^{+2}(docusate), CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), CNP–Zn^{+2}(deoxycholate), CNP–Zn^{+2}(decanoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), CNP–Ca^{+2}(docusate), CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), and CNP–Ca^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(deoxycholate), CNP–Ca^{+2}(decanoate), and CNP–Ca^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(Oleate) and CNP–Ca^{+2}(Pamoate). In various embodiments, the peptide salt is CNP–Ca^{+2}(Oleate). In various embodiments, the peptide salt is CNP–Ca^{+2}(Pamoate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), CNP–Zn^{+2}(deoxycholate), CNP–Zn^{+2}(decanoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Zn^{+2}(deoxycholate), CNP–Zn^{+2}(decanoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Zn^{+2}(Oleate) and CNP–

Zn^{+2}(Pamoate). In various embodiments, the peptide salt is CNP–Zn^{+2}(Oleate). In various embodiments, the peptide salt is CNP–Zn^{+2}(Pamoate).

[0127] In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Ca^{+2}(Oleate), PG-CNP37–Ca^{+2}(Pamoate), PG-CNP37–Ca^{+2}(deoxycholate), PG-CNP37–Ca^{+2}(decanoate), PG-CNP37–Ca^{+2}(docusate), PG-CNP37–Zn^{+2}(Oleate), PG-CNP37–Zn^{+2}(Pamoate), PG-CNP37–Zn^{+2}(deoxycholate), PG-CNP37–Zn^{+2}(decanoate), and PG-CNP37–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Ca^{+2}(Oleate), PG-CNP37–Ca^{+2}(Pamoate), PG-CNP37–Ca^{+2}(docusate), PG-CNP37–Zn^{+2}(Oleate), PG-CNP37–Zn^{+2}(Pamoate), and CNP–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Ca^{+2}(Oleate), PG-CNP37–Ca^{+2}(Pamoate), and PG-CNP37–Ca^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of CNP–Ca^{+2}(deoxycholate), PG-CNP37–Ca^{+2}(decanoate), and PG-CNP37–Ca^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Ca^{+2}(Oleate) and PG-CNP37–Ca^{+2}(Pamoate). In various embodiments, the peptide salt is PG-CNP37–Ca^{+2}(Oleate). In various embodiments, the peptide salt is PG-CNP37–Ca^{+2}(Pamoate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Zn^{+2}(Oleate), PG-CNP37–Zn^{+2}(Pamoate), PG-CNP37–Zn^{+2}(deoxycholate), PG-CNP37–Zn^{+2}(decanoate), and PG-CNP37–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Zn^{+2}(Oleate), PG-CNP37–Zn^{+2}(Pamoate), and PG-CNP37–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Zn^{+2}(deoxycholate), PG-CNP37–Zn^{+2}(decanoate), and PG-CNP37–Zn^{+2}(docusate). In various embodiments, the peptide salt is selected from the group consisting of PG-CNP37–Zn^{+2}(Oleate) and PG-CNP37–Zn^{+2}(Pamoate). In various embodiments, the peptide salt is PG-CNP37–Zn^{+2}(Oleate). In various embodiments, the peptide salt is PG-CNP37–Zn^{+2}(Pamoate).

[0128] *Methods of Making*

[0129] Contemplated herein is also a method of making a composition comprising a hydrophobic peptide salt as described herein.

[0130] In various embodiments, the disclosure provides a method of making a composition comprising a salt of an electrostatically charged peptide comprising, a) contacting an electrostatically charged peptide in an aqueous solution with a hydrophobic counterion in solution; b) mixing the electrostatically charged peptide solution with the hydrophobic counterion

solution in a manner sufficient for the peptide and counterion to form a complex, wherein the formation of the peptide-counterion complex results in formation of a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form comprising the peptide salt. In various embodiments, when the peptide-counterion salt further comprises a polyvalent cation. the method comprises before step (b), contacting the electrostatically charged peptide in solution with a polyvalent cation in aqueous solution, forming a peptide-cation complex. The peptide-cation complex is then contacted with a hydrophobic counterion to form a peptide-cation-counterion complex.

[0131] In various embodiments, the disclosure provides a method of making a composition comprising a salt of an electrostatically charged peptide comprising (a), contacting the electrostatically charged peptide in solution with a polyvalent cation in aqueous solution, forming a peptide-cation complex, b) contacting the peptide-cation complex in an aqueous solution with a hydrophobic counterion in solution; and c) mixing the peptide-cation complex solution with the hydrophobic counterion solution in a manner sufficient for the peptide-cation and counterion to form a complex, wherein the formation of the peptide-cation counterion complex results in formation of a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form comprising the peptide salt.

[0132] In various embodiments, the mixing is carried out by dropwise addition of the hydrophobic counterion solution to the peptide solution. The solutions are mixed by vortexing after addition of each drop of hydrophobic counterion solution, or other means for mixing known in the art.

[0133] In various embodiments, the method further comprises step (c) or (d) washing the peptide salt in buffer or water. In various embodiments, the washing is carried out in an aqueous solution, e.g., buffer or water.

[0134] In various embodiments, the method further comprises step (d) or (e) obtaining the peptide salt by centrifugation to form a peptide salt pellet. In various embodiments, if the salt is in gel form, the salt is obtained by centrifugation or by decanting the liquid phase followed by lyophilization or other drying methods.

[0135] In various embodiments, the method further comprises step (e) or (f) removing water from the peptide salt pellet. It is contemplated that water or another aqueous solution can be removed from the pellet by lyophilization or drying using techniques known in the art.

[0136] In various embodiments, the method further comprises resuspending the pellet in an aqueous solution or oil. In various embodiments, the aqueous solution is water, saline, or buffer. In various embodiments, the oil comprises a triglyceride or a fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. In various embodiments, the fatty acid in the triglyceride is saturated or unsaturated, or combinations thereof.

[0137] The fatty acid may be the oil itself or in a triglyceride. In various embodiments, the fatty acid is a short chain, medium chain or long chain fatty acid. In various embodiments, the fatty acid in a triglyceride is saturated or unsaturated, and can be a medium chain or long chain fatty acid. In various embodiments, the fatty acid is a C-6 to C-20 fatty acid. In various embodiments, the fatty acid is a C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acid. In various embodiments, the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid.

[0138] In various embodiments, the method contemplates use of at least one molar equivalent of hydrophobic counterion, such as a hydrophobic anion, to: 1) the total number of charged amino acids if cation is present; or 2) the total number of positive charges if no cation is present. Accordingly, in various embodiments, the method contemplates the use of at least one equivalent of hydrophobic counterion to the total number of positive charges in the hydrophilic peptide when no cation is present, or to the total number of positive charges in the complex comprising hydrophilic peptide and cation when the polyvalent cation is present. This ratio is referred to herein as the peptide: hydrophobic counterion ratio. In various embodiments, the peptide: hydrophobic counterion ratio is at least one molar equivalent of hydrophobic counterion to total number of positively charged amino acids in the peptide (when no cation is present) or the complex comprising peptide and cation (when cation is present). In various embodiments the peptide: hydrophobic counterion ratio is between 1:1 to 1:20, or from 1:1 to 1:50. The peptide: counterion ratio can be between 1:2 to 1:15, 1:2 to 1:10, 1:2 to 1:8, 1:3 to 1:10, or 1:4 to 1:10. In various embodiments, the peptide: hydrophobic counterion ratio is 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19 or 1:20. In various embodiments, the peptide: hydrophobic counterion ratio is 1:1. In various embodiments, the peptide: hydrophobic counterion ratio is 1:2. In various embodiments, the peptide: hydrophobic counterion ratio is 1:3. In various embodiments, the peptide: hydrophobic counterion ratio is 1:4. In various embodiments, the peptide: hydrophobic counterion ratio is 1:5. In various embodiments, the peptide: hydrophobic counterion ratio is 1:6. In various embodiments, the peptide: hydrophobic counterion ratio is 1:7. In various embodiments, the peptide: hydrophobic counterion ratio is 1:8. In various embodiments, the peptide: hydrophobic

counterion ratio is 1:9. In various embodiments, the peptide: hydrophobic counterion ratio is 1:10. In various embodiments, the peptide: hydrophobic counterion ratio is at least two molar equivalents of hydrophobic counterion to total number of positively charged amino acids in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In various embodiments, the peptide: hydrophobic counterion ratio is at least three molar equivalents of hydrophobic counterion to total number of positively charged amino acids in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In various embodiments, the peptide: hydrophobic counterion ratio is at least four molar equivalents of hydrophobic counterion to total number of positively charged amino acids in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In some embodiments, the peptide: hydrophobic counterion ratio is less than one molar equivalent of hydrophobic counterion to total number of positive charges in the peptide (when no cation is present) or to the complex comprising the peptide and cation (when cation is present). In various embodiments, the peptide salt has one less molar equivalent of hydrophobic counterion to positive charges in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In various embodiments, the peptide salt has two less molar equivalents of hydrophobic counterion to positive charges in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In various embodiments, the peptide salt has three less molar equivalents of hydrophobic counterion to positive charges in the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present). In various embodiments, the method contemplates introducing, e.g., 0.9, 0.8, 0.7, 0.75, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2 or 0.1 molar equivalents of hydrophobic counterion to the total number of positive charges on the peptide (when no cation is present) or the complex comprising the peptide and cation (when cation is present).

[0139] In various embodiments, the method contemplates including at least one molar equivalent of cation to total number of negatively charged amino acids in the peptide. This ratio is referred to herein as the peptide: cation ratio. In various embodiments, the peptide: cation ratio is between 1:1 to 1:10. The peptide: cation ratio can be 1:2 to 1:10, 1:3 to 1:10, 1:1 to 1:5, 1:2 to 1:5, of 1:2 to 1:8. In various embodiments, the peptide: cation ratio is 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, or 1:10. In various embodiments, the peptide: cation ratio is 1:1. In various embodiments, the peptide: cation ratio is 1:2. In various embodiments, the peptide: cation ratio is 1:3. In various embodiments, the peptide: cation ratio is 1:4. In various embodiments, the peptide: cation ratio is 1:5. In various embodiments, the peptide: cation ratio

is 1:6. In various embodiments, the peptide: cation ratio is 1:7. In various embodiments, the peptide: cation ratio is 1:8. In various embodiments, the peptide: cation ratio is 1:9. In various embodiments, the peptide: cation ratio is 1:10. In various embodiments, the method contemplates including less than one molar equivalent of cation to total number of negatively charged amino acids in the peptide. In various embodiments, the method contemplates introducing, e.g., 0.9, 0.8, 0.7, 0.75, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2 or 0.1 molar equivalents of cation to the total number of negative charges on the peptide.

C-type natriuretic peptides

[0140] C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)) is a small, single chain peptide in a family of peptides (ANP, BNP, CNP) having a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. CNP interacts with natriuretic peptide receptor-B (NPR-B, GC-B) to stimulate the generation of cyclic- guanosine monophosphate (cGMP) (J. Hypertens., 10: 1111-1114 (1992)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

[0141] In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). Both CNP-53 and CNP-22 bind similarly to NPR-B.

[0142] In various embodiments, CNP of the disclosure include truncated CNP ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wild-type amino acid sequences derived from hCNP-53. Such truncated CNP peptides include:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-53) (SEQ ID NO: 56);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-52) (SEQ ID NO:15);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
(CNP-51) (SEQ ID NO:16);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO17:);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 18)
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO:19);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO:20);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO:21);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO:22);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO:23);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:24);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:25);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO:26);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:27);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO:28);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO:29);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO:30);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO:31);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO:32);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO:33);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO:34);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO:35);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO:36);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO:37);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO:38);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO:39);

KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO:40);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO:41);
GLSKGCFGLKLDRIGSMSGLGC (CNP-22) (SEQ ID NO: 68);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO:42);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO:43);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO:44);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO:45); and
CFGLKLDRIGSMSGLGC (CNP-17) (SEQ ID NO: 67).

[0143] In various embodiments, the CNP variant peptides are modified CNP-37 or CNP-38 peptides, optionally having mutation(s)/substitution(s) at the furin cleavage site (underlined), and/or containing glycine or proline-glycine at the N-terminus. Exemplary CNP-37 variants include but are not limited to:
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N); SEQ ID NO: 46];
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37; SEQ ID NO:47);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37; SEQ ID NO: 48);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N); SEQ ID NO: 49];
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37; SEQ ID NO: 50); and
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37: SEQ ID NO:51)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:52);
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:53);
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:54); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:55).

[0144] In various embodiments, the CNP is selected from the group consisting of
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 5);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 1);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 6);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 6);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 5); and
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 7).

[0145] In various embodiments, the CNP variant peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various

embodiments, the acetyl group is on an amino acid side chain within the peptide sequence. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus.

[0146] In additional embodiments, for any of the CNP and CNP variants described herein that have asparagine (Asn/N) residue(s) and/or glutamine (Gln/Q) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln. Such substitution(s) are designed in part to minimize or avoid any potential deamidation of asparagine and/or glutamine.

[0147] Additional CNP peptides and variants are disclosed in U.S. Patent 8,198,242, incorporated by reference herein.

[0148] In various embodiments, the electrostatically charged peptide is a C-type natriuretic peptide (CNP) or CNP variant and the hydrophilic peptide salt is a salt of CNP or a CNP variant. In various embodiments, the CNP is a CNP variant as described herein.

[0149] In various embodiments, the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4);
and, pharmaceutical salts thereof. In various embodiments, the CNP is a CNP-acetate.

[0150] In various embodiments, when the hydrophobic peptide salt is a hydrophobic CNP salt, the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate. In various embodiments, if a polyvalent cation is present, the cation comprises zinc, magensium or calcium. In various embodiments, if the cation is present, the cation comprises zinc. In various embodiments, the cation comprises calcium. In various embodiments, if a polyvalent cation is present, the cation is Zn^{2+}, Mg^{2+}, or Ca^{2+}. In various embodiments, if a cation is present, the cation is Zn^{2+}. In various embodiments, if a cation is present, the cation is Ca^{2+}.

[0151] In various embodiments, the disclosure provides a hydrophobic salt of C-type natriuretic peptide comprising a CNP peptide in complex with a hydrophobic counterion. In various embodiments, the salt further comprises a polyvalent cation, optionally a metal cation. In various embodiments, the salt is a purified CNP salt.

[0152] Methods of purifying hydrophobic salts are known in the art and contemplated herein. In various embodiments, the hydrophobic salt has a purity of at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more.

Methods of Use

[0153] Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, CNP and functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias

[0154] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP salts of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, chondrodysplasia congenit, homozygous achondroplasia, chondrodysplasia congenit, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia congenit, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), and PTPN11 mutations (Noonan's syndrome).

[0155] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal

dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, chondrodysplasia congenit, homozygous achondroplasia, chondrodysplasia congenit, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia congenit, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia. Short stature, growth plate disorder, bone-related disorder or skeletal dysplasias contemplated herein include disorders related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome) and IGF1R mutation.

[0156] Short stature, growth plate disorder, bone-related disorder or skeletal dysplasias contemplated herein include disorders related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), and PTPN11 mutations (Noonan's syndrome).

[0157] Additional short stature and growth plate disorders contemplated by the methods include disorders related to mutations in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3.

[0158] Additional short stature and growth plate disorders contemplated by the methods include disorders related to mutations in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or IGF1R.

[0159] Further, the CNP salts are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias described herein.

[0160] Growth plate disorders include disorders that result in short stature or abnormal bone growth and that may be the result of a genetic mutation in a gene involved in bone growth, including collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3. In various embodiments, growth plate disorders include disorders that result in short stature or abnormal bone growth and that may be the result of a genetic mutation in a gene involved in bone growth, including collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or IGF1R. In various embodiments, the growth plate disorder or short stature is

associated with one or more mutations in a gene associated with a RASopathy. In various embodiments, a subject with a growth plate disorder is heterozygous for a mutation in a growth plate gene. In various embodiments, the mutation is a loss-of-function mutation. In various embodiments, the mutation is a gain-of-function mutation. Growth plate disorders include, but are not limited to, familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. See, e.g., Plachy et al., J Clin Endocrinol Metab 104: 4273-4281, 2019.

[0161] Mutations in ACAN can give rise to familial osteochondritis dissecans and short stature and eventually osteoarthritis, characterized by areas of bone damage (or lesions) caused by the detachment of cartilage and sometimes bone from the end of the bone at a joint. It has been suggested that the disorganized cartilage network in growing bones impairs their growth, leading to short stature. A mutation associated with ACAN and short stature includes Val2303Met. See Stattin et al., Am J Hum Genet 86(2):126-37, 2010. It is contemplated that patients with a mutation in ACAN resulting in short stature would benefit from treatment with CNP as administration may be able to increase height in these patients by the known interaction of CNP with FGFR3.

[0162] The natriuretic peptide system, including receptor NPR2, has been shown to be involved in regulation of endochondral bone growth (Vasques et al., Horm Res Pediat 82:222-229, 2014). Studies have shown that homozygous or compound heterozygous loss-of-function mutations in NPR2 cause acromesomelic dysplasia type Maroteaux (AMDM), which is a skeletal dysplasia having extremely short stature (Vasquez et al., 2014, *supra*). There are reports implicating heterozygous loss-of-function (such as dominant negative) NPR2 mutations as a cause of short stature, whereas gain-of-function NPR2 heterozygous mutations have been found to be responsible for tall stature (Vasquez et al., 2014, *supra*). In view of CNP's interaction with NPR2 to stimulate cGMP generation, increasing cGMP levels is desirable in these conditions and would have therapeutic benefit in the management of the complications from these diseases and conditions.

[0163] Heterozygous mutations of NPR2 are believed to result in idiopathic short stature and other forms of short stature. Mutations in the NPR2 gene are set out below and described in Amano et al., J Clin Endocrinol Metab 99:E713-718, 2014, Hisado-Oliva et al., J Clin Endocrinol Metab 100:E1133-1142, 2015 and Vasques et al., J Clin Endocrinol Metab 98:E1636-1644, 2013, hereby incorporated by reference. It is contemplated that a subject having short stature to be treated with a CNP variant as described herein has a height SDS of less than -1.0, -1.5, -

2.0, -2.5, or -3.0, and has at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. However, because *de novo* mutations in NPR2 can result in short stature as defined by a height SDS of less than -1.5, -2.0, -2.5, or -3.0, treatment of individuals who are heterozygous carriers of a deleterious mutation in NPR2 with neither parent having short stature is also contemplated. Further contemplated is treatment of individuals who are heterozygous for deleterious mutations in other growth plate genes with CNP to improve stature and/or enhance bone growth.

[0164] Exemplary NPR2 mutations in patients that may be treated with a CNP variant include:

Disease	Nucleotide	Mutation	Amino acid change
Short stature	1669C>T	missense	Arg557Cys
Short stature	2794C>T	missense	Arg932Cys
Short stature	2905G>C	missense	Val969Leu
Short stature	3058C>T	missense	Arg1020Trp
Short stature	2972A>G	missense	Glu991Gly
Short stature	1262C>T	missense	Thr421Met
Short stature	766G>T	missense	Asp256Tyr
Short stature	1982C>A	missense	Thr661Lys
Short stature	2449G>A	missense	Glu817Lys
Short stature	1517G>A	missense	Arg506His
Short stature	1802G>C	missense	Arg601Pro
Short stature	1481T>G	missense	Ile494Ser
Short stature	142G>T	missense	Ala48Ser
Short stature	1167G>T	missense	Glu389Asp
Short stature	1249C>G	missense	Gln417Glu
Short stature	328C>T	missense	Arg110Cys
Short stature	2455C>T	missense	Arg819Cys
Short stature	788G>C	missense	Arg263Pro
Short stature	226T>C	missense	Ser76Pro
Short stature	2710A>T	nonsense	Lys904Term

9:35809194:C:G	Leu1009Val
9:35802761:G:C	Leu615Phe

9:35799645:C:T	Pro301Ser
9:35792928:C:T	Arg174Cys
9:35801728:C:G	His508Asp
9:35792713:T:C	Val102Ala
9:35793980:T:A	Tyr250Ter
9:35807085:C:T	Thr861Ile
9:35793906:A:G	Ile226Val
9:35808558:G:A	Arg921Gln
9:35802741:G:A	Glu609Lys
9:35802594:G:A	Arg601His
9:35808663:T:A	Leu956Gln
9:35808545:G:C	Gly917Arg

[0165] NPPC's role in skeletal growth is well documented. (Hisado-Oliva et al., Genetics Medicine 20:91-97, 2018). The NPPC knock out mouse showed severe disproportionate form of dwarfism including shortening of limbs and endochondral ossification (Hisado-Oliva et al., 2018, *supra*). Human genome wide studies have shown a link between NPPC and height (Hisado-Oliva et al., 2018, *supra*). Although CNP haploinsufficiency has been believed to be a cause of short stature in humans, a recent study identified heterozygous mutations in families with short stature and hands (Hisado-Oliva et al., 2018, *supra*). These studies observed significant reduction in cGMP production as measured in heterozygous state (Hisado-Oliva et al., 2018, *supra*). Mutations in NPPC include a 355G>T missense mutation causing a Gly119Cys change and a 349C>G missense mutation causing a Arg117Gly change. A CNP variant rescuing CGMP production may provide therapeutic benefit in the management of a disorder in patients having heterozygous loss-of-function NPPC mutations.

[0166] Leri-Weill dyschondrosteosis (LWD) is a rare genetic disorder characterized by shortening of the forearms and lower legs, abnormal misalignment of the wrist (Madelung deformity of the wrist), and associated short stature. LWD is caused by a heterozygous mutation in the short stature homeobox-containing (SHOX) gene or its regulatory elements located on the pseudoautosomal region 1 (PAR1) of the sex chromosomes. (See the Rare Disease Database and Carmona et al., Hum Mol Genet 20:1547-1559, 2011). The disorder Langer mesomelic dysplasia arises when there are two SHOX mutations, and may result from a mutation on each chromosome, either a homozygous or compound heterozygous mutations. A subset of SHOX mutations give rise to idiopathic short stature. Turner syndrome results due to a deletion on the X chromosome that can include the SHOX gene. SHOX has been identified as involved in the regulation of *FGFR3* transcription and contributes to control of bone growth (Marchini et al.,

Endocr Rev. 37: 417–448, 2016). SHOX deficiency leads to increased FGFR3 signaling, and there is some evidence to support that SHOX has direct interactions with CNP/NPR2 as well (Marchini, *supra*). Given the association of SHOX with FGFR3 and bone growth, it is contemplated that a subject having a homozygous or heterozygous SHOX mutation would benefit from treatment with CNP variants as described herein.

[0167] RASopathies are a group of rare genetic conditions caused by mutations in genes of the Ras/mitogen-activated protein kinase (MAPK) pathway. RASopathies are a group of disorders characterized by increased signaling through RAS/MAPK pathway. This pathway leads to downstream activation of the RAF/MEK/ERK pathway. Short stature is a characteristic feature of certain RASopathies. For example, CNP signaling inhibits RAF and leads to decreased MEK and ERK activation.

[0168] Treatment of RASopathies are contemplated herein. RASopathies associated with short stature include Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, and LEOPARD syndrome. Hereditary gingival fibromatosis type 1 is also a RASopathy contemplated herein. RASopathy patients (including Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, LEOPARD syndrome, hereditary gingival fibromatosis type 1) include patients with heterozygous variants in one or more of the following genes: BRAF, CBL, HRAS, KRAS, LZTR1, MAP2K1, MAP2K2, MRAS, NF1, NRAS, PPP1CB, PTPN11, RAF1, RRAS, RIT1, SHOC2, SOS1, or SOS2 (Tajan et al. Endocr. Rev. 2018;39(5):676–700).

[0169] CFC is caused by mutations in several genes in the Ras/MAPK signaling pathway, including K-Ras, B-Raf, Mek1 and Mek2. Costello syndrome, also called faciocutaneoskeletal (FCS) syndrome is caused by activating mutations in the H-Ras gene. Hereditary gingival fibromatosis type I (HGF) is caused by dominant mutations in the SOS1 gene (*Son of Sevenless* homolog 1), which encodes a guanine nucleotide exchange factor (SOS) that acts on the Ras subfamily of small GTPases. Neurofibromatosis type I (NF1) is caused by mutations in the neurofibromin 1 gene, which encodes a negative regulator of the Ras/MAPK signaling pathway. Noonan syndrome (NS) is caused by mutations in one of several genes, including PTPN11, which encodes SHP2, and SOS1, as well as K-Ras and Raf-1.

[0170] CNP has been demonstrated to be an effective therapy in RASopathy models. Ono et al. generated mice deficient in *Nf1* in type II collagen producing cells (Ono et al., Hum. Mol. Genet. 2013;22(15):3048–62). These mice demonstrated constitutive ERK1/2 activation, and decreased chondrocyte proliferation, and maturation. Daily injections of CNP in these mice led

to decreased ERK phosphorylation and corrected the short stature. A mouse model of Cardiofaciocutaneous syndrome using a *Braf* mutation (p.Q241R) (Inoue et al. Hum. Mol. Genet. 2019;28(1):74–83) exhibited decreased body length and reduced growth plate width with smaller proliferative and hypertrophic zones compared to wild type, and CNP administration led to increases in body length in these animals.

[0171] Mutations in multiple genes can cause Noonan syndrome, which is characterized by short stature, heart defects, bleeding problems, and skeletal malformations. Mutations in the PTPN11 gene cause about half of all cases of Noonan's syndrome. SOS1 gene mutations cause an additional 10 to 15 percent, and RAF1 and RIT1 genes each account for about 5 percent of cases. Mutations in other genes each account for a small number of cases. The cause of Noonan syndrome in 15 to 20 percent of people with this disorder is unknown.

[0172] The PTPN11, SOS1, RAF1, and RIT1 genes all encode for proteins that are important in the RAS/MAPK cell signaling pathway, which is needed for cell division and growth (proliferation), differentiation, and cell migration. Many of the mutations in the genes associated with Noonan syndrome cause the resulting protein to be turned on (active) and this prolonged activation alters normal RAS/MAPK signaling, which disrupts the regulation of cell growth and division, leading to the characteristic features of Noonan syndrome. See, e.g., Chen et al., Proc Natl Acad Sci U S A. 111(31):11473-8, 2014, Romano et al., Pediatrics. 126(4):746-59, 2010, and Milosavljević et al., Am J Med Genet 170(7):1874-80, 2016. It is contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve bone growth and short stature. It is also contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve other comorbidities associated with an overactive MAPK pathway in other cells throughout the body where the NPR2 receptor is expressed on its surface.

[0173] Mutations in the PTPN11 gene, which encodes the non-receptor protein tyrosine phosphatase SHP-2, lead to disorders characterized by short stature such as Noonan's Syndrome (Musente et al., Eur J Hum Genet 11:201–206 (2003). Musente (*supra*) identifies numerous mutations in the PTPN11 gene that lead to short stature. Gain of function mutations lead to overactive signaling through SHP2 and inhibit Growth Hormone-induced IGF-1 release, thereby contributing to a decrease in bone growth (Rocca Serra-Nédélec, PNAS 109:4257-4262, 2012). It is contemplated that a subject having a homozygous or heterozygous PTPN11

mutation would benefit from treatment with CNP variants as described herein to improve bone growth and short stature.

[0174] Mutations in the Indian hedgehog (IHH) gene, which is related to regulation of endochondral ossification, have also been associated with short stature syndromes (Vasques et al., J Clin Endocrinol Metab. 103:604-614, 2018). Many IHH mutations identified segregate with short stature in a dominant inheritance pattern. Given the association of IHH with bone growth and ossification, it is contemplated that subjects having a homozygous or heterozygous IHH mutation will benefit from treatment with a CNP variant as described herein.

[0175] Mutations in FGFR3, including N540K and K650N, lead to short stature and hypochondroplasia.

[0176] Insulin-like growth factor 1 receptor (IGF1R) is a heterotetrameric ($\alpha 2\beta 2$) transmembrane glycoprotein with an intrinsic kinase activity. IGF1R has been shown to have a role in prenatal and postnatal growth. Heterozygous mutations in IGF1R have been identified in Small for gestational age children (SGA) and individuals with familial short stature (Kawashima et al., Endocrine J. 59:179-185, 2012). Mutations in IGF1R associated with short stature include R108Q/K115N, R59T, R709Q, G1050K, R481Q, V599E, and G1125A (Kawashima, *supra*).

[0177] Height is a highly heritable trait that can be influenced by the combined effect of hundreds or thousands genes (Wood et al., Nature Genetics, 46:1173-1189, 2014). Short stature in an individual can be the result of the combined effect of these genes, without a single gene being the primary contributor. It is contemplated that such individuals with short stature defined by a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, can be beneficially treated with a CNP variant given the ability of CNP to increase the length of normal animals, for example, enhance bone growth and length of bones.

[0178] In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and having at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. In various embodiments, the short stature is associated with one or more mutations in a gene associated with short stature, such as, collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R), or combinations thereof.

[0179] In various embodiments, the short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0180] In various embodiments, the short stature is a result of mutations in multiple genes as determined by polygenic risk score (PRS). In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in FGFR3 and a low PRS. In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in IGF1R and a low PRS. In various embodiments, the subject has a mutation in NPPC and a low PRS. In various embodiments, the subject has a mutation in SHOX and a low PRS. In various embodiments, the subject has one or more mutation in one or more of FGFR3, IGF1R, NPPC, NPR2 and SHOX, and a low PRS. In various embodiments, the PRS is 1 or 2. In various embodiments, the PRS is 1. In various embodiments, the PRS is 2.

[0181] In addition, the CNP salts are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

[0182] The CNP salts of the disclosure can also be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP compositions are useful for countering the undesired effects of FGF-2 and increasing matrix synthesis in subjects suffering from arthritis, including osteoarthritis, thereby treating arthritis, including osteoarthritis.

[0183] In certain embodiments, the CNP salts and compositions and formulations comprising the same of the present disclosure are useful for improving one or more of the symptom(s) or physiological consequences of a skeletal dysplasia, wherein the improvement may be increased absolute growth, increased growth velocity, increased qualitative computed tomography (QCT) bone mineral density, improvement in growth plate morphology, increased long bone growth, improvement in spinal morphology, improved elbow joint range of motion and/or decreased

sleep apnea. In this regard, it is noted that the terms "improved", "improvement", "increase", "decrease" and grammatical equivalents thereof are all relative terms that when used in relation to a symptom or physiological consequence of a disease state, refer to the state of the symptom or physiological consequence of the disease after treatment with a CNP salt (or composition or formulation comprising the same) of the present invention as compared to the same symptom or physiological consequence of the disease before treatment with a CNP salt (or composition or formulation comprising the same) of the present invention (i.e., as compared to "baseline"). As described above, a "baseline" state can be determined either through measurement of the state in the subject prior to treatment (which can subsequently be compared to the state in the same subject after treatment), or through measurement of that state in a population of subjects suffering from the same affliction that share the same or similar characteristics (e.g., age, sex and/or disease state or progression).

[0184] In yet another embodiment, the disclosure provides salts of CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM). In a still further embodiment, the hydrophobic salt comprising the CNP or CNP variants of the disclosure *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g. 1 uM).

[0185] It is contemplated that any of the CNP variants described herein are useful in the methods.

[0186] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37 (SEQ ID NO: 1)). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the acetyl group is on an amino acid side chain within the peptide sequence. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker. In various embodiments, the peptide comprises a salt of an electrostatically charged peptide, the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion.

[0187] Efficacy of treatment is measured by various parameters. In various embodiments, efficacy is assessed as the change in annualized growth velocity from the baseline period to the

intervention period. Efficacy will also be assessed as the change in height SDS from baseline to end of treatment as measured using the CDC growth curves, and growth velocity SDS will be based on the Bone Mineral Density in Childhood Study (Kelly et al., *J. Clin. Endocrinol. Metab.* 2014;99(6):2104–2112).

[0188] QoLISSY, the Quality of Life in Short Stature Youth, is assessed as directed (*Quality of Life in Short Stature Youth - The QoLISSY Questionnaire User's Manual*. Lengerich: Pabst Science Publishers; 2013).

[0189] Pharmaceutical Compositions

[0190] The disclosure provides pharmaceutical compositions, including modified release compositions, comprising a peptide salt described herein, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (*e.g.*, inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

[0191] The disclosure provides for modified release compositions comprising a hydrophobic peptide salt as described herein. In various embodiments, the modified release composition is an extended release composition. In various embodiments, the extended release composition comprises a hydrophobic CNP salt. In various embodiments, the hydrophobic counterion in the CNP salt is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate. In various embodiments, if a polyvalent cation is present, the cation comprises zinc or calcium. In various embodiments, if a cation is present, the cation is Zn^{2+} or Ca^{2+}.

[0192] Precipitated peptide complexes can display extended release characteristics under pH conditions at which precipitation was formed. Precipitated peptide complexes can also be used for further processing into a matrix that provides additional barriers for sustained release such as slow degrading microspheres, hydrogels, and the like. It is contemplated that the hydrophobic CNP salt is a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle, and is resuspended in an aqueous solution or in oil. In various embodiments, the aqueous solution is water, saline, or buffer. In various embodiments, the particle is between 1 and 10,000 micrometers (um), between 1 um to 2000 um, between 2 um to 1000 um, between 5 um to 500 um, between 10 um to 1000 um, between 50 um to 500 um, between 100 um to 800 um, between 200 to 600 um, between 300um to 500 um, between 100 um to 300 um, between 50 um to 100 um, or between 10 um to 50 um. In various embodiments the particle is a nanoparticle. In various embodiments, the nanoparticle is approximately

between 5 nanometers (nm) to 1000 nm, between 8 nm to 900 nm, between 10 nm-800 nm, between 20 nm to 600 nm, between 50 nm to 500 nm, between 50 to 400 nm, between 20 to 300 nm, between 300 to 800 nm or between 200 to 600 nm.

[0193] In various embodiments, the oil comprises a triglyceride or a fatty acid, which can be saturated or unsaturated. Triglycerides and fatty acids as described herein are also contemplated for use with the hydrophobic CNP salt compositions. In various embodiments, the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid. In various embodiments, the fatty acid is hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid or docusate.

[0194] In various embodiments, for the extended release composition, (i) less than about 20% of peptide is released by day 1; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6.

[0195] In various embodiments, less than about 20% of peptide is released by day 1 at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50%, or about 60% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released weekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released biweekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released monthly at pH 7 to 7.6. It is further contemplated that the release can be at a pH between pH 7.0 to 7.6, between pH 7.1 to 7.5, between pH 7.2 to 7.4, between pH 7.2 to 7.6, or between pH 7.0 to 7.4.

[0196] In various embodiments, (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60% about 65%, about 70%, or about

75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6; or alternatively ii) about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released bi-weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released every three weeks; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released monthly, at pH 7 to 7.6

[0197] In various embodiments, the extended release composition comprises an excipient, diluent or carrier. In various embodiments, the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier.

[0198] Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactants, emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (*e.g.*, water, ethanol); diluents of various buffer content (*e.g.*, Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (*e.g.*, Polysorbate 20, Polysorbate 80); anti-oxidants (*e.g.*, methionine, ascorbic acid, sodium metabisulfite); preservatives (*e.g.*, Thimerosol, benzyl alcohol, m-cresol); and bulking substances (*e.g.*, lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)), which is incorporated herein by reference in its entirety.

[0199] For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (*e.g.*, oil/water emulsions). A carrier can be a solvent or dispersing medium containing, *e.g.*, ethanol, a polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

[0200] In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a hydrophobic CNP salt in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1

mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 0.5 to 5 mg/ml, or from about 0.5 to 3 mg/ml, or from about 1 mg/ml to about 10 mg/ml. In various embodiments, the CNP variant is in a concentration of 0.8 mg/mL to 2 mg/mL. In various embodiments, the CNP variant is at a concentration of 0.8 mg/mL In various embodiments, the CNP variant is at a concentration of 2.0 mg/mL. In various embodiments, the CNP variant is reconstituted from a lyophilized powder.

[0201] In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, and sodium citrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. In some embodiments, the buffering agent has a concentration of about 10 mM ± 5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 9, or from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0 ± 1.0. In various embodiments, the pH is about 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9 or 6.0. In various embodiments, the pH is 5.5.

[0202] In other embodiments, the compositions contain an isotonicity-adjusting agent to render the solution or suspension isotonic and more compatible for administration. Non-limiting examples of isotonicity agents include NaCl, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is NaCl. In certain embodiments, NaCl is in a concentration of about 160 ± 20 mM , or about 140 mM ± 20 mM, or about 120 ± 20 mM , or about 100 mM ± 20 mM, or about 80 mM ± 20 mM, or about 60 mM ± 20 mM.

[0203] In yet other embodiments, the compositions comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4% ± 0.2%, or about 1% ± 0.5%, or about 1.5% ± 0.5%, or about 2.0% ± 0.5%.

[0204] In still other embodiments, the compositions contain an anti-adsorbent (e.g., to mitigate adsorption of a CNP salt to glass or plastic). Anti-adsorbents include without limitation benzyl alcohol, Polysorbate 20, and Polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about

0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

[0205] In additional embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%. In other embodiments, when the stabilizer is methionine, the stabilizer is in a concentration from about 0.01% to about 0.5%, or from about 0.01% to about 0.2%. In still other embodiments, when the stabilizer is glycerin, the stabilizer is in a concentration from about 5% to about 100% (neat).

[0206] In further embodiments, the compositions contain an antioxidant. Exemplary anti-oxidants include without limitation methionine and ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

[0207] Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (*e.g.*, hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (*e.g.*, acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (*e.g.*, isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, (Easton, Pennsylvania (1990)).

[0208] The pharmaceutical compositions can be administered in various forms, such as tablets, capsules, granules, powders, solutions, suspensions, emulsions, ointments, and transdermal patches. The dosage forms of the compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the compositions can take the form of, *e.g.*, a tablet or capsule (including softgel capsule), or can be, *e.g.*, an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or more commonly used excipients, diluents and carriers, such as mannitol, lactose, glucose, sucrose, starch, corn starch, sodium saccharin, talc, cellulose, magnesium carbonate, and lubricating agents (*e.g.*, magnesium stearate, sodium stearyl fumarate). If desired, flavoring, coloring and/or sweetening agents can be added to the solid and liquid formulations. Other optional ingredients for oral formulations include without limitation preservatives, suspending agents, and thickening agents. Oral formulations can also have an enteric coating to protect the CNP salt from the acidic environment of the stomach. Methods of preparing solid

and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, *e.g.*, Remington's Pharmaceutical Sciences, referenced above).

[0209] Formulations for parenteral administration can be prepared, *e.g.*, as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (*e.g.*, buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

[0210] Compositions comprising a hydrophobic CNP salt can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dexran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%. Exemplary anti-oxidants include, but are not limited to, methionine and ascorbic acid.

[0211] In various embodiments, the formulation comprises citric acid, sodium citrate, trehalose, mannitol, methionine, polysorbate 80, and optionally sterile water for injection (WFI).

[0212] The disclosure also provides kits containing, *e.g.*, bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, *e.g.*, sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices, such as aerosol or

injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

[0213] As a non-limiting example, a kit can include syringes having a single chamber or dual chambers. For single-chamber syringes, the single chamber can contain a liquid CNP formulation ready for injection, or a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP salt in a relatively small amount of a suitable solvent system (e.g., glycerin) that can be reconstituted into a solution or suspension for injection. For dual-chamber syringes, one chamber can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP salt in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection.

[0214] As a further example, a kit can include one or more pen injector or autoinjector devices, and dual-chamber cartridges. One chamber of a cartridge can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP salt in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection. A cartridge can comprise an amount of the CNP salt that is sufficient for dosing over a desired time period (e.g., 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, etc.). The pen injector or autoinjector can be adjusted to administer a desired amount of the CNP formulation from a cartridge.

[0215] Administration and Dosing

[0216] The hydrophobic CNP salts, or pharmaceutical compositions or formulations comprising them, can be administered to subjects in various ways such as, e.g., subcutaneously, intraarticularly, intraperitoneally, intramuscularly, intradermally or orally. In one embodiment, the CNP peptide salt composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

[0217] The hydrophobic CNP salts or salt compositions can also be administered by implantation of a depot at the target site of action (e.g., an abnormal or degenerated joint or cartilage area). Alternatively, the CNP salt can be administered sublingually under the tongue (e.g., sublingual tablet) by transdermal delivery (e.g., by means of a patch on the skin) or orally

in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

[0218] The hydrophobic CNP salt compositions described herein can be administered to patients in need thereof at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia). The safety and therapeutic efficacy of the CNP salts can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

[0219] In certain embodiments, the hydrophobic CNP salt compositions described herein are administered at a dose in the range from about 3 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg, or from about 3 nmol/kg to 100 nmol/kg. In some embodiments, the CNP salt compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP salt compositions are administered at a dose of about 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 µg/kg, or about 0.5, 0.8, 1.0, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of hydrophobic CNP salts described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc. In various embodiments, the CNP salt is administered daily subcutaneously. In various embodiments, the CNP salt is administered weekly subcutaneously. In various embodiments, the CNP variant is administered at a dose of 2.5 µg/kg/day to 60 µg/kg/day, 10µg/kg/day to 45 µg/kg/day, or 15µg/kg/day to 30 µg/kg/day. In various embodiments, the CNP variant is administered at a dose of 15 µg/kg/day. In various embodiments, the CNP variant is administered at a dose of 30 µg/kg/day.

[0220] The frequency of dosing/administration of a hydrophobic CNP salt for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The hydrophobic CNP salt can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the hydrophobic CNP salt composition is administered, in a single dose or in multiple doses, once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months, or as deemed appropriate by the treating physician. In various embodiments, the CNP variant is administered for 3 months, 6 months, 12 months or more.

[0221] In some embodiments, a hydrophobic CNP salt composition is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP salt composition may be administered subcutaneously or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP salt composition daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP salt compositions is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

[0222] Biomarkers

[0223] For treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements *in utero* and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, Collagen X, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

[0224] One CNP signaling marker is cGMP (guanosine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (*in vitro*) after CNP exposure, conditioned media from bone ex-plant studies (*ex vivo*) after CNP

exposure, and in the plasma (*in vivo*) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

[0225] Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover precedes new bone formation.

[0226] A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

[0227] Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type II collagen (cartilage-specific marker for cartilage formation), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic *in vivo* studies and from the conditioned media of *ex vivo* studies, using commercially available kits.

[0228] In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP salt or sustained release composition described herein in order to monitor the effects of the CNP composition on bone and cartilage formation and growth *in vivo*. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP salt or sustained release composition has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g., endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, and alkaline phosphatase.

[0229] In various embodiments, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP salt or sustained release composition. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

[0230] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

[0231] Example 1: Generation of Hydrophobic CNP Salts

[0232] In order to determine whether complexing the hydrophilic peptide CNP with a hydrophobic counterion to stabilize its charge would be effective to improve the formulation of CNP for therapeutic purposes, experiments to alter the electrostatic charge of CNP in combination with other ionic components were undertaken.

[0233] To optimize the electrostatic complexation between a CNP peptide and hydrophobic counterion species, and hence controlled precipitation of the peptide complex, the solution environment should allow a large population of both species to be ionized. pH adjustments of both solutions to an intermediate value between 10 (pI of CNP) and the acidic pKa of the counterion (for example, the pKa of an example counterion species oleic acid is ~4-5) is important for the yield of peptide complex precipitation.

[0234] To make hydrophobic CNP salts, including CNP variants, CNP-acetate is placed into water or a buffer. In a first experiment, a stock solution comprising CNP at 20 mg/mL in water was prepared. 100 mM Tris, pH 9.00 is also effective as a solvent. Disodium pamoic acid, oleic acid, or sodium docusate were placed in a buffer at a concentration that is in a molar excess to the number of oppositely charged groups on CNP, in this instance a 12 molar excess of counterion to CNP. CNP has 6 positively charged groups at pH 7, and this is a two-fold excess over the number of positively charged groups. It was found that in buffered solutions (76 mM acetate buffer pH 4.84 and 100 mM phosphate buffer pH 6.61) the counterions were not easily soluble and formed a suspension of what appeared to be insoluble or low solubility counterion in buffer, however in water and in 100 mM Tris pH 9.00 counterions oleic acid, disodium pamoic acid, and sodium docusate were soluble.

[0235] When a metal cation was added to facilitate salt precipitation, the metal cation was dissolved in water and added to the CNP solution and not the counterion solution, it was observed that if the metal cation is added directly to the counterion it induced precipitation of the counterion solutions. The metal cation solution (e.g., $ZnCl_2$) was dissolved in water at high concentration (>100 mg/mL) such that a small volume could be added to the CNP solution and not significantly change the concentration or pH. The metal cation could be added at various concentrations, but in the initial experiment it was added in a 4 molar excess to the CNP concentration (there are two negatively charged amino acids in CNP and this was added such that there were 2 moles of zinc per mol negatively charged amino acid). The counterion solution was then added to the CNP solution containing or not containing metal cation dropwise, vortexing the tube at the highest setting for one second after every addition.

[0236] After enough solution was added for the desired ratio of CNP to counterion, the reaction tube was spun in a centrifuge at 10,000xg for 5 minutes to pellet the salt precipitate. After spinning, the supernatant was then removed and an equal volume of water to supernatant removed was added and the pellet was resuspended. This time the tube was spun for 7500xg for 3-5 minutes and the salt could be observed to pellet. After spinning, the salt pellet was again washed with water and resuspended. The tube was spun again and supernatant removed. The contents were then moved to a vial (e.g., a 6R borosilicate glass vial), stoppered, frozen, and lyophilized.

[0237] The subsequent salt powder was then evaluated for solubility in various buffers. Initially, ~1 mg of powder was weighed into a tube and the proper volume of solvent added to get to 1 mg/mL. The tube was left overnight rocking at about 37° C. It was found that the oleate salts were dissolvable in 20% acetic acid. The pamoate salt was soluble in dimethyl sulfoxide (DMSO).

[0238] These experiments show that precipitation of highly aqueous soluble peptides into water insoluble/low solubility aggregates with sizes ranging from 5 nanometers to 1 millimeter in diameter is possible.

[0239] Example 2--Characterization of CNP Salts

[0240] Hydrophobic salts of CNP were made as in Example 1 and tested for precipitation and solubility. Table 1 describes that CNP complexed with various hydrophobic counterions results in precipitation or formation of solids.

Table 1

Counterion	Solvent for Reaction	PPT
CNP-Oleate	H_2O	Yes
CNP-Pamoate	H_2O	Yes
CNP-Deoxycholate	H_2O	Yes
CNP-Decanoate	H_2O	Gelation
CNP –Oleate	Na-Phos	Yes
CNP -Pamoate	Na-Phos	Yes
CNP -Deoxycholate	Na-Phos	Yes
CNP -Decanoate	Na-Phos	Gelation
CNP -Ca^{+2} (Oleate)	H_2O	Yes
CNP -Ca^{+2} (Pamoate)	H_2O	Yes
CNP -Ca^{+2} (deoxycholate)	H_2O	Yes
CNP -Ca^{+2} (decanoate)	H_2O	Yes
CNP -Zn^{+2} (Oleate)	H_2O	Yes
CNP -Zn^{+2} (Pamoate)	H_2O	Yes
CNP -Zn^{+2} (deoxycholate)	H_2O	Yes
CNP -Zn^{+2} (decanoate)	H_2O	Yes

[0241] Dissolution studies were also carried out with the salt precipitate. 1 mg CNP salt was resuspended in 50 mL 1X PBS, pH 6.5, at 37°C and measured for dissolving of solids and release of CNP into solution for 7 days. Buffer was not replaced daily. Figures 1A-1D and Figure 2 show that dissolution of the hydrophobic salts was slower than that for the CNP-acetate composition.

[0242] Example 3: Heterozygous NPR2 Mutations Are Responsive to CNP Treatment

[0243] To determine the effects of CNP on subjects with short stature resulting from NPR2 mutations, cellular models of NPR2 mutations were developed. Exemplary NPR2 mutations analyzed are set out in Figure 5. Rat chondrosarcoma (RCS) cells having either a knockout of, or heterozygous loss of function mutations in, the NPR2 gene were made by RNP transfection into RCS cells using 125ng NPR2 variants or wild-type NPR2 plasmid DNA transfected into RCS or HEK293 cells. Single cell clones were seeded and genotyped by Sanger sequencing. Cell models are able to reproduce published cGMP phenotypes of the different mutations.

[0244] The NPR2 clones were created by creating insertions and deletions in the first exon of NPR2 in RCS cells. The sequence of the first exon in NPR2 was confirmed by next-generation sequencing and is set out in Figure 4. NPR2 mutant cells were tested for activity in response to CNP administration by a cGMP stimulation assay using a CatchPoint Cyclic-GMP Fluorescent Assay after treatment with 6nM of Pro-Gly CNP37. Briefly, NIH3T3 cells (ATCC, CRL-1658) and NPR-transfected HEK293 cells were seeded at 60,000 cells/well in a 96-well plate (96-well black imaging plates, Grenier, #655090). RCS (rat chondrosarcoma) cells were seeded at 40,000 cells/well. Culture media was as follows: NIH3T3 culture media: DMEM high glucose, pyruvate (Thermo, 11995-073) + 10% FBS + 1x Pen Strep (abbrev P/S, Thermo, cat# 15140122). NIH3T3 was the control system for the cGMP assay HEK293 culture media: EMEM + 10% FBS + 1x P/S + 1x GMAX. RCS culture media: DMEM + 10% FBS + 1x Pen Strep. Serum free NIH3T3 media: DMEM + 1x P/S for treatment of cells with IBMX (CAS 28822-58-4); Serum free NIH3T3 media with BSA: DMEM + 1x P/S + 0.5 mg/mL BSA (Thermo, A9418-100G) for treatment of cells with CNP.

[0245] Cells were incubated for 24 hours at 37° C, 5% CO_2. For cells to be treated with CNP variants, plates were pre-treated with IBMX (Enzo life sciences, 89161-340, 1g) 15 minutes prior to use. IBMX is a potent, non-specific inhibitor of phosphodiesterases. An 800 mM stock solution of IBMX is diluted in IBMX dilution media (serum-free media (DMEM + 1x PBS mixed 1:1 with 1x PBS) to a 0.75 mM working stock.

[0246] For cell treatment, cells were removed from the incubator, growth media was removed from cells and cells treated with IBMX. 80 µL 0.75 mM IBMX was added to each well and cells returned to 37 °C incubator for 15 minutes. After 15 minutes CNP (40 µL/well) is added to each test well and cells returned to 37° C incubator for 15 minutes. The plate was mixed by gentle tapping and imaged on a Solentim cell metric to visualize cells and determine if there is any cell lifting and then placed back into 37° C incubator.

[0247] The reaction was stopped and cells lysed by adding 40 µL lysis buffer (from cGMP kit). Plate was placed on a shaker for 5 minutes to complete lysis. The cell lysate was used in the cGMP assay.

[0248] The cGMP assay was carried out using a cGMP calibrator, rabbit anti-cGMP antibody and HRP-cGMP prepared as according to manufacturer's protocol. 40 µL of calibrator was added to wells of an anti-cGMP antibody coated plated, and 40 µL of lysate to be analyzed added to the appropriate wells. 40 µL of reconstituted rabbit anti-cGMP antibody was added to all wells and plates placed on shaker five minutes for mixing. 40 µL of reconstituted HRP-cGMP was added to each well and incubated for 2 hours at room temperature. Plates were manually aspirated and washed 4x with 300 µL wash buffer. 100 µL of stoplight red substrate was added to each well, the plate, covered and left at room temperature for at least 10 mins, protected from light. The plate was read for fluorescence intensity on a Spectramax M, or similar instrument, at excitation 530 nm and emission at 590 nm.

[0249] Figure 3 shows that adding exogenous Pro-Gly-CNP37 variant rescues cGMP readout in a NPR2 +/- rat chondrosarcoma cell model. Previous activation data reports cGMP EC50 in the range of 40 to 360nM for activation of PRKG2 (Campbell et al., ACS Chem Biol 12, 2388-2398, 2017); Vaandrager et al., J Biol Chem 272, 11816-23, 1997); Pohler et al., FEBS Lett 374, 419-25, 1995). In the heterozygous NPR2 knockout cells, a CNP dose >0.163nM is able to achieve an intracellular concentration exceeding the EC50 range for PRKG2 activation cGMP (Figure 1). Whereas, in wild-type cells a CNP dose of 0.040nM is able to achieve the same cGMP concentration. These results demonstrate that CNP supplementation can achieve the cGMP levels necessary for PRKG2 activation and growth in cells with loss-of-function mutations of NPR2.

[0250] These results also suggest that administration of CNP variants is useful to restore bone growth in subjects with short stature that have reduced activity of NPR2. It is further contemplated that treatment with CNP variants will be beneficial in subjects having mutations in other growth plate genes in which cGMP signaling may be impaired.

Example 4: Identification of Mutations Associated With Short Stature

[0251] It is hypothesized that genes showing clear evidence of genetically-driven bi-directional effects are more likely to represent therapeutic targets that can be effectively modulated in a broad patient population. To identify genes that are core regulators of growth,

the intersections of five gene lists were analyzed including, the list of genes from genome-wide association study (GWAS). Core growth regulators would be the most likely to contain rare coding mutations with bidirectional effects (i.e. short stature or skeletal dysplasia AND tall stature or overgrowth).

[0252] Databases queried include: *GWAS* 2,067 non-repeating closest genes for each of the 3,290 independent genetic variants reported by a large GWAS meta-analysis of height using ~700,000 individuals were extracted; *HGMD* The "allmut" table from HGMD version v2019_2 was queried looking for all pathogenic variants labelled as "DM" having either "short stature" and "tall stature or overgrowth" in the same genes; *OMIM* The list of OMIM genes related to growth disorders was previously described and was created using the keywords: short stature, overgrowth, skeletal dysplasia, brachydactyly.

[0253] First, the Human Gene Mutation Database (HGMD version v2019_2) was queried for genes associated with short or tall stature (Stenson et al., Hum Genet 136:665-677, 2017). There were 47 genes annotated with at least one pathogenic variant reported in the literature to cause "short stature". Only 20 genes were annotated as tall stature or overgrowth genes. Secondly, a manually curated list of 258 OMIM genes was used (248 short, 20 tall) which was created using the keywords: short stature, overgrowth, skeletal dysplasia, brachydactyly (Wood et al., Nat Genet 46:1173-86, 2014). Third, the intersection of these lists was compared with the list of genes from GWAS. At the intersection of these list there were three genes known to be associated with height (IGF1R, NPPC, NPR2) and two additional genes were identified (FGFR3, SHOX).

[0254] Additional analysis led to generation of a new group of five Core genes that showed significantly decreased height (β=-0.20, 95%CI [-0.26 to -0.14], p=4.04x10-11) and significantly increased risk for Idiopathic Short Stature (ISS) (OR=2.75, 95%CI [1.92 - 3.96]. Each of the core five genes (FGFR3, IGF1R, NPPC, NPR2 and SHOX) were associated with height when considered individually, and also were associated with short stature when taken in combination with other mutations. Exemplary mutations in FGFR3, IGF1R, NPPC, NPR2 and SHOX are set out in Figure 6.

[0255] Combined LoF (loss of function) and Missense variants in NPR2 and IGF1R were also associated with increased risk for ISS (OR=3.31, P= 0.001, OR= 2.85, P=0.002, respectively). Entire gene deletions and/or mutations causing loss of protein function in SHOX, IGF1R, NPPC, NPR2 have been reported in familial short stature with various degrees of severity.

[0256] Analysis shows that carriers of variants in any of the five core genes are at approximately a 3-fold increased risk for ISS and account for 6.7% of the total ISS population. Furthermore, a dose-dependent rescue of NPR2 signaling in a cell model of NPR2 haploinsufficiency after adding exogenous CNP was demonstrated.

[0257] According to the omnigenic model (Liu, et al., Cell 177:1022-1034 e6 (2019); Boyle et al., Cell 169:1177-1186 (2017)) if these genes are core human growth genes, then their effects should be modulated by multiple weaker common genetic variants driving regulatory networks. To indirectly test this hypothesis, polygenic risk scores (PRS) were calculated for height using the largest published GWAS meta-analysis for height that did not include any samples from the UK Biobank project. The cohort was divided into five equally-sized (n=6,824) PRS quintiles (PRS 1 being the lowest height, PRS 5 the tallest height). There was a dose-dependent relationship between increasing PRS score and mean height (β=0.30 per each PRS quintile increase) (Fig. 7A). Carriers of LoF variants in the five core genes were consistently shorter than non-carriers across the five different PRS backgrounds. See Figure 7. The data suggest that the combined effect of PRS and rare protein variants is consistent with an additive model: polygenic effects modulated height in both carriers and non-carriers.

[0258] The risk for ISS across PRS groups was calculated using PRS=3 as a reference. The lowest PRS group was associated with increased risk for ISS and the highest PRS group with a decreased risk (OR=5.43, P=8.58x10-34; OR=0.22, P=4.49x10-7 for PRS 1 and PRS 5 respectively). The effect of rare coding variants of the five core genes was evaluated for ISS stratified by PRS group. Carriers of any of the five core genes were at increased risk for ISS in the first three quintiles (OR=2.64, P=3.09x10-5; OR=2.17, P=0.04; OR=5.29, P=1.58x10-5; OR=2.72, P=0.09 Figs. 7C-F). Consistent directions of effect were observed for carriers of each individual core gene on ISS risk stratified by PRS (Figs. 7C-F).

[0259] Further, additive effects of PRS, mostly coming from multiple common genetic variation with individual small effects, predicted 20.1% of the variance in height in the dataset. These additive effects of PRS appeared to have similar magnitude on carriers of rare coding variation of core genes as well as in non-carriers. This observation indicates that PRS may be a strong contributor in the differences in penetrance of rare pathogenic variants (especially in models of haploinsufficiency such as the ones described here). Supporting this idea, it was observed that two of eight NPR2 variant carriers with low NPR2 activity had a low-normal height. This data suggests that most ISS individuals possessing mutations in NPR2 may also

have a polygenic background that made them more susceptible to the pathogenic effect of losing NPR2 activity.

[0260] These results support the idea that CNP-based treatments could be effective in NPR2 haploinsufficient patient populations. Further, results showing a significant bi-directional (LoF and GoF) correlation of cGMP levels and height in NPR2 carriers of the general population suggest that targeting this receptor with CNP analogs could be an effective therapy for all ISS individuals.

[0261] Example 5: Release Profiles of CNP Salts In Vitro

[0262] Release analysis of the hydrophobic CNP salts were also carried out. Briefly, Pro-Gly-CNP37 Zn Pamoate, and Pro-Gly-CNP37 Zn-oleate were made fresh and placed into a dissolution apparatus, a Pion microDiss, in 15 mL media, impeller spinning at 250 RPM, temperature control setpoint 37.4 °C, the same day it was made. Every 24 hours for 4 days, vessel contents were transferred to VWR polypropylene "Falcon" tubes, spun down at 4000 x g for 30 min, RP-UPLC samples were taken/frozen down, and solids resuspended in fresh media (15 mL). The salt was run on RP-UPLC to obtain signal, and concentration was obtained from comparison to calibration curve. Percent salt released was obtained by comparing mass released to initial mass Pro-Gly-CNP37 used in making the salt.

[0263] Figure 8A and Figure 8B show the cumulative release profiles of the CNP peptide salts over 7 days, as a percentage of total amount of CNP peptide salt.

[0264] An additional release profile was run using the protocol above. ~16.6 mg of Pro-Gly-CNP37-acetate (starting material to make salts) or Pro-Gly-CNP37 Zn-Pamoate salt placed into each well. CNP salt was made, lyophilized, sealed, and stored at 4°C before use. For CNP acetate controls, vessel 1 in 1x PBS, Vessel 5 in 1x PBS+0.05% PS80. Every 24 hours for 7 days, vessel contents transferred to VWR polypropylene "Falcon" tubes, spun down at 4000 x g for 30 min, UPLC sample taken/frozen down, and solids resuspended in fresh media (15 mL). CNP salts were run on RP-UPLC to obtain signal and the concentration obtained from comparison to a calibration curve. Cumulative release profiles over 7 days are shown in Figures 9A and 9B.

[0265] Pro-Gly-CNP-37- Docusate and Pro-Gly-CNP-37-Zn-Docusate salts were made fresh, or made and lyophilized, and sealed in glass vial prior to use in Pion for Dissolution. Every 24 hours for 4 days, vessel contents transferred to VWR polypropylene "Falcon" tubes, spun down

at 4000 x g for 30 min, sample taken/frozen down, and solids resuspended in fresh media (15 mL). Salts were run on LC-MS to quantify, and % salt released by comparing to initial salt placed in dissolution vessel.

[0266] Figures 10A to 10C show the cumulative release profiles of the docusate salts over 4 days (Figure 10A) or 7 days (Figures 10B and 10C).

[0267] Example 6: Release Profiles of CNP Salts In Vivo

[0268] The release profile of an sample salt, CNP-pamoate was then analyzed for release profiles in vivo in rats over 7 days after subcutaneous injection of the CNP salt.

[0269] Figure 11 shows the release profile of Pro-Gly-CNP37 from the salt into plasma over 7 days. Release of the salts was observed as an initial burst over 24 hours with some salt release over time.

[0270] Any single embodiment herein may be supplemented with one or more element from any one or more other embodiment herein.

[0271] It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but is intended to cover all modifications which are within the spirit and scope of the invention as defined by the appended claims; the above description; the following numbered paragraphs, and/or shown in the attached drawings.

[0272] Examples of the Embodiments:

[0273] Paragraph 1. A composition comprising a salt of an electrostatically charged peptide, the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion.

[0274] Paragraph 2. The composition of Paragraph 1, wherein the hydrophobic counterion is complexed via a non-covalent bond.

[0275] Paragraph 3. The composition of Paragraph 1, wherein the hydrophobic counterion is complexed to the electrostatically charged peptide via a cleavable linker.

[0276] Paragraph 4. The composition of any one of Paragraphs 1 to 3, wherein the salt further comprises a cation complexed to the peptide-counterion complex.

[0277] Paragraph 5. The composition of Paragraph 4, wherein the electrostatically charged peptide, hydrophobic counterion, and cation are complexed via a non-covalent bond.

[0278] Paragraph 6. The composition of Paragraph 4 or 5, wherein the cation has a charge of +2, +3 or +4.

[0279] Paragraph 7. The composition of any one of Paragraphs 4 to 6, wherein the cation is a metal cation.

[0280] Paragraph 8. The composition of any one of Paragraphs 4 to 7, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), platinum (Pt), copper (Cu) and Gold (Au). In an alternative embodiment, provided is the composition of any one of Paragraphs 4 to 7, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), and Gold (Au).

[0281] Paragraph 9. The composition of any one of Paragraphs 1 to 8, wherein the hydrophobic counterion has a cLogP of about 0 to about 10, or a pKa of about -2 to about 5, or both.

[0282] Paragraph 10. The composition of any one of Paragraphs 1 to 9, wherein the hydrophobic counterion has a cLogP of about 2 to about 9, and a pKa less than about 5.

[0283] Paragraph 11. The composition of any one of Paragraphs 1 to 10, wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL) phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, sodium dodecyl benzenesulfonate. In an alternative embodiment, provided is the composition of any one of Paragraphs 1 to 10, wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL) phosphatidic acid, sodium decanoate, sodium 2-naphthalenesulfonate, sodium 1-heptanesulfonate sodium 1-octanesulfonate monohydrate, sodium 1-decanesulfonate, sodium dodecyl sulfate, sodium dodecyl benzenesulfonate and ionic surfactants.

[0284] Paragraph 12. The composition of any one of Paragraphs 1 to 11, wherein the peptide salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form.

[0285] Paragraph 13. The composition of any one of Paragraphs 1 to 12, wherein the electrostatically charged peptide is a C-type natriuretic peptide (CNP).

[0286] Paragraph 14. The composition of Paragraph 13, wherein the CNP is a CNP variant.

[0287] Paragraph 15. The composition of Paragraphs 13 or 14, wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4) and pharmaceutical salts thereof

[0288] Paragraph 16. The composition of Paragraph 15 wherein the CNP is CNP-acetate.

[0289] Paragraph 17. The composition of any one of Paragraphs 13 to 16 wherein the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

[0290] Paragraph 18. The composition of any one of Paragraphs 13 to 17, wherein if a cation is present, the cation is zinc or calcium.

[0291] Paragraph 19. The composition of any one of Paragraphs 1 to 18 further comprising an excipient, diluent or carrier.

[0292] Paragraph 20. The composition of Paragraph 19 wherein the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier.

[0293] Paragraph 21. A sterile pharmaceutical composition comprising the composition of any one of Paragraphs 1 to 20.

[0294] Paragraph 22. An extended release composition comprising a salt of an electrostatically charged peptide, the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion.

[0295] Paragraph 23. The extended release composition of Paragraph 22, wherein the hydrophobic counterion is complexed via non-covalent bond.

[0296] Paragraph 24. The extended release composition of Paragraph 23, wherein the hydrophobic counterion is complexed to the electrostatically charged peptide via a cleavable linker.

[0297] Paragraph 25. The extended release composition of any one of Paragraphs 22 to 24, wherein the salt further comprises a cation complexed to the peptide-counterion complex.

[0298] Paragraph 26. The extended release composition of Paragraph 25, wherein the electrostatistically charged peptide, hydrophobic counterion, and cation are complexed via a non-covalent bond.

[0299] Paragraph 27. The extended release composition of Paragraph 26, wherein the cation has a charge of +2, +3 or +4.

[0300] Paragraph 28. The extended release composition of any one of Paragraphs 25 to 27, wherein the cation is a metal cation.

[0301] Paragraph 29. The extended release composition of any one of Paragraphs 25 to 28, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), platinum (Pt), copper (Cu) and Gold (Au).

[0302] Paragraph 30. The extended release composition of any one of Paragraphs 22 to 29, wherein the hydrophobic counterion has a cLogP of about 0 to about 10, or a pKa of about -2 to about 5, or both.

[0303] Paragraph 31. The extended release composition of any one of Paragraphs 22 to 30, wherein the hydrophobic counterion has a cLogP of about 2 to about 9, and a pKa less than about 5.

[0304] Paragraph 32. The extended release composition of any one of Paragraphs 22 to 31, wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristate, palmitate,

stearate, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidate, decanoate, 2-naphthalenesulfonate, 1-heptanesulfonate, 1-octanesulfonate monohydrate, 1-decanesulfonate, dodecyl sulfate, dextran sulfate, and dodecyl benzenesulfonate.

[0305] Paragraph 33. The extended release composition of any one of Paragraphs 22 to 32, wherein the CNP salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form.

[0306] Paragraph 34. The extended release composition of any one of Paragraphs 22 to 33, wherein the electrostatically charged peptide is a C-type natriuretic peptide (CNP).

[0307] Paragraph 35. The extended release composition of Paragraph 34, wherein the CNP is a CNP variant.

[0308] Paragraph 36. The composition of Paragraph 34 or 35, wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4); and, pharmaceutical salts thereof.

[0309] Paragraph 37. The extended release composition of Paragraph 36, wherein the CNP is CNP-acetate.

[0310] Paragraph 38. The extended release composition of any one of Paragraphs 34 to 37, wherein the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

[0311] Paragraph 39. The extended release composition of any one of Paragraphs 34 to 38, wherein if a cation is present, the cation is zinc or calcium.

[0312] Paragraph 40. The extended release composition of any one of Paragraphs 33-39, wherein the peptide salt solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle is resuspended in an aqueous solution or in oil.

[0313] Paragraph 41. The extended release composition of Paragraph 40, wherein the aqueous solution is water, saline, or buffer.

[0314] Paragraph 42. The extended release composition of Paragraph 40, wherein the oil comprises a triglyceride or a fatty acid.

[0315] Paragraph 43. The extended release composition of Paragraph 42, wherein the fatty acid is saturated or unsaturated.

[0316] Paragraph 44. The extended release composition of Paragraph 42 or 43, wherein the fatty acid is a C-6 to C-20 fatty acid.

[0317] Paragraph 45. The extended release composition of any one of Paragraphs 42 to 44, wherein the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid.

[0318] Paragraph 46. The extended release composition of any one of Paragraphs 22 to 45, wherein (i) less than 20% of peptide is released by day 1; and

ii) about 90% of peptide is released by day 7, or about 90% of peptide is released by day 14, or about 90% of peptide is released by day 31, at pH 7 to 7.6.

[0319] Paragraph 47. The extended release composition of any one of Paragraphs 22 to 46, wherein about less than 20% of peptide is released by day 1 at pH 7 to 7.6.

[0320] Paragraph 48. The extended release composition of any one of Paragraphs 22 to 47, wherein about 90% of peptide is released by day 7 pH 7 to 7.6.

[0321] Paragraph 49. The extended release composition of any one of Paragraphs 22 to 47, wherein about 90% of peptide is released by day 30 pH 7 to 7.6.

[0322] Paragraph 50. The extended release composition of any one of Paragraphs 22 to 49 further comprising an excipient, diluent or carrier.

[0323] Paragraph 51. The extended release composition of Paragraph 50, wherein the excipient, excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier.

[0324] Paragraph 52. A sterile pharmaceutical composition comprising the extended release composition of any one of Paragraphs 22 to 51.

[0325] Paragraph 53. A method of making a composition comprising a salt of an electrostatically charged peptide comprising,

a) contacting an electrostatically charged peptide in an aqueous solution with a hydrophobic counterion in solution;

b) mixing the electrostatically charged peptide solution with the hydrophobic counterion solution in a manner sufficient for the peptide and counterion to form a complex, wherein the formation of the peptide-counterion complex results in formation of a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form comprising the peptide salt.

[0326] Paragraph 54. The method of Paragraph 53, optionally comprising before step (b), contacting the electrostatically charged peptide in solution with a cation in aqueous solution, forming a peptide-cation complex.

[0327] Paragraph 55. The method of Paragraph 53 or 54 further comprising step (c) washing the peptide salt in buffer or water.

[0328] Paragraph 56. The method of Paragraph 55 further comprising step (d) obtaining the peptide salt by centrifugation to form a peptide salt pellet.

[0329] Paragraph 57. The method of Paragraph 56 further comprising step (e) removing water from the peptide salt pellet.

[0330] Paragraph 58. The method of Paragraph 57 further comprising resuspending the pellet in an aqueous solution or oil.

[0331] Paragraph 59. The method of any one of Paragraphs 53 to 58, wherein the peptide: hydrophobic counterion ratio is between 1:1 to 1:20.

[0332] Paragraph 60. The method of any one of Paragraphs 54 to 59, wherein the peptide: cation ratio is between 1:1 to 1:10.

[0333] Paragraph 61. The method of any one of Paragraphs 53 to 60, wherein the hydrophobic counterion is complexed via non-covalent bond.

[0334] Paragraph 62. The composition of Paragraph 61, wherein the hydrophobic counterion is complexed to the electrostatically charged peptide via a cleavable linker.

[0335] Paragraph 63. The method of any one of Paragraphs 54 to 62, wherein if the salt further comprises a cation complexed to the peptide-counterion complex, the cation is complexed via covalent or non-covalent bond, or a mixture thereof.

[0336] Paragraph 64. The method of Paragraph 63, wherein the cation is complexed to the peptide-hydrophobic counterion complex via non-covalent bond.

[0337] Paragraph 65. The composition of Paragraph 63 or 64, wherein the electrostatistically charged peptide, hydrophobic counterion, and cation are complexed via a non-covalent bond.

[0338] Paragraph 66. The method of any one of Paragraphs 54 to 65, wherein the cation has a charge of +2, +3 or +4.

[0339] Paragraph 67. The method of any one of Paragraphs 54 to 66, wherein the cation is a metal cation.

[0340] Paragraph 68. The method of any one of Paragraphs 54 to 67, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), and Gold (Au). Further contemplated is the method of any one of Paragraphs 54 to 67, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), platinum (Pt), copper (Cu) and Gold (Au).

[0341] Paragraph 69. The method of any one of Paragraph 46 to 58, wherein the hydrophobic counterion has a cLogP of about 0 to about 10, or a pKa of about -2 to about 5, or both.

[0342] Paragraph 70. The method of any one of Paragraphs 53 to 69, wherein the hydrophobic counterion has a cLogP of about 2 to about 9, and a pKa less than about 5.

[0343] Paragraph 71. The method of any one of Paragraphs 53 to 70, wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL) and phosphatidic acid.

[0344] Paragraph 72. The method of any one of Paragraphs 53 to 71, wherein the electrostatically charged peptide is a C-type natriuretic peptide (CNP).

[0345] Paragraph 73. The method of Paragraph 72, wherein the CNP is a CNP variant.

[0346] Paragraph 74. The method of Paragraph 72 or 73, wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4);
and, salts thereof.

[0347] Paragraph 75. The method of Paragraph 74, wherein the CNP is CNP-acetate.

[0348] Paragraph 76. The method of any one of Paragraphs 72 to 75 wherein the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

[0349] Paragraph 77. The method of any one of Paragraphs 72 to 76, wherein if a cation is present, the cation is zinc or calcium.

[0350] Paragraph 78. A method of treating a bone-related disorder or skeletal dysplasia in a subject in need thereof comprising administering to the subject a composition comprising a hydrophobic salt of C-type natriuretic peptide (CNP) of any one of Paragraphs 1 to 52.

[0351] Paragraph 79. The method of Paragraph 78, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), insulin growth factor 1 receptor (IGF1R) mutation, and idiopathic short stature.

[0352] Paragraph 80. A method of elongating a bone or increasing long bone growth in a subject in need thereof, comprising administering to the subject a extended release composition comprising a salt of C-type natriuretic peptide (CNP) of any one of Paragraphs 1 to 52, and wherein the administering elongates a bone or increases long bone growth.

[0353] Paragraph 81. The method of any one of Paragraphs 78 to 80, wherein the CNP is a CNP variant.

[0354] Paragraph 82. The method of any one of Paragraphs 78 to 81, wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4);
and, salts thereof.

[0355] Paragraph 83. The method of Paragraph 83 wherein the CNP is CNP acetate.

[0356] Paragraph 84. The method of any one of Paragraphs 78 to 83, wherein the composition is administered subcutaneously, intradermally, intraarticularly, orally, or intramuscularly.

[0357] Paragraph 85. The method of any one of Paragraphs 78 to 84, wherein the composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

[0358] Paragraph 86. The method of any one of Paragraphs 78 to 85, wherein the composition is an extended release composition.

[0359] Paragraph 87. A salt of C-type natriuretic peptide comprising a CNP peptide in complex with a hydrophobic counterion.

[0360] Paragraph 88. The salt of Paragraph 87, wherein the hydrophobic counterion is complexed via non-covalent bond.

[0361] Paragraph 89. The salt of Paragraph 87 or 88, wherein the hydrophobic counterion has a cLogP of about 2 to about 9, or a pKa less than about 5, or both.

[0362] Paragraph 90. The salt of any one of Paragraphs 87 to 89, wherein the hydrophobic counterion has a cLogP of about 2 to about 9, and a pKa less than about 5.

[0363] Paragraph 91. The salt of any one of Paragraphs 87 to 90, wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristic acid, palmitic acid, stearic acid, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL) and phosphatidic acid.

[0364] Paragraph 92. The salt of any one of Paragraphs 87 to 91 further comprising a cation complexed with the peptide and counterion.

[0365] Paragraph 93. The salt of Paragraph 92, wherein the cation is complexed via a non-covalent bond.

[0366] Paragraph 94. The salt of Paragraph 92 or 93, wherein the cation has a charge of +2, +3 or +4.

[0367] Paragraph 95. The salt of any one of Paragraphs 92 to 94, wherein the cation is a metal cation.

[0368] Paragraph 96. The salt of any one of Paragraphs 92 to 95, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), and Gold (Au). Further contemplated is the salt of any one of Paragraphs 92 to 95, wherein the cation is selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), platinum (Pt), copper (Cu) and Gold (Au).

[0369] Paragraph 97. The salt of any one of Paragraphs 87 to 96, wherein the CNP salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form.

[0370] Paragraph 98. The salt of any one of Paragraphs 87 to 97, wherein the CNP is a CNP variant.

[0371] Paragraph 99. The salt of any one of Paragraphs 87 to 98, wherein the CNP is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4); and, pharmaceutical salts thereof.

[0372] Paragraph 100. The salt of Paragraph 99 wherein the CNP is CNP-acetate.

[0373] Paragraph 101. The salt of any one of Paragraphs 87 to 100 wherein the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

[0374] Paragraph 102. The salt of any one of Paragraphs 87 to 101, wherein if a cation is present, the cation is zinc or calcium.

[0375] Paragraph 103. The salt of any one of Paragraphs 87 to 102 which is purified.

[0376] Paragraph 104. A composition comprising a hydrophobic salt of any one of paragraphs 1 to 52 or 87 to 103 for use in treating a bone-related disorder or skeletal dysplasia, or for elongating a bone or increasing long bone growth.

[0377] Paragraph 105. Use of a composition comprising a hydrophobic salt of any one of paragraphs 1 to 52 or 87 to 103 in the preparation of a medicament for treating a bone-related disorder or skeletal dysplasia, or for elongating a bone or increasing long bone growth.

[0378] Paragraph 106. The composition or use of paragraphs 104 or 105 wherein the composition is an extended release composition.

WHAT IS CLAIMED:

1. A composition comprising a hydrophobic salt of a C-type natriuretic peptide (CNP), the salt comprising the CNP complexed with a hydrophobic counterion.

2. The composition of claim 1, wherein the salt further comprises a polyvalent cation complexed to the peptide-counterion complex.

3. The composition of claim 2, wherein the CNP, hydrophobic counterion, and cation are complexed via a non-covalent bond.

4. The composition of claim 2 or 3 wherein the cation has a charge of +2, +3 or +4.

5. The composition of any one of claims 2 to 4 wherein the cation is a metal cation.

6. The composition of any one of claims 2 to 5 wherein the cation comprises a metal selected from the group consisting of beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba), zinc (Zn), cadmium (Cd), boron (B), aluminum (Al), gallium (Ga), indium (In), thallium (Tl), Iron (Fe), Manganese (Mn), Cobalt (Co), Nickel (Ni), Titanium (Ti), Vanadium (V), platinum (Pt), copper (Cu) and Gold (Au).

7. The composition of any one of claims 2 to 6, wherein the cation comprises zinc or calcium.

8. The composition of any one of claims 1 to 7 wherein the hydrophobic counterion has a cLogP of about 0 to about 10, or its conjugate acid has a pKa of about -2 to about 5, or both.

9. The composition of any one of claims 1 to 8 wherein the hydrophobic counterion has a cLogP of about 2 to about 9, and its conjugate acid has a pKa less than about 5.

10. The composition of any one of claim 1-9, wherein the hydrophobic counterion is selected from the group consisting of a deprotonated fatty acid, a deprotonated bile acid, an ionic surfactant, naphthoate and derivatives thereof, nicotinate and derivatives thereof, an alkyl sulfonate, a dialkyl sulfosuccinate, a phospholipid, an alkyl sulfonate, an aryl sulfonate, an alkylbenzene sulfonate, an alkyl sulfate, an aryl sulfate, a dextran sulfate, an alkylbenzene sulfate, and a combination of any of the foregoing.

11. The composition of any one of claims 1 to 10 wherein the hydrophobic counterion is selected from the group consisting of palmitate, deoxycholate, oleate, pamoate, nicotinate, dodecyl sulfate, docusate, myristate, palmitate, stearate, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PL), phosphatidate,

decanoate, 2-naphthalenesulfonate, 1-heptanesulfonate, 1-octanesulfonate monohydrate, 1-decanesulfonate, dodecyl sulfate, dextran sulfate, and dodecyl benzenesulfonate.

12. The composition of any one of claims 1 to 11, wherein the peptide salt is in solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form.

13. The composition of any one of claims 1 to 12 wherein the CNP is a CNP variant.

14. The composition of any one of claims 1 to 13 wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4), and salts thereof.

15. The composition of claim 14, wherein the CNP is CNP-acetate.

16. The composition of any one of claims 1 to 15 wherein the hydrophobic counterion is oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

17. The composition of any one of claims 2 to 16, wherein cation is Zn^{2+} or Ca^{2+}.

18. The composition of any one of claims 1 to 17 further comprising an excipient, diluent or carrier.

19. The composition of claim 18 wherein the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier.

20. A sterile pharmaceutical composition comprising the composition of any one of claims 1-19.

21. An extended release composition comprising a salt of a C-type natriuretic peptide (CNP), the salt comprising the electrostatically charged peptide complexed with a hydrophobic counterion.

22. The extended release composition of claim 21, wherein the salt further comprises a cation complexed to the peptide-counterion complex.

23. The extended release composition of claims 21 or 22 wherein the peptide salt solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle is resuspended in an aqueous solution or in oil.

24. The extended release composition of claim 23, wherein the oil comprises a triglyceride or a fatty acid, optionally wherein the fatty acid is saturated or unsaturated.

25. The extended release composition of claim 23 or claim 24, wherein the fatty acid is a C-6 to C-20 fatty acid.

26. The extended release composition of any one of claims 23 to 25, wherein the fatty acid is hexanoic acid, octanoic acid, decanoic acid, or dodecanoic acid.

27. The extended release composition of claim 23, wherein the aqueous solution is water, saline, or buffer.

28. The extended release composition of any one of claims 21 to 27, wherein

(i) less than 20% of peptide is released by day 1; and

(ii) about 90% of peptide is released by day 7, or about 90% of peptide is released by day 14, or about 90% of peptide is released by day 31,

at pH 7 to 7.6.

29. The extended release composition of any one of claims 21 to 28 further comprising a pharmaceutically acceptable excipient, diluent or carrier.

30. A method of making a composition comprising a hydrophobic salt of a C-type natriuretic peptide (CNP) comprising,

a) contacting the CNP in an aqueous solution with a hydrophobic counterion in solution;

b) mixing the CNP solution with the hydrophobic counterion solution in a manner sufficient for the peptide and counterion to form a complex, wherein the formation of the peptide-counterion complex results in formation of a solid, semi-solid, gel, crystalline, amorphous, nanoparticle, microparticle, amorphous nanoparticle, amorphous microparticle, crystalline nanoparticle or crystalline microparticle form comprising the CNP salt.

31. The method of claim 30, optionally comprising before step (b), contacting the CNP in solution with a polyvalent cation in aqueous solution, forming a peptide-cation complex.

32. The method of claim 30 or claim 31 further comprising step (c) washing the hydrophobic CNP salt with buffer or water.

33. The method of claim 32 further comprising step (d) obtaining the hydrophobic CNP salt by centrifugation to form a CNP salt pellet.

34. The method of claim 33 further comprising step (e) removing water from the CNP salt pellet.

35. The method of claim 34 further comprising resuspending the pellet in an aqueous solution or oil.

36. The method of any one of claims 30-35, wherein the peptide: hydrophobic counterion ratio is between 1:1 to 1:20.

37. The method of any one of claims 31-36, wherein the peptide:cation ratio is between 1:1 to 1:10.

38. A method of treating a bone-related disorder or skeletal dysplasia in a subject in need thereof comprising administering to the subject a composition comprising a hydrophobic CNP salt of any one of claims 1-29 or 44-52.

39. The method of claim 38, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), insulin growth factor 1 receptor (IGF1R) mutation, and idiopathic short stature.

40. A method of elongating a bone or increasing long bone growth in a subject in need thereof, comprising administering to the subject a composition comprising a hydrophobic

CNP salt of any one of claims 1 to 29 or 44-52, and wherein the administering elongates a bone or increases long bone growth.

41. The method of any one of claims 38 to 40, wherein the composition is administered subcutaneously, intradermally, intraarticularly, orally, or intramuscularly.

42. The method of any one of claims 38 to 41, wherein the composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

43. The method of any one of claims 38 to 42, wherein the composition is an extended release composition.

44. A hydrophobic salt of C-type natriuretic peptide (CNP) comprising a CNP in complex with a hydrophobic counterion.

45. The hydrophobic salt of claim 44 further comprising a cation complexed with the peptide and counterion.

46. The hydrophobic salt of claim 44 or 45, wherein the hydrophobic counterion is selected from the group consisting of oleate, deoxycholate, decanoate, pamoate, docusate or dodecyl sulfate.

47. The hydrophobic salt of any one of claims 44 to 46, wherein cation is Zn^{2+} or Ca^{2+}.

48. The hydrophobic salt of any one of claims 44 to 46, wherein the salt is selected from the group consisting of CNP-oleate, CNP-pamoate, CNP-deoxycholate, CNP-decanoate and CNP-docusate.

49. The hydrophobic salt of any one of claims 45 to 48, wherein the salt is selected from the group consisting of CNP–Ca^{+2}(Oleate), CNP–Ca^{+2}(Pamoate), CNP–Ca^{+2}(deoxycholate), CNP–Ca^{+2}(decanoate), CNP–Ca^{+2}(docusate), CNP–Zn^{+2}(Oleate), CNP–Zn^{+2}(Pamoate), CNP–Zn^{+2}(deoxycholate), CNP–Zn^{+2}(decanoate), and CNP–Zn^{+2}(docusate).

50. The hydrophobic salt of any one of claims 44 to 49, wherein the CNP is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1)

LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4), and salts thereof.

51. The hydrophobic salt of any one of claims 44 to 50, wherein the CNP is CNP-acetate.

52. The hydrophobic salt of any one of claims 44 to 51 which is purified.

53. A composition comprising a hydrophobic CNP salt of any one of claims 1 to 29 or 44-52 for use in treating a bone-related disorder or skeletal dysplasia, or for elongating a bone or increasing long bone growth.

54. Use of a composition comprising a hydrophobic CNP salt of any one of claims 1 to 29 or 44-52 in the preparation of a medicament for treating a bone-related disorder or skeletal dysplasia, or for elongating a bone or increasing long bone growth.

Figure 1A

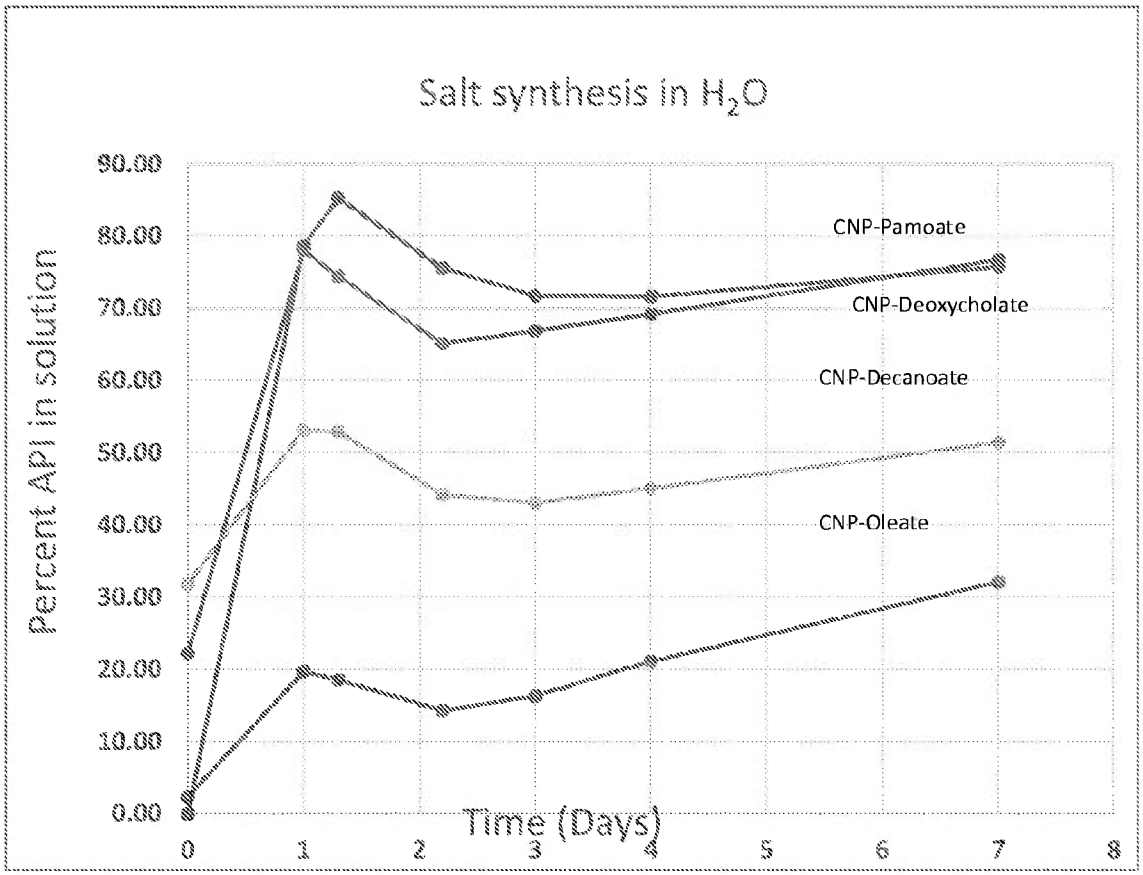
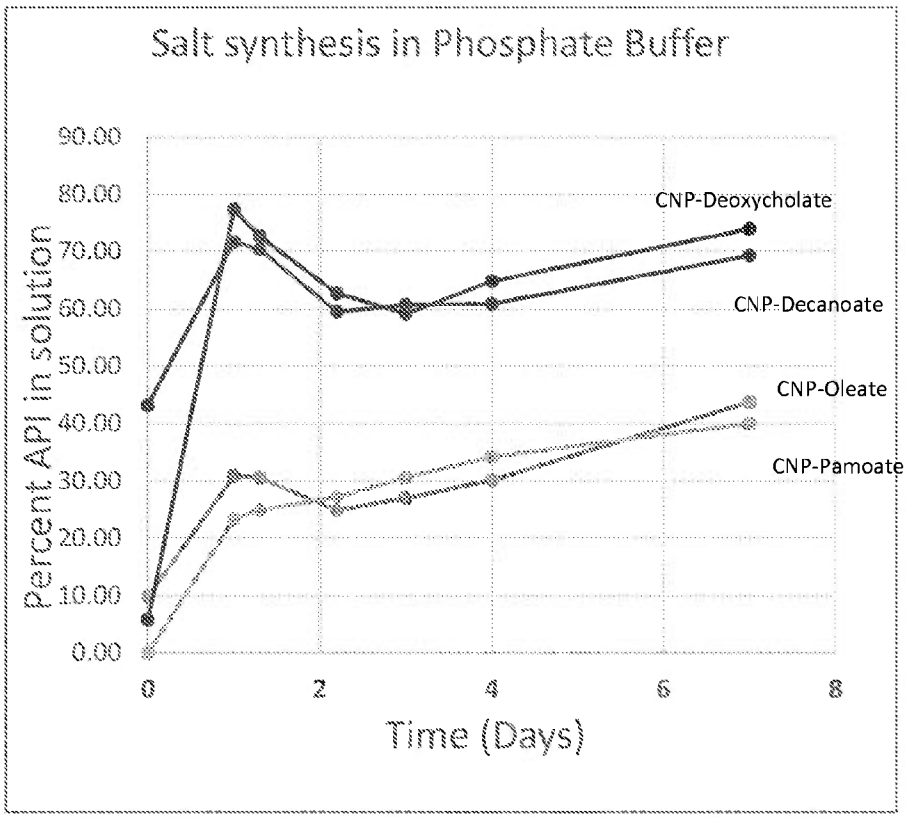

Salt synthesis in H_2O
Percent API in solution
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Time (Days)
0
1
2
3
4
5
6
7
8
CNP-Pamoate
CNP-Deoxycholate
CNP-Decanoate
CNP-Oleate

Figure 1B


Salt synthesis in Phosphate Buffer
Percent API in solution
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
2
4
6
8
Time (Days)
CNP-Deoxycholate
CNP-Decanoate
CNP-Oleate
CNP-Pamoate

Figures 1C

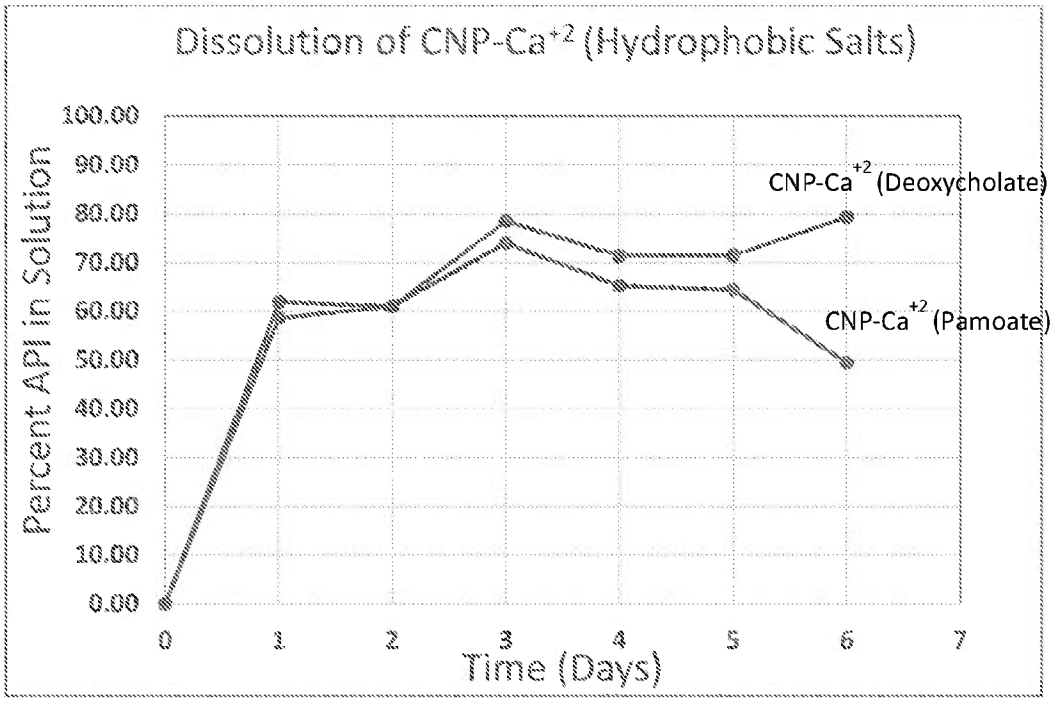
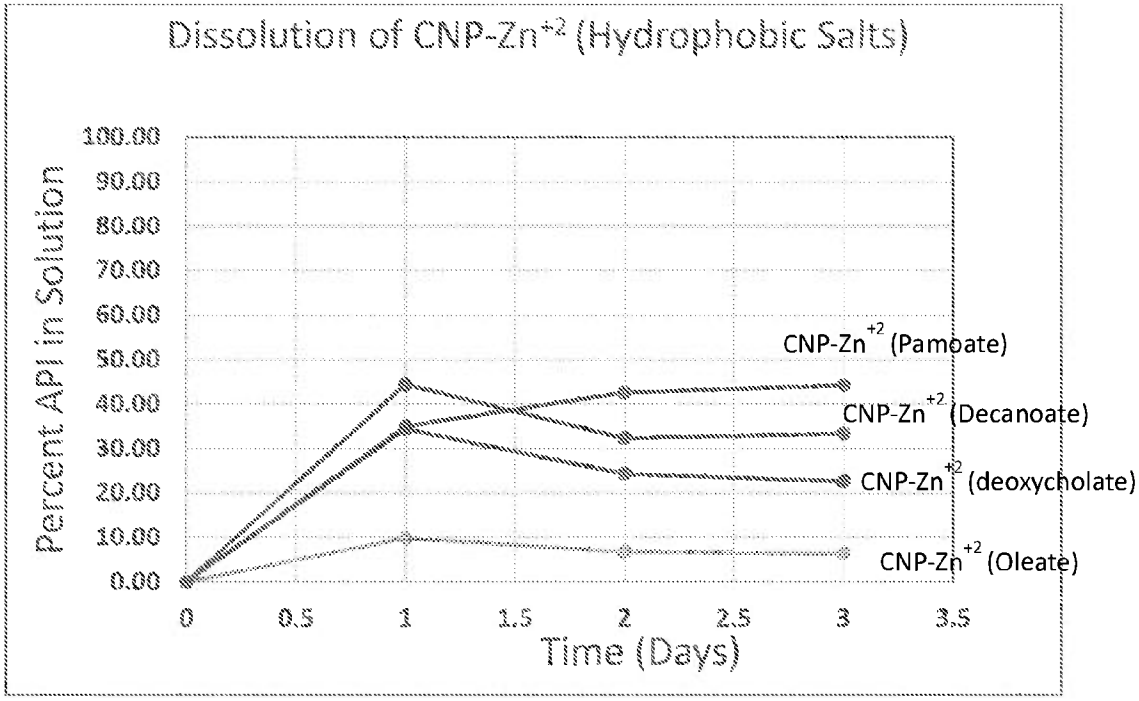

Dissolution of CNP-Ca+2 (Hydrophobic Salts)
Percent API in Solution
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
1
2
3
4
5
6
7
Time (Days)
CNP-Ca+2 (Deoxycholate)
CNP-Ca+2 (Pamoate)

Figure 1D


Dissolution of CNP-Zn+2 (Hydrophobic Salts)
Percent API in Solution
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
0.5
1
1.5
2
2.5
3
3.5
Time (Days)
CNP-Zn+2 (Pamoate)
CNP-Zn+2 (Decanoate)
CNP-Zn+2 (deoxycholate)
CNP-Zn+2 (Oleate)

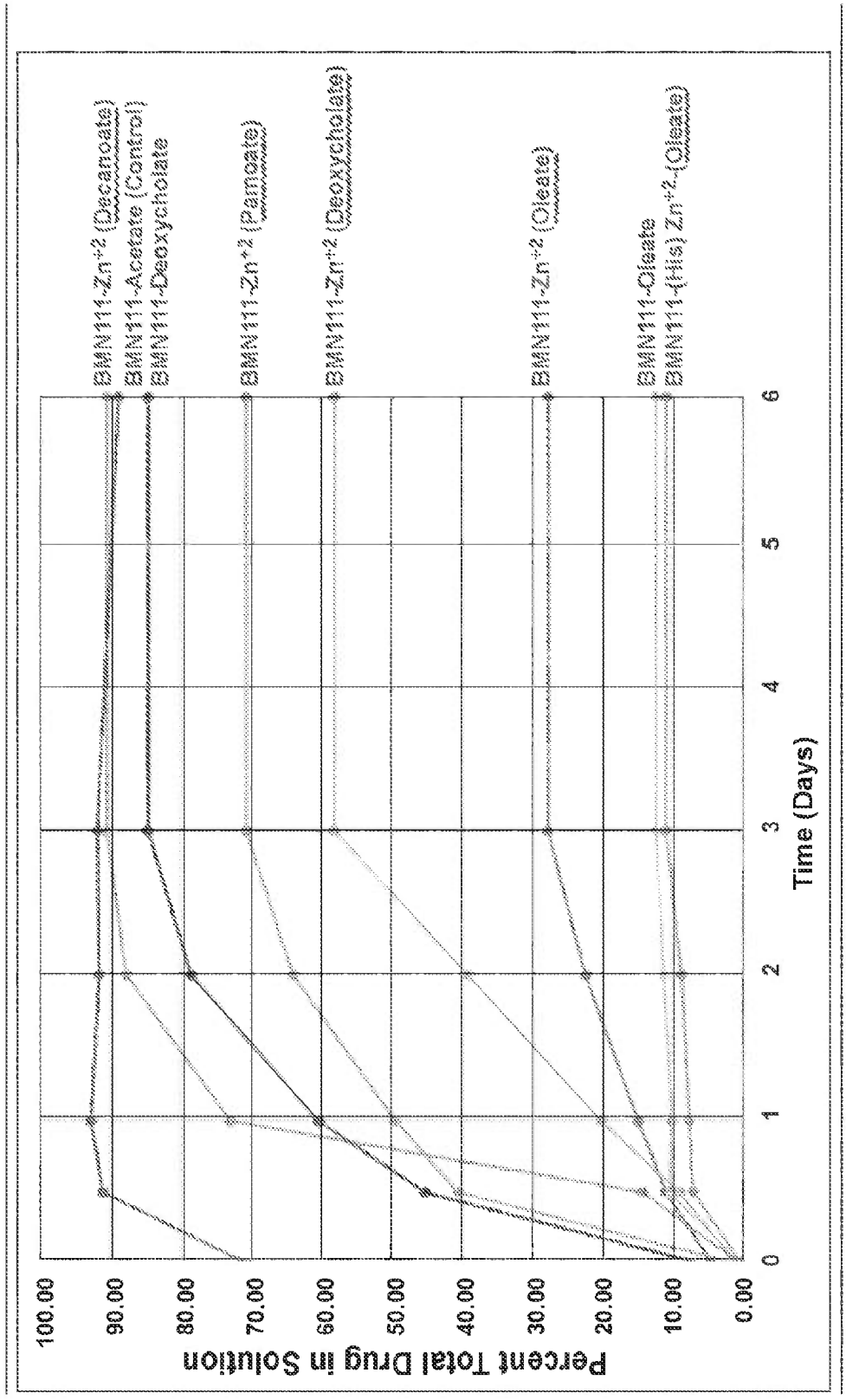

Percent Total Drug in Solution
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
1
2
3
4
5
6
Time (Days)
BMN111-Zn+2 (Decanoate)
BMN111-Acetate (Control)
BMN111-Deoxycholate
BMN111-Zn+2 (Pamoate)
BMN111-Zn+2 (Deoxycholate)
BMN111-Zn+2 (Oleate)
BMN111-Oleate
BMN111-(His) Zn+2-(Oleate)

Figure 2

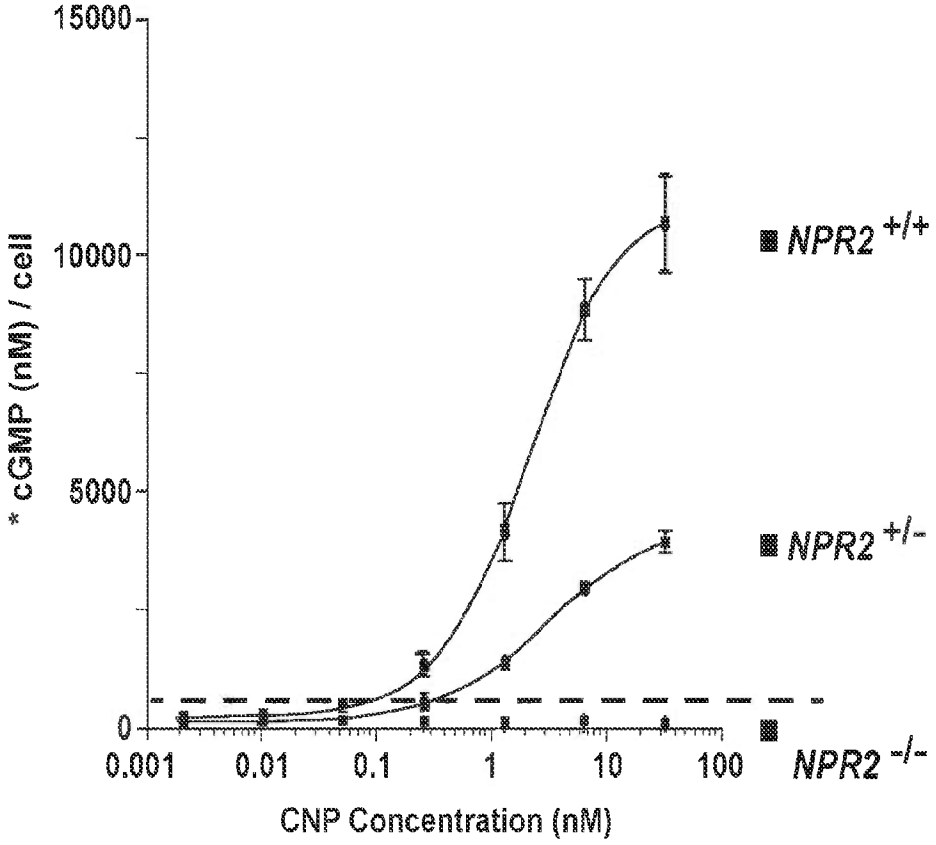

15000
10000
5000
0
* cGMP (nM) / cell
0.001
0.01
0.1
1
10
100
CNP Concentration (nM)
NPR2 +/+
NPR2 +/-
NPR2 -/-
cGMP EC50 = 96 nM

Figure 3

Figure 4

NPR2 Clone, nucleotide sequence

Clone 47, Allele 1 (SEQ ID NO: 57):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATCGA
GGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAG
GTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 35, Allele 1 (SEQ ID NO: 58):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATCGA
GGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAG
GTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 35, Allele 2(SEQ ID NO: 59):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCACA
ATTGGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCAT
CGAGGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGC
CAGGTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 47, Allele 1(SEQ ID NO: 60):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC

Figure 4 Continued

TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATC**C**G
AGGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCA
GGTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 47, Allele 2(SEQ ID NO: 61):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCGGGGTGTTTGAGGCCCTGCAGGGCAGCAAC
CTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAGGTGGCCCTGAGCAAGCCACCCACTTCATCAGA
GCCAACGGGCGCA

NPR2 Clone, predicted protein product sequence

Clone 47, Allele 1 (SEQ ID NO: 62):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIEGVFEALQGSNLSVQHQVYTREPGGPEQAT
HFIRANGRIVYICGPLEMLHEILLQAQRENLTNGDYVFFYLDVFGESLRAGPTRATGRPWQDNRTQEQAQAL
REAFQTVLVITYREPPNPEYQEFQNRLLIRAREDFGVELAPSLMNLIAGCFYDGILLYAQVLNETIQEGGTRED
GLRIVEKMQGRRYHGVTGLVVMDKNNDRETDFVLWAMGDLESGDFQPAAHYSGAEKQIWWTGRPIPW
VKGAPPLDNPPCAFDLDDPSCDKTPLSTLAIVALGTGITFIMFGVSSFLIFRKLMLEKELASMLWRIRWEELQF
GNSDRYHKGAGSRLTLSLRGSSYGSLMTAHGKYQIFANTGHFKGNVVAIKHVNKKRIELTRQVLFELKHMRD
VQFNHLTRFIGACIDPPNICIVTEYCPRGSLQDILENDSINLDWMFRYSLINDLVKGMAFLHNSIISSHGSLKSS
NCVVDSRFVLKITDYGLASFRSTAEPDDSHALYAKKLWTAPELLSGNPLPTTGMQKADVYSFAIILQEIALRSG
PFYLEGLDLSPKEIVQKVRNGQRPYFRPSIDRTQLNEELVLLMERCWAQDPTERPDFGQIKGFIRRFNKEGGT
SILDNLLLRMEQYANNLEKLVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEAFDSVTIYFSDIVGF
TALSAESTPMQVVTLLNDLYTCFDAIIDNFDVYKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVS
SFRIRHRPHDQLRLRIGVHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGQALKIHVSSTTKDALDELGC
FQLELRGDVEMKGKGKMRTYWLLGERKGPPGLL*

Clone 35, Allele 1(SEQ ID NO: 63):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIEGVFEALQGSNLSVQHQVYTREPGGPEQAT
HFIRANGRIVYICGPLEMLHEILLQAQRENLTNGDYVFFYLDVFGESLRAGPTRATGRPWQDNRTQEQAQAL
REAFQTVLVITYREPPNPEYQEFQNRLLIRAREDFGVELAPSLMNLIAGCFYDGILLYAQVLNETIQEGGTRED
GLRIVEKMQGRRYHGVTGLVVMDKNNDRETDFVLWAMGDLESGDFQPAAHYSGAEKQIWWTGRPIPW
VKGAPPLDNPPCAFDLDDPSCDKTPLSTLAIVALGTGITFIMFGVSSFLIFRKLMLEKELASMLWRIRWEELQF

Figure 4 Continued

GNSDRYHKGAGSRLTLSLRGSSYGSLMTAHGKYQIFANTGHFKGNVVAIKHVNKKRIELTRQVLFELKHMRD
VQFNHLTRFIGACIDPPNICIVTEYCPRGSLQDILENDSINLDWMFRYSLINDLVKGMAFLHNSIISSHGSLKSS
NCVVDSRFVLKITDYGLASFRSTAEPDDSHALYAKKLWTAPELLSGNPLPTTGMQKADVYSFAIILQEIALRSG
PFYLEGLDLSPKEIVQKVRNGQRPYFRPSIDRTQLNEELVLLMERCWAQDPTERPDFGQIKGFIRRFNKEGGT
SILDNLLLRMEQYANNLEKLVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEAFDSVTIYFSDIVGF
TALSAESTPMQVVTLLNDLYTCFDAIIDNFDVYKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVS
SFRIRHRPHDQLRLRIGVHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGQALKIHVSSTTKDALDELGC
FQLELRGDVEMKGKGKMRTYWLLGERKGPPGLL*

Clone 35, Allele 2(SEQ ID NO: 64):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFTIGLLGLLCCIWMLAQMTGPTTSPSRGCLRPCRAATSVCNTRCIPESQVALSKPPTS
SEPTGALCISAARWRCFMRSCFRPRGRT*

Clone 47, Allele 1(SEQ ID NO: 65):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIRGGV*

Clone 47, Allele 2(SEQ ID NO: 66):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPGCLRPCRAATSVCNTRCIPESQVALSKPPTSSEPTGALCISAARWRCFMRSCFRPRGRT*

Figure 5

Genetic Variants Found in NPR2 Genes

NPR2 Mutations			
G21R	P301S	Q500X	R745Q
A48S	R318Q	H508D	R819C
A59T	R358W	G541S	T861I
V102A	E359A	A549T	V883M
R110G	I364fs	R562Q	T907M
A164G	E389D	R601H	G917R
I226V	E415K	R601S	R921Q
Y250X	K480N	E609K	R957C
R263H	A488P	S721R	L1009V
T297M	I494S	E727Q	

Figure 6

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
2	231925451	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.355G>C	ENSP00000387159.1:p.Gly119Arg		33
2	231925486	['G', 'GT']	1	1.00	['frameshift_variant'	HIGH	NPPC	ENST00000409852.2:c.319_320insA	ENSP00000387159.1:p.Ser107TyrfsTer37		
2	231925513	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.293A>G	ENSP00000387159.1:p.Tyr98Cys		23
2	231925514	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.292T>G	ENSP00000387159.1:p.Tyr98Asp		18
2	231925523	['C', 'A']	2	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.283G>T	ENSP00000387159.1:p.Ala95Ser		15
2	231925529	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.277C>T	ENSP00000387159.1:p.Pro93Ser	4.04E-06	26
2	231925540	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.266T>A	ENSP00000387159.1:p.Leu89Gln		23
2	231925550	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.256G>C	ENSP00000387159.1:p.Ala86Pro		23
2	231925553	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.253T>C	ENSP00000387159.1:p.Trp85Arg	4.06E-06	23
2	231925580	['G', 'T']	3	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.226C>A	ENSP00000387159.1:p.Arg76Ser	6.96E-05	25
2	231925584	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.222C>G	ENSP00000387159.1:p.Asp74Glu		21
2	231925597	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.209G>T	ENSP00000387159.1:p.Arg70Leu		26
2	231925600	['G', 'T']	1	1.00	['stop_gained']	HIGH	NPPC	ENST00000409852.2:c.206C>A	ENSP00000387159.1:p.Ser69Ter		38
2	231925639	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.167C>T	ENSP00000387159.1:p.Ala56Val	3.95E-05	18
2	231925646	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.160G>T	ENSP00000387159.1:p.Asp54Tyr		23
2	231925649	['CGTT', 'C'	3	1.00	['inframe_deletion']	MODERATE	NPPC	ENST00000409852.2:c.154_156del	ENSP00000387159.1:p.Lys52del	4.44E-06	
2	231925649	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.157G>A	ENSP00000387159.1:p.Gly53Ser		21
2	231925657	['T', 'C']	3	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.149A>G	ENSP00000387159.1:p.Gln50Arg	1.81E-05	21
2	231925663	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.143G>T	ENSP00000387159.1:p.Gly48Val		24
2	231925669	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.137C>G	ENSP00000387159.1:p.Ala46Gly		23
2	231925708	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.98G>A	ENSP00000387159.1:p.Arg33Gln	5.65E-06	24
2	231925711	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.95C>T	ENSP00000387159.1:p.Pro32Leu		13
2	231926189	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.61T>C	ENSP00000387159.1:p.Ser21Pro	3.40E-05	23
2	231926200	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.50C>G	ENSP00000387159.1:p.Ser17Cys		28
2	231926201	['A', 'AGAC	1	1.00	['inframe_insertion']	MODERATE	NPPC	ENST00000409852.2:c.40_48dup	ENSP00000387159.1:p.Thr14_Leu16dup		
4	1793974	['G', 'GTG(	1	1.00	['inframe_insertion']	MODERATE	FGFR3	ENST00000340107.8:c.42_47dup	ENSP00000339824.4:p.Ala15_Ile16insMetAla		
4	1794009	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.75G>C	ENSP00000339824.4:p.Leu25Phe		8
4	1794016	['G', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.82G>C	ENSP00000339824.4:p.Glu28Gln		22
4	1794043	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.109G>A	ENSP00000339824.4:p.Glu37Lys		22
4	1799274	['G', 'A']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.130G>A	ENSP00000339824.4:p.Gly44Ser	0.000225	0
4	1799289	['T', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.145T>G	ENSP00000339824.4:p.Leu49Val	4.06E-06	5
4	1799292	['G', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.148G>T	ENSP00000339824.4:p.Val50Phe		22
4	1799292	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.148G>A	ENSP00000339824.4:p.Val50Ile		13
4	1799303	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.159C>A	ENSP00000339824.4:p.Ser53Arg		11
4	1799304	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.160G>A	ENSP00000339824.4:p.Gly54Arg	4.06E-05	23
4	1799310	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.166G>A	ENSP00000339824.4:p.Ala56Thr		0
4	1799316	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.172G>A	ENSP00000339824.4:p.Glu58Lys	8.13E-06	15
4	1799328	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.184C>A	ENSP00000339824.4:p.Pro62Thr		1
4	1799332	['C', 'G']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.188C>G	ENSP00000339824.4:p.Pro63Arg	3.27E-05	3
4	1799337	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.193G>A	ENSP00000339824.4:p.Gly65Arg	0.000176	11
4	1799389	['T', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.245T>C	ENSP00000339824.4:p.Val82Ala		2
4	1799416	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.272C>T	ENSP00000339824.4:p.Pro91Leu	0.000176	8
4	1799422	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.278G>A	ENSP00000339824.4:p.Arg93Gln	1.69E-05	12
4	1799457	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.313G>A	ENSP00000339824.4:p.Gly105Arg	9.26E-06	24
4	1799472	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.328C>T	ENSP00000339824.4:p.Arg110Trp	1.50E-05	22
4	1799473	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.329G>A	ENSP00000339824.4:p.Arg110Gln	7.05E-05	17
4	1799479	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.335G>A	ENSP00000339824.4:p.Arg112Gln	0.000229	9
4	1799491	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.347G>A	ENSP00000339824.4:p.Arg116His	4.18E-05	3
4	1799493	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.349G>A	ENSP00000339824.4:p.Val117Ile	7.21E-05	0
4	1799496	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.352C>G	ENSP00000339824.4:p.Leu118Val	1.22E-05	12
4	1799756	['C', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.389C>A	ENSP00000339824.4:p.Ser130Tyr		19
4	1799756	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.389C>T	ENSP00000339824.4:p.Ser130Phe	1.20E-05	17
4	1799758	['T', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.391T>C	ENSP00000339824.4:p.Ser131Pro	8.01E-06	22
4	1799770	['G', 'A']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.403G>A	ENSP00000339824.4:p.Glu135Lys	1.20E-05	21
4	1799776	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.409G>A	ENSP00000339824.4:p.Gly137Arg	1.60E-05	15
4	1799792	['AGGACA'	1	1.00	['inframe_deletion']	MODERATE	FGFR3	ENST00000340107.8:c.436_441del	ENSP00000339824.4:p.Arg146_Ala147del	1.20E-05	
4	1801373	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.452C>T	ENSP00000339824.4:p.Pro151Leu		27
4	1801384	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.463C>T	ENSP00000339824.4:p.Arg155Trp	6.53E-06	24
4	1801393	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.472C>T	ENSP00000339824.4:p.Arg158Trp	6.50E-06	23
4	1801394	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.473G>A	ENSP00000339824.4:p.Arg158Gln	5.85E-05	27
4	1801402	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.481A>G	ENSP00000339824.4:p.Lys161Glu		31
4	1801435	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.514G>A	ENSP00000339824.4:p.Val172Ile	6.67E-05	24

Figure 6 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
4	1801439	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.518G>A	ENSP00000339824.4:p.Arg173His	4.00E-05 28
4	1801445	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.524G>A	ENSP00000339824.4:p.Arg175His	3.98E-05 27
4	1801456	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.535G>A	ENSP00000339824.4:p.Ala179Thr	14
4	1801477	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.556A>G	ENSP00000339824.4:p.Ile186Val	23
4	1801478	['T', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.557T>A	ENSP00000339824.4:p.Ile186Asn	25
4	1801481	['C', 'A']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.560C>A	ENSP00000339824.4:p.Ser187Tyr	2.52E-05 19
4	1801495	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.574G>A	ENSP00000339824.4:p.Gly192Ser	26
4	1801506	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.585C>G	ENSP00000339824.4:p.Phe195Leu	1.84E-05 23
4	1801510	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.589G>A	ENSP00000339824.4:p.Gly197Ser	6.04E-06 24
4	1801634	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.630G>T	ENSP00000339824.4:p.Gln210His	16
4	1801689	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.685G>A	ENSP00000339824.4:p.Val229Ile	12
4	1801717	['G', 'A']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.713G>A	ENSP00000339824.4:p.Arg238Gln	0.0005 9
4	1801743	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.739G>A	ENSP00000339824.4:p.Glu247Lys	1.34E-05 34
4	1801841	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.746C>T	ENSP00000339824.4:p.Ser249Phe	1.26E-05 25
4	1801849	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.754C>T	ENSP00000339824.4:p.Arg252Trp	25
4	1801874	['C', 'T']	8	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.779C>T	ENSP00000339824.4:p.Pro260Leu	2.51E-05 28
4	1801903	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.808G>A	ENSP00000339824.4:p.Asp270Asn	9.32E-06 22
4	1801948	['A', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.853A>G	ENSP00000339824.4:p.Ile285Val	2.41E-05 26
4	1802005	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.910C>T	ENSP00000339824.4:p.Pro304Ser	26
4	1802941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.9586>T	ENSP00000339824.4:p.Asp320Asn	4.33E-06 23
4	1802944	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.961G>A	ENSP00000339824.4:p.Val321Met	2.60E-05 20
4	1802948	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.965G>A	ENSP00000339824.4:p.Arg322His	8.68E-06 23
4	1802953	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.970C>T	ENSP00000339824.4:p.Arg324Cys	1.30E-05 22
4	1802954	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.971G>A	ENSP00000339824.4:p.Arg324His	1.73E-05 18
4	1802960	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.986C>T	ENSP00000339824.4:p.Ser329Leu	3.07E-05 24
4	1802975	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.992G>A	ENSP00000339824.4:p.Arg331Gln	2.19E-05 18
4	1802986	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1003G>A	ENSP00000339824.4:p.Glu335Lys	4.85E-05 20
4	1803005	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1022C>T	ENSP00000339824.4:p.Thr341Ile	22
4	1803019	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1036G>A	ENSP00000339824.4:p.Val346Met	1.74E-05 22
4	1803046	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1063G>A	ENSP00000339824.4:p.Val355Ile	1.34E-05 24
4	1803052	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1069G>A	ENSP00000339824.4:p.Gly357Arg	2.25E-05 17
4	1803054	['G', 'GC']	3	1.00	['frameshift_variant'	HIGH	FGFR3	ENST00000340107.8:c.1075dup	ENSP00000339824.4:p.Arg359ProfsTer11	
4	1803058	['C', 'T']	1	1.00	['stop_gained']	HIGH	FGFR3	ENST00000340107.8:c.1075C>T	ENSP00000339824.4:p.Arg359Ter	1.82E-05 39
4	1803059	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1076G>A	ENSP00000339824.4:p.Arg359Gln	3.64E-05 9
4	1803061	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1078G>A	ENSP00000339824.4:p.Ala360Thr	18
4	1804332	['G', 'C']	5	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1084G>C	ENSP00000339824.4:p.Glu362Gln	1.24E-05 15
4	1804353	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1105G>T	ENSP00000339824.4:p.Asp369Tyr	20
4	1804360	['C', 'T']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1112C>T	ENSP00000339824.4:p.Ala371Val	2.81E-05 18
4	1804375	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1127C>G	ENSP00000339824.4:p.Ala376Gly	19
4	1804381	['T', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1133T>G	ENSP00000339824.4:p.Ile378Ser	26
4	1804383	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1135C>A	ENSP00000339824.4:p.Leu379Ile	9
4	1804388	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1140C>A	ENSP00000339824.4:p.Ser380Arg	20
4	1804401	['T', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1153T>C	ENSP00000339824.4:p.Phe385Leu	7.99E-06 19
4	1804404	['T', 'A']	2	0.99	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1156T>A	ENSP00000339824.4:p.Phe386Ile	7.99E-06 18
4	1804413	['A', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1165A>T	ENSP00000339824.4:p.Ile389Phe	18
4	1804414	['T', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1166T>A	ENSP00000339824.4:p.Ile389Asn	24
4	1804423	['T', 'TGGC	1	1.00	['inframe_insertion']	MODERATE	FGFR3	ENST00000340107.8:c.1179_1181dup	ENSP00000339824.4:p.Ala394dup	
4	1804443	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1195C>T	ENSP00000339824.4:p.Arg399Cys	3.21E-05 25
4	1804449	['C', 'T']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1201C>T	ENSP00000339824.4:p.Arg401Cys	1.60E-05 23
4	1804455	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1207C>G	ENSP00000339824.4:p.Pro403Ala	2.40E-05 9
4	1804464	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1216A>G	ENSP00000339824.4:p.Lys406Glu	4.00E-06 23
4	1804486	['T', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1238T>G	ENSP00000339824.4:p.Val413Gly	31
4	1804492	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1244A>G	ENSP00000339824.4:p.Lys415Arg	8.01E-06 25
4	1804493	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1245G>C	ENSP00000339824.4:p.Lys415Asn	1.20E-05 26
4	1804496	['C', 'G']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1248C>G	ENSP00000339824.4:p.Ile416Met	20
4	1804516	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1268G>A	ENSP00000339824.4:p.Arg423Gln	2.41E-05 29
4	1804824	['G', 'C']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1273G>C	ENSP00000339824.4:p.Val425Leu	6.41E-06 28
4	1804828	['C', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1277C>T	ENSP00000339824.4:p.Ser426Phe	1.28E-05 25
4	1804846	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1295C>T	ENSP00000339824.4:p.Ser432Phe	29
4	1804872	['C', 'G']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1321C>G	ENSP00000339824.4:p.Arg441Gly	18
4	1804872	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1321C>T	ENSP00000339824.4:p.Arg441Cys	2.56E-05 23
4	1804873	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1322G>A	ENSP00000339824.4:p.Arg441His	3.84E-05 27

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
4	1804878	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1327G>A	ENSP00000339824.4:p.Ala443Thr	1.28E-05 11
4	1804888	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1337C>T	ENSP00000339824.4:p.Ser446Phe	2.56E-05 24
4	1804902	['C', 'G']	1	0.99	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1351C>G	ENSP00000339824.4:p.Pro451Ala	20
4	1804906	['C', 'T']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1355C>T	ENSP00000339824.4:p.Thr452Met	0.001288 6
4	1804911	['G', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1360G>T	ENSP00000339824.4:p.Ala454Ser	2.56E-05 20
4	1804929	['G', 'C']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1378G>C	ENSPUU00339824.4:p.Glu460Gln	6.39E-06 26
4	1804941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1390G>A	ENSP00000339824.4:p.Asp464Asn	26
4	1804968	['C', 'G']	2	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1417C>G	ENSP00000339824.4:p.Arg473Gly	23
4	1805405	['T', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1469T>C	ENSP00000339824.4:p.Met490Thr	25
4	1805416	['A', 'G']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.1480A>G	ENSP00000339824.4:p.Ile494Val	4.03E-06 13
4	1805428	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1492A>G	ENSP00000339824.4:p.Lys498Glu	8.07E-06 29
4	1805434	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1498C>T	ENSP00000339824.4:p.Arg500Trp	8.09E-05 24
4	1805435	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1499G>A	ENSP00000339824.4:p.Arg500Gln	8.08E-06 23
4	1805440	['G', 'A']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1504G>A	ENSPUU00339824.4:p.Ala502Thr	5.66E-05 21
4	1805561	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENSTUU00340107.8:c.1543G>A	ENSPUU00339824.4:p.Asp515Asn	4.79E-05 23
4	1805571	['A', 'C']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.1553A>C	ENSP00000339824.4:p.Asp518Ala	3.99E-06 27
4	1805571	['A', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1553A>T	ENSP00000339824.4:p.Asp518Val	27
4	1805573	['A', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1555A>C	ENSP00000339824.4:p.Lys519Gln	1.20E-05 25
4	1805573	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1555A>G	ENSP00000339824.4:p.Lys519Glu	3.99E-06 23
4	1805574	['A', 'G']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1556A>G	ENSP00000339824.4:p.Lys519Arg	5.58E-05 24
4	1805583	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1565C>T	ENSP00000339824.4:p.Ser522Leu	7.98E-06 25
4	1805636	['A', 'G']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1618A>G	ENSPUU00339824.4:p.Ile540Val	1.20E-05 22
4	1805642	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1624A>G	ENSP00000339824.4:p.Asn542Asp	3.99E-06 28
4	1805643	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1625A>G	ENSP00000339824.4:p.Asn542Ser	3.99E-06 26
4	1805649	['T', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1631T>G	ENSP00000339824.4:p.Leu544Arg	32
4	1805661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1643C>T	ENSP00000339824.4:p.Thr548Met	25
4	1805666	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1648G>A	ENSP00000339824.4:p.Gly550Ser	25
4	1805761	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1663G>A	ENSP00000339824.4:p.Val555Met	0.000146 25
4	1805761	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1663G>C	ENSP00000339824.4:p.Val555Leu	4.05E-06 24
4	1805774	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1676A>G	ENSPUU00339824.4:p.Tyr559Cys	21
4	1805779	['G', 'C']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1681G>C	ENSP00000339824.4:p.Ala561Pro	2.02E-05 23
4	1805807	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1709G>A	ENSP00000339824.4:p.Arg570Gln	24
4	1805810	['C', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1712C>T	ENSP00000339824.4:p.Ala571Val	0.00015 26
4	1805819	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1721C>T	ENSP00000339824.4:p.Pro574Leu	2.03E-05 25
4	1805822	['C', 'T']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1724C>T	ENSPUU00339824.4:p.Pro575Leu	26
4	1805837	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1739C>T	ENSP00000339824.4:p.Ser580Phe	9.69E-05 24
4	1805860	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1762G>A	ENSP00000339824.4:p.Glu588Lys	6.83E-05 23
4	1805911	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1813C>T	ENSPUU00339824.4:p.Arg605Trp	4.01E-06 25
4	1805920	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1822G>C	ENSP00000339824.4:p.Glu608Gln	27
4	1805921	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1823A>G	ENSP00000339824.4:p.Glu608Gly	4.02E-06 33
4	1805929	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1831G>A	ENSP00000339824.4:p.Ala611Thr	4.03E-06 33
4	1805930	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1832C>T	ENSP00000339824.4:p.Ala611Val	2.42E-05 25
4	1805933	['C', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1835C>A	ENSP00000339824.4:p.Ser612Tyr	1.21E-05 27
4	1806039	['G', 'A']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1885G>A	ENSP00000339824.4:p.Glu629Lys	0.00018 33
4	1806169	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1961C>G	ENSP00000339824.4:p.Thr654Ser	8.04E-06 20
4	1806281	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.1990G>C	ENSPUU00339824.4:p.Ala664Pro	4.04E-06 33
4	1806290	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1999G>T	ENSP00000339824.4:p.Ala667Ser	1.21E-05 25
4	1806300	['ACC', 'A'	1	1.00	['frameshift_variant'	HIGH	FGFR3	ENST00000340107.8:c.2010_2011del	ENSP00000339824.4:p.Asp670GlufsTer8	
4	1806315	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2024A>G	ENSP00000339824.4:p.His675Arg	25
4	1806326	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.2035G>A	ENSP00000339824.4:p.Val679Ile	4.02E-06 27
4	1806548	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2039G>T	ENSP00000339824.4:p.Trp680Leu	34
4	1806581	['C', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2072C>T	ENSP00000339824.4:p.Thr691Met	4.00E-06 26
4	1806593	['C', 'T']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2084C>T	ENSP00000339824.4:p.Ser695Phe	1.20E-05 25
4	1806619	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2110G>A	ENSP00000339824.4:p.Glu704Lys	4.00E-06 27
4	1806668	['A', 'G']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2159A>G	ENSP00000339824.4:p.Asn720Ser	6.81E-05 23
4	1806669	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENSTUU00340107.8:c.2160C>G	ENSPUU00339824.4:p.Asn720Lys	24
4	1806842	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2188C>T	ENSP00000339824.4:p.Arg730Trp	4.20E-06 23
4	1806843	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2189G>A	ENSP00000339824.4:p.Arg730Gln	3.35E-05 26
4	1806855	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2201A>G	ENSP00000339824.4:p.His734Arg	4.12E-06 21
4	1806860	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2206G>A	ENSP00000339824.4:p.Ala736Thr	4.93E-05 21
4	1806861	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2207C>T	ENSP00000339824.4:p.Ala736Val	3.29E-05 3
4	1806876	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2222C>T	ENSP00000339824.4:p.Pro741Leu	26

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
4	1806935	['G', 'A']	2	1.00	['splice_donor_variant'	HIGH	FGFR3	ENST00000340107.8:c.2280-1G>A		4.58E-06	25
4	1807132	['C', 'T']	7	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2297C>T	ENSP00000339824.4:p.Ser766Leu	5.47E-05	27
4	1807135	['C', 'T']	5	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2300C>T	ENSP00000339824.4:p.Ala767Val	6.36E-05	13
4	1807156	['C', 'T']	2	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2321C>T	ENSP00000339824.4:p.Pro774Leu	1.84E-05	24
4	1807159	['G', 'A']	3	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2324G>A	ENSP00000339824.4:p.Gly775Asp	3.19E-05	22
4	1807165	['A', 'G']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2330A>G	ENSP00000339824.4:p.Gln777Arg	4.50E-06	20
4	1807191	['G', 'C']	2	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2356G>C	ENSP00000339824.4:p.Gly786Arg		24
4	1807194	['G', 'GA']	1	1.00	['frameshift_variant'	HIGH	FGFR3	ENST00000340107.8:c.2360dup	ENSP00000339824.4:p.Asp787GlufsTer32		
4	1807197	['G', 'A']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2362G>A	ENSP00000339824.4:p.Asp788Asn	8.61E-06	27
4	1807201	['CCGT', 'C	1	1.00	['inframe_deletion'	MODERATE	FGFR3	ENST00000340107.8:c.2367_2369del	ENSP00000339824.4:p.Val790del		
4	1807204	['T', 'C']	2	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2369T>C	ENSP00000339824.4:p.Val790Ala		27
4	1807230	['G', 'A']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2395G>A	ENSP00000339824.4:p.Ala799Thr		17
4	1807237	['C', 'T']	1	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2402C>T	ENSP00000339824.4:p.Pro801Leu	8.24E-05	22
4	1807254	['C', 'T']	5	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2419C>T	ENSP00000339824.4:p.Arg807Trp	1.83E-05	14
4	1807255	['G', 'A']	2	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2420G>A	ENSP00000339824.4:p.Arg807Gln	2.79E-05	15
4	1807258	['C', 'A']	2	1.00	['missense_variant'	MODERATE	FGFR3	ENST00000340107.8:c.2423C>A	ENSP00000339824.4:p.Thr808Lys	4.67E-06	24
9	35792461	['G', 'A']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.53G>A	ENSP00000341083.2:p.Arg18His		23
9	35792469	['G', 'C']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.61G>C	ENSP00000341083.2:p.Gly21Arg		19
9	35792475	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.67C>T	ENSP00000341083.2:p.Arg23Trp		24
9	35792580	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.172C>T	ENSP00000341083.2:p.Arg58Trp	3.99E-06	25
9	35792581	['G', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.173G>T	ENSP00000341083.2:p.Arg58Leu	1.20E-05	23
9	35792583	['G', 'A']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.175G>A	ENSP00000341083.2:p.Ala59Thr		21
9	35792601	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.193C>T	ENSP00000341083.2:p.Arg65Trp	7.97E-06	23
9	35792652	['C', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.244C>A	ENSP00000341083.2:p.Leu82Met	2.39E-05	23
9	35792688	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.280G>A	ENSP00000341083.2:p.Asp94Asn	7.96E-06	19
9	35792709	['T', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.301T>G	ENSP00000341083.2:p.Cys101Gly		27
9	35792713	['T', 'C']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.305T>C	ENSP00000341083.2:p.Val102Ala		20
9	35792736	['C', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.328C>G	ENSP00000341083.2:p.Arg110Gly		27
9	35792818	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.410G>A	ENSP00000341083.2:p.Arg137His		21
9	35792833	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.425C>T	ENSP00000341083.2:p.Thr142Ile		21
9	35792839	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.431C>T	ENSP00000341083.2:p.Pro144Leu		24
9	35792888	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.475C>T	ENSP00000341083.2:p.His159Tyr		22
9	35792885	['C', 'G']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.477C>G	ENSP00000341083.2:p.His159Gln		19
9	35792890	['A', 'G']	5	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.482A>G	ENSP00000341083.2:p.Asn161Ser	1.59E-05	23
9	35792899	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.491C>T	ENSP00000341083.2:p.Ala164Val		17
9	35792501	['C', 'T']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.493C>T	ENSP00000341083.2:p.Arg165Cys	3.98E-06	26
9	35792928	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.520C>T	ENSP00000341083.2:p.Arg174Cys	3.98E-06	27
9	35792992	['A', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.584A>G	ENSP00000341083.2:p.Asn195Ser	7.99E-06	23
9	35793021	['C', 'T']	1	1.00	['stop_gained'	HIGH	NPR2	ENST00000342694.6:c.613C>T	ENSP00000341083.2:p.Arg205Ter		35
9	35793022	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.614G>A	ENSP00000341083.2:p.Arg205Gln		20
9	35793033	['G', 'A']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.625G>A	ENSP00000341083.2:p.Gly209Ser	4.09E-06	21
9	35793039	['G', 'A']	3	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.631G>A	ENSP00000341083.2:p.Glu211Lys	4.13E-06	21
9	35793904	['A', 'C']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.674A>C	ENSP00000341083.2:p.Tyr225Ser		24
9	35793906	['A', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.676A>G	ENSP00000341083.2:p.Ile226Val	1.19E-05	21
9	35793952	['A', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.722A>T	ENSP00000341083.2:p.Gln241Leu		21
9	35793955	['G', 'A']	6	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.725G>A	ENSP00000341083.2:p.Arg242Lys	2.78E-05	21
9	35793980	['T', 'A']	1	1.00	['stop_gained'	HIGH	NPR2	ENST00000342694.6:c.750T>A	ENSP00000341083.2:p.Tyr250Ter		35
9	35794018	['G', 'A']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.788G>A	ENSP00000341083.2:p.Arg263His	3.58E-05	24
9	35794024	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.794G>A	ENSP00000341083.2:p.Gly265Asp		20
9	35794029	['A', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.799A>G	ENSP00000341083.2:p.Thr267Ala	2.39E-05	21
9	35794044	['C', 'T']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.814C>T	ENSP00000341083.2:p.Arg272Trp	2.78E-05	24
9	35794045	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.815G>A	ENSP00000341083.2:p.Arg272Gln	1.19E-05	20
9	35794056	['G', 'C']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.826G>C	ENSP00000341083.2:p.Asp276His	3.98E-06	24
9	35794060	['A', 'G']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.830A>G	ENSP00000341083.2:p.Asn277Ser	4.38E-05	21
9	35794063	['G', 'A']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.833G>A	ENSP00000341083.2:p.Arg278His	2.79E-05	22
9	35794068	['C', 'T']	3	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.838C>T	ENSP00000341083.2:p.Arg280Trp	1.20E-05	24
9	35794099	['A', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.863A>G	ENSP00000341083.2:p.Glu288Gly	1.20E-05	25
9	35799630	['A', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.886A>G	ENSP00000341083.2:p.Ile296Val		19
9	35799634	['C', 'T']	2	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.890C>T	ENSP00000341083.2:p.Thr297Met	3.98E-06	26
9	35799645	['C', 'T']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.901C>T	ENSP00000341083.2:p.Pro301Ser		25
9	35799660	['T', 'G']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.916T>G	ENSP00000341083.2:p.Tyr306Asp		28
9	35799679	['G', 'C']	1	1.00	['missense_variant'	MODERATE	NPR2	ENST00000342694.6:c.935G>C	ENSP00000341083.2:p.Arg312Pro		24

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
9	35799697	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.953G>A	ENSP00000341083.2:p.Arg318Gln	7.95E-05 22
9	35799699	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.955G>C	ENSP00000341083.2:p.Glu319Gln	17
9	35799714	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.970G>C	ENSP00000341083.2:p.Glu324Gln	1.19E-05 22
9	35799715	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.971A>T	ENSP00000341083.2:p.Glu324Val	23
9	35800041	['G', 'GCTT	1	1.00	['inframe_insertion']	MODERATE	NPR2	ENST00000342694.6:c.1010_1012dup	ENSP00000341083.2:p.Phe337dup	3.98E-06
9	35800104	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1070C>T	ENSP00000341083.2:p.Thr357Ile	5.17E-05 21
9	35800110	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1076A>C	ENSP00000341083.2:p.Glu359Ala	19
9	35800122	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1088G>A	ENSP00000341083.2:p.Arg363Gln	5.57E-05 22
9	35800124	['AT', 'A']	3	1.00	['frameshift_variant'	HIGH	NPR2	ENST00000342694.6:c.1092del	ENSP00000341083.2:p.Ile364Metfs*13	7.95E-06
9	35800133	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1099A>G	ENSP00000341083.2:p.Lys367Glu	24
9	35800142	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1108G>A	ENSP00000341083.2:p.Gly370Arg	1.19E-05 24
9	35800427	['C', 'T']	2	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.1162C>T	ENSP00000341083.2:p.Arg388Ter	1.19E-05 35
9	35800720	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1230C>G	ENSP00000341083.2:p.His410Gln	18
9	35800733	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1243G>A	ENSP00000341083.2:p.Glu415Lys	21
9	35800752	['C', 'T']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1262C>T	ENSP00000341083.2:p.Thr421Met	7.16E-05 21
9	35800757	['C', 'T']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1267C>T	ENSP00000341083.2:p.Arg423Trp	2.39E-05 24
9	35800758	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1268G>A	ENSP00000341083.2:p.Arg423Gln	1.19E-05 22
9	35800782	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1292C>T	ENSP00000341083.2:p.Ala431Val	12
9	35800791	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1301C>T	ENSP00000341083.2:p.Ser434Leu	1.99E-05 15
9	35800803	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1313C>A	ENSP00000341083.2:p.Pro438His	23
9	35800814	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1324G>A	ENSP00000341083.2:p.Asp442Asn	3.98E-06 23
9	35800815	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1325A>G	ENSP00000341083.2:p.Asp442Gly	3.98E-06 24
9	35801090	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1372A>G	ENSP00000341083.2:p.Ile458Val	2.39E-05 21
9	35801091	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1373T>C	ENSP00000341083.2:p.Ile458Thr	7.95E-06 24
9	35801118	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1400T>G	ENSP00000341083.2:p.Phe467Cys	25
9	35801125	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1407G>A	ENSP00000341083.2:p.Met469Ile	1.99E-05 16
9	35801154	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1436G>A	ENSP00000341083.2:p.Arg479Gln	7.96E-06 35
9	35801646	['G', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1440G>C	ENSP00000341083.2:p.Lys480Asn	1.59E-05 23
9	35801684	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1478G>A	ENSP00000341083.2:p.Arg493His	7.96E-06 33
9	35801722	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1516C>T	ENSP00000341083.2:p.Arg506Cys	1.59E-05 24
9	35801723	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1517G>A	ENSP00000341083.2:p.Arg506His	0.000119 28
9	35801728	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1522C>G	ENSP00000341083.2:p.His508Asp	3.98E-06 22
9	35801744	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1538G>A	ENSP00000341083.2:p.Ser513Asn	29
9	35801759	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1553C>T	ENSP00000341083.2:p.Ser518Leu	7.95E-06 25
9	35801932	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1564T>C	ENSP00000341083.2:p.Ser522Pro	31
9	35801941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1573G>A	ENSP00000341083.2:p.Gly525Ser	0.000151 24
9	35801989	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1621G>A	ENSP00000341083.2:p.Gly541Ser	3.18E-05 32
9	35802009	['A', 'T']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1636A>T	ENSP00000341083.2:p.Asn546Tyr	27
9	35802218	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1645G>A	ENSP00000341083.2:p.Ala549Thr	1.19E-05 30
9	35802230	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1657G>C	ENSP00000341083.2:p.Val553Leu	22
9	35802246	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1673T>C	ENSP00000341083.2:p.Ile558Thr	7.97E-06 27
9	35802249	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1676A>G	ENSP00000341083.2:p.Glu559Gly	29
9	35802263	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1690G>C	ENSP00000341083.2:p.Val564Leu	23
9	35802545	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1753G>A	ENSP00000341083.2:p.Ala585Thr	3.98E-06 28
9	35802609	['T', 'G']	1	1.00	['splice_donor_variar	HIGH	NPR2	ENST00000342694.6:c.1815+2T>G		34
9	35802749	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1833C>G	ENSP00000341083.2:p.Asp611Glu	18
9	35802761	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1845G>C	ENSP00000341083.2:p.Leu615Phe	27
9	35802774	['C', 'T']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1858C>T	ENSP00000341083.2:p.Arg620Cys	3.98E-06 28
9	35802802	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1886A>G	ENSP00000341083.2:p.Lys629Arg	28
9	35805565	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1942T>G	ENSP00000341083.2:p.Ser648Ala	26
9	35805638	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2015C>T	ENSP00000341083.2:p.Ala672Val	16
9	35805648	['T', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2025T>A	ENSP00000341083.2:p.Asp675Glu	17
9	35805656	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2033A>G	ENSP00000341083.2:p.His678Arg	24
9	35805661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2038C>T	ENSP00000341083.2:p.Leu680Phe	3.98E-06 21
9	35805664	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2041T>C	ENSP00000341083.2:p.Tyr681His	25
9	35805833	['A', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2051A>C	ENSP00000341083.2:p.Lys684Thr	27
9	35805844	['G', 'T']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2062G>T	ENSP00000341083.2:p.Ala688Ser	7.96E-06 25
9	35805869	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2087C>T	ENSP00000341083.2:p.Pro696Leu	3.98E-06 16
9	35805873	['G', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2091G>T	ENSP00000341083.2:p.Leu697Phe	3.98E-06 19
9	35805895	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2113G>A	ENSP00000341083.2:p.Ala705Thr	3.98E-06 28
9	35805901	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2119G>A	ENSP00000341083.2:p.Val707Ile	1.99E-05 22
9	35805932	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2150T>C	ENSP00000341083.2:p.Ile717Thr	1.20E-05 28

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
9	35805941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2159G>A	ENSP00000341083.2:p.Arg720His	2.39E-05	32
9	35805945	['T', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2163T>A	ENSP00000341083.2:p.Ser721Arg	7.97E-06	18
9	35805961	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2179G>C	ENSP00000341083.2:p.Glu727Gln		24
9	35806069	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2287T>G	ENSP00000341083.2:p.Ile736Met	7.95E-06	24
9	35806090	['TCAG', 'T']	1	1.00	['inframe_deletion']	MODERATE	NPR2	ENST00000342694.6:c.2231_2233del	ENSP00000341083.2:p.Gln744del		
9	35806095	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2234G>A	ENSP00000341083.2:p.Arg745Gln	2.39E-05	22
9	35806103	['T', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2242T>A	ENSP00000341083.2:p.Phe748Ile		27
9	35806121	['C', 'T']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2260C>T	ENSP00000341083.2:p.Arg754Trp	7.95E-05	24
9	35806129	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2268A>C	ENSP00000341083.2:p.Gln756His		15
9	35806157	['G', 'C']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2296G>C	ENSP00000341083.2:p.Glu766Gln	7.95E-06	21
9	35806161	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2300G>A	ENSP00000341083.2:p.Arg767Gln	1.19E-05	26
9	35806187	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2326C>T	ENSP00000341083.2:p.Arg776Trp	7.96E-06	26
9	35806220	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2359C>T	ENSP00000341083.2:p.Arg787Trp	0.000291	28
9	35806221	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2360G>C	ENSP00000341083.2:p.Arg787Pro		28
9	35806221	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2360G>A	ENSP00000341083.2:p.Arg787Gln	3.98E-06	27
9	35806223	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2362C>T	ENSP00000341083.2:p.Arg788Cys	2.79E-05	27
9	35806429	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2410C>T	ENSP00000341083.2:p.Arg804Cys	3.98E-06	28
9	35806448	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2429A>T	ENSP00000341083.2:p.Asn810Ile		25
9	35806474	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2455C>T	ENSP00000341083.2:p.Arg819Cys	1.99E-05	27
9	35806501	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2482C>T	ENSP00000341083.2:p.Arg828Cys		29
9	35806506	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2487G>C	ENSP00000341083.2:p.Lys829Asn		25
9	35807045	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2542C>T	ENSP00000341083.2:p.Arg848Trp	1.59E-05	24
9	35807053	['G', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2550G>C	ENSP00000341083.2:p.Glu850Asp	3.98E-06	24
9	35807085	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2582C>T	ENSP00000341083.2:p.Thr861Ile		27
9	35807129	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2626G>A	ENSP00000341083.2:p.Glu876Lys		31
9	35807144	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2641C>G	ENSP00000341083.2:p.Gln881Glu		23
9	35808509	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2713G>A	ENSP00000341083.2:p.Val905Met	4.02E-06	35
9	35808519	['T', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2723T>C	ENSP00000341083.2:p.Ile908Thr	6.42E-05	29
9	35808545	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2749G>C	ENSP00000341083.2:p.Gly917Arg		32
9	35808558	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2762G>A	ENSP00000341083.2:p.Arg921Gln	1.20E-05	33
9	35808591	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2795G>A	ENSP00000341083.2:p.Arg932His	3.19E-05	29
9	35808608	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2812C>G	ENSP00000341083.2:p.Leu938Val		24
9	35808613	['T', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2817T>A	ENSP00000341083.2:p.Asp939Glu		17
9	35808621	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2825C>A	ENSP00000341083.2:p.Ser942Tyr		18
9	35808629	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2833C>T	ENSP00000341083.2:p.Arg945Cys	3.18E-05	25
9	35808644	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2848C>A	ENSP00000341083.2:p.Pro950Thr		26
9	35808663	['T', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2867T>A	ENSP00000341083.2:p.Leu956Gln	1.99E-05	32
9	35808665	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2869C>T	ENSP00000341083.2:p.Arg957Cys	1.19E-05	31
9	35808669	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2873T>C	ENSP00000341083.2:p.Ile958Thr	7.95E-06	29
9	35809177	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3008C>A	ENSP00000341083.2:p.Ser1003Tyr		25
9	35809180	['C', 'T']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3011C>T	ENSP00000341083.2:p.Thr1004Ile	2.39E-05	24
9	35809186	['A', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3017A>C	ENSP00000341083.2:p.Lys1006Thr	3.98E-06	27
9	35809194	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3025C>G	ENSP00000341083.2:p.Leu1009Val		24
9	35809206	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3037G>A	ENSP00000341083.2:p.Gly1013Arg		28
9	35809227	['CGG', 'C']	1	1.00	['frameshift_variant']	HIGH	NPR2	ENST00000342694.6:c.3063_3064del	ENSP00000341083.2:p.Asp1022CysfsTer35		
9	35809244	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3075G>A	ENSP00000341083.2:p.Met1025Ile	1.19E-05	24
9	35809395	['C', 'T']	2	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.3094C>T	ENSP00000341083.2:p.Arg1032Ter		40
9	35809396	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3095G>A	ENSP00000341083.2:p.Arg1032Gln	3.18E-05	24
9	35809413	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3112G>A	ENSP00000341083.2:p.Gly1038Arg		29
9	35809419	['C', 'T']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3118C>T	ENSP00000341083.2:p.Arg1040Trp	3.18E-05	25
9	35809420	['G', 'A']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3119G>A	ENSP00000341083.2:p.Arg1040Gln	7.56E-05	19
9	35809428	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3127C>T	ENSP00000341083.2:p.Pro1043Ser		18
9	35809434	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3133G>A	ENSP00000341083.2:p.Gly1045Arg		37
9	35809436	['AC', 'A']	1	1.00	['frameshift_variant']	HIGH	NPR2	ENST00000342694.6:c.3136del	ENSP00000341083.2:p.Leu1046SerfsTer7		
9	35809441	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3140T>C	ENSP00000341083.2:p.Leu1047Pro		25
15	98649583	['T', 'C']	1	1.00	['start_lost']	HIGH	IGF1R	ENST00000650285.1:c.2T>C	ENSP00000497069.1:p.Met1?		23
15	98649597	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.16G>A	ENSP00000497069.1:p.Gly6Arg	0.000605	22
15	98649609	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.28C>T	ENSP00000497069.1:p.Pro10Ser	1.21E-05	21
15	98649610	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.29C>T	ENSP00000497069.1:p.Pro10Leu	4.05E-06	20
15	98649636	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.55C>G	ENSP00000497069.1:p.Leu19Val	1.21E-05	15
15	98649636	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.55C>T	ENSP00000497069.1:p.Leu19Phe	1.62E-05	15
15	98649640	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.59C>T	ENSP00000497069.1:p.Ser20Phe	3.24E-05	20

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
15	98649652	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.71C>G	ENSP00000497069.1:p.Ser24Trp	4.05E-06	21
15	98649654	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.73C>T	ENSP00000497069.1:p.Leu25Phe		21
15	98649661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.80C>T	ENSP00000497069.1:p.Pro27Leu	1.62E-05	23
15	98649674	['AAGTG',	1	1.00	['splice_donor_varia	HIGH	IGF1R	ENST00000650285.1:c.94+3_94+6del		3.64E-05	
15	98707651	['C', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.184C>A	ENSP00000497069.1:p.Leu62Met		25
15	98707667	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.200C>G	ENSP00000497069.1:p.Ala67Gly		19
15	98707679	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.212G>A	ENSP00000497069.1:p.Arg71His	2.39E-05	25
15	98707703	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.236C>T	ENSP00000497069.1:p.Thr79Met	1.59E-05	26
15	98707808	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.341A>G	ENSP00000497069.1:p.Asn114Ser		23
15	98707838	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.371A>G	ENSP00000497069.1:p.Asn124Ser	1.59E-05	19
15	98707849	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.382A>G	ENSP00000497069.1:p.Ile128Val	3.98E-06	22
15	98707868	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.401G>A	ENSP00000497069.1:p.Arg134Lys	3.98E-06	21
15	98707958	['T', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.491T>G	ENSP00000497069.1:p.Val164Gly		22
15	98707976	['T', 'C']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.509T>C	ENSP00000497069.1:p.Val170Ala	3.98E-06	23
15	98708024	['TGGA', '	1	1.00	['inframe_deletion']	MODERATE	IGF1R	ENST00000650285.1:c.562_564del	ENSP00000497069.1:p.Glu188del		
15	98708072	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.605A>G	ENSP00000497069.1:p.Asn202Ser	0.000137	17
15	98891348	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.664C>T	ENSP00000497069.1:p.Arg222Trp	0.00011	23
15	98891352	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.668C>T	ENSP00000497069.1:p.Ala223Val	4.05E-06	23
15	98891390	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.706C>G	ENSP00000497069.1:p.Leu236Val		24
15	98891405	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.721G>A	ENSP00000497069.1:p.Ala241Thr	8.00E-06	16
15	98891417	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.733G>A	ENSP00000497069.1:p.Asp245Asn	4.00E-06	18
15	98891420	['A', 'G']	6	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.736A>G	ENSP00000497069.1:p.Thr246Ala	2.80E-05	17
15	98891439	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.755G>A	ENSP00000497069.1:p.Arg252His	2.79E-05	22
15	98891469	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.785C>G	ENSP00000497069.1:p.Pro262Arg		15
15	98891478	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.794C>T	ENSP00000497069.1:p.Pro265Leu	7.96E-06	26
15	98891484	['A', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.800A>C	ENSP00000497069.1:p.Asn267Thr	1.99E-05	16
15	98891540	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.856C>G	ENSP00000497069.1:p.Leu286Val		13
15	98891564	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.880G>A	ENSP00000497069.1:p.Glu294Lys	8.09E-06	17
15	98891606	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.922C>T	ENSP00000497069.1:p.Pro308Ser		26
15	98891622	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.938G>A	ENSP00000497069.1:p.Arg313His	4.18E-06	24
15	98891625	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.941A>G	ENSP00000497069.1:p.Asn314Ser		18
15	98891628	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.944G>A	ENSP00000497069.1:p.Gly315Asp		12
15	98891633	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.949C>G	ENSP00000497069.1:p.Gln317Glu		16
15	98896760	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.957G>A	ENSP00000497069.1:p.Met319Ile		23
15	98896789	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.986C>T	ENSP00000497069.1:p.Pro329Leu	7.96E-06	23
15	98896794	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.991G>A	ENSP00000497069.1:p.Val331Ile	1.99E-05	21
15	98896816	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1013C>G	ENSP00000497069.1:p.Thr338Arg		17
15	98896829	['T', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1026T>G	ENSP00000497069.1:p.Asp342Glu		22
15	98896879	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1076A>G	ENSP00000497069.1:p.Asn359Ser	1.59E-05	23
15	98896884	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1081C>T	ENSP00000497069.1:p.Leu361Phe	3.98E-06	22
15	98896897	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1094G>A	ENSP00000497069.1:p.Arg365Gln		33
15	98896899	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1096C>T	ENSP00000497069.1:p.Arg366Trp	1.19E-05	24
15	98899488	['T', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1114T>A	ENSP00000497069.1:p.Ser372Thr		22
15	98899518	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1144G>A	ENSP00000497069.1:p.Glu382Lys		28
15	98899536	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1162G>A	ENSP00000497069.1:p.Val388Met	0.000577	26
15	98899542	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1168A>G	ENSP00000497069.1:p.Ile390Val		23
15	98899568	['C', 'CT',	1	1.00	['frameshift_variant'	HIGH	IGF1R	ENST00000650285.1:c.1196dup	ENSP00000497069.1:p.Leu399PhefsTer71	3.98E-06	
15	98899591	['G', 'A']	7	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1217G>A	ENSP00000497069.1:p.Arg406His	3.18E-05	24
15	98908740	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1309G>A	ENSP00000497069.1:p.Asp436Asn	7.96E-06	22
15	98908800	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1363T>C	ENSP00000497069.1:p.Cys455Arg		28
15	98908818	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1381C>T	ENSP00000497069.1:p.Arg461Cys		28
15	98908819	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1382G>A	ENSP00000497069.1:p.Arg461His	1.99E-05	23
15	98908834	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1397C>T	ENSP00000497069.1:p.Thr466Met	3.98E-06	27
15	98908848	['C', 'T']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1411C>T	ENSP00000497069.1:p.Arg471Cys	1.20E-05	27
15	98908849	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1412G>A	ENSP00000497069.1:p.Arg471His	4.78E-05	25
15	98911326	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1474G>A	ENSP00000497069.1:p.Val492Ile	0.000569	17
15	98911333	['A', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1481A>T	ENSP00000497069.1:p.His494Leu	3.98E-06	24
15	98911354	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1502C>T	ENSP00000497069.1:p.Ser501Leu	0.000358	15
15	98911357	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1505A>G	ENSP00000497069.1:p.Lys502Arg		23
15	98911362	['C', 'T']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1510C>T	ENSP00000497069.1:p.Arg504Cys	1.19E-05	28
15	98911383	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1531C>T	ENSP00000497069.1:p.Arg511Trp	2.39E-05	25
15	98911390	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1538G>A	ENSP00000497069.1:p.Arg513Gln	7.95E-06	23

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
15	98911414	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1562T>C	ENSP00000497069.1:p.Ile521Thr	25
15	98911417	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1565G>C	ENSP00000497069.1:p.Ser522Thr	2.98E-06 23
15	98911425	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1573G>A	ENSP00000497069.1:p.Val525Ile	3.98E-05 25
15	98911441	['C', 'T']	5	1.00	['missense_variant',	MODERATE	IGF1R	ENST00000650285.1:c.1589C>T	ENSP00000497069.1:p.Ala530Val	1.19E-05 34
15	98913124	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1670C>G	ENSP00000497069.1:p.Pro557Arg	19
15	98913124	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1670C>T	ENSP00000497069.1:p.Pro557Leu	7.95E-06 24
15	98913138	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1684G>A	ENSP00000497069.1:p.Val562Met	1.59E-05 9
15	98913138	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1684G>T	ENSP00000497069.1:p.Val562Leu	3.98E-06 7
15	98913147	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1693G>A	ENSP00000497069.1:p.Gly565Ser	2.78E-05 22
15	98913160	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1706A>G	ENSP00000497069.1:p.His569Arg	1.19E-05 19
15	98913189	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1735G>A	ENSP00000497069.1:p.Val579Ile	6.76E-05 16
15	98913204	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1750G>A	ENSP00000497069.1:p.Val584Met	1.99E-05 24
15	98913216	['A', 'G']	6	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1762A>G	ENSP00000497069.1:p.Met588Val	21
15	98913228	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1774G>A	ENSP00000497069.1:p.Asp592Asn	2.39E-05 22
15	98913237	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1783C>G	ENSP00000497069.1:p.Arg595Gly	23
15	98913264	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1810A>T	ENSP00000497069.1:p.Ile604Phe	22
15	98913277	['C', 'CT']	1	1.00	['frameshift_variant'	HIGH	IGF1R	ENST00000650285.1:c.1825dup	ENSP00000497069.1:p.Ser609PhefsTer73	
15	98915984	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1849G>A	ENSP00000497069.1:p.Val617Ile	2.78E-05 17
15	98916017	['A', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1882A>C	ENSP00000497069.1:p.Ile628Leu	19
15	98916048	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1913A>G	ENSP00000497069.1:p.Asn638Ser	1.19E-05 26
15	98916090	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1955C>G	ENSP00000497069.1:p.Pro652Arg	22
15	98916099	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1963G>A	ENSP00000497069.1:p.Gly655Ser	8.35E-05 15
15	98916111	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1976G>A	ENSP00000497069.1:p.Arg659Gln	0.00066 19
15	98916126	['C', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1991C>T	ENSP00000497069.1:p.Ser664Phe	1.19E-05 23
15	98916701	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2026G>A	ENSP00000497069.1:p.Gly676Ser	1.59E-05 22
15	98916708	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2033T>C	ENSP00000497069.1:p.Ile678Thr	19
15	98916755	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2080G>T	ENSP00000497069.1:p.Gly694Trp	3.98E-06 24
15	98916797	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2122G>A	ENSP00000497069.1:p.Glu708Lys	3.98E-06 23
15	98916797	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2122G>C	ENSP00000497069.1:p.Glu708Gln	3.98E-06 22
15	98916799	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2124G>C	ENSP00000497069.1:p.Glu708Asp	3.98E-06 21
15	98916805	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2130G>C	ENSP00000497069.1:p.Gln710His	3.98E-06 21
15	98916845	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2170A>G	ENSP00000497069.1:p.Asn724Asp	24
15	98916856	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2181C>G	ENSP00000497069.1:p.His727Gln	23
15	98916869	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2194G>A	ENSP00000497069.1:p.Val732Met	7.97E-06 24
15	98922156	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2210G>T	ENSP00000497069.1:p.Arg737Met	29
15	98922204	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2258G>A	ENSP00000497069.1:p.Arg753Gln	3.98E-06 23
15	98922221	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2275G>A	ENSP00000497069.1:p.Ala759Thr	8
15	98922286	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2340C>G	ENSP00000497069.1:p.Ser780Arg	20
15	98922326	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2380C>T	ENSP00000497069.1:p.Arg794Trp	3.56E-05 28
15	98922345	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2399G>A	ENSP00000497069.1:p.Arg800His	3.98E-06 30
15	98922381	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2435A>G	ENSP00000497069.1:p.Lys812Arg	17
15	98922396	['C', 'T']	6	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2450C>T	ENSP00000497069.1:p.Ala817Val	20
15	98922406	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2460C>G	ENSP00000497069.1:p.Phe820Leu	4.00E-06 11
15	98922407	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2461G>A	ENSP00000497069.1:p.Val821Ile	8.00E-06 24
15	98923948	['C', 'T']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2558C>T	ENSP00000497069.1:p.Pro853Leu	0.00167 19
15	98923997	['C', 'G']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.2607C>G	ENSP00000497069.1:p.Tyr869Ter	35
15	98924534	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2632G>A	ENSP00000497069.1:p.Glu878Lys	1.59E-05 23
15	98924551	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2649G>T	ENSP00000497069.1:p.Gln883His	3.98E-06 22
15	98924558	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2656A>G	ENSP00000497069.1:p.Arg886Gly	3.98E-06 21
15	98924595	['C', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2693C>A	ENSP00000497069.1:p.Pro898Gln	1.19E-05 26
15	98924625	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2723C>T	ENSP00000497069.1:p.Thr908Ile	23
15	98929558	['C', 'G']	1	1.00	['missense_variant',	MODERATE	IGF1R	ENST00000650285.1:c.2783C>G	ENSP00000497069.1:p.Thr928Arg	14
15	98929590	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2815G>A	ENSP00000497069.1:p.Ala939Thr	17
15	98929602	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2827G>A	ENSP00000497069.1:p.Ala943Thr	1.59E-05 18
15	98929617	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2842G>A	ENSP00000497069.1:p.Val948Met	1.19E-05 23
15	98929644	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2869G>A	ENSP00000497069.1:p.Val957Ile	19
15	98929648	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2873T>C	ENSP00000497069.1:p.Phe958Ser	24
15	98930252	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2903G>C	ENSP00000497069.1:p.Gly968Ala	24
15	98930266	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2917T>C	ENSP00000497069.1:p.Tyr973His	32
15	98930299	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2950G>A	ENSP00000497069.1:p.Ala984Thr	4.03E-06 23
15	98934863	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2996T>C	ENSP00000497069.1:p.Ile999Thr	30
15	98934875	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3008G>A	ENSP00000497069.1:p.Arg1003Gln	3.18E-05 31

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
15	98934880	['C', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3013C>A	ENSP00000497069.1:p.Leu1005Ile		25
15	98934908	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3041A>G	ENSP00000497069.1:p.Tyr1014Cys	1.19E-05	32
15	98934982	['G', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3115G>T	ENSP00000497069.1:p.Ala1039Ser	7.96E-06	29
15	98934983	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3116C>T	ENSP00000497069.1:p.Ala1039Val		27
15	98934988	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3121A>G	ENSP00000497069.1:p.Met1041Val	3.98E-06	22
15	98935328	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3199G>T	ENSP00000497069.1:p.Gly1067Cys		34
15	98935380	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3251G>A	ENSP00000497069.1:p.Arg1084Gln	6.39E-06	27
15	98935392	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3263A>T	ENSP00000497069.1:p.Lys1088Ile		33
15	98935403	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3274C>T	ENSP00000497069.1:p.Arg1092Trp	6.39E-06	24
15	98935404	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3275G>A	ENSP00000497069.1:p.Arg1092Gln		34
15	98935422	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3293T>C	ENSP00000497069.1:p.Met1098Thr		21
15	98939223	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3320C>T	ENSP00000497069.1:p.Pro1107Leu		28
15	98939233	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3330C>G	ENSP00000497069.1:p.Ser1110Arg	7.95E-06	22
15	98939238	['T', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3335T>G	ENSP00000497069.1:p.Met1112Arg		31
15	98942935	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3470C>T	ENSP00000497069.1:p.Thr1157Met	3.98E-06	27
15	98942947	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3482A>G	ENSP00000497069.1:p.Tyr1161Cys	2.39E-05	33
15	98942959	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3494A>G	ENSP00000497069.1:p.Tyr1165Cys		33
15	98942964	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3499C>T	ENSP00000497069.1:p.Arg1167Trp		32
15	98942970	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3505G>A	ENSP00000497069.1:p.Gly1169Arg		34
15	98948627	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3641A>T	ENSP00000497069.1:p.Gln1214Leu		32
15	98948645	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3659A>G	ENSP00000497069.1:p.Gln1220Arg		30
15	98948653	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3667C>T	ENSP00000497069.1:p.Arg1223Cys		33
15	98948691	['C', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3705C>A	ENSP00000497069.1:p.Asp1235Glu		19
15	98957063	['T', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3725T>A	ENSP00000497069.1:p.Phe1242Tyr		20
15	98957093	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3755A>G	ENSP00000497069.1:p.Asn1252Ser	3.98E-06	24
15	98957146	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3808A>G	ENSP00000497069.1:p.Met1270Val		19
15	98957183	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3845G>A	ENSP00000497069.1:p.Ser1282Asn	7.95E-06	30
15	98957184	['CGAG', 'C	1	1.00	['inframe_deletion']	MODERATE	IGF1R	ENST00000650285.1:c.3850_3852del	ENSP00000497069.1:p.Glu1283del	1.99E-05	
15	98957185	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3847G>A	ENSP00000497069.1:p.Glu1283Lys	0.000103	25
15	98957206	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3868C>T	ENSP00000497069.1:p.Pro1290Ser		9
15	98957207	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3869C>T	ENSP00000497069.1:p.Pro1290Leu	3.58E-05	17
15	98957214	['G', 'GCT'	1	1.00	['inframe_insertion']	MODERATE	IGF1R	ENST00000650285.1:c.3882_3887dup	ENSP00000497069.1:p.Asp1294_Leu1295dup	3.98E-06	
15	98957221	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3882C>G	ENSP00000497069.1:p.Leu1295Val		20
15	98957239	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3901G>A	ENSP00000497069.1:p.Glu1301Lys		32
15	98957270	['C', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3932C>T	ENSP00000497069.1:p.Ser1311Leu	8.00E-05	22
15	98957273	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3935C>T	ENSP00000497069.1:p.Ser1312Phe		23
15	98957290	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3952G>C	ENSP00000497069.1:p.Asp1318His		24
15	98957290	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3952G>T	ENSP00000497069.1:p.Asp1318Tyr	1.20E-05	24
15	98957300	['C', 'A']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.3962C>A	ENSP00000497069.1:p.Ser1321Ter	3.99E-06	47
15	98957307	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3969C>G	ENSP00000497069.1:p.His1323Gln		16
15	98957317	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3979A>G	ENSP00000497069.1:p.Asn1327Asp	3.60E-05	21
15	98957326	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3988G>A	ENSP00000497069.1:p.Gly1330Ser	5.20E-05	14
15	98957341	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4003G>T	ENSP00000497069.1:p.Val1335Phe		23
15	98957348	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4010G>A	ENSP00000497069.1:p.Arg1337His	2.00E-05	26
15	98957362	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4024G>A	ENSP00000497069.1:p.Glu1342Lys	4.00E-06	25
15	98957366	['G', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4028G>T	ENSP00000497069.1:p.Arg1343Ile	2.00E-05	23
15	98957383	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4045A>G	ENSP00000497069.1:p.Met1349Val	8.02E-06	26
15	98957393	['G', 'C']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4055G>C	ENSP00000497069.1:p.Gly1352Ala		28
15	98957395	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4057C>T	ENSP00000497069.1:p.Arg1353Cys	8.04E-06	28
15	98957404	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4066G>A	ENSP00000497069.1:p.Glu1356Lys	4.83E-05	25
15	98957404	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4066G>C	ENSP00000497069.1:p.Glu1356Gln		25
15	98957407	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4069C>T	ENSP00000497069.1:p.Arg1357Trp	0.00025	25
15	98957408	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4070G>A	ENSP00000497069.1:p.Arg1357Gln	3.22E-05	32
15	98957425	['C', 'T']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.4087C>T	ENSP00000497069.1:p.Gln1363Ter	8.07E-06	52
15	98957432	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4094C>T	ENSP00000497069.1:p.Ser1365Leu	8.08E-06	26
X	630310	['A', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.13A>G	ENSP00000370990.1:p.Thr5Ala	7.99E-06	25
X	630922	['T', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.25T>C	ENSP00000370990.1:p.Ser9Pro		25
X	630950	['A', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.53A>T	ENSP00000370990.1:p.Asp18Val	3.99E-06	22
X	630981	['TAAG', 'T	3	1.00	['inframe_deletion']	MODERATE	SHOX	ENST00000381578.6:c.88_90del	ENSP00000370990.1:p.Lys30del		
X	631004	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.1076>A	ENSP00000370990.1:p.Arg366Gln		26
X	631028	['C', 'A']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.1310C>A	ENSP00000370990.1:p.Ala44Glu		23
X	631045	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.1486>A	ENSP00000370990.1:p.Gly50Arg		22

Figure 6 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
X	631074	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.177C>G	ENSP00000370990.1:p.Ile59Met		0
X	631087	['G', 'C']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.190G>C	ENSP00000370990.1:p.Gly64Arg		20
X	631097	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.200C>T	ENSP00000370990.1:p.Pro67Leu		23
X	631108	['T', 'C']	4	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.211T>C	ENSP00000370990.1:p.Phe71Leu		21
X	631115	['A', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.218A>G	ENSP00000370990.1:p.Asp73Gly		20
X	631163	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.266G>C	ENSP00000370990.1:p.Arg89Thr		22
X	631169	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.272C>T	ENSP00000370990.1:p.Ala91Val		10
X	634631	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.291C>G	ENSP00000370990.1:p.Cys97Trp		24
X	634635	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.295G>A	ENSP00000370990.1:p.Glu99Lys		23
X	634637	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.297G>C	ENSP00000370990.1:p.Glu99Asp		7
X	634668	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.328G>T	ENSP00000370990.1:p.Asp110Tyr		26
X	634671	['G', 'C']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.331G>C	ENSP00000370990.1:p.Gly111Arg		22
X	634676	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.336G>C	ENSP00000370990.1:p.Gln112His		24
X	634689	['CAG', 'C']	1	1.00	['frameshift_variant'	HIGH	SHOX	ENST00000381578.6:c.352_353del	ENSP00000370990.1:p.Arg118AlafsTer63		
X	634696	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.356G>A	ENSP00000370990.1:p.Arg119His	4.01E-06	24
X	634705	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.365C>G	ENSP00000370990.1:p.Thr122Ser		24
X	634719	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.379G>C	ENSP00000370990.1:p.Glu127Gln		25
X	634741	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.401G>A	ENSP00000370990.1:p.Arg134Gln		25
X	634743	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.403C>T	ENSP00000370990.1:p.Leu135Phe		23
X	634771	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.431C>G	ENSP00000370990.1:p.Ala144Gly		24
X	634776	['A', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.436A>C	ENSP00000370990.1:p.Met146Leu		24
X	634782	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.442G>A	ENSP00000370990.1:p.Glu148Lys		25
X	640851	['C', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.517C>A	ENSP00000370990.1:p.Arg173Ser		19
X	640877	['A', 'C']	4	1.00	['missense_variant',	MODERATE	SHOX	ENST00000381578.6:c.543A>C	ENSP00000370990.1:p.Lys181Asn	3.58E-05	24
X	641001	['G', 'A']	4	1.00	['missense_variant',	MODERATE	SHOX	ENST00000381578.6:c.547G>A	ENSP00000370990.1:p.Val183Ile		17
X	641037	['C', 'T']	2	1.00	['stop_gained']	HIGH	SHOX	ENST00000381578.6:c.583C>T	ENSP00000370990.1:p.Arg195Ter		35
X	641038	['G', 'A']	2	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.584G>A	ENSP00000370990.1:p.Arg195Gln		25
X	641052	['G', 'A']	3	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.598G>A	ENSP00000370990.1:p.Val200Ile		22
X	641065	['C', 'T']	4	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.611C>T	ENSP00000370990.1:p.Ala204Val		23
X	641065	['C', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.611C>A	ENSP00000370990.1:p.Ala204Asp		23
X	641068	['T', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.614T>C	ENSP00000370990.1:p.Leu205Ser		23
X	644397	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.640G>A	ENSP00000370990.1:p.Ala214Thr		25
X	644418	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.661G>A	ENSP00000370990.1:p.Val221Met		26
X	644422	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.665C>T	ENSP00000370990.1:p.Ala222Val		23
X	644423	['C', 'CCAG	1	1.00	['inframe_insertion']	MODERATE	SHOX	ENST00000381578.6:c.670_681dup	ENSP00000370990.1:p.Ala224_His227dup	3.36E-05	
X	644427	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.676G>A	ENSP00000370990.1:p.Ala226Thr		22
X	644428	['CGCACCC	3	1.00	['inframe_deletion']	MODERATE	SHOX	ENST00000381578.6:c.685_696del	ENSP00000370990.1:p.His229_Leu232del		
X	644434	['C', 'CGCA	1	1.00	['inframe_insertion']	MODERATE	SHOX	ENST00000381578.6:c.683_684dup	ENSP00000370990.1:p.Leu228_His229dup		
X	644464	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.707C>T	ENSP00000370990.1:p.Ala236Val		25
X	644484	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.727C>G	ENSP00000370990.1:p.Pro243Ala		21
X	645524	['CCGCCTC	1	1.00	['inframe_deletion']	MODERATE	SHOX	ENST00000381578.6:c.772_792del	ENSP00000370990.1:p.Ser258_Ala264del		
X	645539	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.782C>G	ENSP00000370990.1:p.Ala261Gly		19
X	645563	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.806G>T	ENSP00000370990.1:p.Ser269Ile		24
X	645578	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.821C>T	ENSP00000370990.1:p.Ser274Phe		26
X	645586	['G', 'A']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.829G>A	ENSP00000370990.1:p.Ala277Thr		27
X	645598	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.841C>T	ENSP00000370990.1:p.Leu281Phe		25
X	646604	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.847G>C	ENSP00000370990.1:p.Ala283Pro		26
X	646608	['G', 'A']	2	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.851G>A	ENSP00000370990.1:p.Arg284Gln		25
X	646622	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.865G>T	ENSP00000370990.1:p.Ala289Ser		24
X	646622	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.865G>T	ENSP00000370990.1:p.Ala289Ser		24

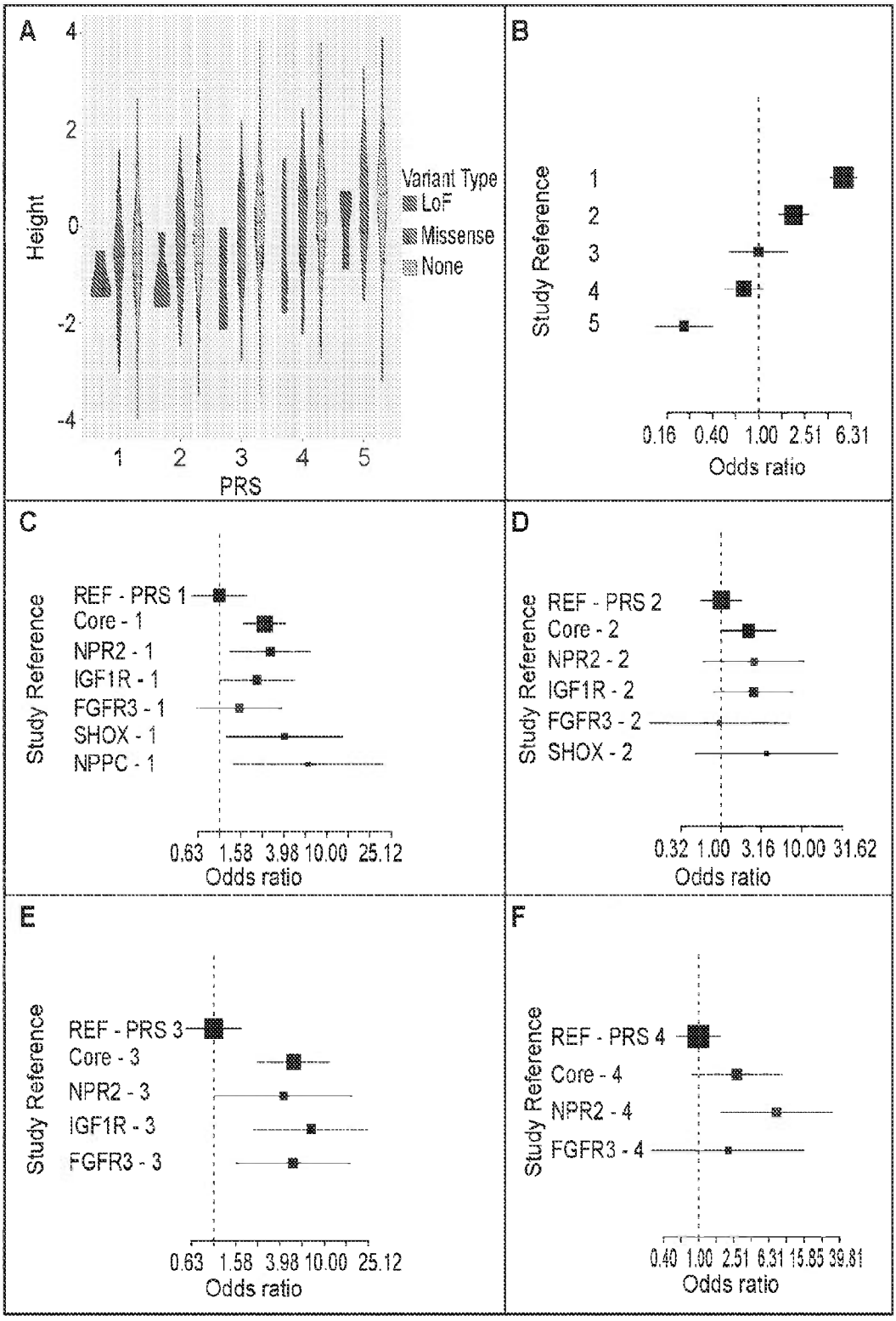


Figure 7A to 7F

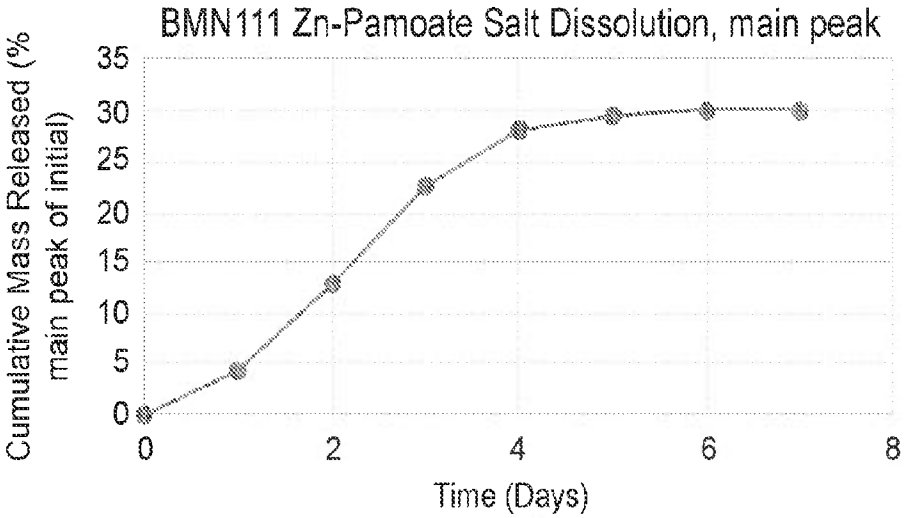


Figure 8A

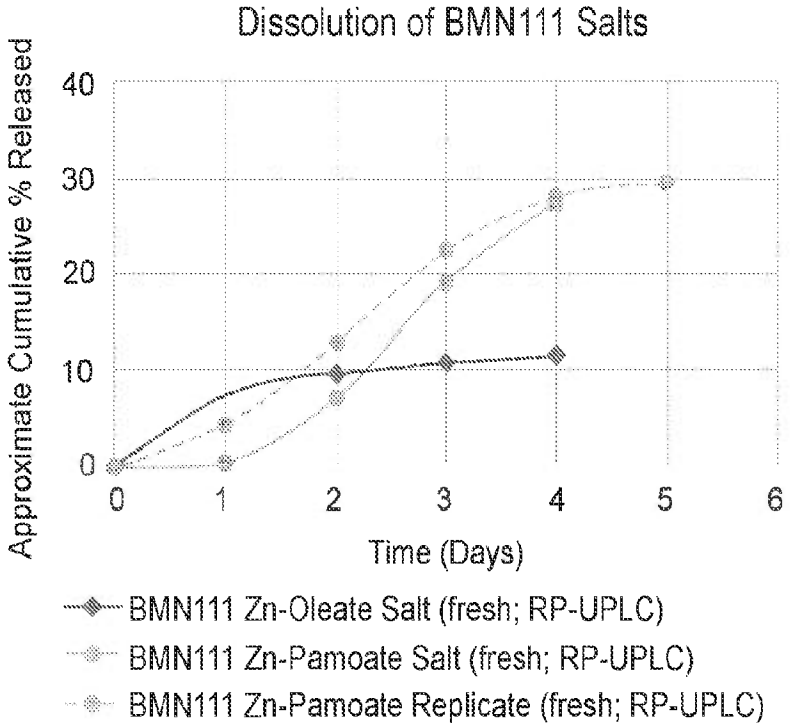


Figure 8B

Figure 9A

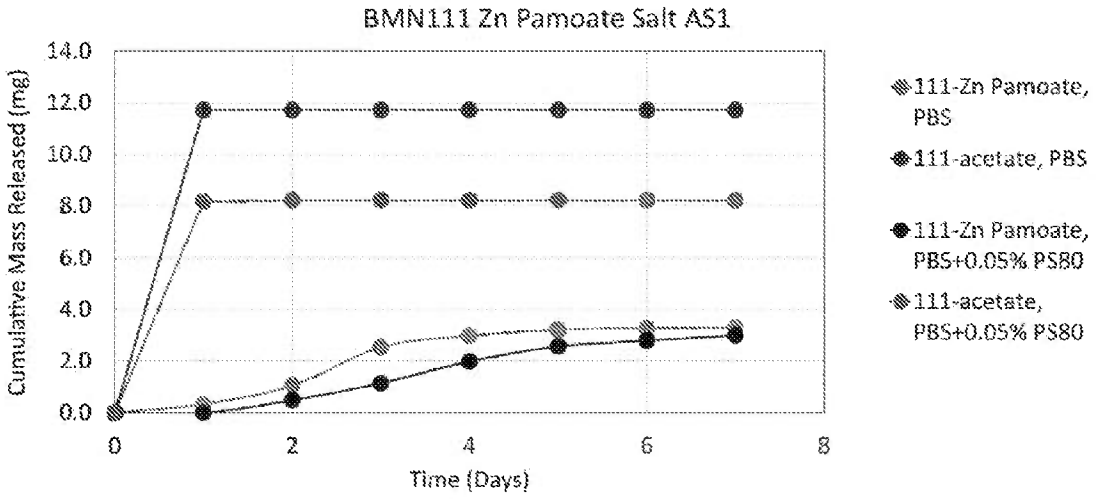
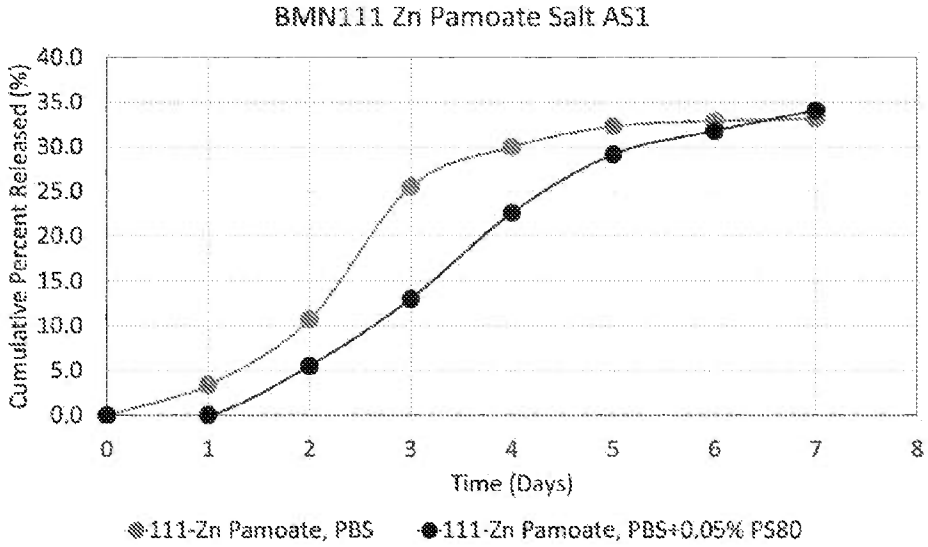


Figure 9B



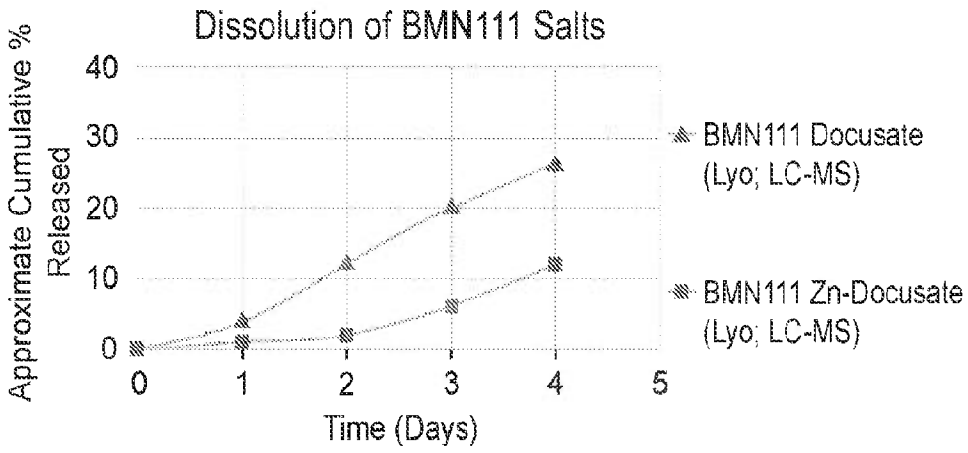


Figure 10A

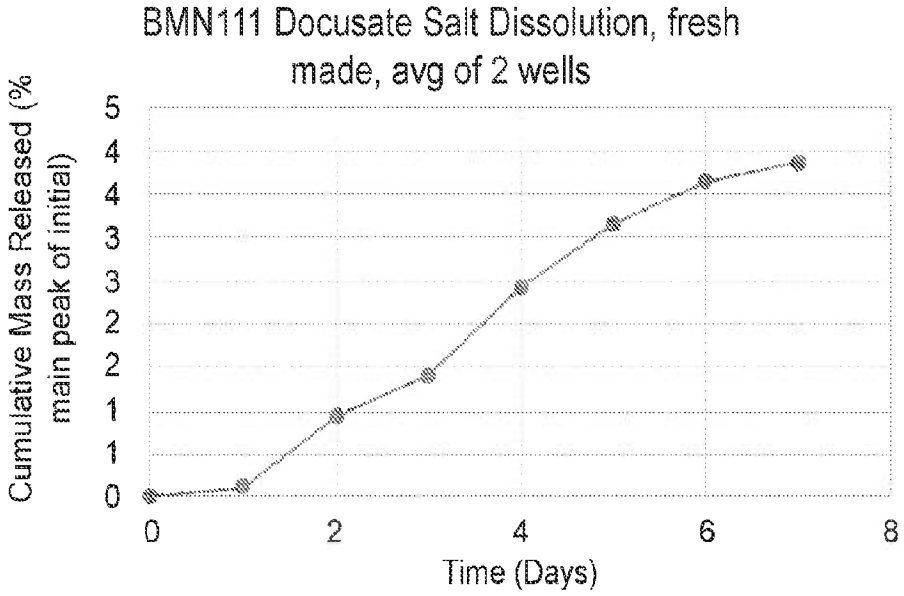


Figure 10B

Figure 10C

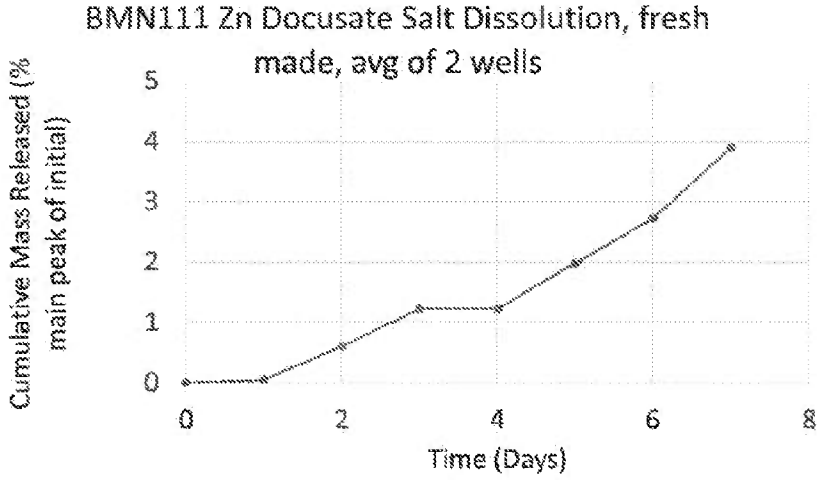
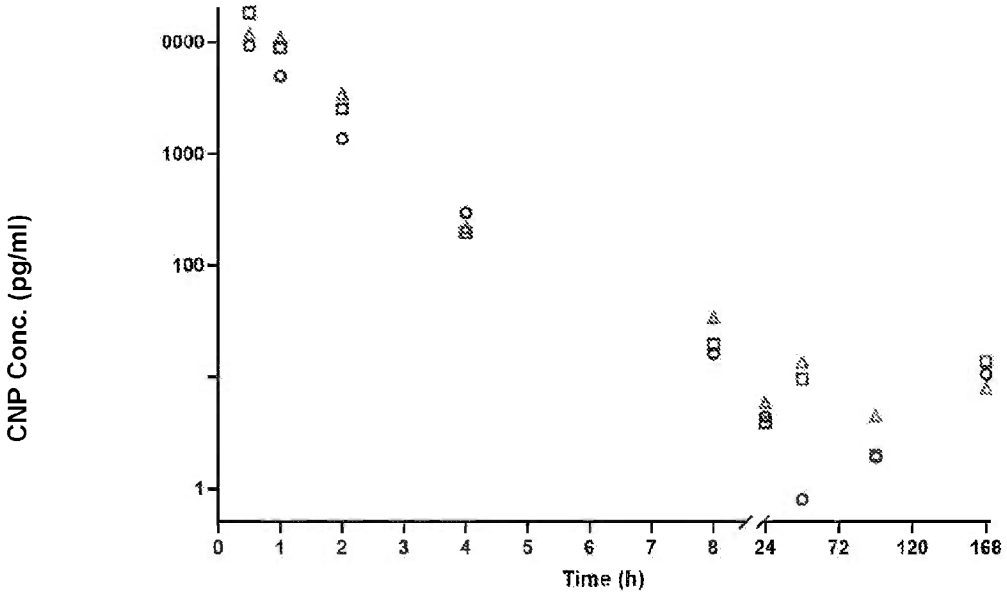

BMN111 Zn Docusate Salt Dissolution, fresh made, avg of 2 wells
Cumulative Mass Released (% main peak of initial)
0
1
2
3
4
5
0
2
4
6
8
Time (Days)

Figure 11



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/045885

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/52 A61K47/54 A61P19/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, COMPENDEX, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/035821 A1 (SHARMA SHUBH D [US] ET AL) 11 February 2010 (2010-02-11)	1-7,10, 11,13, 15-22, 27-29, 44-49, 51,52
Y	paragraphs [0014], [0093], [0094], [0219], [0220], [0227], [0228]; example 6	8,9,12, 14, 23-26, 38-43, 50,53,54

Y	WO 2019/090030 A1 (PRUDHOMME ROBERT K [US]; RISTROPH HURT D [US] ET AL.) 9 May 2019 (2019-05-09) claims 1, 17-20	8,9,12, 23-26

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2020	04/12/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van der Kooij, M

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/045885

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KURT D. RISTROPH ET AL: "Hydrophobic ion pairing: encapsulating small molecules, peptides, and proteins into nanocarriers", NANOSCALE ADVANCES, vol. 1, no. 11, 1 January 2019 (2019-01-01), pages 4207-4237, XP055736481, DOI: 10.1039/C9NA00308H abstract; figure 5	8,9,12, 23-26
Y	US 2010/297021 A1 (WENDT DANIEL J [US] ET AL) 25 November 2010 (2010-11-25) claim 13	14, 38-43, 50,53,54
Y	WO 2017/020034 A1 (BIOMARIN PHARM INC [US]) 2 February 2017 (2017-02-02) claims 1-61; sequences 5,7	14, 38-43, 50,53,54

1

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/045885

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-29, 38-54

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-29, 38-54

   A hydrophobic salt of CNP comprising CNP in complex with a hydrophobic counterion; a composition comprising a hydrophobic salt of CNP, whereby CNP is complexed with a hydrophobic counterion; a sterile pharmaceutical composition thereof; an extended release composition comprising the electrostatically charged peptide complexed with a hydrophobic counterion; medical uses thereof for treating bone-related disorder or skeletal dysplasia or for elongating a bone or increasing long bone growth.

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2. claims: 30-37

   A method of making a composition comprising a hydrophobic salt of a CNP.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/045885

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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Form PCT/ISA/210 (patent family annex) (April 2005)

Publication Number: WO 2021/055497 A1
Publication Date: Mar 25, 2021

Abstract

The present disclosure, relates, in general, to stable variants of C-type natriuretic peptide (CNR) and uses thereof to treat bone-related disorders.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 March 2021 (25.03.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/055497 A1

(51) International Patent Classification:
A61K 47/54 (2017.01) *A61P 19/08* (2006.01)
A61K 38/22 (2006.01) *C07K 14/58* (2006.01)

(21) International Application Number:
PCT/US2020/051100

(22) International Filing Date:
16 September 2020 (16.09.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/901,093	16 September 2019 (16.09.2019)	US
62/935,050	13 November 2019 (13.11.2019)	US
62/963,350	20 January 2020 (20.01.2020)	US
62/964,852	23 January 2020 (23.01.2020)	US
63/038,667	12 June 2020 (12.06.2020)	US

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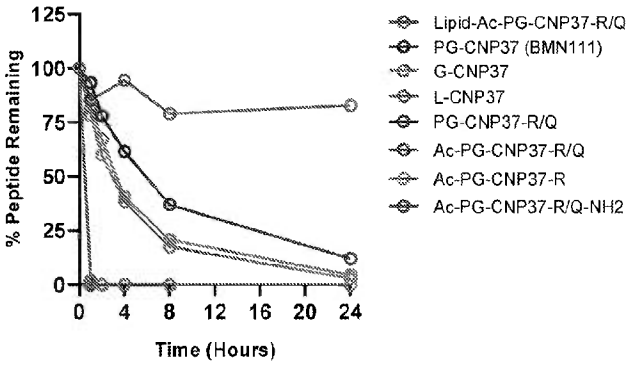
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: CNP VARIANTS AND CONJUGATES THEREOF

Figure 2A



(57) Abstract: The present disclosure, relates, in general, to stable variants of C-type natriuretic peptide (CNR) and uses thereof to treat bone-related disorders.

WO 2021/055497 A1

CNP VARIANTS AND CONJUGATES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. Provisional Patent Application No. 62/901,093, filed September 16, 2019, U.S. Provisional Patent Application No. 62/935,050, filed November 13, 2019, U.S. Provisional Patent Application No. 62/963,350, filed January 20, 2020, U.S. Provisional Patent Application No. 62/964,852, filed January 23, 2020, and U.S. Provisional Patent Application No. 63/038,667, filed June 12, 2020, herein incorporated by reference in their entireties.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification as a text file. The name of the text file containing the Sequence Listing is " 54736_Seqlisting.txt", which was created on August 6, 2020 and is 54,429 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

FIELD OF THE DISCLOSURE

[0003] The present disclosure, relates, in general, to variants of C-type natriuretic peptide (CNP), pharmaceutical compositions comprising CNP variants and methods of use. The CNP variants are useful as therapeutic agents for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders, such as skeletal dysplasias (e.g., achondroplasia).

BACKGROUND

[0004] C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)) is a small, single chain peptide in a family of peptides (ANP, BNP, CNP) having a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. CNP interacts with natriuretic peptide receptor-B (NPR-B, GC-B) to stimulate the generation of cyclic- guanosine monophosphate (cGMP) (J. Hypertens., 10: 1111-1114 (1992)). CNP is expressed widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

[0005] In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). Both CNP-53 and CNP-22 bind similarly to NPR-B.

[0006] Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, *et al.*, J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, *et al.*, Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

[0007] Therapeutic use of CNP (CNP22) has been limited by its short plasma half-life, which has been shown to be 2.6 minutes *in vivo* in humans (J Clin. Endocrinol. Metab., 78: 1428-35 (1994)). A CNP variant having a longer *in vivo* serum half-life and exhibiting similar or improved activity to that of wild-type CNP is important for a sustainable therapeutic strategy.

SUMMARY

[0008] The present disclosure relates to novel variants of C-type natriuretic peptide (CNP) having increased circulating half-life and stability in aqueous media, pharmaceutical compositions comprising such CNP variants, and methods of using such CNP variants to treat disorders responsive to CNP, including but not limited to bone-related disorders such as achondroplasia.

[0009] In various embodiments, the disclosure provides a variant of C-type natriuretic peptide (CNP) selected from the group consisting of
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1);

PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 6); and
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 5).

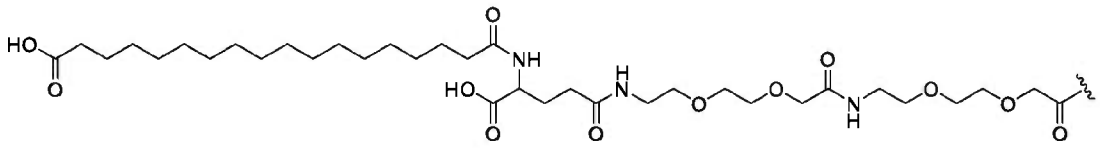
[0010] In various embodiments, the disclosure provides a variant of C-type natriuretic peptide (CNP) selected from the group consisting of
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 5);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 6);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC(SEQ ID NO: 5); and
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 7).

[0011] In various embodiments, the variant peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus.

[0012] In various embodiments, the variant peptide comprises a conjugate moiety. In various embodiments, the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the conjugate moiety is on a lysine residue. In various embodiments, the conjugate moiety comprises one or more acid moieties. In various embodiments, the acid moiety is a hydrophobic acid.

[0013] In various embodiments, the conjugate moiety comprises one or more acid moieties linked to a hydrophilic spacer. In various embodiments, the hydrophilic spacer is any amino acid. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu). In various embodiments, the hydrophilic spacer is OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) or OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) linked to one or two or more OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the acid moiety is a fatty acid. Exemplary fatty acids include short chain, medium chain, or long chain fatty acids, or a dicarboxylic fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. Contemplated are C-6 to C-20 fatty acids, including but not limited to, C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acids, saturated or unsaturated. In various embodiments, the fatty acid is decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid, arachidic acid, or diacids of the same.

[0014] In various embodiments, the acid moiety and the hydrophilic spacer have the structure AEEA-AEEA-γGlu-C18DA. In various embodiments, the acid moiety and the hydrophilic spacer have the structure:



, wherein " ~ " represents the point of attachment to a CNP variant. In various embodiments, " ~ " represents the point of attachment to a hydrolysable linker, wherein the hydrolysable linker is attached to a CNP variant. In various embodiments, the hydrolysable linker is capable of releasing intact CNP variant.

[0015] In various embodiments, the CNP variant with the conjugate moiety is a component of modified release composition. In various embodiments, the modified release composition is an extended release composition. In various embodiments, the CNP variant comprising a conjugate moiety and hydrolysable linker is capable of releasing the CNP variant, wherein (i) less than about 20% of CNP variant is released by day 1; and (ii) about 90% of the CNP variant is released weekly, or about 90% of the CNP variant is released bi-weekly, or about 90% of the CNP variant is released monthly, at pH 7 to 7.6.

[0016] In various embodiments, (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60% about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6; or alternatively ii) about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released bi-weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released every three weeks; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released monthly, at pH 7 to 7.6

[0017] In various embodiments, the variant has the structure:
PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC (SEQ

ID NO: 5), or Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 8).

[0018] In various embodiments, the variant is selected from the group consisting of

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8);

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9);

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10);

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11);

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12);

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 13); and

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 14).

[0019] In various embodiments, the variant comprises one or more linker groups. In various embodiments, the linker is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the linker is on a lysine residue.

[0020] In various embodiments, the linker is a hydrolysable linker.

[0021] In various embodiments, the CNP variant is attached to the conjugate moiety via the linker. In various embodiments, the linker is attached to the conjugate moiety via the hydrophilic spacer of the conjugate moiety. In various embodiments, the linker is aminoethoxy-2-ethoxy acetic acid (AEEA). In various embodiments, the linker is a bicin-type or peptoid linker, which refers to a linker having a similar cleavage mechanism as bicin (bis-2-hydroxyethylglycinamide), but cleaving instead via an asymmetric N-alkyl peptide, i.e., a peptoid. In various embodiments, the linker is an electronic linker based on nonenzymatic β-elimination. In various embodiments, the electronic linker comprises an SO_2 moiety. Examples of linkers as illustrated in a CNP conjugate are set out in Figure 1. See also Santi, et.al., Proc Natl Acad Sci USA 109:6211-6216, 2012).

[0022] In various embodiments, the conjugate moiety is a synthetic polymeric group. In various embodiments, the variant comprises a synthetic polymeric group coupled to the variant through a hydrolysable linker. In various embodiments, the synthetic polymeric group comprises a hydrophilic polymer moiety. In various embodiments, the hydrophilic polymer moiety comprises polyethylene glycol (PEG). In various embodiments, the hydrophilic polymer moiety comprises polyethylene glycol (PEG) having a 6 to 20 atom chain length.

[0023] In various embodiments, the variant peptide is made synthetically.

[0024] In various embodiments, the variant peptide is stable for 10 days at about 37° C, pH 7.0 to 7.6. In various embodiments, the variant peptide is stable for at least 10 days at about 37° C, pH 7.0 to 7.4. In various embodiments, the variant peptide is stable for at least 10 days at about 37° C, pH 7.2 to 7.6.

[0025] In various embodiments, the variant peptides are stable to deamidation. In various embodiments, the variant peptides are stable to oxidation. In various embodiments, the variant peptides are stable to deamidation, and/or oxidation, or combinations thereof. In various embodiments, methionine is replaced by nor-leucine. In various embodiments, there is little to no detectable deamidation after 10 days.

[0026] In various embodiments, the variant peptide has a half-life of about 10 days at about 37° C, pH 7.0 to 7.6. In various embodiments, the variant peptide has a half-life of about 10 days at about 37° C, pH 7.0 to 7.4. In various embodiments, the variant peptide has a half-life of about 10 days at about 37° C, pH 7.2 to 7.6. In various embodiments, the variant peptide has a half-life of at least 10 days at about 37° C, pH 7.0 to 7.6. In various embodiments, the variant peptide has a half-life of at least 10 days at about 37° C, pH 7.0 to 7.4. In various embodiments, the variant peptide has a half-life of at least 10 days at about 37° C, pH 7.2 to 7.6. In various embodiments, the half-life is at least about 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 30 days or more.

[0027] In various embodiments, the variant peptide has an EC50 from 0.1 to 10 nM in a cGMP assay. In various embodiments, the variant peptide has an EC50 from 0.1 to 25 nM in a cGMP assay.

[0028] In various embodiments, greater than 45% of the variant peptide is detected after 10 days in aqueous media at physiological conditions, e.g., about 37° C, pH 7.0 to 7.6. In various embodiments, greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the variant peptide is detected after 10 days in aqueous media at physiological conditions, e.g., about 37° C, pH 7.0 to 7.6.

[0029] In various embodiments, greater than 45% of the variant peptide is detected after 10 days in aqueous media at 37° C, pH 7.4. In various embodiments, greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the variant peptide is detected after 10 days in aqueous media at 37° C, pH 7.4.

[0030] In various embodiments, greater than 45% of the variant peptide is detected after 10 days in plasma at physiological conditions, e.g., about 37° C, pH 7.0 to 7.6. In various embodiments, greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the variant peptide is detected after 10 days in plasma at physiological conditions, e.g., about 37° C, pH 7.0 to 7.6.

[0031] In various embodiments, greater than 45% of the variant peptide is detected after 10 days in plasma at 37° C, pH 7.4. In various embodiments, greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the variant peptide is detected after 10 days in plasma at 37° C, pH 7.4.

[0032] In various embodiments, the variant peptide is conjugated to a lipid, fatty acid, hydrophilic spacer, or linker, or optionally combinations thereof. In various embodiments, the linker is a hydrophilic polymer moiety. In various embodiments, the hydrophilic polymer moiety is a synthetic hydrophilic polymer moiety.

[0033] In various embodiments, the variant peptide has a longer half-life compared to Pro-Gly-CNP37. In various embodiments, the variant peptide has a longer half-life compared to CNP-22. In various embodiments, the variant peptide has a longer half-life compared to Pro-Gly-CNP37 and/or CNP-22. In various embodiments, the variant peptide has a longer half-life compared to Pro-Gly-CNP37 and/or CNP-22 in vitro and/or in vivo.

[0034] The disclosure further provides a pharmaceutical composition comprising a CNP variant described herein, and a pharmaceutically acceptable excipient, carrier or diluent.

[0035] In various embodiments, the composition is a lyophilized formulation prepared from a formulation that comprises a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6. In various embodiments, the lyophilized formulation is prepared from a formulation that further comprises an isotonicity-adjusting agent or a bulking agent selected from the group consisting of mannitol, sucrose, sorbitol, trehalose, polysorbate 80, and combinations thereof. In various embodiments, the lyophilized formulation is prepared from a formulation that further comprises an antioxidant selected from the group consisting of methionine, ascorbic acid, salt forms of ascorbic acid, thioglycerol, and combinations thereof. In various embodiments, the CNP variant composition is supplied as a lyophilized powder for reconstitution from 0.8 mg to 10 mg. In various embodiments, the CNP variant composition is supplied as a 0.8-mg or 2-mg lyophilized, preservative-free powder for reconstitution.

[0036] In various embodiments, the composition is an extended release composition.

[0037] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (BMN111) (SEQ ID NO: 1).

[0038] Also provided is a method of treating a bone-related disorder or skeletal dysplasia in a subject in need thereof comprising administering to the subject a composition comprising a CNP variant as described herein.

[0039] In various embodiments, the bone-related disorder or skeletal dysplasia is selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia.

[0040] In various embodiments the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

[0041] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome).

[0042] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from an NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), or PTPN11 mutations (Noonan's syndrome), or insulin growth factor 1 receptor (IGF1R).

[0043] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1,

COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3.

[0044] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0045] In various embodiments, the bone-related disorder, skeletal dysplasia or short stature disorder results from a RASopathy. In various embodiments, the RASopathy is Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, or LEOPARD syndrome.

[0046] In one embodiment, the RASopathy is hereditary gingival fibromatosis type 1.

[0047] In various embodiments, the CNP variants are useful to treat growth plate disorders and short stature, including familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. In various embodiments, the short stature or growth plate disorder is a result of a mutation in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R).

[0048] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0049] In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and having at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. In various embodiments, the short stature is associated with one or more mutations in a gene associated with short stature, such as, collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R), or combinations thereof. In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0050] In various embodiments, the short stature is a result of mutations in multiple genes as determined by polygenic risk score (PRS). In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in FGFR3 and a

low PRS. In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in IGF1R and a low PRS. In various embodiments, the subject has a mutation in NPPC and a low PRS. In various embodiments, the subject has a mutation in SHOX and a low PRS. In various embodiments, the subject has one or more mutation in one or more of FGFR3, IGF1R, NPPC, NPR2 and SHOX, and a low PRS. In various embodiments, the PRS is 1 or 2. In various embodiments, the PRS is 1. In various embodiments, the PRS is 2. Polygenic risk scores (PRS) were calculated for height as described in Example 4. PRS 1 refers to the lowest height, PRS 5 the tallest height.

[0051] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker.

[0052] In various embodiments, the disclosure provides a method of elongating a bone or increasing long bone growth in a subject in need thereof, comprising administering to the subject a composition comprising a CNP variant described herein, and wherein the administering elongates a bone or increases long bone growth.

[0053] In various embodiments, the composition is administered subcutaneously, intradermally, intraarticularly, orally, or intramuscularly.

[0054] In various embodiments, the composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

[0055] In various embodiments, the composition is an extended release composition.

[0056] Further contemplated is a method of treating a CNP-responsive condition or disorder, comprising administering a CNP variant or composition as described herein to a subject, and monitoring the level of at least one bone- or cartilage-associated biomarker in the subject, wherein an increase in the level of the at least one bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP peptide or variant on the subject or the condition or disorder.

[0057] Further contemplated is a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant or a composition as described herein.

[0058] Further contemplated is a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant or a composition as described herein.

[0059] In various embodiments, the method further comprises adjusting the amount or frequency of administration of the CNP peptide or variant as described herein, wherein i) the amount or frequency of administration of the CNP peptide or variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a target level; or ii) the amount or frequency of administration of the CNP peptide or variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level.

[0060] In various embodiments, the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, Collagen Type I C-Telopeptide (CTx), osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, collagen X, and alkaline phosphatase.

[0061] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the acetyl group is on an amino acid side chain within the peptide sequence. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker.

[0062] Also provided is a method of making a CNP variant described herein comprising synthesizing the peptide on a solid-phase resin using Fmoc amino acids.

[0063] In various embodiments, the method comprises acetylating the peptides by reacting the resin with NMP/Ac_2O/DIEA (10:1:0.1, v/v/v).

[0064] In various embodiments, the method comprises conjugating the peptide to a conjugate moiety, optionally on a lysine residue. In various embodiments, the method comprises cleaving the protective amino group on lysine, reacting the peptide with 2x Fmoc-amino PEG(2) followed by amino acid, followed by conjugation of the lipid or fatty acid moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

[0065] Figure 1 illustrates use of a peptoid or electronic linker in a CNP conjugate described herein.

[0066] Figure 2 shows the stability of CNP variants in human plasma over a period of 24 hrs.

[0067] Figure 3 shows the stability of CNP variants under different culture conditions.

[0068] Figure 43 shows the effects of a CNP variant (Pro-Gly-CNP) on cells carrying either NPR2 homozygous or heterozygous mutations, as measured by cGMP stimulation.

[0069] Figure 5 shows the nucleotide and predicted protein sequence of the first exon in NPR2 mutant clones transfected into RCS cells.

[0070] Figure 6 shows exemplary NPR2 mutations analyzed for response to CNP.

[0071] Figure 7 shows exemplary mutations associated with short stature in FGFR3, IGF1R, NPPC, NPR2 and SHOX.

[0072] Figure 8 illustrates the Combined effect of PRS and rare coding variants on Height. Fig. 8A. Effects on height as a quantitative trait, samples were divided in five groups based on their PRS, violin-plots with horizontal lines representing the 25%, 50% and 75% percentile of height. Samples were grouped by carrying status of missense, loss of function or None in any of the five core genes. Fig. 8B. Effect reflected on Odds ratios for "Idiopathic Short Stature" or ISS. Odds for ISS using PRS =3 as reference vs the other PRS groups. Fig. 8C. Odds for ISS using PRS =1 as reference vs having missense and/or loss of function variants in core genes. Fig. 8D. Odds for ISS using PRS =1 non-carriers as reference vs having missense and/or loss of function variants in core genes. Fig. 8E. Odds for ISS using PRS =2 non-carriers as reference vs having missense and/or loss of function variants in core genes. Fig. 8F. Odds for ISS using PRS =3 non-carriers as reference vs having missense and/or loss of function variants in core genes. G. Odds for ISS using PRS =4 non-carriers as reference vs having missense and/or loss of function variants in core genes.

DETAILED DESCRIPTION

[0073] The present disclosure relates to stable CNP variants useful in treating skeletal dysplasias and bone growth disorders.

[0074] As used in the specification and the appended claims, the indefinite articles "a" and "an" and the definite article "the" include plural as well as singular referents unless the context clearly dictates otherwise.

[0075] The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood that the term "about" or "approximately" applies to each one of the numerical values in that series.

[0076] The term "C-type natriuretic peptide" or "CNP" refers to a small, single chain peptide having a 17-amino acid loop structure at the C-terminal end (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) and variants thereof. The 17-mer CNP loop structure, is also referred to as CNP 17, the CNP ring, or CNP cyclic domain. CNP includes the active 53-amino acid peptide (CNP-53) and the mature 22-amino acid peptide (CNP-22), and peptides of varying lengths between the two peptides.

[0077] In various embodiments, a "CNP variant" is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that a CNP variant peptide may comprise from about 1 to about 53, or 1 to 39, or 1 to 38, or 1 to 37, or 1 to 35, or 1 to 34, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, a CNP variant may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide.

[0078] The term "conjugate moiety" refers to a moiety that is conjugated to the variant peptide. Conjugate moieties include a lipid, fatty acid, hydrophilic spacer, synthetic polymer, linker, or optionally, combinations thereof.

[0079] The term "effective amount" refers to a dosage sufficient to produce a desired result on a health condition, pathology, or disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation. It will be understood that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors, including the activity of the specific compound employed; the bioavailability, metabolic stability, rate of excretion and length of action of that compound; the mode and time of administration of the compound; the age, body weight, general health, sex, and diet of the patient; and the severity of the particular condition.

[0080] "Substantially pure" or "isolated" means an object species is the predominant species present (*i.e.*, on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. In one embodiment, a substantially pure composition means that the species of interest comprises at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or more of the macromolecular species present in the composition on a molar or weight basis. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (*e.g.*, BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In an embodiment, the compounds of the disclosure are substantially pure or isolated. In another embodiment, the compounds of the disclosure are substantially pure or isolated with respect to the macromolecular starting materials used in their production. In yet another embodiment, the pharmaceutical compositions of the disclosure comprise a substantially pure or isolated CNP variant admixed with one or more pharmaceutically acceptable excipients, carriers or diluents, and optionally with another biologically active agent.

[0081] "Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

[0082] A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

[0083] A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

[0084] "Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[0085] "Bone- or cartilage-associated biomarker" or "bone- or cartilage-associated marker" refers to a growth factor, enzyme, protein, or other detectable biological substance or moiety whose level is increased or decreased in association with, e.g., cartilage turnover, cartilage formation, cartilage growth, bone resorption, bone formation, bone growth, or combinations thereof. Such biomarkers may be measured before, during and/or after administration of a CNP variant as described herein. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, propeptides of collagen type I and fragments thereof, collagen type I and fragments thereof, osteocalcin, proliferating cell nuclear antigen (PCNA), aggrecan chondroitin sulfate, collagen X, and alkaline phosphatase. Cartilage- and bone-associated biomarkers can be measured in any appropriate biological sample, including but not limited to tissues, blood, serum, plasma, cerebrospinal fluid, synovial fluid and urine. In some embodiments, the biomarkers are measured in blood, plasma or serum from animals undergoing efficacy/pharmacodynamic *in vivo* studies and/or from the conditioned media of *ex vivo* studies.

[0086] "Pharmaceutical composition" or "formulation" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or

diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

[0087] "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

[0088] A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

[0089] By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

[0090] "Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body

temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

[0091] By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

[0092] As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender. In various embodiments, the subject is human. In various embodiments the subject is a child or adolescent. In various embodiments, the subject is an infant. In various embodiments, the subject is older than 3 , older than 2, older than 1, or older than 6 months in age.

C-type natriuretic peptide variants

[0093] C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)) is a small, single chain peptide in a family of peptides (ANP, BNP, CNP) having a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. CNP interacts with natriuretic peptide receptor-B (NPR-B, GC-B) to stimulate the generation of cyclic- guanosine monophosphate (cGMP) (J. Hypertens., 10: 1111-1114 (1992)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

[0094] Natural CNP gene and polypeptide have been previously described. U.S. Patent No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Patent No. 6,034,231 discloses the human gene and polypeptide of pre-proCNP (126 amino acids) and the human CNP-53 gene and polypeptide. The mature CNP is a 22-amino acid peptide (CNP-22). Certain CNP variants are disclosed in US Patent 8,198,242, incorporated by reference herein.

[0095] In various embodiments, CNP of the disclosure includes truncated CNP ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wild-type amino acid sequences derived from hCNP-53. Such truncated CNP peptides include:

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-53) (SEQ ID NO: 56);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO:15);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO:16);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO:17);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 18)
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO:19);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO:20);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO:21);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO:22);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO:23);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:24);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:25);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO:26);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO:27);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO:28);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:2);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO:3);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO:29);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO:30);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO:4);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO:31);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO:32);

RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO:33);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO:34);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO:35);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO:36);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO:37);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO:38);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO:39);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO:40);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO:41);
GLSKGCFGLKLDRIGSMSGLGC (CNP-22) (SEQ ID NO: 68);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO:42);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO:43);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO:44);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO:45); and
CFGLKLDRIGSMSGLGC (CNP-17) (SEQ ID NO: 67).

[0096] In various embodiments, the CNP variant peptides are modified CNP-37 or CNP-38 peptides, optionally having mutation(s)/substitution(s) at the furin cleavage site (underlined), and/or containing glycine or proline-glycine at the N-terminus. Exemplary CNP-37 variants include but are not limited to:

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N); SEQ ID NO: 46];
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37; SEQ ID NO: 47);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37; SEQ ID NO: 48);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N); SEQ ID NO: 49];
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO:1);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37; SEQ ID NO: 50);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37: SEQ ID NO:51)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:52);
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:53);
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:54); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO:55);

[0097] In various embodiments, CNP variants of the disclosure include
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5);

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5);
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 7);

PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC (SEQ ID NO:5); and

PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC (SEQ ID NO: 1) .

[0098] It is further contemplated that any of the variants described herein having fewer than 53 amino acids, can be extended by addition of amino acids to the N-terminal end of the peptide. For example, if the CNP variant is a 34-, 35-, 36-, 37-, 38- or 39-mer having altered amino acids compared to wt CNP, such a variant can be extended on the N-terminal end with either wild type or modified residues from CNP53, or from other peptides.

[0099] In various embodiments, the CNP variant further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus, C-terminus or attached to an internal amino acid side group. In various embodiments, the acetyl group is on the N-terminus of the peptide.

[0100] In various embodiments, the peptide variant further comprises an OH or an NH_2 group at the C-terminus.

[0101] In various embodiments, the CNP variants are selected from the group consisting of:

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8),

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9),

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10),

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11), and

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12).

[0102] In various embodiments, the CNP variants are selected from the group consisting of:

PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8),

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9),

PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10),

PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11), and

PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12)

PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 7).

[0103] In various embodiments, the CNP variants are selected from the group consisting of:

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8),

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9),

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10),

Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11),

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12);

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 13); and

Ac- PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 14).

[0104] In various embodiments, the CNP variant is selected from the group consisting of Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO:8). In various embodiments, the CNP variant is Ac-PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1). In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1).

[0105] In additional embodiments, for any of the CNP variants described herein that have asparagine (Asn/N) residue(s) and/or glutamine (Gln/Q) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Asn residue(s) and/or any Gln residue(s) can independently be substituted with any other natural or unnatural amino acids, including conservative substitutions such as Asn to Gln. Such substitution(s) are designed in part to minimize or avoid any potential deamidation of asparagine and/or glutamine.

[0106] In additional embodiments, for any of the CNP variants described herein that have lysine (Lys/K) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Lys residue(s) can independently be substituted with any other natural or unnatural amino acids, including substitutions such as Lys to Arg. In various embodiments, all lysine residues are independently substituted with any other natural or unnatural amino acids,

including substitutions such as Lys to Arg, except the Lys residue in the CNP variant cyclic domain is not substituted with any other natural or unnatural amino acids.

[0107] In one embodiment, the CNP variants are cyclized via formation of a disulfide bond between Cys^6 and $Cys^{22,}$ as designated in the wtCNP22 peptide. Cys^6 can be a cysteine analog such as, e.g., homocysteine or penicillamine. In a further embodiment, the CNP variants can be cyclized by a covalent bond formed head-to-tail, side chain-to-side chain, side chain-to-head, or side chain-to-tail. In an embodiment, the covalent bond is formed between an amino acid at or toward the N-terminus and an amino acid at or toward the C-terminus of the peptide (referred to as "terminal" amino acids in this context). In another embodiment, the covalent bond is formed between the side chains of the two terminal amino acids. In yet another embodiment, the covalent bond is formed between the side chain of one terminal amino acid and the terminal group of the other terminal amino acid, or between the terminal groups of the two terminal amino acids.

[0108] Head-to-tail cyclization of the terminal amine to the terminal carboxyl group can be carried out using a number of methods, e.g., using p-nitrophenyl ester, 2,4,5-trichlorophenyl ester, pentafluorophenyl ester, the azide method, the mixed anhydride method, HATU, a carbodimide (e.g., DIC, EDC or DCC) with a catalyst such as HOBt, HONSu or HOAt, or on-resin cyclization.

[0109] In addition, the cyclic structure can be formed via a bridging group involving the side chains of amino acid residues of the CNP variant and/or the terminal amino acid residues. A bridging group is a chemical moiety that allows cyclization of two portions of the peptide. Non-limiting examples of bridging groups include amides, thioethers, thioesters, disulfides, ureas, carbamates, sulfonamides, and the like. A variety of methods are known in the art for incorporation of units having such bridging groups. For example, a lactam bridge (i.e., a cyclic amide) can be formed between the N-terminal amino group or an amino group on a side chain and the C-terminal carboxylic acid or a carboxyl group on a side chain, e.g., the side chain of lysine or ornithine and the side chain of glutamic acid or aspartic acid. A thioester can be formed between the C-terminal carboxyl group or a carboxyl group on a side chain and the thiol group on the side chain of cysteine or a cysteine analog.

[0110] Alternatively, a cross link can be formed by incorporating a lanthionine (thio-dialanine) residue to link alanine residues that are covalently bonded together by a thioether bond. In another method, a cross-linking agent, such as a dicarboxylic acid (e.g., suberic acid

(octanedioic acid)), can link the functional groups of amino acid side chains, such as free amino, hydroxyl, and thiol groups.

[0111] Enzyme-catalyzed cyclization can also be used. For example, it has been reported that the thioesterase domain of tyrocidine synthetase can be used to cyclize a thioester precursor, a subtilisin mutant can be utilized to cyclize peptide glycolate phenylalanylamide esters, and the antibody ligase 16G3 can be employed to cyclize a p-nitrophenylester. For a review of peptide cyclization, see Davies, J. Peptide Sci., 9: 471-501 (2003), incorporated herein by reference in its entirety.

[0112] In certain embodiments, the final product has a purity of at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99%.

Peptide Conjugates

[0113] Peptide therapeutics are attractive biological therapeutic agents, but are often disadvantaged by low stability and short half-life in solution (Tang et al., Eur J Pharm Sci. 102:63-70, 2017). Attempts to improve efficacy of peptide therapeutics, by enhancing stability and/or increasing the half-life, include attempts to encapsulate hydrophilic peptides into biodegradable particles such as liposomes or polymer particles. However, this has been difficult due to the cationic nature of these peptides and their ability to electrostatically interact with liposomes of negatively charged polymers (Griesser et al., Int J Pharmaceutics 520:267-274, 2017). Generation of peptide conjugates has been one means used to enable better encapsulation of hydrophilic polymers into microparticles or liposomes (Lu et al., Mol. Pharmaceutics 15:216-225, 2018).

[0114] Peptides can be a string of amino acids from 5 to 100 amino acids. The peptide can have positively charged amino acids, negatively charged amino acids, or a mixture of both, such that the peptide is capable of interacting with charged moieties, e.g., a cation, anion or a combination thereof having charged species opposite to those in the peptide.

[0115] It is contemplated that the peptide is complexed to a moiety, e.g., a conjugate moiety, that confers increased stability or half-life. In various embodiments, the conjugate moiety is complexed via a non-covalent bond or is attached by a covalent bond. The moiety may be non-covalently attached with the peptide via electrostatic interactions. Alternatively, the moiety may be covalently associated to the peptide via one or more linker moieties. Linkers can be cleavable and non-cleavable linkers. Cleavable linkers may be cleaved via enzymes, nucleophilic/basic reagents, reducing agents, photo-irradiation, electrophilic/acidic reagents,

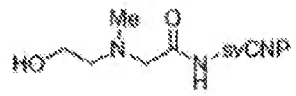
organometallic and metal reagents, or oxidizing reagents. Linkers may also be self-immolative linkers. Exemplary linkers include, but are not limited to, N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene), beta alanine, 4-aminobutyric acid (GABA), 2-aminoethoxy acid (AEA), aminoethoxy-2-ethoxy acetic acid (AEEA), 5 aminovaleric acid (AVA), 6-aminocaproic acid (Abx), a vicinal diol cleavable linker, Trimethyl Lock Lactonization, p-alkoxyphenyl carbamate, bicin, peptoid or bicin-type linkers, and electronic linkers as described herein.

[0116] In various embodiments, the linker is attached to a residue of the CNP variant within the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the linker is attached to a lysine residue. In various embodiments, the linker is attached to a lysine residue in the CNP cyclic domain.

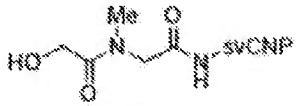
[0117] In various embodiments, the CNP variant is attached to the conjugate moiety via the linker. In various embodiments, the linker is attached to the conjugate moiety via the hydrophilic spacer of the conjugate moiety.

[0118] In various embodiments, the linker is a hydrolysable linker.

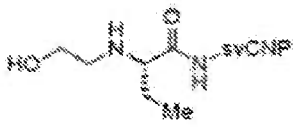
[0119] In various embodiments the linker is a peptoid or electronic linker. In various embodiments the linker is a peptoid linker. In various embodiments the linker is an electronic linker. In various embodiments, the linker comprises an SO_2 moiety. Exemplary linkers are illustrated in Figure 1. It is further contemplated that linkers in Figure 1 are modified by substitution on the R groups. For example, bicin-type linkers include the structures as set out below:



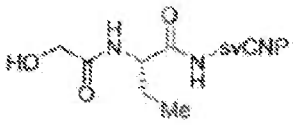
BCC-1



BCC-2



BCC-3



BCC-4

[0120] In various embodiments, the moiety conjugated to the peptide is a synthetic polymer such as polyethylene glycol, a linker, a lipid moiety or fatty acid, or a combination thereof. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a spacer and a linker. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a polyethylene glycol spacer or a polyethylene glycol derivative spacer, and a linker. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a spacer, and a linker, wherein the spacer comprises a substituted C-6 to C-20 alkyl chain or any amino acid, or a combination of both, wherein the carbon atoms of the alkyl chain can be replaced by one or more of O, NH, N(C-1 to C-6 alkyl), or carbonyl groups.

[0121] In various embodiments, the CNP variant is conjugated with a fatty acid. It is hypothesized that the lipid technology increases the serum half-life of the CNP variant allowing for less frequent injections and/or improved oral delivery. In various embodiments, the fatty acid is a short chain, medium chain, long chain fatty acid, or a dicarboxylic fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. In various embodiments, the fatty acid is a C-6 to C-20 fatty acid. In various embodiments, the fatty acid is a C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acid. In various embodiments, the fatty acid is decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid, arachidic acid, or diacids of the same. In various embodiments, the fatty acid is conjugated to a lysine residue.

[0122] In various embodiments, it is contemplated that the CNP variants described herein comprise a conjugate moiety as described herein. It is contemplated that the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the conjugate moiety is on a lysine residue. In various embodiments,

the conjugate moiety comprises one or more acid moieties. In various embodiments, the acid moiety is a fatty acid.

[0123] In various embodiments, the conjugate moiety comprises an acid moiety linked to a hydrophilic spacer. In various embodiments, the hydrophilic spacer is a substituted C-6 to C-20 alkyl chain or any amino acid, or a combination of both, wherein the carbon atoms of the alkyl chain can be replaced by one or more of O, NH, N(C-1 to C-6 alkyl), or carbonyl groups. In various embodiments, the hydrophilic spacer is any amino acid. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu). In various embodiments, the hydrophilic spacer is a substituted C-6 to C-20 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-9 to C-18 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-18 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-9 alkyl chain. In various embodiments, the hydrophilic spacer is one or more OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is one or two OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the spacer is OEG (8-amino-3,6-dioxaoctanoic acid) or γGlu. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) linked to one or more OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γGlu) linked to one or two OEG (8-amino-3,6-dioxaoctanoic acid) groups (diEG). In various embodiments, the acid moiety and the hydrophilic spacer have the structure AEEA-AEEA-γGlu-C18DA.

[0124] In various embodiments, the CNP variant has the structure:

PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC (SEQ ID NO: 5), or

[0125] Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 8).In various embodiments, the CNP variant has the structure PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC, Ac-PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1), or PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1). In various embodiments, the CNP variant comprises Asn to Glu variants of the above peptides.

[0126] In various embodiments, the disclosure contemplates use of hydrophilic or water soluble polymers (e.g., oxygenated alkyl chains, wherein the carbon atoms can be replaced with one or more oxygen atoms, such as polyethylene glycol (PEG) or polyethylene oxide (PEO) and the like). In various embodiments, the water soluble polymers can vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus, internal, and/or C-terminus), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 5, 10, 25, 50 or 100 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP variant by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. In various embodiments, negatively charged PEG-CNP variants can be designed for reduced renal clearance, including but not limited to use of carboxylated, sulfated and phosphorylated compounds (Caliceti, Adv. Drug Deliv. Rev., 55: 1261-77 (2003); Perlman, J. Clin. Endo. Metab., 88: 3227-35 (2003); Pitkin, Antimicrob. Ag. Chemo., 29: 440-444 (1986); Vehaskari, Kidney Int'l, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

[0127] In another embodiment, the hydrophilic polymer (e.g., PEG or PEO) moieties conjugated to the N-terminus, C-terminus and/or internal site(s) of CNP variants described herein contain one or more functional groups that are positively charged under physiological conditions. Such moieties are designed, *inter alia*, to improve distribution of such conjugated CNP variants to cartilage tissues. In one embodiment, PEG moieties contain one or more primary, secondary or tertiary amino groups, quaternary ammonium groups, and/or other amine-containing (e.g., urea) groups.

Methods of Making

[0128] Contemplated herein is also a method of making a composition comprising a CNP variant and optionally a conjugate moiety as described herein.

[0129] In various embodiments, the CNP variant is made synthetically using standard protein synthesis chemistry. For example, peptides are synthesized step-wise using a solid-phase resin and standard Fmoc chemistry. Peptides are cleaved from the resin using tri-fluroacetic acid (TFA) and purified by reverse phase high-performance liquid chromatography (RP-HPLC).

[0130] In various embodiments, the method further comprises, acetylating the peptides by reacting the resin with NMP/Ac_2O/DIEA, optionally at 10:1:0.1, v/v/v.

[0131] Further provided is a method wherein the peptide is conjugated to a conjugate moiety, optionally on a lysine residue. The step comprising cleaving the protective amino group on the lysine, reacting the peptide with 2x Fmoc-amino PEG(2) followed by amino acid, followed by conjugation of the lipid or fatty acid moiety. In various embodiments, the conjugate moiety comprises one or more lipids or fatty acids and a hydrophobic spacer.

[0132] The method further provides a step of cleaving the peptide from the resin by contacting with tri-fluoroacetic acid, and a step of purifying the peptide by reverse phase-HPLC.

[0133] In certain embodiments, the CNP variants described herein are produced by a recombinant process that comprises culturing in a medium a host cell comprising a first polynucleotide encoding a CNP variant polypeptide, optionally linked to a second polynucleotide encoding a cleavable peptide or protein under conditions that result in expression of a fusion polypeptide encoded by the polynucleotides. In some embodiments, the host cell is transformed with an expression vector comprising the polynucleotide encoding the CNP variant polypeptide, optionally linked to the polynucleotide encoding the cleavable peptide or protein. In certain embodiments, the fusion polypeptide is expressed as a soluble protein or as an inclusion body. The expressed fusion polypeptide can be isolated from the host cell or culture medium, and the isolated fusion polypeptide can be contacted with a cleaving agent to release the CNP variant.

[0134] Methods of making CNP variant peptides, including use of host cells, expression vectors, cleavable peptides, and culture parameters, are disclosed in U.S. Patent 8,198,242, hereby incorporated by reference.

Methods of Use

[0135] Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias

[0136] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, chondrodysplasia congenit, homozygous achondroplasia, chondrodysplasia congenit, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia congenit, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia. Short stature, growth plate disorder, bone-related disorder or skeletal dysplasias contemplated herein include disorders related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), and PTPN11 mutations (Noonan's syndrome).

[0137] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia. Non-limiting examples of CNP-responsive bone-related disorders and skeletal dysplasias include achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, chondrodysplasia congenit, homozygous achondroplasia, chondrodysplasia congenit, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia congenit, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia. Short stature, growth plate disorder, bone-related disorder or skeletal dysplasias contemplated herein include disorders

related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome) and IGF1R mutation.

[0138] Additional short stature and growth plate disorders contemplated by the methods include disorders related to mutations in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3.

[0139] Additional short stature and growth plate disorders contemplated by the methods include disorders related to mutations in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or IGF1R.

[0140] Further, the CNP variants are useful as an adjunct or alternative to growth hormone for treating idiopathic short stature and other skeletal dysplasias.

[0141] Growth plate disorders include disorders that result in short stature or abnormal bone growth and that may be the result of a genetic mutation in a gene involved in bone growth, including collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, or FGFR3. In various embodiments, growth plate disorders include disorders that result in short stature or abnormal bone growth and that may be the result of a genetic mutation in a gene involved in bone growth, including collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or IGF1R. In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy. In various embodiments, a subject with a growth plate disorder is heterozygous for a mutation in a growth plate gene. In various embodiments, the mutation is a loss-of-function mutation. In various embodiments, the mutation is a gain-of-function mutation. Growth plate disorders include, but are not limited to, familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. See, e.g., Plachy et al., J Clin Endocrinol Metab 104: 4273-4281, 2019.

[0142] Mutations in ACAN can give rise to familial osteochondritis dissecans and short stature and eventually osteoarthritis, characterized by areas of bone damage (or lesions) caused by the detachment of cartilage and sometimes bone from the end of the bone at a joint. It has been suggested that the disorganized cartilage network in growing bones impairs their growth, leading to short stature. A mutation associated with ACAN and short stature includes Val2303Met. See Stattin et al., Am J Hum Genet 86(2):126-37, 2010. It is contemplated that patients with a mutation in ACAN resulting in short stature would benefit from treatment with

CNP as administration may be able to increase height in these patients by the known interaction of CNP with FGFR3.

[0143] The natriuretic peptide system, including receptor NPR2, has been shown to be involved in regulation of endochondral bone growth (Vasques et al., Horm Res Pediat 82:222-229, 2014). Studies have shown that homozygous or compound heterozygous loss-of-function mutations in NPR2 cause acromesomelic dysplasia type Maroteaux (AMDM), which is a skeletal dysplasia having extremely short stature (Vasquez et al., 2014, *supra*). There are reports implicating heterozygous loss-of-function (such as dominant negative) NPR2 mutations as a cause of short stature, whereas gain-of-function NPR2 heterozygous mutations have been found to be responsible for tall stature (Vasquez et al., 2014, *supra*). In view of CNP's interaction with NPR2 to stimulate cGMP generation, increasing cGMP levels is desirable in these conditions and would have therapeutic benefit in the management of the complications from these diseases and conditions.

[0144] Heterozygous mutations of NPR2 are believed to result in idiopathic short stature and other forms of short stature. Mutations in the NPR2 gene are set out below and described in Amano et al., J Clin Endocrinol Metab 99:E713-718, 2014, Hisado-Oliva et al., J Clin Endocrinol Metab 100:E1133-1142, 2015 and Vasques et al., J Clin Endocrinol Metab 98:E1636-1644, 2013, hereby incorporated by reference. It is contemplated that a subject having short stature to be treated with a CNP variant as described herein has a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and has at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. However, because de novo mutations in NPR2 can result in short stature as defined by a height SDS of less than -1.5, -2.0, -2.5, or -3.0, treatment of individuals who are heterozygous carriers of a deleterious mutation in NPR2 with neither parent having short stature is also contemplated. Further contemplated is treatment of individuals who are heterozygous for deleterious mutations in other growth plate genes with CNP to improve stature and/or enhance bone growth.

[0145] Exemplary NPR2 mutations in patients that may be treated with a CNP variant include:

Disease	Nucleotide	Mutation	Amino acid change
Short stature	1669C>T	missense	Arg557Cys

Short stature	2794C>T	missense	Arg932Cys
Short stature	2905G>C	missense	Val969Leu
Short stature	3058C>T	missense	Arg1020Trp
Short stature	2972A>G	missense	Glu991Gly
Short stature	1262C>T	missense	Thr421Met
Short stature	766G>T	missense	Asp256Tyr
Short stature	1982C>A	missense	Thr661Lys
Short stature	2449G>A	missense	Glu817Lys
Short stature	1517G>A	missense	Arg506His
Short stature	1802G>C	missense	Arg601Pro
Short stature	1481T>G	missense	Ile494Ser
Short stature	142G>T	missense	Ala48Ser
Short stature	1167G>T	missense	Glu389Asp
Short stature	1249C>G	missense	Gln417Glu
Short stature	328C>T	missense	Arg110Cys
Short stature	2455C>T	missense	Arg819Cys
Short stature	788G>C	missense	Arg263Pro
Short stature	226T>C	missense	Ser76Pro
Short stature	2710A>T	nonsense	Lys904Term

9:35809194:C:G	Leu1009Val
9:35802761:G:C	Leu615Phe
9:35799645:C:T	Pro301Ser
9:35792928:C:T	Arg174Cys
9:35801728:C:G	His508Asp
9:35792713:T:C	Val102Ala
9:35793980:T:A	Tyr250Ter
9:35807085:C:T	Thr861Ile
9:35793906:A:G	Ile226Val
9:35808558:G:A	Arg921Gln
9:35802741:G:A	Glu609Lys
9:35802594:G:A	Arg601His
9:35808663:T:A	Leu956Gln
9:35808545:G:C	Gly917Arg

[0146] NPPC's role in skeletal growth is well documented (Hisado-Oliva et al., Genetics Medicine 20:91-97, 2018). The NPPC knock out mouse showed severe disproportionate form of dwarfism including shortening of limbs and endochondral ossification (Hisado-Oliva et al., 2018, *supra*). Human genome wide studies have shown a link between NPPC and height

(Hisado-Oliva et al., 2018, *supra*). Although CNP haploinsufficiency has been believed to be a cause of short stature in humans, a recent study identified heterozygous mutations in families with short stature and hands (Hisado-Oliva et al., 2018, *supra*). These studies observed significant reduction in cGMP production as measured in heterozygous state (Hisado-Oliva et al., 2018, *supra*). Mutations in NPPC include a 355G>T missense mutation causing a Gly119Cys change and a 349C>G missense mutation causing a Arg117Gly change. A CNP variant rescuing CGMP production may provide therapeutic benefit in the management of a disorder in patients having heterozygous loss-of-function NPPC mutations.

[0147] Leri-Weill dyschondrosteosis (LWD) is a rare genetic disorder characterized by shortening of the forearms and lower legs, abnormal misalignment of the wrist (Madelung deformity of the wrist), and associated short stature. LWD is caused by a heterozygous mutation in the short stature homeobox-containing (SHOX) gene or its regulatory elements located on the pseudoautosomal region 1 (PAR1) of the sex chromosomes. (See the Rare Disease Database and Carmona et al., Hum Mol Genet 20:1547-1559, 2011). The disorder Langer mesomelic dysplasia arises when there are two SHOX mutations, and may result from a mutation on each chromosome, either a homozygous or compound heterozygous mutations. A subset of SHOX mutations give rise to idiopathic short stature. Turner syndrome results due to a deletion on the X chromosome that can include the SHOX gene. SHOX has been identified as involved in the regulation of *FGFR3* transcription and contributes to control of bone growth (Marchini et al., Endocr Rev. 37: 417–448, 2016). SHOX deficiency leads to increased FGFR3 signaling, and there is some evidence to support that SHOX has direct interactions with CNP/NPR2 as well (Marchini, *supra*). Given the association of SHOX with FGFR3 and bone growth, it is contemplated that a subject having a homozygous or heterozygous SHOX mutation would benefit from treatment with CNP variants as described herein.

[0148] RASopathies are a group of rare genetic conditions caused by mutations in genes of the Ras/mitogen-activated protein kinase (MAPK) pathway. RASopathies are a group of disorders characterized by increased signaling through RAS/MAPK pathway. This pathway leads to downstream activation of the RAF/MEK/ERK pathway. Short stature is a characteristic feature of certain RASopathies. For example, CNP signaling inhibits RAF and leads to decreased MEK and ERK activation.

[0149] Treatment of RASopathies are contemplated herein. RASopathies associated with short stature include Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, and LEOPARD syndrome. Hereditary gingival fibromatosis type 1 is

also a RASopathy contemplated herein. RASopathy patients (including Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, LEOPARD syndrome, hereditary gingival fibromatosis type 1) include patients with heterozygous variants in one or more of the following genes: BRAF, CBL, HRAS, KRAS, LZTR1, MAP2K1, MAP2K2, MRAS, NF1, NRAS, PPP1CB, PTPN11, RAF1, RRAS, RIT1, SHOC2, SOS1, or SOS2 (Tajan et al. Endocr. Rev. 2018;39(5):676–700).

[0150] CFC is caused by mutations in several genes in the Ras/MAPK signaling pathway, including K-Ras, B-Raf, Mek1 and Mek2. Costello syndrome, also called faciocutaneoskeletal (FCS) syndrome is caused by activating mutations in the H-Ras gene. Hereditary gingival fibromatosis type I (HGF) is caused by dominant mutations in the SOS1 gene (*Son of Sevenless* homolog 1), which encodes a guanine nucleotide exchange factor (SOS) that acts on the Ras subfamily of small GTPases. Neurofibromatosis type I (NF1) is caused by mutations in the neurofibromin 1 gene, which encodes a negative regulator of the Ras/MAPK signaling pathway. Noonan syndrome (NS) is caused by mutations in one of several genes, including PTPN11, which encodes SHP2, and SOS1, as well as K-Ras and Raf-1.

[0151] CNP has been demonstrated to be an effective therapy in RASopathy models. Ono et al. generated mice deficient in *Nf1* in type II collagen producing cells (Ono et al., Hum. Mol. Genet. 2013;22(15):3048–62). These mice demonstrated constitutive ERK1/2 activation, and decreased chondrocyte proliferation, and maturation. Daily injections of CNP in these mice led to decreased ERK phosphorylation and corrected the short stature. A mouse model of Cardiofaciocutaneous syndrome using a *Braf* mutation (p.Q241R) (Inoue et al. Hum. Mol. Genet. 2019;28(1):74–83). exhibited decreased body length and reduced growth plate width with smaller proliferative and hypertrophic zones compared to wild type, and CNP administration led to increases in body length in these animals.

[0152] Mutations in multiple genes can cause Noonan syndrome, which is characterized by short stature, heart defects, bleeding problems, and skeletal malformations. Mutations in the PTPN11 gene cause about half of all cases of Noonan's syndrome. SOS1 gene mutations cause an additional 10 to 15 percent, and RAF1 and RIT1 genes each account for about 5 percent of cases. Mutations in other genes each account for a small number of cases. The cause of Noonan syndrome in 15 to 20 percent of people with this disorder is unknown.

[0153] The PTPN11, SOS1, RAF1, and RIT1 genes all encode for proteins that are important in the RAS/MAPK cell signaling pathway, which is needed for cell division and growth (proliferation), differentiation, and cell migration. Many of the mutations in the genes associated

with Noonan syndrome cause the resulting protein to be turned on (active) and this prolonged activation alters normal RAS/MAPK signaling, which disrupts the regulation of cell growth and division, leading to the characteristic features of Noonan syndrome. See, e.g., Chen et al., Proc Natl Acad Sci U S A. 111(31):11473-8, 2014, Romano et al., Pediatrics. 126(4):746-59, 2010, and Milosavljević et al., Am J Med Genet 170(7):1874-80, 2016. It is contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve bone growth and short stature. It is also contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve other comorbidities associated with an overactive MAPK pathway in other cells throughout the body where the NPR2 receptor is expressed on its surface.

[0154] Mutations in the PTPN11 gene, which encodes the non-receptor protein tyrosine phosphatase SHP-2, lead to disorders characterized by short stature such as Noonan's Syndrome (Musente et al., Eur J Hum Genet 11:201–206 (2003). Musente (*supra*) identifies numerous mutations in the PTPN11 gene that lead to short stature. Gain of function mutations lead to overactive signaling through SHP2 and inhibit Growth Hormone-induced IGF-1 release, thereby contributing to a decrease in bone growth (Rocca Serra-Nédélec, PNAS 109:4257-4262, 2012). It is contemplated that a subject having a homozygous or heterozygous PTPN11 mutation would benefit from treatment with CNP variants as described herein to improve bone growth and short stature.

[0155] Mutations in the Indian hedgehog (IHH) gene, which is related to regulation of endochondral ossification, have also been associated with short stature syndromes (Vasques et al., J Clin Endocrinol Metab. 103:604-614, 2018). Many IHH mutations identified segregate with short stature in a dominant inheritance pattern. Given the association of IHH with bone growth and ossification, it is contemplated that subjects having a homozygous or heterozygous IHH mutation will benefit from treatment with a CNP variant as described herein.

[0156] Mutations in FGFR3, including N540K and K650N, lead to short stature and hypochondroplasia.

[0157] Insulin-like growth factor 1 receptor (IGF1R) is a heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein with an intrinsic kinase activity. IGF1R has been shown to have a role in prenatal and postnatal growth. Heterozygous mutations in IGF1R have been identified in Small for gestational age children (SGA) and individuals with familial short stature (Kawashima

et al., Endocrine J. 59:179-185, 2012). Mutations in IGF1R associated with short stature include R108Q/K115N, R59T, R709Q, G1050K, R481Q, V599E, and G1125A (Kawashima, *supra*).

[0158] Height is a highly heritable trait that can be influenced by the combined effect of hundreds or thousands genes (Wood et al, 2014, Nature Genetics, 46:1173-1189. Short stature in an individual can be the result of the combined effect of these genes, without a single gene being the primary contributor. It is contemplated that such individuals with short stature defined by a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, can be beneficially treated with a CNP variant given the ability of CNP to increase the length of normal animals, for example, enhance bone growth and length of bones.

[0159] In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and having at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. In various embodiments, the short stature is associated with one or more mutations in a gene associated with short stature, such as, collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or insulin growth factor 1 receptor (IGF1R), or combinations thereof.

[0160] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[0161] In various embodiments, the short stature is a result of mutations in multiple genes as determined by polygenic risk score (PRS). Polygenic risk scores (PRS) were calculated for height using the largest published GWAS meta-analysis for height that did not include any samples from the UK Biobank project as described in Example 4. The cohort was divided into five PRS quintiles (PRS 1 being the lowest height, PRS 5 the tallest height). In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in FGFR3 and a low PRS. In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in IGF1R and a low PRS. In various embodiments, the subject has a mutation in NPPC and a low PRS. In various embodiments, the subject has a mutation in SHOX and a low PRS. In various embodiments, the subject has one or more mutation in one or more of FGFR3, IGF1R, NPPC,

NPR2 and SHOX, and a low PRS. In various embodiments, the PRS is 1 or 2. In various embodiments, the PRS is 1. In various embodiments, the PRS is 2.

[0162] In addition, the CNP variants are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

[0163] The CNP variants of the disclosure can also be used to treat osteoarthritis. Osteoarthritis is a degenerative disease of the articular cartilage and occurs frequently in the elderly. Osteoarthritis involves destruction of the cartilage and proliferative change in the bone and cartilage resulting from degeneration of articular components, with the change resulting in a secondary arthritis (e.g., synovitis). The extracellular matrix proteins, which are the functional entity of the cartilage, are reduced, and the number of chondrocytes decreases in osteoarthritis (Arth. Rheum. 46(8): 1986-1996 (2002)). By promoting the matrix production, growth and differentiation of chondrocytes, the CNP compositions are useful for countering the undesired effects of FGF-2 and increasing matrix synthesis in subjects suffering from arthritis, including osteoarthritis, thereby treating arthritis, including osteoarthritis.

[0164] In certain embodiments, the CNP variants and compositions and formulations comprising the same of the present disclosure are useful for improving one or more of the symptom(s) or physiological consequences of a skeletal dysplasia, wherein the improvement may be increased absolute growth, increased growth velocity, increased qualitative computed tomography (QCT) bone mineral density, improvement in growth plate morphology, increased long bone growth, improvement in spinal morphology, improved elbow joint range of motion and/or decreased sleep apnea. In this regard, it is noted that the terms "improved", "improvement", "increase", "decrease" and grammatical equivalents thereof are all relative terms that when used in relation to a symptom or physiological consequence of a disease state, refer to the state of the symptom or physiological consequence of the disease after treatment with a CNP variant (or composition or formulation comprising the same) of the present invention as compared to the same symptom or physiological consequence of the disease before treatment with a CNP variant (or composition or formulation comprising the same) of the present invention (i.e., as compared to "baseline"). As described above, a "baseline" state can be determined either through measurement of the state in the subject prior to treatment (which can subsequently be compared to the state in the same subject after treatment), or through

measurement of that state in a population of subjects suffering from the same affliction that share the same or similar characteristics (e.g., age, sex and/or disease state or progression).

[0165] Also provided is a method of overcoming cell growth arrest induced by a constitutively active mutant fibroblast growth factor receptor 3 (FGFR-3) comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant or a composition as described herein.

[0166] Further provided is a method of stimulating cGMP production in a cell expressing natriuretic peptide receptor B (NPR-B) comprising contacting the cell expressing NPR-B with a CNP variant or a composition as described herein.

[0167] In yet another embodiment, the disclosure provides CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM). In a still further embodiment, the CNP variants of the disclosure *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 uM).

[0168] It is contemplated that any of the CNP variants described herein are useful in the methods.

[0169] In various embodiments, the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1). In various embodiments, the peptide further comprises an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the peptide further comprises an OH or an NH_2 group at the C-terminus. In various embodiments, the variant comprises one or more linker groups as described herein. In various embodiments, the linker is a hydrolysable linker.

[0170] Efficacy of treatment is measured by various parameters. In various embodiments, efficacy is assessed as the change in annualized growth velocity from the baseline period to the intervention period. Efficacy will also be assessed as the change in height SDS from baseline to end of treatment as measured using the CDC growth curves, and growth velocity SDS will be based on the Bone Mineral Density in Childhood Study (Kelly et al., *J. Clin. Endocrinol. Metab.* 2014;99(6):2104–2112).

[0171] QoLISSY, the Quality of Life in Short Stature Youth, is assessed as directed (*Quality of Life in Short Stature Youth - The QoLISSY Questionnaire User's Manual.* Lengerich: Pabst Science Publishers; 2013).

Pharmaceutical Compositions

[0172] The disclosure provides pharmaceutical compositions, including modified release compositions, comprising a CNP variant described herein, and one or more pharmaceutically acceptable excipients, carriers and/or diluents. In certain embodiments, the compositions further comprise one or more other biologically active agents (*e.g.*, inhibitors of proteases, receptor tyrosine kinases, and/or the clearance receptor NPR-C).

[0173] The disclosure provides for modified release compositions comprising a conjugate moiety as described herein. Modified-release compositions include those that deliver a drug with a delay after its administration (delayed-release dosage) or for a prolonged period of time (extended-release dosage). Various embodiments of a CNP peptide conjugate provided herein include modified-release compositions, such as extended release, sustained or controlled release, and delayed release. The term "extended release composition" refers to a composition formulated in a manner in order to make the active ingredient/drug available over an extended period of time following administration (US Pharmacopeia). Extended-release dosage include sustained-release (SR) or controlled-release (CR) forms in which. Sustained release maintains drug release over a sustained period but not necessarily at a constant rate, while CR maintains drug release over a sustained period at a nearly constant rate (Pharmaceutics: Drug Delivery and Targeting, Yvonne Perrie, Thomas Rades, Pharmaceutical Press, 2009). Delayed-release compositions or products are modified to delay release of the drug substance for some period of time after initial administration.

[0174] In various embodiments, the modified release composition is an extended release composition.

[0175] In various embodiments, for the extended release composition, (i) less than about 20% of peptide is released by day 1; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6.

[0176] In various embodiments, less than about 20% of peptide is released by day 1 at pH 7 to 7.6. In various embodiments, less than about 10% of peptide is released by day 1 at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50% of

peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 30%, or about 40%, or about 50%, or about 60% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released weekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released biweekly, at pH 7 to 7.6. In various embodiments, about 90% of peptide is released monthly at pH 7 to 7.6. It is further contemplated that the release can be at a pH between pH 7.0 to 7.6, between pH 7.1 to 7.5, between pH 7.2 to 7.4, between pH 7.2 to 7.6, or between pH 7.0 to 7.4.

[0177] In various embodiments, (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 90% of peptide is released weekly, or about 90% of peptide is released bi-weekly, or about 90% of peptide is released monthly, at pH 7 to 7.6. It is further contemplated that (i) less than about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60% about 65%, about 70%, or about 75% of peptide is released by day 1, at pH 7.0 to 7.6; and (ii) about 70%, about 80%, or about 90% of peptide is released weekly; or about 70%, about 80%, or about 90% of peptide is released bi-weekly; or about 70%, about 80%, or about 90% of peptide is released every three weeks; or about 70%, about 80%, or about 90% of peptide is released monthly, at pH 7 to 7.6; or alternatively ii) about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released bi-weekly; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released every three weeks; or about 70%, about 75%, about 80%, about 85%, or about 90% of peptide is released monthly, at pH 7 to 7.6

[0178] In various embodiments, the composition comprises an excipient, diluent or carrier. In various embodiments, the extended release composition comprises an excipient, diluent or carrier. In various embodiments, the excipient, diluent or carrier is a pharmaceutically acceptable excipient, diluent or carrier.

[0179] Non-limiting examples of excipients, carriers and diluents include vehicles, liquids, buffers, isotonicity agents, additives, stabilizers, preservatives, solubilizers, surfactants,

emulsifiers, wetting agents, adjuvants, and so on. The compositions can contain liquids (*e.g.*, water, ethanol); diluents of various buffer content (*e.g.*, Tris-HCl, phosphate, acetate buffers, citrate buffers), pH and ionic strength; detergents and solubilizing agents (*e.g.*, Polysorbate 20, Polysorbate 80); anti-oxidants (*e.g.*, methionine, ascorbic acid, sodium metabisulfite); preservatives (*e.g.*, Thimerosol, benzyl alcohol, m-cresol); and bulking substances (*e.g.*, lactose, mannitol, sucrose). The use of excipients, diluents and carriers in the formulation of pharmaceutical compositions is known in the art; see, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)), which is incorporated herein by reference in its entirety.

[0180] For example, carriers include without limitation diluents, vehicles and adjuvants, as well as implant carriers, and inert, non-toxic solid or liquid fillers and encapsulating materials that do not react with the active ingredient(s). Non-limiting examples of carriers include phosphate buffered saline, physiological saline, water, and emulsions (*e.g.*, oil/water emulsions). A carrier can be a solvent or dispersing medium containing, *e.g.*, ethanol, a polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, and the like), a vegetable oil, and mixtures thereof.

[0181] In some embodiments, the compositions are liquid formulations. In certain embodiments, the formulations comprise a CNP variant in a concentration range from about 0.1 mg/ml to about 20 mg/ml, or from about 0.5 mg/ml to about 20 mg/ml, or from about 1 mg/ml to about 20 mg/ml, or from about 0.1 mg/ml to about 10 mg/ml, or from about 0.5 mg/ml to about 10 mg/ml, or from about 0.5 to 5 mg/ml, or from about 0.5 to 3 mg/ml, or from about 1 mg/ml to about 10 mg/ml. In various embodiments, the CNP variant is in a concentration of 0.8 mg/mL to 2 mg/mL. In various embodiments, the CNP variant is at a concentration of 0.8 mg/mL. In various embodiments, the CNP variant is at a concentration of 2.0 mg/mL. In various embodiments, the CNP variant is reconstituted from a lyophilized powder.

[0182] In further embodiments, the compositions comprise a buffer solution or buffering agent to maintain the pH of a CNP-containing solution or suspension within a desired range. Non-limiting examples of buffer solutions include phosphate buffered saline, Tris buffered saline, and Hank's buffered saline. Buffering agents include without limitation sodium acetate, sodium phosphate, and sodium citrate. Mixtures of buffering agents can also be used. In certain embodiments, the buffering agent is acetic acid/acetate or citric acid/citrate. The amount of buffering agent suitable in a composition depends in part on the particular buffer used and the desired pH of the solution or suspension. In some embodiments, the buffering agent has a

concentration of about 10 mM ± 5 mM. In certain embodiments, the pH of a composition is from about pH 3 to about pH 9, or from about pH 3 to about pH 7.5, or from about pH 3.5 to about pH 7, or from about pH 3.5 to about pH 6.5, or from about pH 4 to about pH 6, or from about pH 4 to about pH 5, or is at about pH 5.0 ± 1.0. In various embodiments, the pH is about 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9 or 6.0. In various embodiments, the pH is 5.5.

[0183] In other embodiments, the compositions contain an isotonicity-adjusting agent to render the solution or suspension isotonic and more compatible for administration. Non-limiting examples of isotonicity agents include NaCl, dextrose, glucose, glycerin, sorbitol, xylitol, and ethanol. In certain embodiments, the isotonicity agent is NaCl. In certain embodiments, NaCl is in a concentration of about 160 ± 20 mM , or about 140 mM ± 20 mM, or about 120 ± 20 mM , or about 100 mM ± 20 mM, or about 80 mM ± 20 mM, or about 60 mM ± 20 mM.

[0184] In yet other embodiments, the compositions comprise a preservative. Preservatives include, but are not limited to, m-cresol and benzyl alcohol. In certain embodiments, the preservative is in a concentration of about 0.4% ± 0.2%, or about 1% ± 0.5%, or about 1.5% ± 0.5%, or about 2.0% ± 0.5%.

[0185] In still other embodiments, the compositions contain an anti-adsorbent (e.g., to mitigate adsorption of a CNP variant to glass or plastic). Anti-adsorbents include without limitation benzyl alcohol, Polysorbate 20, and Polysorbate 80. In certain embodiments, the anti-adsorbent is in a concentration from about 0.001% to about 0.5%, or from about 0.01% to about 0.5%, or from about 0.1% to about 1%, or from about 0.5% to about 1%, or from about 0.5% to about 1.5%, or from about 0.5% to about 2%, or from about 1% to about 2%.

[0186] In additional embodiments, the compositions comprise a stabilizer. Non-limiting examples of stabilizers include glycerin, glycerol, thioglycerol, methionine, and ascorbic acid and salts thereof. In some embodiments, when the stabilizer is thioglycerol or ascorbic acid or a salt thereof, the stabilizer is in a concentration from about 0.1% to about 1%. In other embodiments, when the stabilizer is methionine, the stabilizer is in a concentration from about 0.01% to about 0.5%, or from about 0.01% to about 0.2%. In still other embodiments, when the stabilizer is glycerin, the stabilizer is in a concentration from about 5% to about 100% (neat).

[0187] In further embodiments, the compositions contain an antioxidant. Exemplary anti-oxidants include without limitation methionine and ascorbic acid. In certain embodiments, the molar ratio of antioxidant to CNP is from about 0.1:1 to about 15:1, or from about 1:1 to about 15:1, or from about 0.5:1 to about 10:1, or from about 1:1 to about 10:1 or from about 3:1 to about 10:1.

[0188] Pharmaceutically acceptable salts can be used in the compositions, including without limitation mineral acid salts (*e.g.*, hydrochloride, hydrobromide, phosphate, sulfate), salts of organic acids (*e.g.*, acetate, propionate, malonate, benzoate, mesylate, tosylate), and salts of amines (*e.g.*, isopropylamine, trimethylamine, dicyclohexylamine, diethanolamine). A thorough discussion of pharmaceutically acceptable salts is found in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, (Easton, Pennsylvania (1990)).

[0189] The pharmaceutical compositions can be administered in various forms, such as tablets, capsules, granules, powders, solutions, suspensions, emulsions, ointments, and transdermal patches. The dosage forms of the compositions can be tailored to the desired mode of administration of the compositions. For oral administration, the compositions can take the form of, *e.g.*, a tablet or capsule (including softgel capsule), or can be, *e.g.*, an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules for oral administration can include one or more commonly used excipients, diluents and carriers, such as mannitol, lactose, glucose, sucrose, starch, corn starch, sodium saccharin, talc, cellulose, magnesium carbonate, and lubricating agents (*e.g.*, magnesium stearate, sodium stearyl fumarate). If desired, flavoring, coloring and/or sweetening agents can be added to the solid and liquid formulations. Other optional ingredients for oral formulations include without limitation preservatives, suspending agents, and thickening agents. Oral formulations can also have an enteric coating to protect the CNP variant from the acidic environment of the stomach. Methods of preparing solid and liquid dosage forms are known, or will be apparent, to those skilled in this art (see, *e.g.*, Remington's Pharmaceutical Sciences, referenced above).

[0190] Formulations for parenteral administration can be prepared, *e.g.*, as liquid solutions or suspensions, as solid forms suitable for solubilization or suspension in a liquid medium prior to injection, or as emulsions. For example, sterile injectable solutions and suspensions can be formulated according to techniques known in the art using suitable diluents, carriers, solvents (*e.g.*, buffered aqueous solution, Ringer's solution, isotonic sodium chloride solution), dispersing agents, wetting agents, emulsifying agents, suspending agents, and the like. In addition, sterile fixed oils, fatty esters, polyols and/or other inactive ingredients can be used. As further examples, formulations for parenteral administration include aqueous sterile injectable solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can contain suspending agents and thickening agents.

[0191] Compositions comprising a CNP variant can also be lyophilized formulations. In certain embodiments, the lyophilized formulations comprise a buffer and bulking agent, and optionally an antioxidant. Exemplary buffers include without limitation acetate buffers and citrate buffers. Exemplary bulking agents include without limitation mannitol, sucrose, dexran, lactose, trehalose, and povidone (PVP K24). In certain embodiments, mannitol is in an amount from about 3% to about 10%, or from about 4% to about 8%, or from about 4% to about 6%. In certain embodiments, sucrose is in an amount from about 6% to about 20%, or from about 6% to about 15%, or from about 8% to about 12%. Exemplary anti-oxidants include, but are not limited to, methionine and ascorbic acid.

[0192] In various embodiments, the formulation comprises citric acid, sodium citrate, trehalose, mannitol, methionine, polysorbate 80, and optionally sterile water for injection (WFI).

[0193] The disclosure also provides kits containing, *e.g.*, bottles, vials, ampoules, tubes, cartridges and/or syringes that comprise a liquid (e.g., sterile injectable) formulation or a solid (e.g., lyophilized) formulation. The kits can also contain pharmaceutically acceptable vehicles or carriers (e.g., solvents, solutions and/or buffers) for reconstituting a solid (e.g., lyophilized) formulation into a solution or suspension for administration (e.g., by injection), including without limitation reconstituting a lyophilized formulation in a syringe for injection or for diluting concentrate to a lower concentration. Furthermore, extemporaneous injection solutions and suspensions can be prepared from, *e.g.*, sterile powder, granules, or tablets comprising a CNP-containing composition. The kits can also include dispensing devices, such as aerosol or injection dispensing devices, pen injectors, autoinjectors, needleless injectors, syringes, and/or needles.

[0194] As a non-limiting example, a kit can include syringes having a single chamber or dual chambers. For single-chamber syringes, the single chamber can contain a liquid CNP formulation ready for injection, or a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) that can be reconstituted into a solution or suspension for injection. For dual-chamber syringes, one chamber can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection.

[0195] As a further example, a kit can include one or more pen injector or autoinjector devices, and dual-chamber cartridges. One chamber of a cartridge can contain a pharmaceutically acceptable vehicle or carrier (e.g., solvent system, solution or buffer), and the other chamber can contain a solid (e.g., lyophilized) CNP formulation or a liquid formulation of a CNP variant in a relatively small amount of a suitable solvent system (e.g., glycerin) which can be reconstituted into a solution or suspension, using the vehicle or carrier from the first chamber, for injection. A cartridge can comprise an amount of the CNP variant that is sufficient for dosing over a desired time period (e.g., 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, etc.). The pen injector or autoinjector can be adjusted to administer a desired amount of the CNP formulation from a cartridge.

Administration and Dosing

[0196] The CNP variants, or pharmaceutical compositions or formulations comprising them, can be administered to subjects in various ways such as, e.g., subcutaneously, intraarticularly, intraperitoneally, intramuscularly, intradermally or orally. In one embodiment, the CNP variant composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

[0197] The CNP variants or compositions thereof can also be administered by implantation of a depot at the target site of action (e.g., an abnormal or degenerated joint or cartilage area). Alternatively, the CNP variant can be administered sublingually under the tongue (e.g., sublingual tablet) by transdermal delivery (e.g., by means of a patch on the skin) or orally in the form of microspheres, microcapsules, liposomes (uncharged or charged (e.g., cationic)), polymeric microparticles (e.g., polyamides, polylactide, polyglycolide, poly(lactide-glycolide)), microemulsions, and the like.

[0198] The CNP variant compositions described herein can be administered to patients in need thereof at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia). The safety and therapeutic efficacy of the CNP variant can be determined by standard pharmacological procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Active agents exhibiting a large therapeutic index are normally preferred.

[0199] In certain embodiments, the CNP variant compositions described herein are administered at a dose in the range from about 3, 4, 5, 6, 7, 8, 9 or 10 nmol/kg to about 300 nmol/kg, or from about 20 nmol/kg to about 200 nmol/kg. In some embodiments, the CNP compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 125, 130, 140, 150, 160, 170, 175, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 750, 1000, 1250, 1500, 1750 or 2000 nmol/kg or other dose deemed appropriate by the treating physician. In other embodiments, the CNP variant compositions are administered at a dose of about 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 µg/kg, or about 0.5, 0.8, 1.0, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg/kg, or other dose deemed appropriate by the treating physician. The doses of CNP or CNP variant described herein can be administered according to the dosing frequency/frequency of administration described herein, including without limitation daily, 2 or 3 times per week, weekly, every 2 weeks, every 3 weeks, monthly, etc. In various embodiments, the CNP or CNP variant is administered daily subcutaneously. In various embodiments, the CNP or CNP variant is administered weekly subcutaneously. In various embodiments, the CNP variant is administered at a dose of 2.5 µg/kg/day to 60 µg/kg/day, 10µg/kg/day to 45 µg/kg/day, or 15µg/kg/day to 30 µg/kg/day. In various embodiments, the CNP variant is administered at a dose of 15 µg/kg/day. In various embodiments, the CNP variant is administered at a dose of 30 µg/kg/day.

[0200] The frequency of dosing/administration of a CNP variant for a particular subject may vary depending upon various factors, including the disorder being treated and the condition and response of the subject to the therapy. The CNP variant can be administered in a single dose or in multiple doses per dosing. In certain embodiments, the CNP variant composition is administered, in a single dose or in multiple doses, once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months, or as deemed appropriate by the treating physician. In various embodiments, the CNP variant is administered for 3 month, 6 months, 12 months or more.

[0201] In some embodiments, a CNP variant composition is administered so as to allow for periods of growth (e.g., chondrogenesis), followed by a recovery period (e.g., osteogenesis). For example, the CNP composition may be administered subcutaneously or by another mode daily or multiple times per week for a period of time, followed by a period of no treatment, then

the cycle is repeated. In some embodiments, the initial period of treatment (e.g., administration of the CNP variant composition daily or multiple times per week) is for 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks or 12 weeks. In a related embodiment, the period of no treatment lasts for 3 days, 1 week, 2 weeks, 3 weeks or 4 weeks. In certain embodiments, the dosing regimen of the CNP variant compositions is daily for 3 days followed by 3 days off; or daily or multiple times per week for 1 week followed by 3 days or 1 week off; or daily or multiple times per week for 2 weeks followed by 1 or 2 weeks off; or daily or multiple times per week for 3 weeks followed by 1, 2 or 3 weeks off; or daily or multiple times per week for 4, 5, 6, 7, 8, 9, 10, 11 or 12 weeks followed by 1, 2, 3 or 4 weeks off.

Biomarkers

[0202] For treatment of bone-related disorders, indicators of growth can be measured, such as long bone growth measurements *in utero* and neonatal and measurements of bone growth biomarkers such as CNP, cGMP, Collagen II, Collagen X, osteocalcin, and Proliferating Cell Nuclear Antigen (PCNA).

[0203] One CNP signaling marker is cGMP (guanosine 3',5' cyclic monophosphate). The level of this intracellular signaling molecule increases after CNP binds to and activates its cognate receptor NPR-B. Elevated levels of cGMP can be measured from cell culture extracts (*in vitro*) after CNP exposure, conditioned media from bone ex-plant studies (ex *vivo*) after CNP exposure, and in the plasma (*in vivo*) within minutes of CNP administration subcutaneously, intravenously, or via other routes of administration known in the art.

[0204] Cartilage and bone-specific analytes (or cartilage- and bone-associated markers) can also be measured to assess CNP efficacy. For example, fragments of cleaved collagen type II are a cartilage-specific marker for cartilage turnover. Type II collagen is the major organic constituent of cartilage and fragments of type II collagen (cleaved collagen) are released into circulation, and subsequently secreted into the urine, following cartilage turnover. Cartilage turnover precedes new bone formation.

[0205] A bone-specific biomarker for bone formation which can be measured is N-terminal propeptides of type I procollagen (PINP). The synthesis of type I collagen is an important step in bone formation, as type I collagen is the major organic component in bone matrix. During collagen synthesis, propeptides are released from the procollagen molecule and can be detected in serum. In addition, fragments of collagen type I can be measured as a marker for bone resorption.

[0206] Other potential biomarkers for cartilage and bone formation and growth include aggrecan chondroitin sulfate (cartilage-specific marker for cartilage turnover), propeptides of type II collagen (cartilage-specific marker for cartilage formation), collagen type I C-telopeptide (CTx), alkaline phosphatase (bone-specific) and osteocalcin (bone-specific marker for bone formation). Cartilage- and bone-associated biomarkers can be measured, e.g., in serum from efficacy/pharmacodynamic *in vivo* studies and from the conditioned media of *ex vivo* studies, using commercially available kits.

[0207] In one embodiment, the level of at least one bone- or cartilage-associated biomarker is assayed or measured in a subject that has been administered a CNP variant or composition described herein in order to monitor the effects of the CNP composition on bone and cartilage formation and growth *in vivo*. For example, an increase in the level of at least one bone- or cartilage-associated biomarker may indicate that administration of a CNP variant or composition has a positive effect on bone growth and is a useful treatment for skeletal dysplasias and other bone- or cartilage-related diseases or disorders associated with decreased CNP activity. Exemplary bone- or cartilage-associated biomarkers include, but are not limited to, CNP (e.g., endogenous levels of CNP), cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, collagen type I C-telopeptide (CTx), osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, collagen X, aggrecan chondroitin sulfate, and alkaline phosphatase.

[0208] In various embodiments, biomarkers are measured by obtaining a biological sample from a subject who will be administered, is being administered or has been administered a CNP variant. Biomarkers can be measured using techniques known in the art, including, but not limited to, Western Blot, enzyme linked immunosorbant assay (ELISA), and enzymatic activity assay. The biological sample can be blood, serum, urine, or other biological fluids.

[0209] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

Example 1: Synthesis of CNP Variants

[0210] CNP variant peptides were synthesized on solid-phase using a resin that would leave a C-terminal COOH on a Symphony/Prelude (Protein Technologies Inc., USA), Voyager (CEM GmbH, Germany), or SyroII (MultiSyntech, Germany) synthesizer.

[0211] All Fmoc-amino acids were purchased from Biosolve (Netherlands) or Bachem GmbH (Germany) with side-chain functional groups protected with N-*t*-Boc (KW), O-*t*-Bu (DESTY), N-Trt (HNQ), S-Trt (C), or N-Pbf (R) groups. A 5-fold excess of HBTU/HOBt/amino acid/DIPEA (1:1:1:2) in NMP with a 20 minute activation time using double couplings was employed for every amino acid coupling step.

[0212] Acetylation (Ac) of the peptide was performed by reacting the resin with NMP/Ac_2O/DIEA (10:1:0.1, v/v/v) for 30 min at room temperature.

[0213] For conjugation of a moiety, the protective amino group on lysine was cleaved to create a reactive group. Standard Fmoc synthesis was used to react 2x Fmoc-amino PEG(2) followed by glutamic acid, followed by C18-diacid.

[0214] The completed peptide was cleaved from the resin by reaction with a TFA (40 mL/mmol resin) for 2 hours at room temperature. Crude peptide was filtered, precipitated with ice-cold Et_2O followed by lyophilization and ultimately purification by preparative reverse phase-high-performance liquid chromatography (RP-HPLC). Final products and purity were confirmed by mass spectrometry.

[0215] CNP variants were generated based on the sequence of Pro-Gly CNP-37 (PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(SEQ ID NO: 1)), optionally having changes in different amino acid residues, and/or acetylation at the N-terminus, and/or other modifications, and include (amino acid changes underlined):

CNP-8: Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8),

CNP-5: Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH2(SEQ ID NO: 9),

CNP-6: Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10),

CNP-7: Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH2(SEQ ID NO: 11),
CNP-9: Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH2(SEQ ID NO: 12),

Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 8), and

PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 7).

[0216] Fully reduced peptides were dissolved in 0.1 M Tris-buffer (pH 8.0) with or without guanidine HCl, containing 1 mM cysteine (SS-form) and 8 mM cysteine (SH-form) in a final concentration of 0.1 mg/mL and stirred at room temperature. Disuflide formation was monitored by HPLC-analysis until no further peak changes were observed. Mixtures were loaded on preparative RP-HPLC for purification.

Example 2: Characterization of Synthesized CNP Variants

[0217] CNP variants synthesized as in Example 1 were analyzed by mass spectrometry and UV spectroscopy to determine purity and stability after 10 days.

[0218] Stability was measured using RP-HPLC analysis of purity over time T0 to T10. Briefly, the CNP variants were diluted 1:5 in buffer: 5mM citrate, 6% sucrose, 1.5% mannitol, 0.7mg/ml methionine and 0.005% tween80, pH 5.6 An injection volume of 10 µL was injected onto a Phenomenex Aeris XB-C18 RP column (2 µm, 100A, 2.1 x 250 mm; p/n 00G-4505-AN) HPLC conditions were: Mobile Phase A: H_2O / 0.05% TFA, pH 3 w/NH_4OH (~3mM final), Mobile Phase B: 70% CH_3CN in H_2O / 0.05% TFA, pH 3 w/NH_4OH (~3mM final) and a flow rate of 0.25mL/min. Measurement was carried out with a column temperature of 55° C and UV: 214nm (bw 4nm); ref 360nm (bw 20nm), UV: 280nm (bw 4nm); ref 360nm (bw 20nm). CNP variants were held 10 days in PBS, pH 7.4, at 37° C and stability measurements obtained. Results of stability measurements are shown in Table 1 below as % variant detected at T0 (0 days) or T10 (10 days).

Table 1

Peptide	T0, %	T10, %	EC50, T0, nm
Pro-Gly CNP37	76.7	39.5	1.7
CNP-5	83	47	3
CNP-6	90	58	0.4
CNP-7	77	53	2.1
CNP-8	91	88	0.2
CNP-9	92	77	0.9

[0219] CNP variants were tested for activity by a cGMP stimulation assay using a CatchPoint Cyclic-GMP Fluorescent Assay Bulk kit (Molecular Devices, R8075). Briefly, NIH3T3 cells (ATCC, CRL-1658) and HEK293 cells were seeded at 60,000 cells/well in a 96-well plate (96-well black imaging plates, Grenier, #655090). Culture media was as follows: NIH3T3 culture media: DMEM high glucose, pyruvate (Thermo, 11995-073) + 10% FBS + 1x Pen Strep (abbrev P/S, Thermo, cat# 15140122). NIH3T3 was the control system for the cGMP assay HEK293 culture media: EMEM + 10% FBS + 1x P/S + 1x GMAX. Serum free NIH3T3 media: DMEM + 1x P/S for treatment of cells with IBMX (CAS 28822-58-4); Serum free NIH3T3 media with BSA: DMEM + 1x P/S + 0.5 mg/mL BSA (Thermo, A9418-100G) for treatment of cells with CNP.

[0220] Cells were incubated for 24 hours at 37° C, 5% CO_2. For cells to be treated with CNP variants, plates were pre-treated with IBMX (Enzo life sciences, 89161-340, 1g) 15 minutes prior to use. IBMX is a potent, non-specific inhibitor of phosphodiesterases. An 800 mM stock solution of IBMX is diluted in IBMX dilution media (serum-free media (DMEM + 1x PBS mixed 1:1 with 1x PBS) to a 0.75 mM working stock.

[0221] CNP variants were prepared as follows: Dilute 10 mg/ml CNP solution 1:1000 in CNP dilution media (DMEM + 1x P/S + 0.5 mg/mL BSA). This solution is further diluted to obtain a CNP starting solution plated at 100 nM CNP/well. This 100 nM CNP solution is further serially diluted 1:5 for six dilutions to obtain a lower end concentration of 0.0064 nM CNP /well. This provides a 7-point dose curve for analysis.

[0222] For cell treatment, cells were removed from the incubator, growth media was removed from cells and cells treated with IBMX. 80 µL 0.75 mM IBMX was added to each well and cells returned to 37° C incubator for 15 minutes. After 15 minutes CNP (40 µL/well) was added to each test well and cells returned to 37° C incubator for 15 minutes. The plate was mixed by gentle tapping. The plate was imaged on a Solentim cell metric to visualize cells and determine if there is any cell lifting and then placed back into 37° C incubator.

[0223] The reaction was stopped and cells lysed by adding 40 µL lysis buffer (from cGMP kit). Plate was placed on a shaker for 5 minutes to complete lysis. The cell lysate was used in the cGMP assay.

[0224] The cGMP assay was carried out using a cGMP calibrator, rabbit anti-cGMP antibody and HRP-cGMP prepared as according to manufacturer's protocol. 40 µL of calibrator was added to wells of an anti-cGMP antibody coated plate, and 40 µL of lysate to be analyzed added to the appropriate wells. 40 µL of reconstituted rabbit anti-cGMP antibody was added to all wells and plates placed on shaker five minutes for mixing. 40 µL of reconstituted HRP-cGMP

was added to each well and incubated for 2 hours at room temperature. Plates were manually aspirated and washed 4x with 300 µL wash buffer. 100 µL of stoplight red substrate was added to each well, the plate covered and left at room temperature for at least 10 mins, protected from light. The plate was read for fluorescence intensity on a Spectramax M, or similar instrument, at excitation 530 nm and emission at 590 nm.

[0225] Table 1 shows that the CNP variants stimulate cGMP production, suggesting that the stable variants described herein are useful as therapeutics to treat bone-related disorders.

[0226] Example 2A: Stability of CNP Variants or Conjugates in Plasma

[0227] Stability of different CNP variants or CNP conjugates were tested for stability in human plasma over a 24 hour period. Briefly, a C18 fatty acid was fused to gamma glutamic acid using 2 OEG spacers (see e.g., Lau et al., J. Med. Chem 58:7370-7380, 2015). For the chromatography, Waters UPLC H-Class connected with BEH C18 1.7 um, 2.1 x 150 mm; Mobile Phase A: 1% DMSO 0.1% Formic Acid in Water; Mobile Phase B: 1% DMSO 0.1% Formic Acid in CAN. Mass Spectrometry was carried out using an AB Sciex QTRAP.

[0228] each CNP variant was prepared at 200nM in Human Plasma Li Heparin and smaples Incubated at 37° C 5% CO_2. The reaction was quenched at 0, 1, 2, 4, 8, 24 hours with 0.5M Sodium Citrate pH 4. Plasma proteins were precipitated with 0.2% Formic Acid in MeOH and samples prepared using WCX 96-well uElution plate and analyzed by LC-MS/MS 6500.

[0229] Variants CNP-R refers to CNP37 variants in which the K residues not in the ring portion are changed to R residues, and CNP Q/R refers to CNP37 variants in which N residues are changed to Q and K residues not in the ring portion are changed to R.

[0230] Figure 1 illustrates CNP conjugates having different linker structures. Figure 2 shows that lipidated conjugate has improved stability in plasma compared to PEGylated or non-conjugated peptides in plasma.

[0231] Stability of different conjugates was also analyzed at varying conditions. Briefly, the same protocol as above was used at 37° C (control), 37° C+ 0.5M NaCl, 37°C + protease inhibitors or 4° C. Figure 3 shows that PG-CNP37 and variants are prone to proteolysis in human plasma and PG-CNP37 shows greater stability compared to other variants.

Example 3: Heterozygous NPR2 Mutations Are Responsive to CNP Treatment

[0232] To determine the effects of CNP on subjects with short stature resulting from NPR2 mutations, cellular models of NPR2 mutations were developed. Exemplary NPR2 mutations analyzed are set out in Figure 6. Rat chondrosarcoma (RCS) cells having either a knockout of, or heterozygous loss of function mutations in, the NPR2 gene were made by RNP transfection into RCS cells using 125ng NPR2 variants or wild-type NPR2 plasmid DNA transfected into RCS or HEK293 cells. Single cell clones were seeded and genotyped by Sanger sequencing. Cell models are able to reproduce published cGMP phenotypes of the different mutations.

[0233] The NPR2 clones were created by creating insertions and deletions in the first exon of NPR2 in RCS cells. The sequence of the first exon in NPR2 was confirmed by next-generation sequencing and is set out in Figure 5. NPR2 mutant cells were tested for activity in response to CNP administration by a cGMP stimulation assay using a CatchPoint Cyclic-GMP Fluorescent Assay after treatment with 6nM of Pro-Gly CNP37. RCS (rat chondrosarcoma) cells were seeded at 40,000 cells/well in RCS culture media: DMEM + 10% FBS + 1x Pen Strep. Figure 4 shows that adding exogenous Pro-Gly-CNP37 variant rescues cGMP readout in a NPR2 +/- rat chondrosarcoma cell model.

[0234] Previous activation data reports cGMP EC50 in the range of 40 to 360nM for activation of PRKG2 (Campbell et al., ACS Chem Biol 12, 2388-2398, 2017); Vaandrager et al., J Biol Chem 272, 11816-23, 1997); Pohler et al., FEBS Lett 374, 419-25, 1995). In the heterozygous NPR2 knockout cells, a CNP dose >0.163nM is able to achieve an intracellular concentration exceeding the EC50 range for PRKG2 activation cGMP (Figure 4). Whereas, in wild-type cells a CNP dose of 0.040nM is able to achieve the same cGMP concentration. These results demonstrate that CNP supplementation can achieve the cGMP levels necessary for PRKG2 activation and growth in cells with loss-of-function mutations of NPR2.

[0235] These results also suggest that administration of CNP variants is useful to restore bone growth in subjects with short stature that have reduced activity of NPR2. It is further contemplated that treatment with CNP variants will be beneficial in subjects having mutations in other growth plate genes in which cGMP signaling may be impaired.

Example 4: Identification of Mutations Associated With Short Stature

[0236] It is hypothesized that genes showing clear evidence of genetically-driven bi-directional effects are more likely to represent therapeutic targets that can be effectively modulated in a broad patient population. To identify genes that are core regulators of growth,

the intersections of five gene lists were analyzed including, the list of genes from genome-wide association study (GWAS). Core growth regulators would be the most likely to contain rare coding mutations with bidirectional effects (i.e. short stature or skeletal dysplasia AND tall stature or overgrowth).

[0237] Databases queried include: *GWAS* 2,067 non-repeating closest genes for each of the 3,290 independent genetic variants reported by a large GWAS meta-analysis of height using ~700,000 individuals were extracted; *HGMD* The "allmut" table from HGMD version v2019_2 was queried looking for all pathogenic variants labelled as "DM" having either "short stature" and "tall stature or overgrowth" in the same genes; *OMIM* The list of OMIM genes related to growth disorders was previously described and was created using the keywords: short stature, overgrowth, skeletal dysplasia, brachydactyly.

[0238] First, the Human Gene Mutation Database (HGMD version v2019_2) was queried for genes associated with short or tall stature (Stenson et al., Hum Genet 136:665-677, 2017). There were 47 genes annotated with at least one pathogenic variant reported in the literature to cause "short stature". Only 20 genes were annotated as tall stature or overgrowth genes. Secondly, a manually curated list of 258 OMIM genes was used (248 short, 20 tall) which was created using the keywords: short stature, overgrowth, skeletal dysplasia, brachydactyly (Wood et al., Nat Genet 46:1173-86, 2014). Third, the intersection of these lists was compared with the list of genes from GWAS. At the intersection of these list there were three genes known to be associated with height (IGF1R, NPPC, NPR2) and two additional genes were identified (FGFR3, SHOX).

[0239] Additional analysis led to generation of a new group of five Core genes that showed significantly decreased height (β=-0.20, 95%CI [-0.26 to -0.14], p=4.04x10-11) and significantly increased risk for Idiopathic Short Stature (ISS) (OR=2.75, 95%CI [1.92 - 3.96]. Each of the core five genes (FGFR3, IGF1R, NPPC, NPR2 and SHOX) were associated with height when considered individually, and also were associated with short stature when taken in combination with other mutations. Exemplary mutations in FGFR3, IGF1R, NPPC, NPR2 and SHOX are set out in Figure 7.

[0240] Combined LoF (loss of function) and Missense variants in NPR2 and IGF1R were also associated with increased risk for ISS (OR=3.31, P= 0.001, OR= 2.85, P=0.002, respectively). Entire gene deletions and/or mutations causing loss of protein function in SHOX, IGF1R, NPPC, NPR2 have been reported in familial short stature with various degrees of severity.

[0241] Analysis shows that carriers of variants in any of the five core genes are at approximately a 3-fold increased risk for ISS and account for 6.7% of the total ISS population. Furthermore, a dose-dependent rescue of NPR2 signaling in a cell model of NPR2 haploinsufficiency after adding exogenous CNP was demonstrated.

[0242] According to the omnigenic model (Liu, et al., Cell 177:1022-1034 e6 (2019); Boyle et al., Cell 169:1177-1186 (2017)) if these genes are core human growth genes, then their effects should be modulated by multiple weaker common genetic variants driving regulatory networks. To indirectly test this hypothesis, polygenic risk scores (PRS) were calculated for height using the largest published GWAS meta-analysis for height that did not include any samples from the UK Biobank project. The cohort was divided into five equally-sized (n=6,824) PRS quintiles (PRS 1 being the lowest height, PRS 5 the tallest height). There was a dose-dependent relationship between increasing PRS score and mean height (β=0.30 per each PRS quintile increase) (Fig. 8A). Carriers of LoF variants in the five core genes were consistently shorter than non-carriers across the five different PRS backgrounds. See Figure 8. The data suggest that the combined effect of PRS and rare protein variants is consistent with an additive model: polygenic effects modulated height in both carriers and non-carriers.

[0243] The risk for ISS across PRS groups was calculated using PRS=3 as a reference. The lowest PRS group was associated with increased risk for ISS and the highest PRS group with a decreased risk (OR=5.43, P=8.58x10-34; OR=0.22, P=4.49x10-7 for PRS 1 and PRS 5 respectively). The effect of rare coding variants of the five core genes was evaluated for ISS stratified by PRS group. Carriers of any of the five core genes were at increased risk for ISS in the first three quintiles (OR=2.64, P=3.09x10-5; OR=2.17, P=0.04; OR=5.29, P=1.58x10-5; OR=2.72, P=0.09 Figs. 8C-F). Consistent directions of effect were observed for carriers of each individual core gene on ISS risk stratified by PRS (Figs. 8C-F).

[0244] Further, additive effects of PRS, mostly coming from multiple common genetic variation with individual small effects, predicted 20.1% of the variance in height in the dataset. These additive effects of PRS appeared to have similar magnitude on carriers of rare coding variation of core genes as well as in non-carriers. This observation indicates that PRS may be a strong contributor in the differences in penetrance of rare pathogenic variants (especially in models of haploinsufficiency such as the ones described here). Supporting this idea, it was observed that two of eight NPR2 variant carriers with low NPR2 activity had a low-normal height. This data suggests that most ISS individuals possessing mutations in NPR2 may also

have a polygenic background that made them more susceptible to the pathogenic effect of losing NPR2 activity.

[0245] These results support the idea that CNP-based treatments could be effective in NPR2 haploinsufficient patient populations. Further, results showing a significant bi-directional (LoF and GoF) correlation of cGMP levels and height in NPR2 carriers of the general population suggest that targeting this receptor with CNP analogs could be an effective therapy for all ISS individuals.

[0246] It is understood that every embodiment of the disclosure described herein may optionally be combined with any one or more of the other embodiments described herein. Every patent literature and every non-patent literature cited herein are incorporated herein by reference in their entirety.

[0247] It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but is intended to cover all modifications which are within the spirit and scope of the invention as defined by the appended claims; the above description, and/or shown in the attached drawings. Consequently only such limitations as appear in the appended claims should be placed on the disclosure.

WHAT IS CLAIMED:

1. A variant of C-type natriuretic peptide selected from the group consisting of PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 5); PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 1); PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 6); and PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 7).

2. The variant of claim 1, wherein the peptide further comprises an acetyl group.

3 The variant of claim 1 or 2, wherein the acetyl group is on the N-terminus of the peptide.

4. The variant of any one of the preceding claims wherein the peptide further comprises an OH or an NH_2 group at the C-terminus.

5. The variant of any one of the preceding claims, comprising a conjugate moiety.

6. The variant of claim 5, wherein the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain.

7. The variant of claim 5 or 6, wherein the conjugate moiety is on a lysine residue.

8. The variant of any one of claims 5 to 7, wherein the conjugate moiety comprises an acid moiety.

9. The variant of any one of claims 5 to 8, wherein the conjugate moiety comprises an acid moiety linked to a hydrophilic spacer.

10. The variant of any one of claims 8 or 9, wherein the acid moiety and the hydrophilic spacer have the structure AEEA-AEEA-γGlu-C18DA.

11. The variant of any one of the preceding claims wherein the peptide is selected from the group consisting of
Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 8);

Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 9);
Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 10);
Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 11); and
Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH_2 (SEQ ID NO: 12).

12. The variant of any one of claims 10 or 11 wherein the CNP variant is Ac-PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1) or PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 1).

13. The variant of any one of the preceding claims comprising a linker.

14. The variant of claim 13, wherein the linker is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain.

15. The variant of any one of claims 13 or 14, wherein the linker is a hydrolysable linker.

16. The variant of any one of claims 13 to 15, wherein the linker is on a lysine residue.

17. The variant of any one of claims 5 to 16, wherein the CNP variant is linked to the conjugate moiety via the linker.

18. The variant of claim 17, wherein the linker is attached to the conjugate moiety via the hydrophilic spacer of the conjugate moiety.

19. The variant of any one of claims 13 to 18 wherein the linker is a peptoid linker or an electronic linker.

20. The variant of any one of preceding claims, wherein the peptide is made synthetically.

21. The variant of any one of the preceding claims, wherein the peptide is stable for 10 days at 37° C, pH 7 to 7.4.

22. The variant of any one of the preceding claims, wherein the peptide has an EC50 from 0.1 to 10 nm in a cGMP assay.

23. The variant of any one of the preceding claims wherein greater than 45% of the peptide is detected after 10 days in aqueous media at 37° C, pH 7.4.

24. The variant of any one of the preceding claims wherein the peptide is conjugated to one or more lipid, fatty acid, hydrophilic spacer moiety, or optionally, combinations thereof.

25. The variant of any one of the preceding claims, wherein the peptide has a longer half-life compared to Pro-Gly-CNP37, CNP-22, or both Pro-Gly CNP and CNP-22.

26. A pharmaceutical composition comprising a CNP variant according to any one of claims 1 to 25, and a pharmaceutically acceptable excipient, carrier or diluent.

27. The composition of claim 26, which is a lyophilized formulation prepared from a formulation that comprises a citric acid/citrate buffer or an acetic acid/acetate buffer having a pH from about 4 to about 6.

28. The composition of claim 27, wherein the lyophilized formulation is prepared from a formulation that further comprises an isotonicity-adjusting agent or a bulking agent selected from the group consisting of mannitol, sucrose, sorbitol, and combinations thereof.

29. The composition of any one of claims 27 to 28, wherein the lyophilized formulation is prepared from a formulation that further comprises an antioxidant selected from the group consisting of methionine, ascorbic acid, salt forms of ascorbic acid, thioglycerol, and combinations thereof.

30. A method of treating a bone-related disorder or skeletal dysplasia in a subject in need thereof comprising administering to the subject a composition comprising a CNP variant or composition of any one of claims 1-29.

31. The method of claim 30, wherein the bone-related disorder or skeletal dysplasia is selected from the group consisting of osteoarthritis, hypophosphatemic rickets, achondroplasia,

hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, chondrodysplasia punctata, camptomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), insulin growth factor 1 receptor (IGF1R) mutation, and idiopathic short stature.

32. A method of elongating a bone or increasing long bone growth in a subject in need thereof, comprising administering to the subject a composition comprising a CNP variant or composition of any one of claims 1 to 29, and wherein the administering elongates a bone or increases long bone growth.

33. The method of any one of claims 30 to 32, wherein the composition is administered subcutaneously, intradermally, intraarticularly, orally, or intramuscularly.

34. The method of any one of claims 30 to 33, wherein the composition is administered once daily, once weekly, once every two weeks, once every three weeks, once every 4 weeks, once every 6 weeks, once every two months, once every three months or once every six months.

35. The method of any one of claims 30 to 34 wherein the composition is an extended release composition.

36. A method of treating a CNP-responsive condition or disorder, comprising

administering a CNP variant or composition of any one of claims 1 to 29 to a subject, and

monitoring the level of at least one bone- or cartilage-associated biomarker in the subject,

wherein an increase in the level of the at least one bone- or cartilage-associated biomarker indicates a therapeutic effect of the CNP variant on the subject or the condition or disorder.

37. The method of claim 36, further comprising adjusting the amount or frequency of administration of the CNP variant, wherein

i) the amount or frequency of administration of the CNP variant is increased if the level of the at least one bone- or cartilage-associated biomarker is below a target level; or

ii) the amount or frequency of administration of the CNP variant is decreased if the level of the at least one bone- or cartilage-associated biomarker is above a target level.

38. The method of claim 36 or 37, wherein the at least one bone- or cartilage-associated biomarker is selected from the group consisting of CNP, cGMP, propeptides of collagen type II and fragments thereof, collagen type II and fragments thereof, collagen type I C-telopeptide (CTx), osteocalcin, proliferating cell nuclear antigen (PCNA), propeptides of type I procollagen (PINP) and fragments thereof, collagen type I and fragments thereof, aggrecan chondroitin sulfate, collagen X, and alkaline phosphatase.

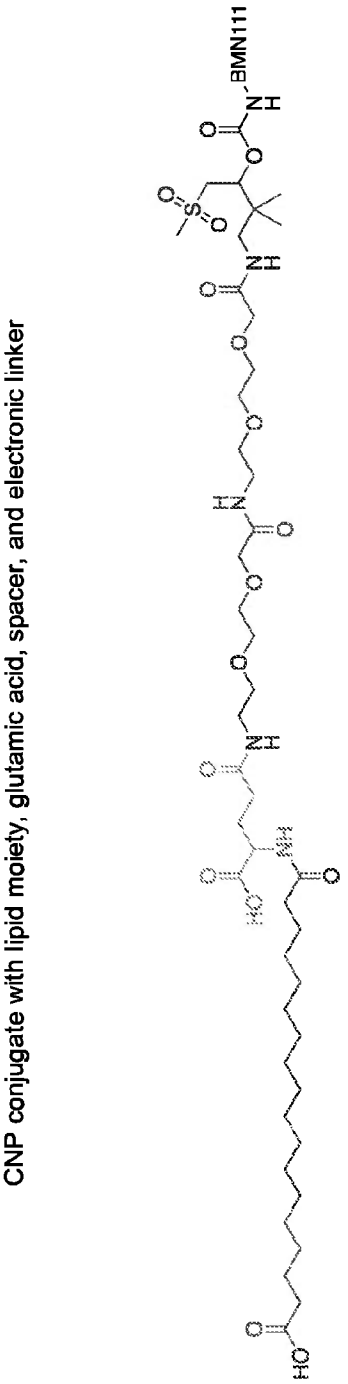
39. A method of making a CNP variant of any one of claims 1 to 25 comprising synthesizing the peptide on a solid-phase resin using Fmoc amino acids.

40. The method of claim 39, wherein the peptides are acetylated by reacting the resin with NMP/Ac_2O/DIEA (10:1:0.1, v/v/v).

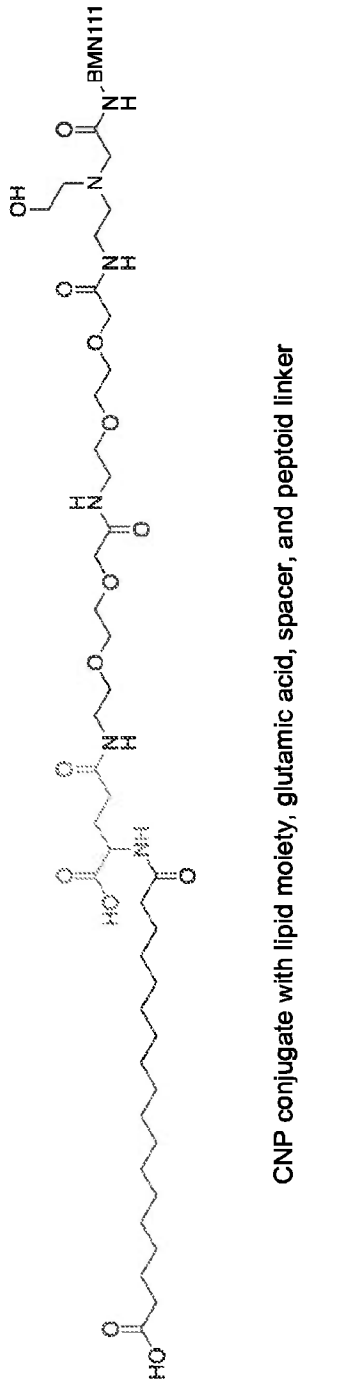
41. The method of claim 39 or 40, wherein the peptide is conjugated to a lipid moiety, optionally on a lysine residue.

42. The method of claim 41, comprising cleaving the protective amino group on the lysine, reacting the peptide with 2x Fmoc-amino PEG(2) followed by amino acid, followed by conjugation of the lipid or fatty acid moiety.

Figure 1



CNP conjugate with lipid moiety, glutamic acid, spacer, and electronic linker



CNP conjugate with lipid moiety, glutamic acid, spacer, and peptoid linker

Figure 2A

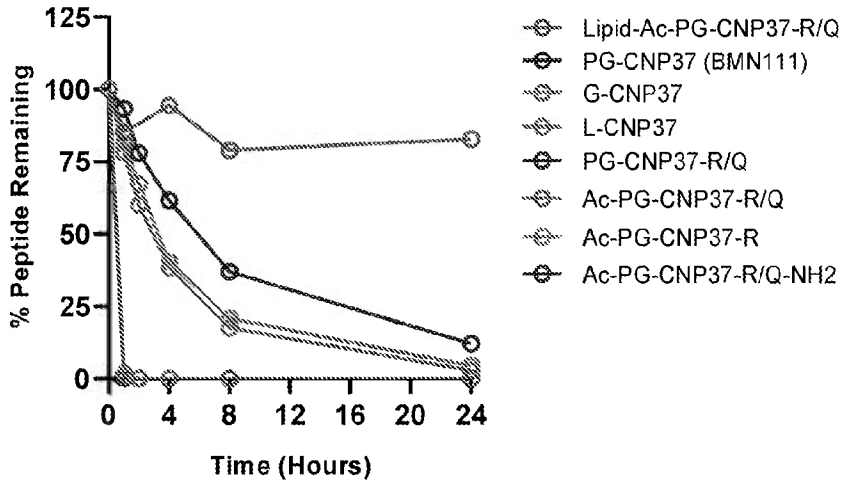
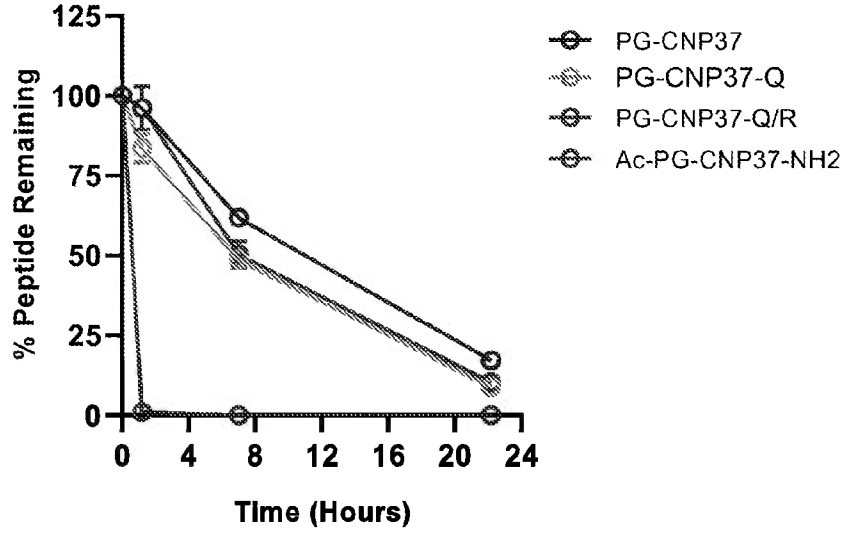


Figure 2B



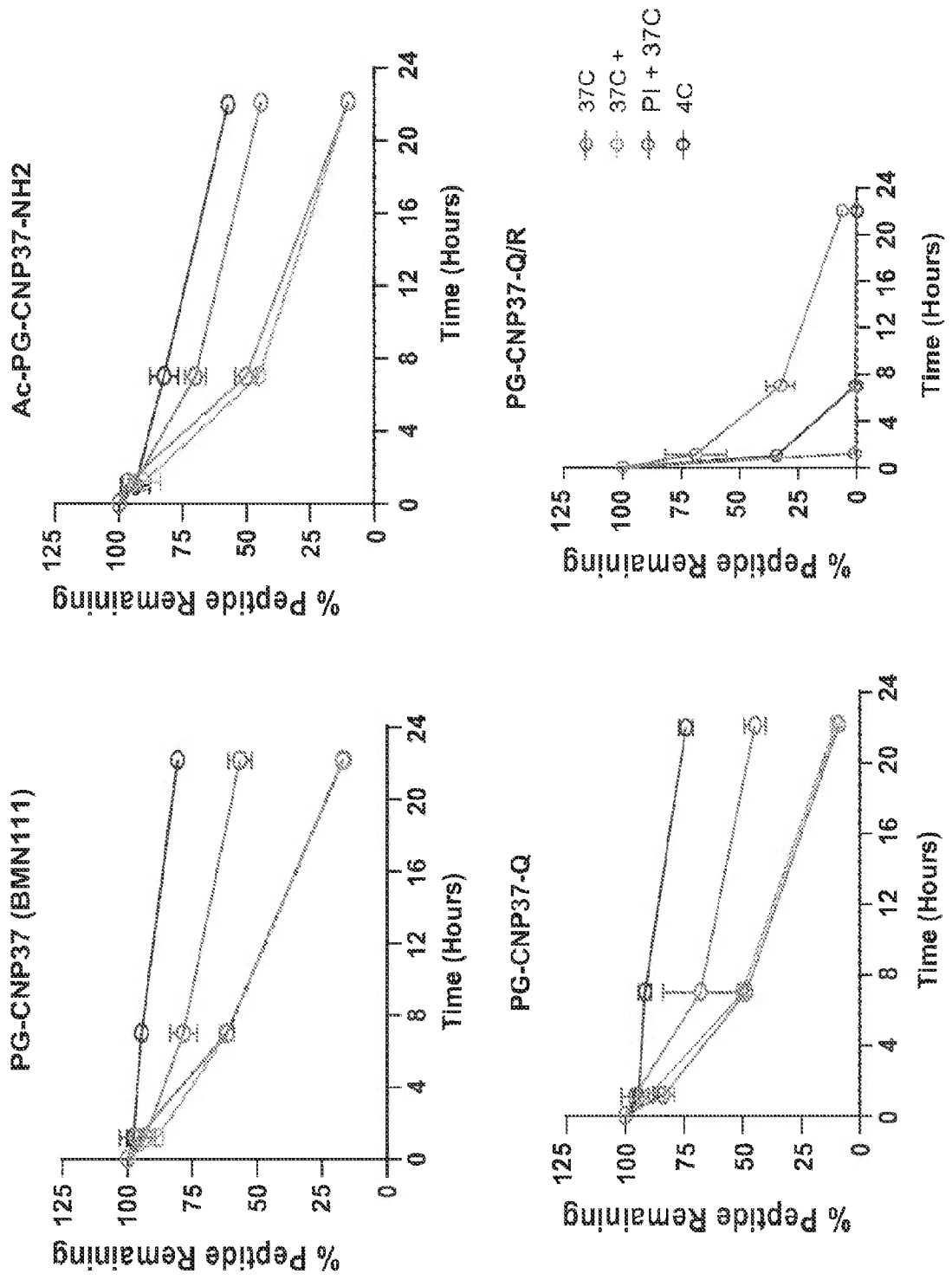

Ac-PG-CNP37-NH2
PG-CNP37 (BMN111)
PG-CNP37-Q/R
PG-CNP37-Q
% Peptide Remaining
Time (Hours)
0
25
50
75
100
125
0
4
8
12
16
20
24
37C
37C +
PI + 37C
4C

Figure 3

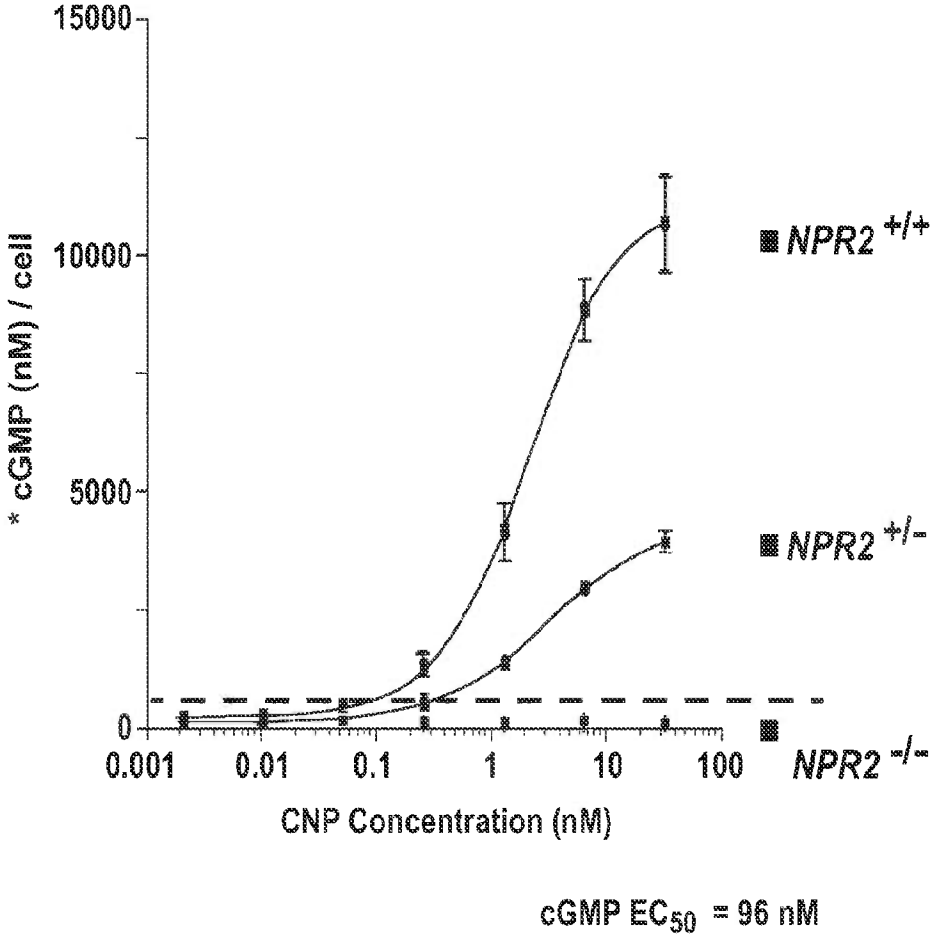

15000
10000
5000
0
* cGMP (nM) / cell
0.001
0.01
0.1
1
10
100
CNP Concentration (nM)
NPR2 +/+
NPR2 +/-
NPR2 -/-
cGMP EC50 = 96 nM

Figure 4

Figure 5

NPR2 Clone, nucleotide sequence

Clone 47, Allele 1(SEQ ID NO: 57):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATCGA
GGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAG
GTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 35, Allele 1(SEQ ID NO: 58):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATCGA
GGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAG
GTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 35, Allele 2(SEQ ID NO: 59):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCACA
ATTGGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCAT
CGAGGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGC
CAGGTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 47, Allele 1(SEQ ID NO: 60)
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG

Figure 5 (cont'd)

GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCACTGCGCGCTGTGGATCTCAAGCTGTACCAT
GACCCCGACCTTCTGTTGGGCCCTGGTTGTGTGTACCCTGCTGCCTCTGTGGCTCGCTTTGCCTCGCACTG
GCACCTTCCCCTCCTGACTGCGGGTGCAGTGGCCTCTGGCTTTGCAGCTAAGAATGAGCATTATCGTACC
CTGGTTCGCACTGGCCCCTCTGCGCCCAAGCTGGGTGAGTTCGTAGTGACATTACACGGGCACTTCAATT
GGACTGCTCGGGCTGCTTTGCTGTATCTGGATGCTCGCACAGATGACCGGCCCCACTACTTCACCATCCG
AGGGGGTGTTTGAGGCCCTGCAGGGCAGCAACCTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCA
GGTGGCCCTGAGCAAGCCACCCACTTCATCAGAGCCAACGGGCGCA

Clone 47, Allele 2(SEQ ID NO: 61):
ATGGCACTGCCATCCCTGCTACTGGTGGTGGCAGCCCTGGCAGGTGGGGTGCGTCCTCCGGGGGCACG
GAACCTGACGCTGGCGGTGGTGCTGCCAGAACACAACCTGAGCTATGCCTGGGCCTGGCCACGGGTGG
GTCCTGCTGTGGCACTGGCTGTGGAGGCGCTGGGCCGGGCACTGCCCGTGGACCTGCGGTTTGTCAGC
TCCGAACTAGACGGCGCCTGCTCTGAGTACCTGGCACCGGGGTGTTTGAGGCCCTGCAGGGCAGCAAC
CTCAGTGTGCAACACCAGGTGTATACCCGAGAGCCAGGTGGCCCTGAGCAAGCCACCCACTTCATCAGA
GCCAACGGGCGCA

NPR2 Clone, predicted protein product sequence

Clone 47, Allele 1(SEQ ID NO: 62):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIEGVFEALQGSNLSVQHQVYTREPGGPEQAT
HFIRANGRIVYICGPLEMLHEILLQAQRENLTNGDYVFFYLDVFGESLRAGPTRATGRPWQDNRTQEQAQAL
REAFQTVLVITYREPPNPEYQEFQNRLLIRAREDFGVELAPSLMNLIAGCFYDGILLYAQVLNETIQEGGTRED
GLRIVEKMQGRRYHGVTGLVVMDKNNDRETDFVLWAMGDLESGDFQPAAHYSGAEKQIWWTGRPIPW
VKGAPPLDNPPCAFDLDDPSCDKTPLSTLAIVALGTGITFIMFGVVSSFLIFRKLMLEKELASMLWRIRWEELQF
GNSDRYHKGAGSRLTLSLRGSSYGSLMTAHGKYQIFANTGHFKGNVVAIKHVNKKRIELTRQVLFELKHMRD
VQFNHLTRFIGACIDPPNICIVTEYCPRGSLQDILENDSINLDWMFRYSLINDLVKGMAFLHNSIISSHGSLKSS
NCVVDSRFVLKITDYGLASFRSTAEPDDSHALYAKKLWTAPELLSGNPLPTTGMQKADVYSFAIILQEIALRSG
PFYLEGLDLSPKEIVQKVRNGQRPYFRPSIDRTQLNEELVLLMERCWAQDPTERPDFGQIKGFIRRFNKEGGT
SILDNLLLRMEQYANNLEKLVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEAFDSVTIYFSDIVGF
TALSAESTPMQVVTLLNDLYTCFDAIIDNFDVYKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVS
SFRIRHRPHDQLRLRIGVHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGQALKIHVSSTTKDALDELGC
FQLELRGDVEMKGKGKMRTYWLLGERKGPPGLL*

Clone 35, Allele 1(SEQ ID NO: 63):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIEGVFEALQGSNLSVQHQVYTREPGGPEQAT

Figure 5 (cont'd)

HFIRANGRIVYICGPLEMLHEILLQAQRENLTNGDYVFFYLDVFGESLRAGPTRATGRPWQDNRTQEQAQAL
REAFQTVLVITYREPPNPEYQEFQNRLLIRAREDFGVELAPSLMNLIAGCFYDGILLYAQVLNETIQEGGTRED
GLRIVEKMQGRRYHGVTGLVVMDKNNDRETDFVLWAMGDLESGDFQPAAHYSGAEKQIWWTGRPIPW
VKGAPPLDNPPCAFDLDDPSCDKTPLSTLAIVALGTGITFIMFGVSSFLIFRKLMLEKELASMLWRIRWEELQF
GNSDRYHKGAGSRLTLSLRGSSYGSLMTAHGKYQIFANTGHFKGNVVAIKHVNKKRIELTRQVLFELKHMRD
VQFNHLTRFIGACIDPPNICIVTEYCPRGSLQDILENDSINLDWMFRYSLINDLVKGMAFLHNSIISSHGSLKSS
NCVVDSRFVLKITDYGLASFRSTAEPDDSHALYAKKLWTAPELLSGNPLPTTGMQKADVYSFAIILQEIALRSG
PFYLEGLDLSPKEIVQKVRNGQRPYFRPSIDRTQLNEELVLLMERCWAQDPTERPDFGQIKGFIRRFNKEGGT
SILDNLLLRMEQYANNLEKLVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEAFDSVTIYFSDIVGF
TALSAESTPMQVVTLLNDLYTCFDAIIDNFDVYKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVS
SFRIRHRPHDQLRLRIGVHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGQALKIHVSSTTKDALDELGC
FQLELRGDVEMKGKGKMRTYWLLGERKGPPGLL*

Clone 35, Allele 2(SEQ ID NO: 64):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFTIGLLGLLCCIWMLAQMTGPTTSPSRGCLRPCRAATSVCNTRCIPESQVALSKPPTS
SEPTGALCISAARWRCFMRSCFRPRGRT*

Clone 47, Allele 1(SEQ ID NO: 65):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPLRAVDLKLYHDPDLLLGPGCVYPAASVARFASHWHLPLLTAGAVASGFAAKNEHYRTLVRTG
PSAPKLGEFVVTLHGHFNWTARAALLYLDARTDDRPHYFTIRGGV*

Clone 47, Allele 2(SEQ ID NO: 66):
MALPSLLLVVAALAGGVRPPGARNLTLAVVLPEHNLSYAWAWPRVGPAVALAVEALGRALPVDLRFVSSEL
DGACSEYLAPGCLRPCRAATSVCNTRCIPESQVALSKPPTSSEPTGALCISAARWRCFMRSCFRPRGRT*

Figure 6

Genetic Variants Found in NPR2 Genes

NPR2 Mutations			
G21R	P301S	Q500X	R745Q
A48S	R318Q	H508D	R819C
A59T	R358W	G541S	T861I
V102A	E359A	A549T	V883M
R110G	I364fs	R562Q	T907M
A164G	E389D	R601H	G917R
I226V	E415K	R601S	R921Q
Y250X	K480N	E609K	R957C
R263H	A488P	S721R	L1009V
T297M	I494S	E727Q	

Figure 7

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom.vep.cadd_phred
2	231925451	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409857.2:c.455C>G	ENSP00000387159.1:p.Gly119Arg	33
2	231925486	['G', 'GT']	1	1.00	['frameshift_variant'	HIGH	NPPC	ENST00000409852.2:c.319_320insA	ENSP00000387159.1:p.Ser107Tyrfs*37	
2	231925513	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.293A>G	ENSP00000387159.1:p.Tyr98Cys	23
2	231925514	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.292T>G	ENSP00000387159.1:p.Tyr98Asp	18
2	231925523	['C', 'A']	2	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.283G>T	ENSP00000387159.1:p.Ala95Ser	15
2	231925529	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.277C>T	ENSP00000387159.1:p.Pro93Ser	4.04E-06 26
2	231925540	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.266T>A	ENSP00000387159.1:p.Leu89Gln	23
2	231925550	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.256G>C	ENSP00000387159.1:p.Ala86Pro	23
2	231925553	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.253T>C	ENSP00000387159.1:p.Trp85Arg	4.06E-06 23
2	231925580	['G', 'T']	3	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.226C>A	ENSP00000387159.1:p.Arg76Ser	6.96E-05 25
2	231925584	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.222C>G	ENSP00000387159.1:p.Asp74Glu	21
2	231925597	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.209G>T	ENSP00000387159.1:p.Arg70Leu	26
2	231925600	['G', 'T']	1	1.00	['stop_gained']	HIGH	NPPC	ENST00000409852.2:c.206C>A	ENSP00000387159.1:p.Ser69Ter	38
2	231925639	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.167C>T	ENSP00000387159.1:p.Ala56Val	3.95E-05 18
2	231925646	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.160G>T	ENSP00000387159.1:p.Asp54Tyr	23
2	231925649	['CCTT', 'C'	3	1.00	['inframe_deletion']	MODERATE	NPPC	ENST00000409852.2:c.154_156del	ENSP00000387159.1:p.Lys52del	4.44E-06
2	231925649	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.157G>A	ENSP00000387159.1:p.Gly53Ser	21
2	231925657	['T', 'C']	3	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.149A>G	ENSP00000387159.1:p.Gln50Arg	1.81E-05 21
2	231925663	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.143G>T	ENSP00000387159.1:p.Gly48Val	24
2	231925669	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.137C>G	ENSP00000387159.1:p.Ala46Gly	23
2	231925708	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.98G>A	ENSP00000387159.1:p.Arg33Gln	5.65E-06 24
2	231925711	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.95C>T	ENSP00000387159.1:p.Pro32Leu	13
2	231926189	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.61T>C	ENSP00000387159.1:p.Ser21Pro	3.40E-05 23
2	231926200	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPPC	ENST00000409852.2:c.50C>G	ENSP00000387159.1:p.Ser17Cys	28
2	231926201	['A', 'AGAC	1	1.00	['inframe_insertion']	MODERATE	NPPC	ENST00000409852.2:c.40_48dup	ENSP00000387159.1:p.Thr14_Leu16dup	
4	1793974	['G', 'GTGT	1	1.00	['inframe_insertion']	MODERATE	FGFR3	ENST00000340107.8:c.42_47dup	ENSP00000339824.4:p.Ala15_Ile16InsMetAla	
4	1794009	['G', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.75G>C	ENSP00000339824.4:p.Leu25Phe	8
4	1794016	['G', 'C']	2	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.82G>C	ENSP00000339824.4:p.Glu28Gln	22
4	1794043	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.109G>A	ENSP00000339824.4:p.Glu37Lys	22
4	1794274	['A', 'T']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.130G>A	ENSP00000339824.4:p.Gly44Ser	0.000225 0
4	1794289	['T', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.145T>G	ENSP00000339824.4:p.Leu49Val	4.06E-06 5
4	1794292	['G', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.148G>T	ENSP00000339824.4:p.Val50Phe	22
4	1794292	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.148G>A	ENSP00000339824.4:p.Val50Ile	13
4	1794303	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.159C>A	ENSP00000339824.4:p.Ser53Arg	11
4	1794304	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.160G>A	ENSP00000339824.4:p.Gly54Arg	4.06E-05 23
4	1794310	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.166G>A	ENSP00000339824.4:p.Ala56Thr	0
4	1794316	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.172G>A	ENSP00000339824.4:p.Glu58Lys	8.13E-06 15
4	1794328	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.184C>A	ENSP00000339824.4:p.Pro62Thr	1
4	1794332	['C', 'G']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.188C>G	ENSP00000339824.4:p.Pro63Arg	3.27E-05 3
4	1794337	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.193G>A	ENSP00000339824.4:p.Val65Arg	0.001076 11
4	1794389	['T', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.245T>C	ENSP00000339824.4:p.Val82Ala	2
4	1794416	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.272C>T	ENSP00000339824.4:p.Pro91Leu	0.000176 8
4	1794422	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.278G>A	ENSP00000339824.4:p.Arg93Gln	1.69E-05 12
4	1794457	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.313G>A	ENSP00000339824.4:p.Gly105Arg	9.26E-06 24
4	1794472	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.328C>T	ENSP00000339824.4:p.Arg110Trp	1.50E-05 22
4	1794473	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.329G>A	ENSP00000339824.4:p.Arg110Gln	7.05E-05 17
4	1794479	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.335G>A	ENSP00000339824.4:p.Arg112Gln	0.000229 9
4	1794491	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.347G>A	ENSP00000339824.4:p.Arg116His	4.18E-05 3
4	1794493	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.349G>A	ENSP00000339824.4:p.Val117Ile	7.21E-05 0
4	1794496	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.352C>G	ENSP00000339824.4:p.Leu118Val	1.22E-05 12
4	1794756	['C', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.389C>A	ENSP00000339824.4:p.Ser130Tyr	19
4	1794756	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.389C>T	ENSP00000339824.4:p.Ser130Phe	1.20E-05 17
4	1794758	['T', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.391T>C	ENSP00000339824.4:p.Ser131Pro	8.01E-06 22
4	1794770	['G', 'A']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.403G>A	ENSP00000339824.4:p.Glu135Lys	1.20E-05 21
4	1794776	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.409G>A	ENSP00000339824.4:p.Gly137Arg	1.60E-05 15
4	1795792	['ACGACA'	1	1.00	['inframe_deletion']	MODERATE	FGFR3	ENST00000340107.8:c.436_445+2del		1.20E-05
4	1801373	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.452C>T	ENSP00000339824.4:p.Pro151Leu	27
4	1801384	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.463C>T	ENSP00000339824.4:p.Arg155Trp	6.53E-06 24
4	1801393	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.472C>T	ENSP00000339824.4:p.Arg158Trp	6.50E-06 23
4	1801394	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.473G>A	ENSP00000339824.4:p.Arg158Gln	5.85E-05 27
4	1801402	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.481A>G	ENSP00000339824.4:p.Lys161Glu	31
4	1801435	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.514G>A	ENSP00000339824.4:p.Val172Ile	6.67E-05 24

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
4	1801439	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.518G>A	ENSP00000339824.4:p.Arg173His	4.00E-05	28
4	1801445	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.524G>A	ENSP00000339824.4:p.Arg175His	3.98E-05	27
4	1801456	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.535G>A	ENSP00000339824.4:p.Ala179Thr		14
4	1801477	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.556A>G	ENSP00000339824.4:p.Ile186Val		23
4	1801478	['T', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.557T>A	ENSP00000339824.4:p.Ile186Asn		25
4	1801481	['C', 'A']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.560C>A	ENSP00000339824.4:p.Ser187Tyr	2.52E-05	19
4	1801495	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.574G>A	ENSP00000339824.4:p.Gly192Ser		26
4	1801506	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.585C>G	ENSP00000339824.4:p.Phe195Leu	1.84E-05	23
4	1801510	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.589G>A	ENSP00000339824.4:p.Gly197Ser	6.04E-06	24
4	1801634	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.630G>T	ENSP00000339824.4:p.Gln210His		16
4	1801689	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.685G>A	ENSP00000339824.4:p.Val229Ile		12
4	1801717	['G', 'A']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.713G>A	ENSP00000339824.4:p.Arg238Gln	0.0005	9
4	1801743	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.739G>A	ENSP00000339824.4:p.Glu247Lys	1.34E-05	34
4	1801841	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.746C>T	ENSP00000339824.4:p.Ser249Phe	1.26E-05	25
4	1801849	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.754C>T	ENSP00000339824.4:p.Arg252Trp		25
4	1801874	['C', 'T']	8	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.779C>T	ENSP00000339824.4:p.Pro260Leu	2.51E-05	28
4	1801903	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.808G>A	ENSP00000339824.4:p.Asp270Asn	9.32E-05	22
4	1801948	['A', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.853A>G	ENSP00000339824.4:p.Ile285Val	2.41E-05	26
4	1802005	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.910C>T	ENSP00000339824.4:p.Pro304Ser		26
4	1802941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.958G>A	ENSP00000339824.4:p.Asp320Asn	4.33E-06	23
4	1802944	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.961G>A	ENSP00000339824.4:p.Val321Met	2.60E-05	20
4	1802948	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.965G>A	ENSP00000339824.4:p.Arg322His	8.68E-06	23
4	1802953	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.970C>T	ENSP00000339824.4:p.Arg324Cys	1.30E-05	22
4	1802954	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.971G>A	ENSP00000339824.4:p.Arg324His	1.73E-05	18
4	1802969	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.986C>T	ENSP00000339824.4:p.Ser329Leu	3.07E-05	24
4	1802975	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.992G>A	ENSP00000339824.4:p.Arg331Gln	2.19E-05	18
4	1802986	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1003G>A	ENSP00000339824.4:p.Glu335Lys	4.89E-05	20
4	1803005	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1022C>T	ENSP00000339824.4:p.Thr341Ile		22
4	1803019	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1036G>A	ENSP00000339824.4:p.Val346Met	1.74E-05	22
4	1803046	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1063G>A	ENSP00000339824.4:p.Val355Ile	1.34E-05	24
4	1803052	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1069G>A	ENSP00000339824.4:p.Gly357Arg	2.25E-05	17
4	1803054	['G', 'GC']	3	1.00	['frameshift_variant'	HIGH	FGFR3	ENST00000340107.8:c.1075dup	ENSP00000339824.4:p.Arg359ProfsTer11		
4	1803058	['C', 'T']	1	1.00	['stop_gained']	HIGH	FGFR3	ENST00000340107.8:c.1075C>T	ENSP00000339824.4:p.Arg359Ter	1.82E-05	39
4	1803059	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1076G>A	ENSP00000339824.4:p.Arg359Gln	3.64E-05	9
4	1803061	['G', 'A']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1078G>A	ENSP00000339824.4:p.Ala360Thr		18
4	1803332	['G', 'C']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1084G>C	ENSP00000339824.4:p.Glu362Gln	1.24E-05	15
4	1803353	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1105G>T	ENSP00000339824.4:p.Asp369Tyr		20
4	1803360	['C', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1112C>T	ENSP00000339824.4:p.Ala371Val	2.81E-05	18
4	1803375	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1127C>G	ENSP00000339824.4:p.Ala376Gly		19
4	1803381	['T', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1133T>G	ENSP00000339824.4:p.Ile378Ser		26
4	1803383	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1135C>A	ENSP00000339824.4:p.Leu379Ile		9
4	1803388	['C', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1140C>A	ENSP00000339824.4:p.Ser380Arg		20
4	1803401	['T', 'C']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1153T>C	ENSP00000339824.4:p.Phe385Leu	7.99E-06	19
4	1803404	['T', 'A']	2	0.99	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1156T>A	ENSP00000339824.4:p.Phe386Ile	7.99E-06	18
4	1803413	['A', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1165A>T	ENSP00000339824.4:p.Ile389Phe		18
4	1803414	['T', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1166T>A	ENSP00000339824.4:p.Ile389Asn		24
4	1803423	['T', 'TGGC	1	1.00	['inframe_insertion']	MODERATE	FGFR3	ENST00000340107.8:c.1179_1181dup	ENSP00000339824.4:p.Ala394dup		
4	1803443	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1195C>T	ENSP00000339824.4:p.Arg399Cys	3.21E-05	25
4	1803449	['C', 'T']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1201C>T	ENSP00000339824.4:p.Arg401Cys	1.60E-05	23
4	1803455	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1207C>G	ENSP00000339824.4:p.Pro403Ala	2.40E-05	9
4	1803464	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1216A>G	ENSP00000339824.4:p.Lys406Glu	4.00E-06	23
4	1803486	['T', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1238T>C	ENSP00000339824.4:p.Val413Gly		31
4	1803492	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1244A>G	ENSP00000339824.4:p.Lys415Arg	8.01E-06	25
4	1803493	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1245G>C	ENSP00000339824.4:p.Lys415Asn	1.20E-05	26
4	1803496	['C', 'G']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1248C>G	ENSP00000339824.4:p.Ile416Met		20
4	1803516	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1268G>A	ENSP00000339824.4:p.Arg423Gln	2.41E-05	29
4	1803824	['G', 'C']	1	1.00	['missense_variant',	MODERATE	FGFR3	ENST00000340107.8:c.1273G>C	ENSP00000339824.4:p.Val425Leu	6.41E-06	28
4	1803828	['C', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1277C>T	ENSP00000339824.4:p.Ser426Phe	1.28E-05	25
4	1804846	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1295C>T	ENSP00000339824.4:p.Ser432Phe		29
4	1804872	['C', 'G']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1321C>G	ENSP00000339824.4:p.Arg441Gly		18
4	1804872	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1321C>T	ENSP00000339824.4:p.Arg441Cys	2.56E-05	23
4	1804873	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1322G>A	ENSP00000339824.4:p.Arg441His	3.84E-05	27

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom	vep.cadd_phred
4	1804878	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1327G>A	ENSP00000339824.4:p.Ala443Thr	1.79E-05	11
4	1804888	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1337C>T	ENSP00000339824.4:p.Ser446Phe	2.56E-05	24
4	1804902	['C', 'G']	1	0.99	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1351C>G	ENSP00000339824.4:p.Pro451Ala		20
4	1804906	['C', 'T']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1355C>T	ENSP00000339824.4:p.Thr452Met	0.001288	6
4	1804911	['G', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1360G>T	ENSP00000339824.4:p.Ala454Ser	2.56E-05	20
4	1804929	['G', 'C']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1378G>C	ENSP00000339824.4:p.Glu460Gln	6.39E-06	26
4	1804941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1390G>A	ENSP00000339824.4:p.Asp464Asn		26
4	1804968	['C', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1417C>G	ENSP00000339824.4:p.Arg473Gly		23
4	1805405	['T', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1469T>C	ENSP00000339824.4:p.Met490Thr		25
4	1805416	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1480A>G	ENSP00000339824.4:p.Ile494Val	4.03E-06	13
4	1805428	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1492A>G	ENSP00000339824.4:p.Lys498Glu	8.07E-06	29
4	1805434	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1498C>T	ENSP00000339824.4:p.Arg500Trp	8.09E-05	24
4	1805435	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1499G>A	ENSP00000339824.4:p.Arg500Gln	8.08E-06	23
4	1805440	['G', 'A']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1504G>A	ENSP00000339824.4:p.Ala502Thr	5.66E-05	21
4	1805561	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1543G>A	ENSP00000339824.4:p.Asp515Asn	4.79E-05	23
4	1805571	['A', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1553A>C	ENSP00000339824.4:p.Asp518Ala	3.99E-06	27
4	1805571	['A', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1553A>T	ENSP00000339824.4:p.Asp518Val		27
4	1805573	['A', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1555A>C	ENSP00000339824.4:p.Lys519Gln	1.20E-05	25
4	1805573	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1555A>G	ENSP00000339824.4:p.Lys519Glu	3.99E-06	23
4	1805574	['A', 'G']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1556A>G	ENSP00000339824.4:p.Lys519Arg	5.58E-05	24
4	1805583	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1565C>T	ENSP00000339824.4:p.Ser522Leu	7.98E-06	25
4	1805636	['A', 'G']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1618A>G	ENSP00000339824.4:p.Ile540Val	1.20E-05	22
4	1805642	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1624A>G	ENSP00000339824.4:p.Asn542Asp	3.99E-06	28
4	1805643	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1625A>G	ENSP00000339824.4:p.Asn542Ser	3.99E-06	26
4	1805649	['T', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1631T>G	ENSP00000339824.4:p.Leu544Arg		32
4	1805661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1643C>T	ENSP00000339824.4:p.Thr548Met		25
4	1805666	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1648G>A	ENSP00000339824.4:p.Gly550Ser		25
4	1805761	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1663G>C	ENSP00000339824.4:p.Val555Leu	0.000146	25
4	1805774	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1676A>G	ENSP00000339824.4:p.Tyr559Cys	4.05E-06	24
4	1805779	['G', 'C']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1681G>C	ENSP00000339824.4:p.Ala561Pro		21
4	1805807	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1709G>A	ENSP00000339824.4:p.Arg570Gln	2.02E-05	23
4	1805810	['C', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1712C>T	ENSP00000339824.4:p.Ala571Val		24
4	1805819	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1721C>T	ENSP00000339824.4:p.Pro574Leu	0.00015	26
4	1805822	['C', 'T']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1724C>T	ENSP00000339824.4:p.Pro575Leu	2.03E-05	25
4	1805837	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1739C>T	ENSP00000339824.4:p.Ser580Phe		26
4	1805860	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1762G>A	ENSP00000339824.4:p.Glu588Lys	9.69E-05	24
4	1805911	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1813C>T	ENSP00000339824.4:p.Arg605Trp	6.83E-05	23
4	1805920	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1822G>C	ENSP00000339824.4:p.Glu608Gln	1.01E-06	25
4	1805921	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1823A>G	ENSP00000339824.4:p.Glu608Gly	4.02E-06	27
4	1805929	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1831G>A	ENSP00000339824.4:p.Ala611Thr	4.03E-06	33
4	1805930	['C', 'T']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1832C>T	ENSP00000339824.4:p.Ala611Val	2.42E-05	25
4	1805933	['C', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1835C>A	ENSP00000339824.4:p.Ser612Tyr	1.21E-05	27
4	1806093	['G', 'A']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1885G>A	ENSP00000339824.4:p.Glu629Lys	0.00018	33
4	1806169	['C', 'G']	4	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1961C>G	ENSP00000339824.4:p.Thr654Ser	8.04E-06	20
4	1806281	['G', 'C']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1990G>C	ENSP00000339824.4:p.Ala664Pro	4.04E-06	33
4	1806290	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.1999G>T	ENSP00000339824.4:p.Ala667Ser	1.21E-05	25
4	1806300	['ACC', 'A']	1	1.00	['frameshift_variant']	HIGH	FGFR3	ENST00000340107.8:c.2010_2011del	ENSP00000339824.4:p.Asp670GlufsTer8		
4	1806315	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2024A>G	ENSP00000339824.4:p.His675Arg		25
4	1806326	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2035G>A	ENSP00000339824.4:p.Val679Ile	4.02E-06	27
4	1806548	['G', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2039G>T	ENSP00000339824.4:p.Trp680Leu		34
4	1806581	['C', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2072C>T	ENSP00000339824.4:p.Thr691Met	4.00E-06	26
4	1806593	['C', 'T']	6	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2084C>T	ENSP00000339824.4:p.Ser695Phe	1.20E-05	25
4	1806619	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2110G>A	ENSP00000339824.4:p.Glu704Lys	4.00E-06	27
4	1806668	['A', 'G']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2159A>G	ENSP00000339824.4:p.Asn720Ser	6.81E-05	23
4	1806669	['C', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2160C>G	ENSP00000339824.4:p.Asn720Lys		24
4	1806842	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2188C>T	ENSP00000339824.4:p.Arg730Trp	4.20E-06	23
4	1806843	['G', 'A']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2189G>A	ENSP00000339824.4:p.Arg730Gln	3.35E-05	26
4	1806855	['A', 'G']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2201A>G	ENSP00000339824.4:p.His734Arg	4.12E-06	21
4	1806860	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2206G>A	ENSP00000339824.4:p.Ala736Thr	4.93E-05	22
4	1806861	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2207C>T	ENSP00000339824.4:p.Ala736Val	3.29E-05	3
4	1806876	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2222C>T	ENSP00000339824.4:p.Pro741Leu		26

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom.vep.cadd_phred
4	1806935	['G', 'A']	7	1.00	['splice_donor_variant'	HIGH	FGFR3	ENST00000340107.8:c.2280+1G>A		4.58E-06 25
4	1807132	['C', 'T']	7	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2297C>T	ENSP00000339824.4:p.Ser766Leu	5.47E-05 27
4	1807135	['C', 'T']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2300C>T	ENSP00000339824.4:p.Ala767Val	6.36E-05 13
4	1807156	['C', 'T']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2321C>T	ENSP00000339824.4:p.Pro774Leu	1.84E-05 24
4	1807159	['G', 'A']	3	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2324G>A	ENSP00000339824.4:p.Gly775Asp	3.19E-05 22
4	1807165	['A', 'G']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2330A>G	ENSP00000339824.4:p.Gln777Arg	4.50E-06 20
4	1807191	['G', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2356G>C	ENSP00000339824.4:p.Gly786Arg	24
4	1807194	['G', 'GA']	1	1.00	['frameshift_variant'	HIGH	FGFR3	ENST00000340107.8:c.2360dup	ENSP00000339824.4:p.Asp787GlufsTer32	
4	1807197	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2362G>A	ENSP00000339824.4:p.Asp788Asn	8.61E-06 27
4	1807201	['CCGT, 'C	1	1.00	['inframe_deletion']	MODERATE	FGFR3	ENST00000340107.8:c.2367_2369del	ENSP00000339821.4:p.Val790del	
4	1807204	['T', 'C']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2369T>C	ENSP00000339824.4:p.Val790Ala	27
4	1807230	['G', 'A']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2395G>A	ENSP00000339824.4:p.Ala799Thr	17
4	1807237	['C', 'T']	1	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2402C>T	ENSP00000339824.4:p.Pro801Leu	8.24E-05 22
4	1807254	['C', 'T']	5	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2419C>T	ENSP00000339824.4:p.Arg807Trp	1.83E-05 14
4	1807255	['G', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2420G>A	ENSP00000339824.4:p.Arg807Gln	2.79E-05 15
4	1807258	['C', 'A']	2	1.00	['missense_variant']	MODERATE	FGFR3	ENST00000340107.8:c.2423C>A	ENSP00000339824.4:p.Thr808Lys	4.67E-06 24
9	35792461	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.53G>A	ENSP00000341083.2:p.Arg18His	23
9	35792469	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.61G>C	ENSP00000341083.2:p.Gly21Arg	19
9	35792475	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.67C>T	ENSP00000341083.2:p.Arg23Trp	24
9	35792580	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.172C>T	ENSP00000341083.2:p.Arg58Trp	3.99E-06 25
9	35792581	['G', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.173G>T	ENSP00000341083.2:p.Arg58Leu	1.20E-05 23
9	35792583	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.175G>A	ENSP00000341083.2:p.Ala59Thr	21
9	35792601	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.193C>T	ENSP00000341083.2:p.Arg65Trp	7.97E-06 23
9	35792652	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.244C>A	ENSP00000341083.2:p.Leu82Met	2.39E-05 23
9	35792688	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.280G>A	ENSP00000341083.2:p.Asp94Asn	7.96E-06 19
9	35792709	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.301T>G	ENSP00000341083.2:p.Cys101Gly	27
9	35792713	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.305T>C	ENSP00000341083.2:p.Val102Ala	20
9	35792736	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.328C>G	ENSP00000341083.2:p.Arg110Gly	27
9	35792818	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.410G>A	ENSP00000341083.2:p.Arg137His	21
9	35792833	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.425C>T	ENSP00000341083.2:p.Thr142Ile	21
9	35792839	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.431C>T	ENSP00000341083.2:p.Pro144Leu	24
9	35792883	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.475C>T	ENSP00000341083.2:p.His159Tyr	22
9	35792885	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.477C>G	ENSP00000341083.2:p.His159Gln	19
9	35792890	['A', 'G']	5	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.482A>G	ENSP00000341083.2:p.Asn161Ser	1.59E-05 23
9	35792899	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.491C>T	ENSP00000341083.2:p.Ala164Val	17
9	35792901	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.493C>T	ENSP00000341083.2:p.Arg165Cys	3.98E-06 26
9	35792928	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.520C>T	ENSP00000341083.2:p.Arg174Cys	3.98E-06 27
9	35792992	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.584A>G	ENSP00000341083.2:p.Asn195Ser	7.99E-06 23
9	35793021	['C', 'T']	1	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.613C>T	ENSP00000341083.2:p.Arg205Ter	35
9	35793022	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.614G>A	ENSP00000341083.2:p.Arg205Gln	20
9	35793033	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.625G>A	ENSP00000341083.2:p.Gly209Ser	4.09E-06 21
9	35793039	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.631G>A	ENSP00000341083.2:p.Glu211Lys	4.13E-06 21
9	35793904	['A', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.674A>C	ENSP00000341083.2:p.Tyr225Ser	24
9	35793906	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.676A>G	ENSP00000341083.2:p.Ile226Val	1.19E-05 21
9	35793952	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.722A>T	ENSP00000341083.2:p.Gln241Leu	21
9	35793955	['G', 'A']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.725G>A	ENSP00000341083.2:p.Arg242Lys	2.78E-05 21
9	35793980	['T', 'A']	1	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.750T>A	ENSP00000341083.2:p.Tyr250Ter	35
9	35794018	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.788G>A	ENSP00000341083.2:p.Arg263His	3.58E-05 24
9	35794024	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.794G>A	ENSP00000341083.2:p.Gly265Asp	20
9	35794029	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.799A>G	ENSP00000341083.2:p.Thr267Ala	2.39E-05 21
9	35794044	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.814C>T	ENSP00000341083.2:p.Arg272Trp	2.78E-05 24
9	35794045	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.815G>A	ENSP00000341083.2:p.Arg272Gln	1.19E-05 20
9	35794056	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.826G>C	ENSP00000341083.2:p.Asp276His	3.98E-06 24
9	35794060	['A', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.830A>G	ENSP00000341083.2:p.Asn277Ser	4.38E-05 21
9	35794063	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.833G>A	ENSP00000341083.2:p.Arg278His	2.79E-05 22
9	35794068	['C', 'T']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.838C>T	ENSP00000341083.2:p.Arg280Trp	1.20E-05 24
9	35794093	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.863A>G	ENSP00000341083.2:p.Glu288Gly	1.20E-05 25
9	35794630	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.886A>G	ENSP00000341083.2:p.Ile296Val	19
9	35794634	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.890C>T	ENSP00000341083.2:p.Thr297Met	3.98E-06 26
9	35794645	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.901C>T	ENSP00000341083.2:p.Pro301Ser	25
9	35794660	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.916T>G	ENSP00000341083.2:p.Tyr306Asp	28
9	35794679	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.935G>C	ENSP00000341083.2:p.Arg312Pro	24

Figure 7 cont'd Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
9	35794697	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.953G>A	ENSP00000341083.2:p.Arg318Gln	7.95E-05
9	35794699	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.955G>C	ENSP00000341083.2:p.Glu319Gln	17
9	35799714	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.970G>C	ENSP00000341083.2:p.Glu324Gln	1.19E-05 22
9	35799715	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.971A>T	ENSP00000341083.2:p.Glu324Val	23
9	35800041	['G', 'GCTT	1	1.00	['inframe_insertion']	MODERATE	NPR2	ENST00000342694.6:c.1010_1012dup	ENSP00000341083.2:p.Phe337dup	3.98E-06
9	35800104	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1070C>T	ENSP00000341083.2:p.Thr357Ile	5.17E-05 21
9	35800110	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1076A>C	ENSP00000341083.2:p.Glu359Ala	19
9	35800122	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1088G>A	ENSP00000341083.2:p.Arg363Gln	5.57E-05 22
9	35800124	['AT', 'A']	3	1.00	['frameshift_variant'	HIGH	NPR2	ENST00000342694.6:c.1092del	ENSP00000341083.2:p.Ile364MetfsTer13	7.95E-06
9	35800133	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1099A>G	ENSP00000341083.2:p.Lys367Glu	21
9	35800142	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1108G>A	ENSP00000341083.2:p.Gly370Arg	1.19E-05 24
9	35800427	['C', 'T']	2	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.1162C>T	ENSP00000341083.2:p.Arg388Ter	1.19E-05 35
9	35800720	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1230C>G	ENSP00000341083.2:p.His410Gln	18
9	35800733	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1243G>A	ENSP00000341083.2:p.Glu415Lys	21
9	35800752	['C', 'T']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1262C>T	ENSP00000341083.2:p.Thr421Met	7.16E-05 21
9	35800757	['C', 'T']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1267C>T	ENSP00000341083.2:p.Arg423Trp	2.39E-05 24
9	35800758	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1268G>A	ENSP00000341083.2:p.Arg423Gln	1.19E-05 22
9	35800782	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1292C>T	ENSP00000341083.2:p.Ala431Val	12
9	35800791	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1301C>T	ENSP00000341083.2:p.Ser434Leu	1.99E-05 15
9	35800803	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1313C>A	ENSP00000341083.2:p.Pro438His	23
9	35800814	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1324G>A	ENSP00000341083.2:p.Asp442Asn	3.98E-06 23
9	35800815	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1325A>G	ENSP00000341083.2:p.Asp442Gly	3.98E-06 24
9	35801090	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1372A>G	ENSP00000341083.2:p.Ile458Val	2.39E-05 21
9	35801091	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1373T>C	ENSP00000341083.2:p.Ile458Thr	7.95E-06 24
9	35801118	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1400T>G	ENSP00000341083.2:p.Phe467Cys	25
9	35801125	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1407G>A	ENSP00000341083.2:p.Met469Ile	1.99E-05 16
9	35801154	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1436G>A	ENSP00000341083.2:p.Arg479Gln	7.96E-06 35
9	35801646	['G', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1440G>C	ENSP00000341083.2:p.Lys480Asn	1.59E-05 23
9	35801684	['G', 'A']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1478G>A	ENSP00000341083.2:p.Arg493His	7.96E-06 33
9	35801722	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1516C>T	ENSP00000341083.2:p.Arg506Cys	1.59E-05 24
9	35801723	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1517G>A	ENSP00000341083.2:p.Arg506His	0.000119 28
9	35801728	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1522C>G	ENSP00000341083.2:p.His508Asp	3.98E-06 22
9	35801744	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1538G>A	ENSP00000341083.2:p.Ser513Asn	29
9	35801759	['C', 'T']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1553C>T	ENSP00000341083.2:p.Ser518Leu	7.95E-06 25
9	35801941	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1561T>C	ENSP00000341083.2:p.Ser522Pro	31
9	35801942	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1573G>A	ENSP00000341083.2:p.Gly525Ser	24
9	35801989	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1621G>A	ENSP00000341083.2:p.Gly541Ser	0.000151 32
9	35802209	['A', 'T']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1636A>T	ENSP00000341083.2:p.Asn546Tyr	3.18E-05 27
9	35802218	['G', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1645G>A	ENSP00000341083.2:p.Ala549Thr	1.19E-05 30
9	35802230	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1657G>C	ENSP00000341083.2:p.Val553Leu	22
9	35802246	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1673T>C	ENSP00000341083.2:p.Ile558Thr	7.97E-06 27
9	35802249	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1676A>G	ENSP00000341083.2:p.Glu559Gly	29
9	35802263	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1690G>C	ENSP00000341083.2:p.Val564Leu	23
9	35802545	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1753G>A	ENSP00000341083.2:p.Ala585Thr	3.98E-06 28
9	35802609	['T', 'G']	1	1.00	['splice_donor_varia	HIGH	NPR2	ENST00000342694.6:c.1815+2T>G		34
9	35802749	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1883C>G	ENSP00000341083.2:p.Asp611Glu	18
9	35802761	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1895G>C	ENSP00000341083.2:p.Leu615Phe	27
9	35802774	['C', 'T']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1858C>T	ENSP00000341083.2:p.Arg620Cys	3.98E-06 28
9	35802802	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1886A>G	ENSP00000341083.2:p.Lys629Arg	28
9	35805565	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.1942T>G	ENSP00000341083.2:p.Ser648Ala	26
9	35805638	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2015C>T	ENSP00000341083.2:p.Ala672Val	16
9	35805648	['T', 'A']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2025T>A	ENSP00000341083.2:p.Asp675Glu	17
9	35805656	['A', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2033A>G	ENSP00000341083.2:p.His678Arg	24
9	35805661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2038C>T	ENSP00000341083.2:p.Leu680Phe	3.98E-06 21
9	35805664	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2041T>C	ENSP00000341083.2:p.Tyr681His	25
9	35805803	['A', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2051A>C	ENSP00000341083.2:p.Lys684Thr	27
9	35805844	['G', 'T']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2062G>T	ENSP00000341083.2:p.Ala688Ser	7.96E-06 25
9	35805869	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2087C>T	ENSP00000341083.2:p.Pro696Leu	3.98E-06 16
9	35805873	['G', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2091G>T	ENSP00000341083.2:p.Leu697Phe	3.98E-06 19
9	35805895	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2113G>A	ENSP00000341083.2:p.Ala705Thr	3.98E-06 28
9	35805901	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2119G>A	ENSP00000341083.2:p.Val707Ile	1.99E-05 22
9	35805932	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2150T>C	ENSP00000341083.2:p.Ile717Thr	1.20E-05 28

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
9	35805941	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2159G>A	ENSP00000341083.2:p.Arg720His	7.39E-05 37
9	35805945	['T', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2163T>A	ENSP00000341083.2:p.Ser721Arg	7.97E-06 18
9	35805961	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2179G>C	ENSP00000341083.2:p.Glu727Gln	24
9	35806069	['T', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2287T>G	ENSP00000341083.2:p.Ile736Met	7.95E-06 24
9	35806090	['TCAG', 'T	1	1.00	['inframe_deletion']	MODERATE	NPR2	ENST00000342694.6:c.2231_2233del	ENSP00000341083.2:p.Gln744del	24
9	35806095	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2234G>A	ENSP00000341083.2:p.Arg745Gln	2.39E-05 22
9	35806103	['T', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2242T>A	ENSP00000341083.2:p.Phe748Ile	27
9	35806121	['C', 'T']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2260C>T	ENSP00000341083.2:p.Arg754Trp	7.95E-05 24
9	35806129	['A', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2268A>C	ENSP00000341083.2:p.Gln756His	15
9	35806157	['G', 'C']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2296G>C	ENSP00000341083.2:p.Glu766Gln	7.95E-06 21
9	35806161	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2300G>A	ENSP00000341083.2:p.Arg767Gln	1.19E-05 26
9	35806187	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2326C>T	ENSP00000341083.2:p.Arg776Trp	7.96E-06 26
9	35806220	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2359C>T	ENSP00000341083.2:p.Arg787Trp	0.000291 28
9	35806221	['G', 'C']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2360G>C	ENSP00000341083.2:p.Arg787Pro	28
9	35806223	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2360G>A	ENSP00000341083.2:p.Arg787Gln	3.98E-06 27
9	35806223	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2362C>T	ENSP00000341083.2:p.Arg788Cys	2.79E-05 27
9	35806429	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2410C>T	ENSP00000341083.2:p.Arg804Cys	3.98E-06 28
9	35806448	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2429A>T	ENSP00000341083.2:p.Asn810Ile	25
9	35806474	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2455C>T	ENSP00000341083.2:p.Arg819Cys	1.99E-05 27
9	35806501	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2482C>T	ENSP00000341083.2:p.Arg828Cys	29
9	35806506	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2487G>C	ENSP00000341083.2:p.Lys829Asn	25
9	35807045	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2542C>T	ENSP00000341083.2:p.Arg848Trp	1.59E-05 24
9	35807053	['G', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2550G>C	ENSP00000341083.2:p.Glu850Asp	3.98E-06 24
9	35806448	['A', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2582C>T	ENSP00000341083.2:p.Thr861Ile	27
9	35807129	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2626G>A	ENSP00000341083.2:p.Glu876Lys	31
9	35807144	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2641C>G	ENSP00000341083.2:p.Gln881Glu	23
9	35808509	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2713G>A	ENSP00000341083.2:p.Val905Met	4.02E-06 35
9	35808519	['T', 'C']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2723T>C	ENSP00000341083.2:p.Ile908Thr	6.42E-05 29
9	35808545	['G', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2749G>C	ENSP00000341083.2:p.Gly917Arg	32
9	35808558	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2762G>A	ENSP00000341083.2:p.Arg921Gln	1.20E-05 33
9	35808591	['G', 'A']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2795G>A	ENSP00000341083.2:p.Arg932His	3.19E-05 29
9	35808608	['C', 'G']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2812C>G	ENSP00000341083.2:p.Leu938Val	24
9	35808613	['T', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2817T>A	ENSP00000341083.2:p.Asp939Glu	17
9	35808621	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2825C>A	ENSP00000341083.2:p.Ser942Tyr	18
9	35808629	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2833C>T	ENSP00000341083.2:p.Arg945Cys	3.18E-05 25
9	35808644	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2848C>A	ENSP00000341083.2:p.Pro950Thr	26
9	35808663	['T', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2867T>A	ENSP00000341083.2:p.Leu956Gln	1.99E-05 32
9	35808665	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2869C>T	ENSP00000341083.2:p.Arg957Cys	1.19E-05 31
9	35808669	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.2873T>C	ENSP00000341083.2:p.Ile958Thr	7.95E-06 29
9	35809177	['C', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3008C>A	ENSP00000341083.2:p.Ser1003Tyr	25
9	35809180	['C', 'T']	4	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3011C>T	ENSP00000341083.2:p.Thr1004Ile	2.39E-05 24
9	35809186	['A', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3017A>C	ENSP00000341083.2:p.Lys1006Thr	3.98E-06 27
9	35809194	['C', 'G']	2	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3025C>G	ENSP00000341083.2:p.Leu1009Val	24
9	35809206	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3037G>A	ENSP00000341083.2:p.Gly1013Arg	28
9	35809227	['CGG', 'C	1	1.00	['frameshift_variant'	HIGH	NPR2	ENST00000342694.6:c.3063_3064del	ENSP00000341083.2:p.Asp1022CysfsTer35	
9	35809244	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3075G>A	ENSP00000341083.2:p.Met1025Ile	1.19E-05 24
9	35809395	['C', 'T']	2	1.00	['stop_gained']	HIGH	NPR2	ENST00000342694.6:c.3094C>T	ENSP00000341083.2:p.Arg1032Ter	40
9	35809396	['G', 'A']	3	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3095G>A	ENSP00000341083.2:p.Arg1032Gln	3.18E-05 24
9	35809413	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3112G>A	ENSP00000341083.2:p.Gly1038Arg	29
9	35809419	['C', 'T']	7	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3118C>T	ENSP00000341083.2:p.Arg1040Trp	3.18E-05 25
9	35809420	['G', 'A']	6	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3119G>A	ENSP00000341083.2:p.Arg1040Gln	7.56E-05 19
9	35809428	['C', 'T']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3127C>T	ENSP00000341083.2:p.Pro1043Ser	18
9	35809434	['G', 'A']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3133G>A	ENSP00000341083.2:p.Gly1045Arg	37
9	35809436	['AC', 'A']	1	1.00	['frameshift_variant'	HIGH	NPR2	ENST00000342694.6:c.3136del	ENSP00000341083.2:p.Leu1046SerfsTer4	
9	35809441	['T', 'C']	1	1.00	['missense_variant']	MODERATE	NPR2	ENST00000342694.6:c.3140T>C	ENSP00000341083.2:p.Leu1047Pro	25
15	98649583	['T', 'C']	1	1.00	['start_lost']	HIGH	IGF1R	ENST00000650285.1:c.2T>C	ENSP00000497069.1:p.Met1?	23
15	98649597	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.16G>A	ENSP00000497069.1:p.Gly6Arg	0.000605 22
15	98649609	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.28C>T	ENSP00000497069.1:p.Pro10Ser	1.21E-05 21
15	98649610	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.29C>T	ENSP00000497069.1:p.Pro10Leu	4.05E-06 20
15	98649636	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.55C>G	ENSP00000497069.1:p.Leu19Val	1.21E-05 15
15	98649636	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.55C>T	ENSP00000497069.1:p.Leu19Phe	1.62E-05 15
15	98649640	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.59C>T	ENSP00000497069.1:p.Ser20Phe	3.24E-05 20

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnomAD_AF	vep.cadd_phred
15	98649657	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.71C>G	ENSP00000497069.1:p.Ser24Trp	4.05E-06	21
15	98649634	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.73C>T	ENSP00000497069.1:p.Leu25Phe		21
15	98649661	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.80C>T	ENSP00000497069.1:p.Pro27Leu	1.62E-05	23
15	98649674	['AAGTG',	1	1.00	['splice_donor_variar	HIGH	IGF1R	ENST00000650285.1:c.94+3_94+6del		3.64E-05	
15	98707651	['C', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.181C>A	ENSP00000497069.1:p.Leu62Met		25
15	98707667	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.200C>G	ENSP00000497069.1:p.Ala67Gly		19
15	98707679	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.212G>A	ENSP00000497069.1:p.Arg71His	2.39E-05	25
15	98707703	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.236C>T	ENSP00000497069.1:p.Thr79Met	1.59E-05	26
15	98707808	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.341A>G	ENSP00000497069.1:p.Asn114Ser		23
15	98707838	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.371A>G	ENSP00000497069.1:p.Asn124Ser	1.59E-05	19
15	98707849	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.382A>G	ENSP00000497069.1:p.Ile128Val	3.98E-06	22
15	98707868	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.401G>A	ENSP00000497069.1:p.Arg134Lys	3.98E-06	21
15	98707938	['T', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.491T>G	ENSP00000497069.1:p.Val164Gly		22
15	98707976	['T', 'C']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.509T>C	ENSP00000497069.1:p.Val170Ala	3.98E-06	23
15	98708024	['TGGA', 'T	1	1.00	['inframe_deletion']	MODERATE	IGF1R	ENST00000650285.1:c.562_564del	ENSP00000497069.1:p.Glu188del		
15	98708072	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.605A>G	ENSP00000497069.1:p.Asn202Ser	0.000137	17
15	98891348	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.664C>T	ENSP00000497069.1:p.Arg222Trp	0.00011	23
15	98891352	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.668C>T	ENSP00000497069.1:p.Ala223Val	4.05E-06	23
15	98891390	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.706C>G	ENSP00000497069.1:p.Leu236Val		24
15	98891405	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.721G>A	ENSP00000497069.1:p.Ala241Thr	8.00E-06	16
15	98891417	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.733G>A	ENSP00000497069.1:p.Asp245Asn	4.00E-06	18
15	98891420	['A', 'G']	6	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.736A>G	ENSP00000497069.1:p.Thr246Ala	2.80E-05	17
15	98891439	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.755G>A	ENSP00000497069.1:p.Arg252His	2.79E-05	22
15	98891469	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.785C>G	ENSP00000497069.1:p.Pro262Arg		15
15	98891478	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.794C>T	ENSP00000497069.1:p.Pro265Leu	7.96E-06	26
15	98891484	['A', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.800A>C	ENSP00000497069.1:p.Asn267Thr	1.99E-05	16
15	98891540	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.856C>G	ENSP00000497069.1:p.Leu286Val		13
15	98891564	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.880G>A	ENSP00000497069.1:p.Glu294Lys	8.09E-06	17
15	98891606	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.922C>T	ENSP00000497069.1:p.Pro308Ser		26
15	98891622	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.938G>A	ENSP00000497069.1:p.Arg313His	4.18E-06	24
15	98891625	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.941A>G	ENSP00000497069.1:p.Asn314Ser		18
15	98891628	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.944G>A	ENSP00000497069.1:p.Gly315Asp		12
15	98891633	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.949C>G	ENSP00000497069.1:p.Gln317Glu		16
15	98896760	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.957G>A	ENSP00000497069.1:p.Met319Ile		23
15	98896789	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.986C>T	ENSP00000497069.1:p.Pro329Leu	7.96E-06	23
15	98896794	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.991G>A	ENSP00000497069.1:p.Val331Ile	1.99E-05	21
15	98896816	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1013C>G	ENSP00000497069.1:p.Thr338Arg		17
15	98896829	['T', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1026T>G	ENSP00000497069.1:p.Asp342Glu		22
15	98896879	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1076A>G	ENSP00000497069.1:p.Asn359Ser	1.59E-05	23
15	98896884	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1081C>T	ENSP00000497069.1:p.Leu361Phe	3.98E-06	22
15	98896897	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1094G>A	ENSP00000497069.1:p.Arg365Gln		33
15	98896899	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1096C>T	ENSP00000497069.1:p.Arg366Trp	1.19E-05	24
15	98896988	['T', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1114T>A	ENSP00000497069.1:p.Ser372Thr		22
15	98899518	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1144G>A	ENSP00000497069.1:p.Glu382Lys		28
15	98899536	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1162G>A	ENSP00000497069.1:p.Val388Met	0.000577	26
15	98899542	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1168A>G	ENSP00000497069.1:p.Ile390Val		23
15	98899568	['C', 'CT']	1	1.00	['frameshift_variant'	HIGH	IGF1R	ENST00000650285.1:c.1196dup	ENSP00000497069.1:p.Leu399ProfsTer71	3.98E-06	
15	98899591	['G', 'A']	7	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1217G>A	ENSP00000497069.1:p.Arg406His	3.18E-05	24
15	98908740	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1303G>A	ENSP00000497069.1:p.Asp435Asn	7.96E-06	22
15	98908800	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1363T>C	ENSP00000497069.1:p.Cys455Arg		28
15	98908818	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1381C>T	ENSP00000497069.1:p.Arg461Cys		28
15	98908819	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1382G>A	ENSP00000497069.1:p.Arg461His	1.99E-05	23
15	98908834	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1397C>T	ENSP00000497069.1:p.Thr466Met	3.98E-06	27
15	98908848	['C', 'T']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1411C>T	ENSP00000497069.1:p.Arg471Cys	1.20E-05	27
15	98908849	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1412G>A	ENSP00000497069.1:p.Arg471His	4.78E-05	25
15	98911326	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1474G>A	ENSP00000497069.1:p.Val492Ile	0.000569	17
15	98911333	['A', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1481A>T	ENSP00000497069.1:p.His494Leu	3.98E-06	24
15	98911354	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1502C>T	ENSP00000497069.1:p.Ser501Leu	0.000358	15
15	98911357	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1505A>G	ENSP00000497069.1:p.Lys502Arg		23
15	98911362	['C', 'T']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1510C>T	ENSP00000497069.1:p.Arg504Cys	1.19E-05	28
15	98911383	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1531C>T	ENSP00000497069.1:p.Arg511Trp	2.39E-05	25
15	98911390	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1538G>A	ENSP00000497069.1:p.Arg513Gln	7.95E-06	23

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom.vep.cadd_phred
15	98911414	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1567T>C	ENSP00000497069.1:p.Ile523Thr	25
15	98911417	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1565G>C	ENSP00000497069.1:p.Ser522Thr	23
15	98911425	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1573G>A	ENSP00000497069.1:p.Val525Ile	25
15	98911441	['C', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1589C>T	ENSP00000497069.1:p.Ala530Val	34
15	98913124	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1670C>G	ENSP00000497069.1:p.Pro557Arg	19
15	98913124	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1670C>T	ENSP00000497069.1:p.Pro557Leu	24
15	98913138	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1684G>A	ENSP00000497069.1:p.Val562Met	9
15	98913138	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1684G>T	ENSP00000497069.1:p.Val562Leu	7
15	98913147	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1693G>A	ENSP00000497069.1:p.Gly565Ser	22
15	98913160	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1706A>G	ENSP00000497069.1:p.His569Arg	19
15	98913189	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1735G>A	ENSP00000497069.1:p.Val579Ile	16
15	98913204	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1750G>A	ENSP00000497069.1:p.Val584Met	24
15	98913216	['A', 'G']	6	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1762A>G	ENSP00000497069.1:p.Met588Val	21
15	98913228	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1774G>A	ENSP00000497069.1:p.Asp592Asn	22
15	98913237	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1783C>G	ENSP00000497069.1:p.Arg595Gly	23
15	98913264	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1810A>T	ENSP00000497069.1:p.Ile604Phe	22
15	98913277	['C', 'CT']	1	1.00	['frameshift_variant'	HIGH	IGF1R	ENST00000650285.1:c.1825dup	ENSP00000497069.1:p.Ser609PhefsTer73	
15	98915984	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1849G>A	ENSP00000497069.1:p.Val617Ile	17
15	98916017	['A', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1882A>C	ENSP00000497069.1:p.Ile628Leu	19
15	98916048	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1913A>G	ENSP00000497069.1:p.Asn638Ser	26
15	98916090	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1955C>G	ENSP00000497069.1:p.Pro652Arg	22
15	98916098	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1963G>A	ENSP00000497069.1:p.Gly655Ser	15
15	98916111	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1976G>A	ENSP00000497069.1:p.Arg659Gln	19
15	98916126	['C', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.1991C>T	ENSP00000497069.1:p.Ser664Phe	23
15	98916701	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2026G>A	ENSP00000497069.1:p.Gly676Ser	22
15	98916708	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2033T>C	ENSP00000497069.1:p.Ile678Thr	19
15	98916755	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2080G>T	ENSP00000497069.1:p.Gly694Trp	24
15	98916797	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2122G>A	ENSP00000497069.1:p.Glu708Lys	23
15	98916797	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2122G>C	ENSP00000497069.1:p.Glu708Gln	22
15	98916799	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2124G>C	ENSP00000497069.1:p.Glu708Asp	21
15	98916805	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2130G>C	ENSP00000497069.1:p.Gln710His	21
15	98916845	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2170A>G	ENSP00000497069.1:p.Asn724Asp	24
15	98916856	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2181C>G	ENSP00000497069.1:p.His727Gln	23
15	98916869	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2194G>A	ENSP00000497069.1:p.Val732Met	24
15	98922156	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2210G>T	ENSP00000497069.1:p.Arg737Met	29
15	98922204	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2258G>A	ENSP00000497069.1:p.Arg753Gln	23
15	98922221	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2275G>A	ENSP00000497069.1:p.Ala759Thr	8
15	98922286	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2340C>G	ENSP00000497069.1:p.Ser780Arg	20
15	98922326	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2380C>T	ENSP00000497069.1:p.Arg794Trp	28
15	98922345	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2399G>A	ENSP00000497069.1:p.Arg800His	30
15	98922381	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2435A>G	ENSP00000497069.1:p.Lys812Arg	17
15	98922396	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2450C>T	ENSP00000497069.1:p.Ala817Val	20
15	98922406	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2460C>G	ENSP00000497069.1:p.Phe820Leu	11
15	98922407	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2461G>A	ENSP00000497069.1:p.Val821Ile	24
15	98923948	['C', 'T']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2558C>T	ENSP00000497069.1:p.Pro853Leu	19
15	98923997	['C', 'G']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.2607C>G	ENSP00000497069.1:p.Tyr869Ter	35
15	98924534	['G', 'A']	4	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2632G>A	ENSP00000497069.1:p.Gly878Lys	23
15	98924551	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2649G>T	ENSP00000497069.1:p.Gln883His	22
15	98924558	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2656A>G	ENSP00000497069.1:p.Arg886Gly	21
15	98924595	['C', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2693C>A	ENSP00000497069.1:p.Pro898Gln	26
15	98924625	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2723C>T	ENSP00000497069.1:p.Thr908Ile	23
15	98925538	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2783C>G	ENSP00000497069.1:p.Thr928Arg	14
15	98925590	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2815G>A	ENSP00000497069.1:p.Ala939Thr	17
15	98925602	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2827G>A	ENSP00000497069.1:p.Ala943Thr	18
15	98929617	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2842G>A	ENSP00000497069.1:p.Val948Met	23
15	98929644	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2869G>A	ENSP00000497069.1:p.Val957Ile	19
15	98929648	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2873T>C	ENSP00000497069.1:p.Phe958Ser	21
15	98930252	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2903G>C	ENSP00000497069.1:p.Gly968Ala	24
15	98930266	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2917T>C	ENSP00000497069.1:p.Tyr973His	32
15	98930299	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2950G>A	ENSP00000497069.1:p.Ala984Thr	23
15	98934863	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.2996T>C	ENSP00000497069.1:p.Ile999Thr	30
15	98934875	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3008G>A	ENSP00000497069.1:p.Arg1003Gln	31

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom.vep.cadd_phred
15	98934880	['C', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3013C>A	ENSP00000497069.1:p.Leu1005Ile	25
15	98934908	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3041A>G	ENSP00000497069.1:p.Tyr1014Cys	1.19E-05 32
15	98934982	['G', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3115G>T	ENSP00000497069.1:p.Ala1039Ser	7.96E-06 29
15	98934983	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3116C>T	ENSP00000497069.1:p.Ala1039Val	27
15	98934988	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3121A>G	ENSP00000497069.1:p.Met1041Val	3.98E-06 22
15	98935328	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3199G>T	ENSP00000497069.1:p.Gly1067Cys	34
15	98935380	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3251G>A	ENSP00000497069.1:p.Arg1084Gln	6.39E-06 27
15	98935392	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3263A>T	ENSP00000497069.1:p.Lys1088Ile	33
15	98935403	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3274C>T	ENSP00000497069.1:p.Arg1092Trp	6.39E-06 24
15	98935404	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3275G>A	ENSP00000497069.1:p.Arg1092Gln	31
15	98935422	['T', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3293T>C	ENSP00000497069.1:p.Met1098Thr	21
15	98939223	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3320C>T	ENSP00000497069.1:p.Pro1107Leu	28
15	98939233	['C', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3330C>G	ENSP00000497069.1:p.Ser1110Arg	7.95E-06 22
15	98939238	['T', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3335T>G	ENSP00000497069.1:p.Met1112Arg	31
15	98942935	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3470C>T	ENSP00000497069.1:p.Thr1157Met	3.98E-06 27
15	98942947	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3482A>G	ENSP00000497069.1:p.Tyr1161Cys	2.39E-05 33
15	98942959	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3494A>G	ENSP00000497069.1:p.Tyr1165Cys	33
15	98942964	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3499C>T	ENSP00000497069.1:p.Arg1167Trp	32
15	98942970	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3505G>A	ENSP00000497069.1:p.Gly1169Arg	34
15	98948627	['A', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3641A>T	ENSP00000497069.1:p.Gln1214Leu	32
15	98948645	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3659A>G	ENSP00000497069.1:p.Gln1220Arg	30
15	98948653	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3667C>T	ENSP00000497069.1:p.Arg1223Cys	33
15	98948691	['C', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3705C>A	ENSP00000497069.1:p.Asp1235Glu	19
15	98942964	['T', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3725T>A	ENSP00000497069.1:p.Phe1242Tyr	20
15	98957093	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3755A>G	ENSP00000497069.1:p.Asn1252Ser	3.98E-06 24
15	98957146	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3808A>G	ENSP00000497069.1:p.Met1270Val	19
15	98957183	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3845G>A	ENSP00000497069.1:p.Ser1282Asn	30
15	98957184	['CGAG', 'C	1	1.00	['inframe_deletion']	MODERATE	IGF1R	ENST00000650285.1:c.3850_3852del	ENSP00000497069.1:p.Glu1284del	1.99E-05
15	98957185	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3847G>A	ENSP00000497069.1:p.Glu1283Lys	0.000103 25
15	98957206	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3868C>T	ENSP00000497069.1:p.Pro1290Ser	9
15	98957207	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3869C>T	ENSP00000497069.1:p.Pro1290Leu	3.58E-05 17
15	98957214	['G', 'GCTC	1	1.00	['inframe_insertion']	MODERATE	IGF1R	ENST00000650285.1:c.3882_3887dup	ENSP00000497069.1:p.Asp1294_Leu1295dup	3.98E-06
15	98957221	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3883C>G	ENSP00000497069.1:p.Leu1295Val	20
15	98957239	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3901G>A	ENSP00000497069.1:p.Glu1301Lys	32
15	98957270	['C', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3932C>T	ENSP00000497069.1:p.Ser1311Leu	8.00E-05 22
15	98957273	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3935C>T	ENSP00000497069.1:p.Ser1312Phe	23
15	98957290	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3952G>C	ENSP00000497069.1:p.Asp1318His	24
15	98957290	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3952G>T	ENSP00000497069.1:p.Asp1318Tyr	1.20E-05 21
15	98957300	['C', 'A']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.3962C>A	ENSP00000497069.1:p.Ser1321Ter	3.99E-06 47
15	98957307	['C', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3969C>G	ENSP00000497069.1:p.His1323Gln	16
15	98957317	['A', 'G']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3979A>G	ENSP00000497069.1:p.Asn1327Asp	3.60E-05 21
15	98957326	['G', 'A']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.3988G>A	ENSP00000497069.1:p.Gly1330Ser	5.20E-05 14
15	98957341	['G', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4003G>T	ENSP00000497069.1:p.Val1335Phe	23
15	98957348	['G', 'A']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4010G>A	ENSP00000497069.1:p.Arg1337His	2.00E-05 26
15	98957362	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4024G>A	ENSP00000497069.1:p.Glu1342Lys	4.00E-06 25
15	98957366	['G', 'T']	5	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4028G>T	ENSP00000497069.1:p.Arg1343Ile	2.00E-05 23
15	98957383	['A', 'G']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4045A>G	ENSP00000497069.1:p.Met1349Val	8.02E-06 26
15	98957393	['G', 'C']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4055G>C	ENSP00000497069.1:p.Gly1352Ala	28
15	98957395	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4057C>T	ENSP00000497069.1:p.Arg1353Cys	8.04E-06 28
15	98957404	['G', 'A']	3	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4066G>A	ENSP00000497069.1:p.Glu1356Lys	4.83E-05 25
15	98957404	['G', 'C']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4066G>C	ENSP00000497069.1:p.Glu1356Gln	25
15	98957407	['C', 'T']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4069C>T	ENSP00000497069.1:p.Arg1357Trp	0.00025 25
15	98957408	['G', 'A']	2	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4070G>A	ENSP00000497069.1:p.Arg1357Gln	3.22E-05 32
15	98957425	['C', 'T']	1	1.00	['stop_gained']	HIGH	IGF1R	ENST00000650285.1:c.4087C>T	ENSP00000497069.1:p.Gln1363Ter	8.07E-06 52
15	98957432	['C', 'T']	1	1.00	['missense_variant']	MODERATE	IGF1R	ENST00000650285.1:c.4094C>T	ENSP00000497069.1:p.Ser1365Leu	8.08E-06 26
X	630910	['A', 'G']	1	1.00	['missense_variant']	MODERATE	5UTR	ENST00003815786.c.13A>G	ENSP00003815786.p.Thr5Ala	7.99E-06 25
X	630922	['T', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00003815786.c.25T>C	ENSP00003815786.p.Ser9Pro	25
X	630950	['A', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00003815786.c.53A>T	ENSP00003815786.p.Asp18Val	22
X	630981	['TAAG', 'T	3	1.00	['inframe_deletion']	MODERATE	SHOX	ENST00003815786.c.88_90del	ENSP00003815786.p.Lys30del	3.99E-06
X	631004	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00003815786.c.107G>A	ENSP00003815786.p.Arg36Gln	26
X	631028	['C', 'A']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00003815786.c.131C>A	ENSP00003815786.p.Ala44Glu	23
X	631045	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00003815786.c.148G>A	ENSP00003815786.p.Gly50Arg	22

Figure 7 cont'd

Genetic variants found in the 5 Core genes

chr	position	alleles	AC	variant_q	vep.consequence	vep.impact	vep.symbol	vep.hgvsc	vep.hgvsp	vep.gnom:vep.cadd_phred
X	631074	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.177C>G	ENSP00000370990.1:p.Ile59Met	0
X	631087	['G', 'C']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.190G>C	ENSP00000370990.1:p.Gly64Arg	20
X	631097	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.200C>T	ENSP00000370990.1:p.Pro67Leu	23
X	631108	['T', 'C']	4	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.211T>C	ENSP00000370990.1:p.Phe71Leu	21
X	631115	['A', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.218A>G	ENSP00000370990.1:p.Asp73Gly	20
X	631163	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.266G>C	ENSP00000370990.1:p.Arg89Thr	22
X	631169	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.272C>T	ENSP00000370990.1:p.Ala91Val	10
X	634631	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.291C>G	ENSP00000370990.1:p.Cys97Trp	24
X	634635	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.295G>A	ENSP00000370990.1:p.Glu99Lys	23
X	634637	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.297G>C	ENSP00000370990.1:p.Glu99Asp	7
X	634668	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.328G>T	ENSP00000370990.1:p.Asp110Tyr	26
X	634671	['G', 'C']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.331G>C	ENSP00000370990.1:p.Gly111Arg	22
X	634676	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.336G>C	ENSP00000370990.1:p.Gln112His	24
X	634689	['CAG', 'C']	1	1.00	['frameshift_variant'	HIGH	SHOX	ENST00000381578.6:c.352_353del	ENSP00000370990.1:p.Arg118AlafsTer63	
X	634696	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.356G>A	ENSP00000370990.1:p.Arg119His	24
X	634705	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.365C>G	ENSP00000370990.1:p.Thr122Ser	4.01E-06 24
X	634719	['G', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.379G>C	ENSP00000370990.1:p.Glu127Gln	25
X	634741	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.401G>A	ENSP00000370990.1:p.Arg134Gln	25
X	634743	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.403C>T	ENSP00000370990.1:p.Leu135Phe	23
X	634771	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.431C>G	ENSP00000370990.1:p.Ala144Gly	24
X	634776	['A', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.436A>C	ENSP00000370990.1:p.Met146Leu	24
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X	640851	['C', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.517C>A	ENSP00000370990.1:p.Arg173Ser	19
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X	641037	['C', 'T']	2	1.00	['stop_gained']	HIGH	SHOX	ENST00000381578.6:c.583C>T	ENSP00000370990.1:p.Arg195Ter	35
X	641038	['G', 'A']	2	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.584G>A	ENSP00000370990.1:p.Arg195Gln	25
X	641052	['G', 'A']	3	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.598G>A	ENSP00000370990.1:p.Val200Ile	22
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X	641065	['C', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.611C>A	ENSP00000370990.1:p.Ala204Asp	23
X	641068	['T', 'C']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.614T>C	ENSP00000370990.1:p.Leu205Ser	23
X	644397	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.640G>A	ENSP00000370990.1:p.Ala214Thr	25
X	644418	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.661G>A	ENSP00000370990.1:p.Val221Met	26
X	644422	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.665C>T	ENSP00000370990.1:p.Ala222Val	23
X	644423	['C', 'CCAG	1	1.00	['inframe_insertion']	MODERATE	SHOX	ENSTUU0003815786:c.670_681dup	ENSP00000370990.1:p.Ala224_His227dup	3.36E-05
X	644427	['G', 'A']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.670G>A	ENSP00000370990.1:p.Ala224Thr	22
X	644428	['CGCACCC	3	1.00	['inframe_deletion']	MODERATE	SHOX	ENST00000381578.6:c.685_696del	ENSP00000370990.1:p.His229_Leu232del	
X	644434	['C', CGCA	1	1.00	['inframe_insertion']	MODERATE	SHOX	ENST00000381578.6:c.683_684dup	ENSP00000370990.1:p.Leu228_His229dup	
X	644464	['C', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.707C>T	ENSP00000370990.1:p.Ala236Val	25
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X	644539	['C', 'G']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.782C>G	ENSP00000370990.1:p.Ala261Gly	19
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X	644586	['G', 'A']	5	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.829G>A	ENSP00000370990.1:p.Ala277Thr	27
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X	644608	['G', 'A']	2	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.851G>A	ENSP00000370990.1:p.Arg284Gln	25
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X	644622	['G', 'T']	1	1.00	['missense_variant']	MODERATE	SHOX	ENST00000381578.6:c.865G>T	ENSP00000370990.1:p.Ala289Ser	24

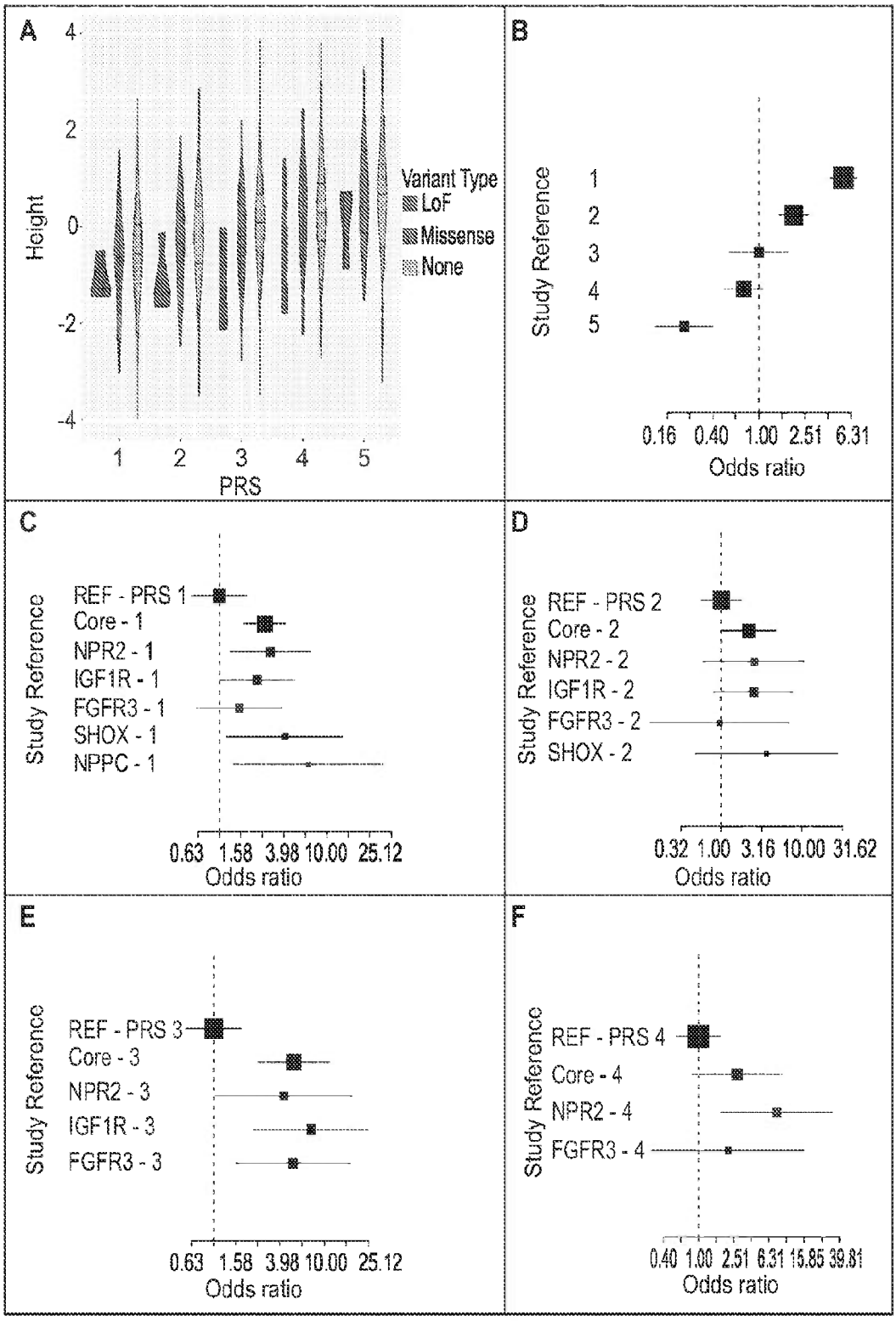


Figure 8A to 8F

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/051100

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/54 A61K38/22 A61P19/08 C07K14/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/135541 A2 (BIOMARIN PHARM INC [US]; WENDT DANIEL J [US] ET AL.) 25 November 2010 (2010-11-25) cited in the application eg [0550], [0747]; sequence 145 -----	1-6,8,9, 13,14, 17, 20-23, 25,39-42
X	WO 2017/118698 A1 (ASCENDIS PHARMA GROWTH DISORDERS AS [DK]) 13 July 2017 (2017-07-13) eg page 35, line 14, page 134, lines 11, 12 -----	1-9, 13-37, 39-42

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 2020	11/12/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schleifenbaum, A

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/051100

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		ZA 201804415 B	27-03-2019

Electronic Acknowledgement Receipt

EFS ID:	47388346
Application Number:	17811748
International Application Number:	
Confirmation Number:	2988
Title of Invention:	C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children
First Named Inventor/Applicant Name:	Jonathan Day
Customer Number:	90849
Filer:	Katherine Lynn Neville/Peter Adams
Filer Authorized By:	Katherine Lynn Neville
Attorney Docket Number:	30610/57038
Receipt Date:	18-JAN-2023
Filing Date:	11-JUL-2022
Time Stamp:	15:03:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	NPL_29_Olney_et_al_779908.pdf	151404 3142fbdce6ccd8e2d51dff374a395607231f4349	no	4

Warnings:

Information:					
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Information:					
3	Foreign Reference	FOR_1_WO9420534A1_779970.pdf	1415062 5c48dc0defaee6d76eb13efc1c3206c7ca0260a3	no	37
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4	Foreign Reference	FOR_2_WO2009067639A2_779966.pdf	6484297 1732f81182d1673384c5c77c5d8730eeaa776f3c	no	125
Warnings:					
Information:					
5	Foreign Reference	FOR_3_WO2010135541A2_779967.pdf	15782580 de17ec53864ab00f6f47d07f3c4ad93023fba525	no	257
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Information:					
6	Foreign Reference	FOR_4_WO2021030411A1_779968.pdf	6285835 10f5f0f3d624459e66843ff05bb085dd7085fe59	no	115
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7	Foreign Reference	FOR_5_WO2021055497A1_779969.pdf	4696917 a2614fdcc059e4edf5f5df907a741bb175fa5f74	no	84
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8	Non Patent Literature	NPL_3_Bartels_et_al_779925.pdf	710103 f13011ffc518c6a87b86382ed4620786fc5d53fd	no	8
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Information:					
10	Non Patent Literature	NPL_5_Chen_et_al_779930.pdf	432553 4d20f153aa7bc4df63fcc9516847988678985527c	no	6
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12	Non Patent Literature	NPL_7_Colvin_et_al_779954.pdf	12977896 95f2b7f2d96ce4fbc521b0783c89ea7601a6b361	no	8
Warnings:					
Information:					
13	Non Patent Literature	NPL_8_Feng_et_al_779934.pdf	796897 4d692bdf6301ef17fab22832bc7462e4509f996a	no	10
Warnings:					
Information:					
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17	Non Patent Literature	NPL_32_Remington_18Edi_779911_Part1.pdf	11691636 d90681116d0235bb73673211720af2f48c819932	no	89
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18	Non Patent Literature	NPL_33_Romano_et_a_779912.pdf	755556 f0459b24f68591c46b9dc9565dbe15e6735d4f8e	no	16
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19	Non Patent Literature	NPL_34_Rousseau_et_a_779939.pdf	686773 805d8692c89512df05d76ac59a25c6d2edd812f0	no	4
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 17811748 Document Date: 01/19/2023

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

Sequence Listing Report for Application No: 17811748

File Name: 17811748_2_1.txt (29371 B), **Created:** January 19, 2023, 01:51 PM
USPTO Receipt Date: January 19, 2023, 01:50 PM
Validation Date: January 19, 2023, 01:51 PM
Reviewed by: bWTwX1h6tBgtuLGCV8RZdgN7Pd03KI+O

DEFECTIVE: This validation report concerns the sequence listing filed 01-19-2023 13:50:11 and indicates the errors found by the USPTO during validation of the sequence listing. Any resolution of the errors identified in this validation report should not be construed as meaning that the sequence listing will comply with all of the requirements of 37 CFR §§ 1.821- 1.825, WIPO Standard ST.25, 37 CFR §§ 1.831-1.835 and 1.839, or WIPO Standard ST.26.
For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV

Reviewer's Comments

1. ST.25 filed when ST.26 is required:

This sequence listing was submitted in a non-provisional application with a filing date of 07/11/2022. Therefore, ST.26 format is required.

* Sequence listings submitted in either non-provisional applications filed under 35 U.S.C. 111(a) or provisional applications filed under 111(b) that have a filing date ON OR AFTER July 1, 2022 must be in XML format and comply with WIPO Standard ST.26 and 37 CFR 1.831 through 1.835.

* Sequence listings submitted in either PCT applications or U.S. national phase applications submitted under 35 U.S.C. 371(c) that have an international filing date ON OR AFTER July 1, 2022 must be in XML format and comply with WIPO Standard ST.26 and/or 37 CFR 1.831 through 1.835.

The messages shown below do not indicate non-compliance with the sequence rules, and do not require correction:

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Validated By CRFValidator v 1.5.0

Application No: 17811748 Version No: 2.1

CRF Copy Request from application Version :

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Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
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Arg Lys Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe

20 25 30

Gly Leu Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
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Tyr Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu

20 25 30

Lys Leu Asp Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
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Lys Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys
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Gly Ala Asn Lys Lys Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu
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Arg Ile Gly Ser Met Ser Gly Leu Gly Cys
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Ile Gly Ser Met Ser Gly Leu Gly Cys
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Gly Ser Met Ser Gly Leu Gly Cys
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<210> 19
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Leu Leu Gln Glu His Pro Asn Ala Arg Lys Tyr Lys Gly Ala Asn Lys
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Ser Met Ser Gly Leu Gly Cys
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Met Ser Gly Leu Gly Cys
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Ser Gly Leu Gly Cys
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Gly Leu Gly Cys
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Cys

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Gly Leu Ser Lys Gly Cys P
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Met Ser Gly Leu Gly Cys
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Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
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Met Ser Gly Leu Gly Cys
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Asn Ser Gly Leu Gly Cys

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Ser Met Ser Gly Leu Gly Cys
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Gly Leu Ser Lys Gly Cys Phe Gly Leu Lys Leu Asp Arg Ile Gly Ser
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Met Ser Gly Leu Gly Cys
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<210> 47
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G

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Jonathan Day et al.

Application No.: 17/811,748 | Confirmation No.: 2988

Filed: July 11, 2022 | Art Unit: Not Yet Assigned

For: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children | Examiner: Not Yet Assigned

RESPONSE TO NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATION CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

MS Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This paper is filed in response to the Notice to Comply With Requirements For Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, dated November 23, 2022. Please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims begin on page 3 of this paper.

Remarks/Arguments begin on page 14 of this paper.

AMENDMENT TO THE SPECIFICATION

Please replace paragraph **[0002]** in the specification filed July 11, 2022 with the following paragraph:

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification as a text file. The name of the text file containing the Sequence Listing is "~~57038_Seqlisting.xml~~ 57038_SubSeqlisting.txt", which was created on ~~July 7, 2022~~ January 18, 2023 and is ~~65,087~~ 29,400 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

IN THE SEQUENCE LISTING

Please replace the sequence listing currently of record with the computer-readable form of the replacement sequence listing (filename: 57038_SubSeqlisting.txt) submitted herewith.

AMENDMENT TO THE CLAIMS

1. (Original) A method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO:

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HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48);MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53);
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO:

54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64): and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

2. (Original) A method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO:);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO:40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59);PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

3. (Original) A method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in the subject wherein the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
15);WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
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KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC(CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC[CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
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(SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-
Gly-CNP37) (SEQ ID NO: 45);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID
NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-
CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ
ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-
CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC
[CNP27(K4,5,9R)]) (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC
[CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53);
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ
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4. (Original) The method of claim 3, wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 25% above baseline in said subject or wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 40% above baseline in said subject.

5. (Currently amended) The method of claim 3 ~~or 4~~, wherein the increase in growth velocity is assessed by measuring standing height, sitting height, weight, head circumference, upper arm length, lower arm length, upper leg length, lower leg length, hand length or foot length.

6. (Currently amended) The method of claim 3 ~~any one of claims 3 to 5~~ that results in no clinically significant change in blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration or blood aspartate aminotransferase concentration in said subject.

7. (Currently amended) The method of claim 3 ~~any one of claims 3 to 6~~ that results in

(a) a change in the upper body length to lower body length ratio of between -0.05 and 0.05;

(b) a change in the upper arm length to forearm length ratio of between -0.05 to 0.05; and/or

(c) a change in the upper leg length to lower leg length ratio of between -0.05 and 0.05.

8. (Currently amended) The method of claim 1 ~~any one of claims 1 to 7~~, wherein

(a) said composition is administered once daily;

(b) said composition is administered once daily over a period of at least 6 months; or

(c) said composition is administered once daily over a period of at least 12 months.

9. (Currently amended) The method of claim 1 ~~any one of the preceding claims~~, wherein the composition is administered to the subject via subcutaneous administration.

10. (Currently amended) The method of claim 1 ~~any one of the preceding claims~~, wherein the composition is administered daily, every other day, 3 times weekly, 2 times weekly, or once weekly.

11. (Currently amended) The method of claim 1 ~~any one of the preceding claims~~, wherein the composition is administered at a dose of 30 µg/kg.

12. (Currently amended) The method of claim 1 ~~any one of the preceding claims~~, wherein the subject is about 6 months to about 2 years old.

13. (Currently amended) The method of claim 1 ~~any one of the preceding claims~~, wherein the subject is about 0 to about 6 months old.

14. (Currently amended) The method of claim 11 ~~any one of claims 11-13~~, wherein the dosage may be decreased to 15 µg/kg when the subject is 2 years old.

15. (Currently amended) The method of claim 1, ~~any one of the preceding claims~~ wherein the skeletal dysplasia is selected from the group consisting of hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism,

osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata, homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature.

16. (Original) The method of claim 9, wherein the skeletal dysplasia is achondroplasia.

17. (Currently amended) The method of claim 1, ~~any one of the preceding claims~~ wherein the composition further comprises one or more pharmaceutical excipients.

18. (Original) The method of claim 17 wherein the composition comprises citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80.

19. (Currently amended) The method of claim 1, ~~any one of the preceding claims~~ wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); and PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24).

20. (Currently amended) The method of claim 1, ~~any one of the preceding claims~~ wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20).

21-22. (Canceled)

REMARKS

This paper is filed in response to a Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, dated November 23, 2022. The Notice also indicated that excess claims fees were due. This response is timely filed.

Amendment to the Specification

The Office suggested that the "Sequence Listing XML" filed with the application was found to contain an error or errors because the Sequence Listing was generated using WIPO Sequence version 1.0.0. Applicant submits herewith a Substitute Sequence Listing in computer-readable form that is in compliance with 37 C.F.R. §§ 1.821-1.825, and respectfully requests that the Substitute Sequence Listing be entered. No new matter has been added by way of this amendment.

The specification has been amended to update the "reference to the sequence listing" paragraph, which does not add new matter.

Amendment to the Claims

The claims are amended solely to reduce filing fees and not for reasons related to patentability. The amendments include no new matter.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 13-2855, under Order No. 30610/57038 from which the undersigned is authorized to draw.

Dated: January 19, 2023

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
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UNITED STATES
PATENT AND TRADEMARK OFFICE

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Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**01/19/2023 01:50:11 PM ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	61459173	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 5

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl.pdf		14	-	55 KB
Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl-PEFRSEQ.pdf	(1-1)	1	Response to Pre-Exam Sequence Notice	15 KB
Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl-SPEC.pdf	(2-2)	1	Specification	13 KB

Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl-CLM.pdf	(3-13)	11	Claims	44 KB
Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl-REM.pdf	(14-14)	1	Applicant Arguments/Remarks Made in an Amendment	15 KB
57038_SubSeqlisting.txt		-	Sequence Listing (Text File)	29 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Response to Notice to Comply with Requirements for Nucleotide or Amino Acid Sequence Discl.pdf	102C30A714F5D8B5798594B7F2CD65A79E7E286A0266ECDFEEA0B9F72C23D8CD510AEFE9D7CA8D74A9188760080F8751EC334097EF341C76903F621B2557D42F
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Response to Notice to Comply with Requirements for Nucleotide or Amino Acid	AE31A57A005693E5A747B3D177ED1D07369BEBFA39721BAEA37AD9016D04B91536BBA91B844C6DE8BF6913A12CD05F17CA35A6BBC25C772562BC19D69AD17D74

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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code - SEQ.XML

WIPO Type Code -

SLIC Placeholder Sheet for P-ELP (CMS) Content

Application Number: 17811748 Document Date: 01/20/2023

The presence of this form in the CMS record indicates that the following document type was received in electronic format on the date identified above. This content is stored in CMS.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access CMS content via DAV or via the SLIC web page.
- External customers may access CMS content via PAIR/Patent Center Retrieval using the Supplemental Content tab.

Form Revision Date: 07 January 2022

Application Number: 17811748

Reviewed by: 97ClN/eHe4jhmn5GMimwpAP70alPRU2kNiDwvOWHBrv8bqgC4kdzMg==

Note: This validation report concerns the sequence listing filed 01-20-2023 18:30:19 and indicates that NO errors were found by the USPTO during validation of the sequence listing. This validation report should not be construed as meaning that the sequence listing complies with all of the requirements of 37 CFR §§ 1.821-1.825, 37 CFR §§ 1.831-1.835 and 1.839, WIPO Standard ST.25, or WIPO Standard ST.26.

For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Listing Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV.

Timestamp: [year=2023; month=1; day=20; hr=18; min=47; sec=25; ms=761]

Validated By CRFValidator v 1.5.0

Application No: 17811748 Version No: 3.1

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Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Jonathan Day et al.

Application No.: 17/811,748 | Confirmation No.: 2988

Filed: July 11, 2022 | Art Unit: Not Yet Assigned

For: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children | Examiner: Not Yet Assigned

SUPPLEMENTAL RESPONSE TO NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATION CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

MS Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This paper is filed in response to a Sequence Listing Error Report dated January 19, 2023 in the above-mentioned application and received subsequent to filing a response to the Notice to Comply With Requirements For Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, dated November 23, 2022.

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**01/20/2023 06:30:19 PM ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Jeanne Brashear
PATENT CENTER #	61471756	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	-

Documents

TOTAL DOCUMENTS: 3

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
57038_SubSeqlisting.xml		-	Sequence Listing (ST.26 XML File)	65 KB
Warning: Please note that ST.23 and ST.25 sequence listings are required for applications with a filing date before July 1, 2022.				
57038_SuppResponse v.1.pdf		3	-	21 KB
57038_SuppResponse v.1-PEFRSEQ.pdf	(1-1)	1	Response to Pre-Exam Sequence Notice	14 KB
57038_SuppResponse v.1-REM.pdf	(2-3)	2	Applicant Arguments/Remarks Made in an Amendment	16 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

AMENDMENT TO THE SPECIFICATION

Please replace paragraph **[0002]** in the specification filed July 11, 2022 with the following paragraph:

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification as a text file. The name of the text file containing the Sequence Listing is "~~57038_Seqlisting.xml~~ 57038_SubSeqlisting.xml", which was created on ~~July 7, 2022~~ January 19, 2023 and is ~~65,087~~ 66,072 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

IN THE SEQUENCE LISTING

Please replace the sequence listing currently of record with the computer-readable form of the replacement sequence listing (filename: 57038_SubSeqlisting.xml) submitted herewith.

REMARKS

This paper is filed in response to a Sequence Listing Report Error received January 19, 2023 after Applicant filed a response to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, dated November 23, 2022.

Amendment to the Specification

The Office suggested that the "Sequence Listing XML" filed with the application on 7-11-22 was found to contain an error or errors because the Sequence Listing was generated using WIPO Sequence version 1.0.0. Applicant submits herewith a Substitute Sequence Listing in computer-readable form that is in compliance with 37 C.F.R. §§ 1.821-1.825, and respectfully requests that the Substitute Sequence Listing be entered. No new matter has been added by way of this amendment.

The specification has been amended to update the "reference to the sequence listing" paragraph, which does not add new matter.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 13-2855, under Order No. 30610/57038 from which the undersigned is authorized to draw.

Dated: January 20, 2023

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
17/811,748	

Applicant(s) Jonathan Day

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
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2	1					
3	1					
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/811,748

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	* 0			OR	x =	0
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x =	0
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
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	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/811,748	07/11/2022	Jonathan Day	30610/57038

CONFIRMATION NO. 2988

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

FORMALITIES LETTER

OC000000052901783

Date Mailed: 01/23/2023

NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

Filing Date Granted

The U.S. Patent and Trademark Office received your reply on 01/19/2023 to the NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES (Notice) mailed 11/23/2022 and it has been entered into the application. The reply, however, is not a complete reply because all of the items required by the NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES have not been received. See "Items Required to Avoid Abandonment" section below. A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application.

The period for reply continues to run from the date of the NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES mailed 11/23/2022. Applicant must submit all required items and pay any fees required below within two months from the date of the NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES to avoid abandonment. If the reply is submitted after two months from the date of the NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES, extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a). See "Time Period for Reply" section below.

Items Required to Avoid Abandonment:

The required items noted below SHOULD be filed along with any items required above.

Summary of Requirements for Patent Applications Filed On Or After July 1, 2022, That Have Sequence Disclosures

37 CFR 1.831(a) requires that patent applications which contain disclosures of nucleotide and/or amino acid sequences that fall within the definitions of 37 CFR 1.831(b) must contain a "Sequence Listing XML", *as a separate part of the disclosure*, which presents the nucleotide and/or amino acid sequences and associated information using the symbols and format in accordance with the requirements of 37 CFR 1.831-1.835. This "Sequence Listing XML" part of the disclosure may be submitted:

1. In accordance with 37 CFR 1.831(a) using the symbols and format requirements of 37 CFR 1.832 through 1.834 via the USPTO's patent electronic filing system (currently Sequence Listing XML files may **only** be properly filed electronically using **Patent Center**) (see Section I.1 of the Legal Framework for Patent Electronic System (https://www.uspto.gov/patents-application-process/filing-online/legal-framework-efs-web), hereinafter "Legal Framework") in XML format, together with an incorporation by reference

statement of the material in the XML file in a separate paragraph of the specification as required by 37 CFR 1.835(a)(2) or 1.835(b)(2) identifying:

a. the name of the XML file
b. the date of creation; and
c. the size of the XML file in bytes; or

2. In accordance with 37 CFR 1.831(a) using the symbols and format requirements of 37 CFR 1.832 through 1.834 on read-only optical disc(s) as permitted by 37 CFR 1.52(e)(1)(ii), labeled according to 37 CFR 1.52(e)(5), with an incorporation-by-reference statement of the material in the XML format according to 37 CFR 1.52(e)(8) and 37 CFR 1.835(a)(2) or 1.835(b)(2) in a separate paragraph of the specification identifying:
   a. the name of the XML file;
   b. the date of creation; and
   c. the size of the XML file in bytes.

SPECIFIC DEFICIENCIES AND THE REQUIRED RESPONSE TO THIS NOTICE ARE AS FOLLOWS:

- This application contains a "Sequence Listing" as a PDF file (37 CFR 1.821(c)(2)) or as physical sheets of paper (37 CFR 1.821(c)(3)). A copy of the "Sequence Listing" in computer readable form (CRF) has been submitted. However, the content of the CRF does not comply with one or more of the requirements of 37 CFR 1.822 through 1.824, as indicated in the "Error Report" that indicates the "Sequence Listing" could not be accepted. Refer to attachment or PAIR document "Computer Readable Form (CRF) for Sequence Listing – Defective" dated 01/19/2023.

  Applicant must provide:

  - A replacement "Sequence Listing" part of the disclosure, as described above in item 1); **together with**
  - **An amendment specifically directing its entry into the application in accordance with 37 CFR 1.825(b)(2)**;
  - A statement that identifies the locations of any deletions, replacements, or additions to the "Sequence Listing" as required by 37 CFR 1.825(b)(3);
  - A statement that the "Sequence Listing" includes no new matter as required b y 37 CFR 1.825(b)(5); and
  - A statement that indicates support for the amendment in the application, as filed, as required by 37 CFR 1.825(b)(4).
- If the replacement "Sequence Listing" part of the disclosure is submitted according to item 1) a) or b) above, Applicant must also provide:
  - A substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125 inserting the required incorporation-by-reference paragraph, consisting of:
    - A copy of the previously-submitted specification, with deletions shown with strikethrough or brackets and insertions shown with underlining (marked-up version);
    - A copy of the amended specification without markings (clean version); and
    - A statement that the substitute specification contains no new matter; and
    - An amendment to the specification to remove the "Sequence Listing" previously submitted as a PDF filed (37 CFR 1.821(c)(2)) or as physical sheets of paper (37 CFR 1.821(c)(3)).
- If the replacement "Sequence Listing" part of the disclosure is submitted according to item 1) c) or d) above, Applicant must also provide:
  - A CRF in accordance with 1.821(e)(1) or 1.821(e)(2) as required by 37 CFR 1.825(b)(6); and

- Statement according to item 2) a) or b) above.

Where to obtain assistance:

USPTO Sequence listing Resource Center at: https://www.uspto.gov/patents/apply/sequence-listing-resource-center.

For rules interpretation and filing requirements, call the Sequence Systems Service Center at (571) 272-2510, the Office of Patent Legal Administration at (571) 272-7701, or, for 35 U.S.C. 371 applications, the PCT Help Desk at (571) 272-4300.

For WIPO Standard ST.26 questions, call (571) 272-2510.

WIPO Sequence software is available at: https://www.wipo.int/standards/en/sequence/index.html.

For assistance with submission of a Sequence Listing XML file via Patent Center, call the Electronic Business Center at (866) 217-9197 or email ebc@uspto.gov.

Time Period for Reply:

Mail date of Notice:	11/23/2022
Last date that extension may be obtained: (Note: The petition and fee must be received by this date, or include a proper certificate of mailing under 37 CFR 1.8 with a date on or before this date, and extend the time to include this date.)	06/23/2023

The following table sets forth the possible extensions of time available, depending on when the reply and corresponding extension of time fee are received.

Length of Extension of Time	Fee under 37 CFR 1.17(a) effective March 19, 2013		
(response due on or before)	Undiscounted	Small Entity	Micro Entity
One Month (02/23/2023)	$220	$88	$44
Two Months (03/23/2023)	$640	$256	$128
Three Months (04/24/2023)	$1480	$592	$296
Four Months (05/23/2023)	$2320	$928	$464
Five Months (06/23/2023)	$3160	$1264	$632

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be addressed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

REPLY VIA ELECTRONIC FILING

A reply may be submitted via electronic filing only by registered users of the appropriate USPTO patent electronic filing system and must include:

- A separate document having the document description, "**Applicant response to Pre-Exam Formalities Notice**"; and
- the item(s) for which a reply period is set forth above.

For more information about electronic filing, contact the Patent Electronic Business Center (EBC) 1-866-217-9197 or visit https://www.uspto.gov/ebc

NOTE: If the reply is not being submitted electronically, a copy of this notice must be included.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/snguyen/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
17/811,748	07/11/2022		1980	30610/57038	20	3

CONFIRMATION NO. 2988

UPDATED FILING RECEIPT

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

OC00000052951257

Date Mailed: 01/24/2023

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Jonathan Day, Buckinghamshire, UNITED KINGDOM;
Elena Fisheleva, Novato, CA;

Applicant(s)

BIOMARIN PHARMACEUTICAL INC., Novato, CA;

Power of Attorney: The patent practitioners associated with Customer Number 90849

Domestic Priority data as claimed by applicant

This appln claims benefit of 63/350,196 06/08/2022
and claims benefit of 63/220,275 07/09/2021

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 11/22/2022

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/811,748**

Projected Publication Date: 05/04/2023

Non-Publication Request: No

Early Publication Request: No
Title

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
17/811,748	

Applicant(s) Jonathan Day

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
2	1					
3	1					
4		1				
5		2				
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Total Claims			0		0	

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/811,748

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	* 0			OR	x =	0
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x =	0
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/811,748	07/11/2022	Jonathan Day	30610/57038

CONFIRMATION NO. 2988

90849
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

PUBLICATION NOTICE



Title:C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Publication No.US-2023-0140311-A1
Publication Date:05/04/2023

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 10/06/2023

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER
HA, JULIE

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
10/06/2023	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mgbdocket@marshallip.com

Office Action Summary	Application No. 17/811,748	Applicant(s) Day et al.	
	Examiner JULIE HA	Art Unit 1654	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 01/19/2023.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-20 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-20 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claims 21-22 have been cancelled.

Claims 1-20 are pending in this application.

DETAILED ACTION

Election/Restriction

2. Restriction to one of the following inventions is required under 35 U.S.C. 121:

1. Claims 1 and 8-20, drawn to a method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, classified in A61P19/08, for example.

2. Claims 2, drawn to a method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth, classified in A61K38/2242, for example.

3. Claims 3 and 4-7, drawn to a method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in the subject, classified in A61K38/2242, for example.

The inventions are independent or distinct, each from the other because:

3. Inventions 1-3 are directed to related Method Inventions. The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed are mutually exclusive. For example, the patient population and the end points are different. Further, search for one would not necessarily lead to the other. Furthermore, the inventions as claimed do not encompass overlapping subject matter and there is nothing of record to show them to be obvious variants.

4. Restriction for examination purposes as indicated is proper because all the inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and/or examination burden if restriction were not required because one or more of the following reasons apply:

> Each group of invention has separate classification and separate status in the art. Further, each group of invention requires a different field of search and different search terms and databases because each group of invention possesses a distinct element not possessed by the other groups of invention as set forth above.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other invention.

5. This application contains claims directed to the following patentably distinct species:

A single disclosed species of a fully defined CNP variant (e.g., SEQ ID NO: 1);

A single disclosed species of how the increase in growth velocity if measured (e.g., measuring standing height);

A single disclosed species of the end results of administering to increase growth velocity (e.g., no clinically significant change in blood hemoglobin concentration);

A single disclosed species of frequency and duration of treatment (e.g., once daily for 12 months);

A single disclosed species of route of administration (e.g., subcutaneous);

A single disclosed species of a dose amount and age of the patient (e.g., 15 μg/kg for 2 year old);

A single disclosed species of skeletal dysplasia (e.g., dwarfism);

A single disclosed species of pharmaceutical excipient (e.g., D-mannitol).

6. The species are independent or distinct because the different CNP variants involve different amino acid contents, leading to different structures. Further, search for one would not necessarily lead to the other. Different species of how growth in velocity is measured is different due to the different methods of measuring the growth velocity. Further, search for one would not necessarily lead to the other. Different end results are different because the different results being measured. Further, search for one would not necessarily lead to the other. Different species of frequency and duration of treatment involve different patient population and end points. Further, search for one would not necessarily lead to the other. Different route of administration involve different formulations, leading to different properties. Further, search for one would not necessarily lead to the other. Different skeletal dysplasia are different due to the different patient population and end points involved. Further, search for one would not necessarily lead to the other. Different pharmaceutical excipients are different due to the different components involved, leading to different properties. Further, search for one would not necessarily lead to the other. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species, or a single grouping of patentably indistinct species, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-20 are generic.

7. There is a serious search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply: There is an examination and search burden for these patentably distinct species due to their mutually exclusive characteristics. The species require a different field of search (e.g., searching different classes/subclasses or electronic resources, or employing different search queries); and/or the prior art applicable to one species would not likely be applicable to another species; and/or the species are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

FOR ANY GROUP ELECTED, Applicant is required to elect a single disclosed species of a fully defined CNP variant sequence (e.g., SEQ I DNO: 1). **If Group 1 is elected**, Applicant is **further required to elect** a single disclosed species of a skeletal dysplasia (e.g., dwarfism), a single disclosed species of the frequency and duration of administration (e.g., once daily for 12 months), a single disclosed species of dose and

age (e.g., 15 μg/kg to 2 years old), and a single disclosed species of a pharmaceutical excipient (e.g., D-mannitol). **If Group 3 is elected**, Applicant is **further required to elect** a single disclosed species of how the growth velocity is measured (e.g., by measuring standing height), and a single disclosed species of end results (e.g., no change in blood hemoglobin concentration).

The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103 or pre-AIA 35 U.S.C. 103(a) of the other species.

8. Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

9. Applicant is reminded that upon the cancelation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

CONCLUSION

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JULIE HA whose telephone number is (571)272-5982. The examiner can normally be reached Monday-Thursday 5:00 am- 6:30 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Alstrum-Acevedo can be reached on 571-272-5548. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center,

visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JULIE HA/
Primary Examiner, Art Unit 1654

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/811,748	Filing Date 07/11/2022	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	12/06/2023	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/LAJUAN D HICKSON/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**12/06/2023 11:00:30 AM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	63506192	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
Response to Restriction Requirement without Traverse.pdf	14	Response to Election / Restriction Filed	52 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Response to Restriction Requirement without Traverse.pdf	0256686CA9099F26AB4BAC253929EB8B519767732E0200FB0E E2C8B8562D2E4D54B88FB2E75A85B3631979709D1AF22DEB5 211A772A501B7AB219419C318CDD6

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Jonathan Day et al.

Application No.: 17/811,748 | Confirmation No.: 2988

Filed: July 11, 2022 | Art Unit: 1654

For: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children | Examiner: Julie Ha

RESPONSE TO RESTRICTION REQUIREMENT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This paper is filed in response to the restriction requirement set forth in the Office Action mailed October 6, 2023.

Amendments to the Claims begin on page **2** of this paper.

Remarks begin on page 13.

Amendments to the Claims

1. (Original) A method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO:

19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48);MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53);

PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64): and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

2. (Original) A method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO:);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50)

(SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO:40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59);PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

3. (Original) A method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in the subject wherein the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
15);WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);

NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC(CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC[CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)]
(SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-
Gly-CNP37) (SEQ ID NO: 45);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID
NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-
CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ
ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-
CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC
[CNP27(K4,5,9R)]) (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC
[CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53);
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ
ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-
CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-

PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

4. (Original) The method of claim 3, wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 25% above baseline in said subject or wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 40% above baseline in said subject.

5. (Previously presented) The method of claim 3, wherein the increase in growth velocity is assessed by measuring standing height, sitting height, weight, head circumference, upper arm length, lower arm length, upper leg length, lower leg length, hand length or foot length.

6. (Previously presented) The method of claim 3 that results in no clinically significant change in blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration or blood aspartate aminotransferase concentration in said subject.

7. (Previously presented) The method of claim 3 that results in

(a) a change in the upper body length to lower body length ratio of between -0.05 and 0.05;

(b) a change in the upper arm length to forearm length ratio of between -0.05 to 0.05; and/or

(c) a change in the upper leg length to lower leg length ratio of between -0.05 and 0.05.

8. (Previously presented) The method of claim 1, wherein

(a) said composition is administered once daily;

(b) said composition is administered once daily over a period of at least 6 months; or

(c) said composition is administered once daily over a period of at least 12 months.

9. (Previously presented) The method of claim 1, wherein the composition is administered to the subject via subcutaneous administration.

10. (Previously presented) The method of claim 1, wherein the composition is administered daily, every other day, 3 times weekly, 2 times weekly, or once weekly.

11. (Currently amended) The method of claim 1, wherein the composition is administered at a dose of 15 ~~30~~ µg/kg.

12. (Currently amended) The method of claim 1, wherein the subject is less than about 5 years old ~~about 6 months to about 2 years old~~.

13. (Previously presented) The method of claim 1, wherein the subject is about 0 to about 6 months old.

14. (Previously presented) The method of claim 11, wherein the dosage may be decreased to 15 µg/kg when the subject is 2 years old.

15. (Previously presented) The method of claim 1, wherein the skeletal dysplasia is selected from the group consisting of hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia

punctata, homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), and idiopathic short stature.

16. (Original) The method of claim 9, wherein the skeletal dysplasia is achondroplasia.

17. (Previously presented) The method of claim 1, wherein the composition further comprises one or more pharmaceutical excipients.

18. (Original) The method of claim 17 wherein the composition comprises citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80.

19. (Previously presented) The method of claim 1, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); and PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24).

20. (Previously presented) The method of claim 1, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20).

21-22. (Canceled)

REMARKS

Amendment to the claims

Support for the amendment to the claims is found throughout the application. Support for amendment to claim 11 is found, for example, in paragraphs [0025], [0144] and Example 1. Support for claim 12 is found, for example, in paragraphs [0013], [0016], and [0162]. The amendments include no new matter.

Election

The Examiner has required restriction between the following inventions:

Group I: Claims 1 and 8-20, drawn to a method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject;

Group II: Claim 2, drawn to a method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth;

Group III: Claims 3 and 4-7, drawn to a method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in the subject.

Applicant elects Group I for further prosecution.

The Action also required election of certain species for search purposes.

Applicant elects the following species:

-a species of CNP variant: Pro-Gly-CNP37 (SEQ ID NO: 1)

-a species of frequency of administration: daily

-a species of route of administration: subcutaneous

-a species of dose amount and age: 15 μg/kg of body weight in patients aged less than 5 years old

-a species of skeletal dysplasia: achondroplasia

-a species of pharmaceutical excipient: sodium citrate

Claims 1 and 8-20 read on the elected subject matter.

Applicant believes no fee is due with this response. The Office is invited to contact the undersigned with any questions regarding this submission.

Dated: December 6, 2023

Respectfully submitted,

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	Art Unit
	JULIE HA	1654

CPC - Searched*		
Symbol	**Date**	**Examiner**
A61K 38/2242, 38/22, 38/00, 9/0019, 38/1709, 47/10, 47/12, 47/183, 9/19	01/26/2024	JH
A61P 19/08	01/26/2024	JH
C07K 7/00, 14/00	01/26/2024	JH

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**
None	01/26/2024	JH

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**
None		01/26/2024	JH

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	**Date**	**Examiner**
STIC: sequence searched: can be accessed via PE2E or SCORE	01/23/2024;01/25/2024	JH
PE2E all inventors named searched	01/26/2024	JH
PE2E assignee searched	01/26/2024	JH
PE2E please see attached	01/26/2024	JH
PDP all inventors named searched: please see attached	01/25/2024	JH
GOOGLE search history: please see attached	01/25/2024	JH
CONTINUITY MAP searched: please see attached	01/23/2024	JH
STN search: please see attached	01/28/2024	JH

/JULIE HA/ Primary Examiner, Art Unit 1654	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	Art Unit
	JULIE HA	1654

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
STIC: sequence searched	can be accessed via PE2E or SCORE	01/23/2024;01/25/2024	JH

/JULIE HA/ Primary Examiner, Art Unit 1654	

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=> d his

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               E C-TYPE NATRIURETIC PEPTIDE/CN 5
L1             1 S E3
               E PRO-GLY-CNP37/CN 5
               E CNP37/CN 5
L2            21 S PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC/SQSP
L3           282 S LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC/SQSP
L4             0 S PQENHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC/SQSP
L5             9 S PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC/SQSP

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L7            38 S L2
L8            82 S L3
L9             0 S L4
L10         1958 S L6 OR L7 OR L8 OR L9
L11           30 S L10 AND (SKELETAL DYSPLASIA)
L12            1 S L11 AND INFANT
L13           72 S L10 AND ACHONDROPLASIA
L14            1 S L13 AND INFANT
L15            8 S L7 AND (SKELETAL DYSPLASIA)
L16           26 S L7 AND ACHONDROPLASIA
L17            1 S L15 AND INFANT
L18            1 S L16 AND INFANT
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PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
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L2	137	Day-Jonathan.in.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:28 AM
L3	2	Fisheleva-Elena.in.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:29 AM
L4	342	(Biomarin Pharmaceutical INC).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:29 AM
L5	342	(("Biomarin") near3 ("Pharmaceutical") near3 ("INC")).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:30 AM
L6	343	(("Biomarin") near3 ("Pharmaceutical")).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:30 AM
L7	24	(("Biomarin") near3 ("Pharmaceutical")).as,aanm. AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:30 AM
L8	2	L2 AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:30 AM
L9	2	L3 AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:31 AM
L10	20	(Pro-Gly-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:32 AM
L11	11	(Pro-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:32 AM
L12	0	(Pro-CNP37) AND (Gly-Pro-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:32 AM
L13	11	(Pro-CNP37) AND (Pro-Gly-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:33 AM
L14	10	"9907834".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:34 AM
L15	8	"2009067639".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:35 AM
L16	10	("7276481" OR	(US-PGPUB; USPAT;	ADJ	ON	ON	2024/01/26

		"1994020534" OR "8198242" OR "8598121" OR "7642243" OR "8658373").pn.	FPRS; EPO; JPO; DERWENT)				08:37 AM
L17	0	"1994020534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:38 AM
L18	0	"19940020534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:38 AM
L19	0	"199420534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:38 AM
L20	6	"9420534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/01/26 08:38 AM
L21	696274	(A61K38/2242 or A61K9/0019 or A61K38/1709 or A61K47/10 or A61K47/12 or A61K47/183 or A61K9/19 or A61P19/08).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:40 AM
L22	869413	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:41 AM
L23	1653	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:42 AM

		C07K7/00 OR C07K14/00).cpc. AND ((c-type natriuretic peptide) OR CNP)					
L27	19	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:43 AM
L28	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:43 AM
L29	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37) AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:43 AM
L30	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:44 AM

		(PRO-CNP37) AND (PRO-GLY-CNP37) AND ((c-type natriuretic peptide) OR CNP)					
L31	19	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:45 AM
L32	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:45 AM
L33	11	(Pro-Gly-CNP37) AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:45 AM
L34	11	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:46 AM
L35	11	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia) AND (achondroplasia)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/01/26 08:46 AM

PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

Web Search History

date, time	web site	search string
1/25/2024 8:35:10 AM	Google	skeletal dysplasia
1/25/2024 8:36:57 AM	Google	pgqehpnarkykgankkglskgcfglkldrigsmsglgc
1/25/2024 8:37:43 AM	Google	pgqehpnarkykgankkglskgcfglkldrigsmsglgc (skeletal dysplasia)
1/25/2024 8:38:45 AM	Google	(C-type natriuretic peptide) (skeletal dysplasia)
1/25/2024 9:13:21 AM	Google	(C-type natriuretic peptide) (skeletal dysplasia) (less than 2 year old)
1/25/2024 9:13:35 AM	Google	(C-type natriuretic peptide) (skeletal dysplasia) (0 to 2 year old)
1/25/2024 9:13:46 AM	Google	(C-type natriuretic peptide) (skeletal dysplasia) (infants)

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	17811748
Filing Date	2022-07-11
First Named Inventor	Jonathan Day
Art Unit	NA
Examiner Name	Not Yet Assigned
Attorney Docket Number	30610/57038

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
/J.H/	1	5352770	A	1994-10-04	MATSUO HISAYUKI	
/J.H/	2	6034231	A	2000-03-07	TANAKA et al.	
/J.H/	3	7276481	B2	2007-10-02	GOLEMBO et al.	
/J.H/	4	7642243	B2	2010-01-05	NAKAO et al.	
/J.H/	5	8198242	B2	2012-06-12	WENDT et al.	
/J.H/	6	8377884	B2	2013-02-19	WENDT et al.	
/J.H/	7	8598121	B2	2013-12-03	WENDT et al.	
/J.H/	8	8658373	B2	2014-02-25	NAKAO et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.H/

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/J.H/	9	9907834	B2	2018-03-06	BULLENS et al.	
/J.H/	10	10646550	B2	2020-05-12	BULLENS et al.	

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U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
/J.H/	1	94/20534	WO	A1	1994-09-15	MAYO FOUNDATION		☐
/J.H/	2	2009/067639	WO	A2	2009-05-28	BIOMARIN PHARM INC		☐
/J.H/	3	2010/135541	WO	A2	2010-11-25	BIOMARIN PHARM INC		☐
/J.H/	4	2021/030411	WO	A1	2021-02-18	BIOMARIN PHARM INC		☐

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/J.H/	5	2021/055497	WO	A1	2021-03-25	BIOMARIN PHARM INC		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
/J.H/	1	ALFONZO et al., Characterization of a G protein-coupled guanylyl cyclase-B receptor from bovine tracheal smooth muscle, J. Recept. Signal. Transduct. Res., 26:269-297 (2006).	☐
/J.H/	2	ALTSCHUL et al., Basic local alignment search tool, J. Mol. Biol., 215(3):403-410 (1990).	☐
/J.H/	3	BARTELS et al., Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux, Am. J. Hum. Genet., 75:27-34 (2004).	☐
/J.H/	4	BELLUS et al., Hypochondroplasia: molecular analysis of the fibroblast growth factor receptor 3 gene, Ann. N.Y. Acad. Sci., 785:182-187 (1996).	☐
/J.H/	5	CHEN et al., Next-generation sequencing identifies rare variants associated with Noonan syndrome, Proc. Natl. Acad. Sci. USA., 111(31):11473-8 (2014).	☐
/J.H/	6	COFFIN et al., Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice, Mol. Biol. Cell., 6:1861-73 (1995).	☐
/J.H/	7	COLVIN et al., Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3, Nature Genet., 12:390-397 (1996).	☐
/J.H/	8	FENG et al., Progressive sequence alignment as a prerequisite to correct phylogenetic trees, J. Mol. Evol., 25:351-360 (1987).	☐

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/J.H/	9	GARDNER et al., Molecular biology of the natriuretic peptide system: implications for physiology and hypertension, Hypertension, 49:419-426 (2007).	☐
/J.H/	10	GenBank: Accession No. NP_002512: natriuretic peptides B preproprotein [Homo sapiens], dated November 27, 2022.	☐
/J.H/	11	GenBank: Accession No. NP_006163: natriuretic peptides A preproprotein [Homo sapiens], dated December 27, 2022.	☐
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/J.H/	15	HUNT et al., Bioactivity and metabolism of C-type natriuretic peptide in normal man, J. Clin. Endocrinol. Metab., 78:1428-35 (1994).	☐
/J.H/	16	International Application No. PCT/US2022/073605, International Search Report and Written Opinion, mailed December 15, 2022.	☐
/J.H/	17	KARLIN et al., Applications and statistics for multiple high-scoring segments in molecular sequences, Proc. Natl. Acad. Sci. U.S.A., 90(12):5873-5877 (1993).	☐
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/J.H/	20	KOLLER et al., Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP), Science, 252:120-123 (1991).	☐
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/J.H/	22	LEHNINGER, Biochemistry (Worth Publishers, Inc., 4th Edition, (2004).	☐
/J.H/	23	LEVIN et al., Natriuretic Peptides, N. Engl. J. Med., 339: 321-328 (1998).	☐
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/J.H/	31	PEARSON et al., Improved tools for biological sequence comparison, Proc. Natl. Acad. Sci. U.S.A., 85(8):2444-2448 (1988).	☐
/J.H/	32	REMINGTON'S Pharmaceutical Sciences, 18th Edition, pages 1435-1712, Mack Publishing Co. (Easton, Pennsylvania (1990)),	☐
/J.H/	33	ROMANO et al., Noonan syndrome: clinical features, diagnosis, and management guidelines, Pediatrics, 126(4):746-59 (2010).	☐
/J.H/	34	ROUSSEAU et al., Missense FGFR3 mutations create cysteine residues in thanatophoric dwarfism type I (TD1), Hum. Mol. Genet., 5:509-512 (1996).	☐
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EXAMINER SIGNATURE

Examiner Signature	/JULIE HA/	Date Considered	01/25/2024

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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	Attorney Docket Number	30610/57038

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Katherine L. Neville/	Date (YYYY-MM-DD)	2023-01-18
Name/Print	Katherine L. Neville	Registration Number	53,379

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.H/

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.H/

Title: C-TYPE NATRIURETIC PEPTIDE VARIANT... **Application number:** 17/811,748

Status: 71/RESPONSE TO NON-FINAL OFFICE ACTION ENTERED AND FORWARDED TO ...

Examiner: 82939 / HA,JULIE

Filing date: 07/11/2022

GAU: 1654

Continuity/foreign: YES

IDS flag clearance

Inventor information

Inventor name	City	State/country	Inventor oath compliant?
Day, Jonathan	Buckinghamshire	UNITED KINGDOM	N/A
Fisheleva, Elena	Novato	CALIFORNIA, UNITED STATES	N/A

Applicant information

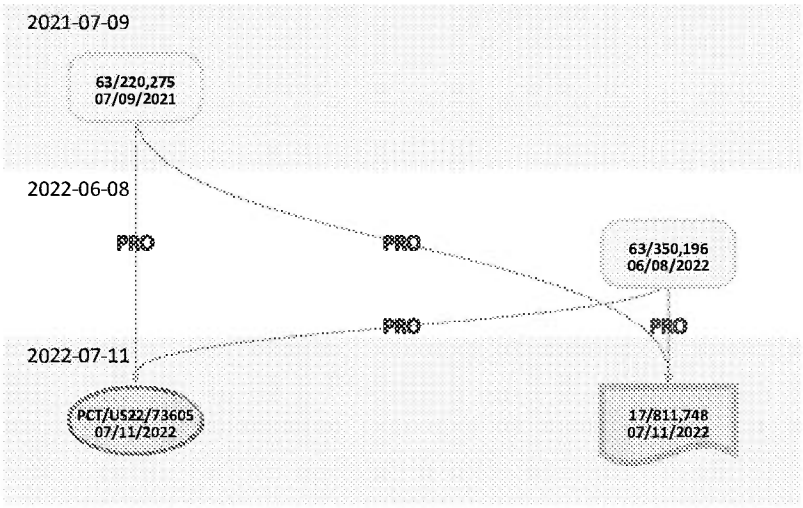
Applicant name	City	State/country
BIOMARIN PHARMACEUTICAL INC.	Novato	CALIFORNIA, UNITED STATES

Assignee information

No assignee information

Application URL: https://opsg-portal.uspto.gov/OPSGPortal/?externalurl=true&General Information=applicantInventor&applicationNo=17811748 (https://opsg-portal.uspto.gov/OPSGPortal/?externalurl=true&General Information=applicantInventor&applicationNo=17811748)

Application: 17/811,748 (01/23/2024)
Total: 4 nodes
Filter: No Filters



Bibliographic Data

Application No: 17/811,748

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /JULIE HA/ Examiner's Signature | Initials

Title: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/11/2022	514	1654	30610/57038
RULE			

APPLICANTS

BIOMARIN PHARMACEUTICAL INC., Novato, CA, UNITED STATES

INVENTORS

Jonathan Day, Buckinghamshire, UNITED KINGDOM

Elena Fisheleva, Novato, CA, UNITED STATES

CONTINUING DATA

This application has PRO of 63350196 06/08/2022

This application has PRO of 63220275 07/09/2021

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

11/22/2022

STATE OR COUNTRY

UNITED KINGDOM

ADDRESS

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606
UNITED STATES

FILING FEE RECEIVED

$1,980

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 02/01/2024

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EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
02/01/2024	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mgbdocket@marshallip.com

Office Action Summary	Application No. 17/811,748	Applicant(s) Day et al.	
	Examiner JULIE HA	Art Unit 1654	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 12/06/2023.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-20 is/are pending in the application.

5a) Of the above claim(s) 2-7 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1 and 8-20 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 07/11/2022 is/are: a)☐ accepted or b)☑ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Response to Election/Restriction filed on December 6, 2023 is acknowledged.

3. Claims 1-20 are pending in this application.

Restriction

4. Applicant's election of Group 1 (claims 1 and 8-20) and the election of the species SEQ ID NO: 1 as the species of the CNP variant, daily administration as the species of frequency of administration, subcutaneous injection as the species of route of administration, 15 μg/kg of body weight in patients aged less than 5 years old as the species of dose amount and age, achondroplasia as the species of skeletal dysplasia, sodium citrate as the species of pharmaceutical excipient in the reply filed on December 6, 2023 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.01(a)). Restriction is deemed to be proper and is made FINAL in this office action. Claims 2-7 are withdrawn from consideration pursuant to 37 CFR 1.142(b), as being drawn to nonelected inventions, there being no allowable generic or linking claim. **Claims 1 and 8-20 are examined on the merits in this office action.**

Objections

5. The abstract is objected to for the following: Applicant is reminded of the proper language and format for an abstract of the disclosure.

The abstract should be in narrative form and generally limited to a single paragraph on a separate sheet within the range of 50 to 150 words. It is important that the abstract not exceed 150 words in length since the space provided for the abstract on the computer tape used by the printer is limited. The form and legal phraseology often used in patent claims, such as "means" and "said," should be avoided. The abstract should describe the disclosure sufficiently to assist readers in deciding whether there is a need for consulting the full patent text for details.

The language should be clear and concise and should not repeat information given in the title. It should avoid using phrases which can be implied, such as, "The disclosure concerns," "The disclosure defined by this invention," "The disclosure describes," etc.

In the instant case, the abstract recites, "The present disclosure provides for use of variant..." at line 1 of the abstract. Applicant should correct these informalities. See MPEP 608.01(b). For example, the abstract may be amended to recite, "Treatment of skeletal dysplasias in children, and improvements in one or more symptoms of skeletal dysplasias by administering variants of C-type natriuretic peptide (CNP)...is described."

6. The drawings are objected to because of the following: Figure 1 has shadings in the figure. The shadings make the numbers hard to see. Corrected drawing sheets in compliance with 37 CFR 1.121(d) are required in reply to the Office action to avoid abandonment of the application. Any amended replacement drawing sheet should include all of the figures appearing on the immediate prior version of the sheet, even if only one figure is being amended. The figure or figure number of an amended drawing should not be labeled as "amended." If a drawing figure is to be canceled, the appropriate figure must be removed from the replacement sheet, and where necessary,

the remaining figures must be renumbered and appropriate changes made to the brief description of the several views of the drawings for consistency. Additional replacement sheets may be necessary to show the renumbering of the remaining figures. Each drawing sheet submitted after the filing date of an application must be labeled in the top margin as either "Replacement Sheet" or "New Sheet" pursuant to 37 CFR 1.121(d). If the changes are not accepted by the examiner, the applicant will be notified and informed of any required corrective action in the next Office action. The objection to the drawings will not be held in abeyance.

Please note, the specification has not been checked to the extent necessary to determine the presence of all possible error. Applicant's cooperation is required in correcting any errors of which applicant may become aware in the specification. MPEP § 608.01.

7. Claim 18 is objected to for the following: Claim 18 recites, "The method of claim 17 wherein the composition comprises..." Applicant is advised to amend the claim to recite, "The method of claim 17, wherein the composition comprises..."

Rejections

35 U.S.C. 112(d)

8. The following is a quotation of 35 U.S.C. 112(d):

> (d) REFERENCE IN DEPENDENT FORMS.—Subject to subsection (e), a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

The following is a quotation of pre-AIA 35 U.S.C. 112, fourth paragraph:

> Subject to the following paragraph [i.e., the fifth paragraph of pre-AIA 35 U.S.C. 112], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

9. Claims 12 and 14 are rejected under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends. Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements.

10. Claim 12 recites, "The method of claim 1, wherein the subject is less than about 5 years old". Claim 1 recites "in a subject **less than or about 2 years old**..." Claim 12 recites patient population that is broader than "in a subject less than or about 2 years old". Therefore, claim 12 does not further limit instant claim 1.

11. Claim 14 recites, "The method of claim 11, wherein the dosage may be decreased to 15 μg/kg when the subject is 2 years old." Claim 14 depends from claim 11. Claim 11 recites, "The method of claim 1, wherein the composition is administered at a dose of 15 μg/kg." Claim 11 already recites "15 μg/kg", therefore, claim 14 does not further limit instant claim 11.

35 U.S.C. 112(b)

12. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

13. Claim 15 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

14. Claim 15 recites parenthetical expressions "(Turner's syndrome/Leri Weill)" and "(Noonan's syndrome)". The metes and bounds of claim 15 is rendered vague and indefinite by the parenthetical recitations of "(Turner's syndrome/Leri Weill)" and "(Noonan's syndrome)" because it is unclear as to whether the limitation is part of the instantly claimed subject matter. See MPEP § 2173.05(d).

35 U.S.C. 103

15. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis (i.e., changing from AIA to pre-AIA) for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

16. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having

ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

17. The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

18. This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

Claim(s) 1 and 8-20 is/are rejected under 35 U.S.C. 103 as being unpatentable over Bullens et al (US Patent No. 9907834, filed with IDS) in view of Wendt et al (WO 2009/067639, filed with IDS). **Please note: during the search, instant SEQ ID NO: 41 was also found in the Bullens et al.**

19. Bullens et al teach instant SEQ ID NO: 1 (see SEQ ID NO: 5) and instant SEQ ID NO: 41 (see SEQ ID NO: 3), for treating patients with skeletal dysplasia (see column 3, lines 25-35), meeting the limitation of instant claims 1 and 19-20, in part. Bullens et al

teach that the skeletal dysplasia is selected from the group consisting of achondroplasia (see column 3, lines 51-52) and the preferred embodiment is that the subject has achondroplasia (see column 4, lines 8-9), meeting the limitation of instant claims 15-16. Bullens et al teach that the amount of CNP variant peptide administered is at least about 7.5 μg/kg, at least about 15 μg/kg, at least about 30 μg/kg or at least about 60 μg/kg (see column 4, lines 58-61), meeting the limitation of instant claims 11 and 14. Bullens et al teach that the CNP variant peptide or composition or formulation comprising the same is administered either subcutaneously or parenterally, preferably subcutaneously (see column 4, lines 62-65), meeting the limitation of instant claim 9. Bullens et al teach that the CNP variant peptide may be administered once daily, or in multiple doses over the course of treatment...in a single dose or in multiple doses, daily, every other day, every 3 days, 2 times per week...every 2 months, every 3 months...six months...twelve months, or longer (see column 5, lines 4-22), meeting the limitation of instant claims 8 and 10. Bullens et al teach that the composition or formulation comprising a CNP variant peptide comprises a pharmaceutically acceptable excipient, carrier or diluent...citric acid/citrate buffer or an acetic acid/acetate buffer (see column 6, lines 5-15)...buffering agents in the formulations may be citric acid monohydrate, sodium citrate dihydrate, or a combination of the two (see column 8, lines 10-13), meeting the limitation of instant claim 17. Bullens et al further teach that CNP variant peptide formulations comprises citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80 (see column 8, lines 38-42), meeting the limitation of instant claim 18.

The difference between the reference and the instant claims is that the reference does not teach the patient population age of less than or about 2 years of age (claim 1), the subject is less than about 5 years old (claim 12), and the subject is about 0 to about 6 months old (claim 13).

20. However, Wendt et al teach CNP variants that are useful as therapeutics for the treatment of diseases responsive to CNP, including but not limited to bone-related disorders such as, e.g., skeletal dysplasias and achondroplasia (see abstract). Wendt et al teach that the amount of CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children ages 0-17 years with achondroplasia (see p. 82, lines 12-14). Wendt et al further teach that the CNP variants described can be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) (see p. 82, lines 28-30). Wendt et al teach that the patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder...bone-related disorder is achondroplasia...the ideal age range of achondroplasia patients will be from **infant (< 1 year of age) to pre-adolescent (< 13 years of age)** (see p. 105, lines 10-15).

21. Therefore, it would have been obvious to one of ordinary skill in the art to combine the teachings of Bullens et al and Wendt et al to treat patients with diagnosed skeletal dysplasia, including achondroplasia, with CNP variant peptides, since both references teach treating achondroplasia patients with CNP variant peptides. One of ordinary skill in the art would be motivated to combine the teachings with a reasonable expectation of success, since Wendt et al teach that the amount of CNP variant used for therapy should give an acceptable rate of growth based on growth charts for children

ages 0-17 years with achondroplasia, may be administered to patients at therapeutically effective doses to treat, ameliorate or prevent bone-related disorders (e.g., skeletal dysplasias, including achondroplasia) and that the patients may be male or female, with a documented diagnosis of a potentially CNP-responsive disorder...bone-related disorder is achondroplasia...the ideal age range of achondroplasia patients will be from **infant (< 1 year of age) to pre-adolescent (< 13 years of age)**.

Therefore, the combined references is *prima facie* obvious over instant claims 1 and 8-20.

CONCLUSION

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JULIE HA whose telephone number is (571)272-5982. The examiner can normally be reached Monday-Thursday 5:00 am- 6:30 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Alstrum-Acevedo can be reached on 571-272-5548. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JULIE HA/
Primary Examiner, Art Unit 1654

DocCode - SCORE

Placeholder Sheet for Supplemental Content

Application Number: 17811748 Document Date: 08/01/2024

The presence of this form indicates that the following document type was received in electronic format on the date identified above. This document is stored separately from the typical case contents which are stored in image format.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded and no paper documents or physical media exist.

- Drawing

At the time of document entry (noted above):

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- External customers may access this supplemental content via Patent Center.

Form Revision Date: March 1, 2024

REPLACEMENT SHEET

Figure 1

	Overall		Cohort 1		Cohort 2		Cohort 3	
	Vosoritide (N=32)	**Placebo (N=32)**	**Vosoritide (N=15)**	**Placebo (N=16)**	**Vosoritide (N=8)**	**Placebo (N=8)**	**Vosoritide (N=9)**	**Placebo (N=8)**
Age on Day 1, Months								
Mean (SD)	24.39 (16.83)	27.82 (19.25)	39.62 (10.11)	44.33 (11.54)	17.00 (5.79)	16.87 (6.21)	5.56 (0.44)	5.76 (0.59)
Median	23.29	26.43	36.86	40.39	17.92	18.56	5.78	5.91
Q1, Q3	5.93, 36.58	7.67, 40.39	30.19, 49.38	34.55, 56.36	11.50, 22.52	10.46, 22.16	5.32, 5.88	5.72, 5.95
Sex, n(%)								
Male	17 (53.1)	13 (40.6)	7 (46.7)	7 (43.8)	5 (62.5)	5 (62.5)	5 (55.6)	1 (12.5)
Female	15 (46.9)	19 (59.4)	8 (53.3)	9 (56.3)	3 (37.5)	3 (37.5)	4 (44.4)	7 (87.5)
Race*								
White	21 (65.6)	25 (78.1)	8 (53.3)	13 (81.3)	6 (75.0)	6 (75.0)	7 (77.8)	6 (75.0)
Asian	10 (31.3)	6 (18.8)	6 (40.0)	3 (18.8)	2 (25.0)	1 (12.5)	2 (22.2)	2 (25.0)
Japanese	4 (12.5)	4 (12.5)	2 (13.3)	3 (18.8)	1 (12.5)	1 (12.5)	1 (11.1)	0

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Jonathan Day et al.

Application No.: 17/811,748 | Confirmation No.: 2988

Filed: July 11, 2022 | Art Unit: 1654

For: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children | Examiner: J. Ha

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. § 1.111

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This paper is filed in response to the Office Action dated February 1, 2024, in the above-identified U.S. patent application.

This paper is filed with a Petition for Three-Month Extension of Time and the requisite fee. If any additional fees are deemed necessary in connection with this filing, the Commissioner is hereby authorized to charge such fees to our deposit account no. 13-2855.

Amendments to the Specification begin on page **2** of this paper.

Amendments to the Claims begin on page **3** of this paper.

Amendments to the Drawings begin on page **14** of this paper.

Remarks begin on page **15** of this paper.

AMENDMENTS TO THE SPECIFICATION

Please update the Abstract on page 74 as follows:

~~The present disclosure provides for use of variants of C-type natriuretic peptide (CNP), for the~~ treatment of skeletal dysplasias in children, and improvement in one or more symptoms of skeletal dysplasias, such as long bone growth or growth velocity, by administering variants of C-type natriuretic peptide (CNP) is described ~~and use in other disorders having a skeletal dysplasia and/or CNP-associated symptom or component~~.

AMENDMENTS TO THE CLAIMS

1. (Original) A method of treating skeletal dysplasia in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID

NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48);MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64): and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

2. (Original) A method of increasing long bone growth in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase long bone growth, wherein the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO:);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO:16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);

GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO:40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52); MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59);PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

3. (Original) A method of increasing growth velocity in a subject less than or about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to increase growth velocity in

the subject wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9); TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11); SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14); AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO:
15);WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23);

PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30);
GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31);
ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32);
NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33);
KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34);
KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35);
LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36);
SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37);
KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38);
GCFGLKLDRIGSMSGLGC(CNP-18) (SEQ ID NO: 39);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC[CNP37(M32N) (SEQ ID NO: 40);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Pro-CNP37) (SEQ ID NO: 41);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC(Met-CNP37) (SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)]
(SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-
Gly-CNP37) (SEQ ID NO: 45);
GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID
NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-
CNP27(M22N)] (SEQ ID NO: 47);
PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ
ID NO: 48); MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-
CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC
[CNP27(K4,5,9R)]) (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC
[CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51);
PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);
MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53);
PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ
ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-

CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

4. (Original) The method of claim 3, wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 25% above baseline in said subject or wherein the increase in growth velocity is an increase in annualized growth velocity as measured by standing height of at least 40% above baseline in said subject.

5. (Previously presented) The method of claim 3, wherein the increase in growth velocity is assessed by measuring standing height, sitting height, weight, head circumference, upper arm length, lower arm length, upper leg length, lower leg length, hand length or foot length.

6. (Previously presented) The method of claim 3 that results in no clinically significant change in blood hemoglobin concentration, blood platelet number, blood electrolyte concentration, blood urea nitrogen concentration, blood creatinine concentration, blood alkaline phosphatase concentration, blood alanine amino transferase concentration or blood aspartate aminotransferase concentration in said subject.

7. (Previously presented) The method of claim 3 that results in

(a) a change in the upper body length to lower body length ratio of between -0.05 and 0.05;

(b) a change in the upper arm length to forearm length ratio of between -0.05 to 0.05; and/or

(c) a change in the upper leg length to lower leg length ratio of between -0.05 and 0.05.

8. (Previously presented) The method of claim 1, wherein

(a) said composition is administered once daily;

(b) said composition is administered once daily over a period of at least 6 months; or

(c) said composition is administered once daily over a period of at least 12 months.

9. (Previously presented) The method of claim 1, wherein the composition is administered to the subject via subcutaneous administration.

10. (Previously presented) The method of claim 1, wherein the composition is administered daily, every other day, 3 times weekly, 2 times weekly, or once weekly.

11. (Currently amended) The method of claim 1, wherein the composition is administered at a dose of ~~15~~ 30 µg/kg.

12. (Currently amended) The method of claim 1, wherein the subject is about 6 months to about 2 years old ~~less than about 5 years old~~.

13. (Previously presented) The method of claim 1, wherein the subject is about 0 to about 6 months old.

14. (Previously presented) The method of claim 11, wherein the dosage may be decreased to 15 µg/kg when the subject is 2 years old.

15. (Currently amended) The method of claim 1, wherein the skeletal dysplasia is selected from the group consisting of hypophosphatemic rickets, achondroplasia, hypochondroplasia, short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis imperfecta, achondrogenesis, chondrodysplasia punctata,

homozygous achondroplasia, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis imperfecta, short-rib polydactyly syndromes, hypochondroplasia, rhizomelic type of chondrodysplasia punctata, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, NPR2 mutation, SHOX mutation, Turner's syndrome/Leri Weill, ~~(Turner's syndrome/Leri Weill)~~, PTPN11 mutations, Noonan's syndrome ~~(Noonan's syndrome)~~, and idiopathic short stature.

16. (Original) The method of claim 9, wherein the skeletal dysplasia is achondroplasia.

17. (Previously presented) The method of claim 1, wherein the composition further comprises one or more pharmaceutical excipients.

18. (Currently amended) The method of claim 17, wherein the composition comprises citric acid monohydrate, sodium citrate dihydrate, trehalose dihydrate, D-mannitol, L-methionine and polysorbate 80.

19. (Previously presented) The method of claim 1, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 43); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N)] (SEQ ID NO: 40); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37) (SEQ ID NO: 42); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37) (SEQ ID NO: 41); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N) (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); and PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24).

20. (Previously presented) The method of claim 1, wherein the CNP variant is selected from the group consisting of PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37) (SEQ ID NO: 1); and LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20).

21-22. (Canceled)

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**08/01/2024 04:05:35 PM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	66615845	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 8

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
Amendment in Response to Non-Final Office Action Under 37CFR1111.pdf		18	-	73 KB
Amendment in Response to Non-Final Office Action Under 37CFR1111-A....pdf	(1-1)	1	Amendment/Request for Reconsideration-After Non-Final Rejection	16 KB
Amendment in Response to Non-Final Office Action Under 37CFR1111-SPEC.pdf	(2-2)	1	Specification	15 KB
Amendment in Response to Non-Final Office Action	(3-13)	11	Claims	46 KB

Under 37CFR1111-CLM.pdf				
Amendment in Response to Non-Final Office Action Under 37CFR1111-DRW.pdf	(14-14)	1	Drawings-only black and white line drawings	13 KB
Amendment in Response to Non-Final Office Action Under 37CFR1111-REM.pdf	(15-18)	4	Applicant Arguments/Remarks Made in an Amendment	31 KB
Three Month Request for Extension of Time Under 37 CFR 1136 a.pdf		1	Extension of Time	22 KB
Figure 1 REPLACEMENT SHEET.pdf		1	Drawings-only black and white line drawings	39 KB
Savarirayan-2023-NPL.pdf		11	Non Patent Literature	652 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Amendment in Response to Non-Final Office Action Under 37CFR1111.pdf	7FF998901A3508CA0711D2A8DD0254AC9D47ACFB2E31577C6F3D4B08E4F09D8224F1FF8ED247EA14839EFFE6A874945A44C1559F3EE2A353E1D1F0CEAC0FB348
Amendment in Response to Non-Final Office Action Under 37CFR1111-A...pdf	A64EC048D2260194BFBA8E84C490BC89AB0BBD9267B85CD4ABE6971F80515553C1E4B29F5D7A93AC427B09C39C82D7B2E5A61DF10EEEEC4858545E5D9BC0BE27
Amendment in Response to Non-Final Office Action Under 37CFR1111-SPEC.pdf	6B07B6F5922108D74CAE4F952B6468EE21CA56D46EF09803EC3F7F4248FC6FC7BF5BF15E5DE9CE8036D5B2B6C4F1E8A21352205E8031F8F368C532AB576AE803
Amendment in Response to Non-Final Office Action Under	94A14C1A6A9BD2AECD2F5EC924FAEC832CC4F8FA2C6D68B09C2B8D372C257F911D0401FF533FE0DF898B22AC3FDFBA8D

37CFR1111-CLM.pdf	F9B1F2D3D2F8D8ADBE532B57DD03F09F
Amendment in Response to Non-Final Office Action Under 37CFR1111-DRW.pdf	5ED24CE88D177A7AC258EE710E36ABA47DA9B19DC006023F5EE6790763AE6CF751FC820F07B356CAEDA3B6B8EFBB6CDFC17CDA5F432822C2012421E4E8988823
Amendment in Response to Non-Final Office Action Under 37CFR1111-REM.pdf	21FCE4792F8DACFD35748FA41714503776F91B1B91121847D4DADA6DB53C9DC1E0F96061CFDDE14BDB1267993243C34772C855223F329A6AEC1A2FFD83A55357
Three Month Request for Extension of Time Under 37 CFR 1136 a.pdf	8301CECD3270CD40AF5FF404091BAA0F3D92264E0E170362F3B990DCEEA36502F367A660A457C3E54B656B2A5244BC9B51D310ACF294ACC9D056B440AE0113C7
Figure 1 REPLACEMENT SHEET.pdf	A760C26DC94F716A8558C52BED425DD93BD3930B304414FD8E88FF0A5723C2856D82A2AEC394F7C8F63343D96444AB101BFD09AA9543A95755F482ADDC1FA2AE
Savarirayan-2023-NPL.pdf	8ADD12B0964F2EA6A1B7CDA9864C3420B0CE547E70C1D3DA897DF617B8C17944F4176A29774F8C2650B277A50481B38071A003350D0B42645FC90ED6DA4E7EEC

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/22 (11-23)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 30610/57038
Application Number 17/811,748-Conf. #2988	Filed July 11, 2022
For C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children	
Art Unit 1654	Examiner J. Ha

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$220	$88	$44	$
☐ Two months (37 CFR 1.17(a)(2))	$640	$256	$128	$
☒ Three months (37 CFR 1.17(a)(3))	$1,480	$592	$296	$ 1,480.00
☐ Four months (37 CFR 1.17(a)(4))	$2,320	$928	$464	$
☐ Five months (37 CFR 1.17(a)(5))	$3,160	$1,264	$632	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 13-2855 .

☒ Payment made via USPTO patent electronic filing system.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 53,379 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Katherine L. Neville/ — Signature
August 1, 2024 — Date

Katherine L. Neville — Typed or printed name
(312) 474-6300 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	08/01/2024 04:05:35 PM Z ET	30610/57038

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2968	FILED BY	Valeria Rodriguez
PATENT CENTER #	66615845	AUTHORIZED BY	Katherine Neville
CUSTOMER #	90849	FILING DATE	07/11/2022
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Jonathan Day

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0402	E202481G06389290	Katherine Neville

PRE-AUTHORIZED ACCOUNT
132855

PRE-AUTHORIZED CATEGORY
37 CFR 1.17 (Patent application and reexamination processing fees)

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1253	EXTENSION FOR RESPONSE WITHIN THIRD MONTH	1480.00	1	1480.00

TOTAL AMOUNT: $1,480.00

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

AMENDMENTS TO THE DRAWINGS

Please replace Figure 1 as filed with Replacement Figure 1 filed herewith.

REMARKS

Claims 1-20 are pending and under consideration with claims 2-7 being withdrawn from consideration.

In the Action, the Examiner objected to claim 18 due to informalities. The Examiner rejected claims 12 and 14 under 35 U.S.C. §112(d), fourth paragraph, as assertedly being of improper dependent form. Claim 15 was rejected under 35 U.S.C. §112(b), second paragraph, as assertedly being indefinite. Claims 1 and 8-20 were rejected under 35 U.S.C. §103 as assertedly being unpatentable over Bullens et al. (U.S. Patent No. 9,907,834) ("Bullens") in view of Wendt et al. (WO 2009/067639) ("Wendt"). Reconsideration is requested in light of the following remarks.

I. Objections to the Specification and Drawings

The Examiner objected to the Abstract of the specification as not being clear and concise. The Abstract is amended herein for clarity as suggested by the Examiner. The amendment includes no new matter.

The Examiner objected to Figure 1 for having shading in the Table making it difficult to read. Submitted herewith is a Replacement Figure 1 presenting a Table with no shading. The amendment to the figure includes no new matter.

II. Support for the Amendment to the Claims

Support for the amendments to the claims is found throughout the specification. Clai m11 is amended to recite that the dose of administration is 30 µg/kg as recited in original claim 11 as filed. Claim 12 is amended to recite the patient age range, as recited in original claim 12 as filed. Claim 15 is amended for clarity to remove terms in parentheses. Claim 18 is amended to include missing punctuation. The amendments include no new matter.

III. The rejection of claims 12 and 14 under 35 U.S.C. §112(d), fourth paragraph, as assertedly being of improper dependent form, should be withdrawn.

The Examiner rejected claims 12 and 14 as not further limiting the claims from which they depend. Claim 12 is amended to recite that the subject is from 6 months to 2 years old, as recited in original claim 12. Claim 14 depends from claim 11. Claim 11 is amended to recite that the dose of administration is 30 µg/kg, as such claim 14 now correctly

limits claim 11. As such, Applicant believes the claims are in proper form and the rejection should be withdrawn.

IV. The rejection of claim 15 under 35 U.S.C. §112(b), second paragraph, as assertedly being indefinite, should be withdrawn.

The Examiner rejected claim 15 as assertedly unclear for reciting terms within parentheses. Applicant submits that the terms in parentheses are specific names of diseases within the category described in claim 15. Claim 15 is amended for clarity to recite the indications separately.

V. The rejection of claims 1 and 8-20 under 35 U.S.C. §103 as assertedly being unpatentable over Bullens and Wendt, should be withdrawn.

The Examiner rejected claims 1 and 8-20 as assertedly obvious over Bullens in view of Wendt, asserting that Bullens discloses methods of treating skeletal dysplasia with CNP variants which are recited in the claims, and Wendt describes that the CNP treatment can be carried out in subjects age 0 to 17, and therefore it would have been obvious to carry out the claimed methods. Applicant respectfully disagrees.

The claims are directed to treatment of skeletal dysplasia patients less than or about 2 years old with the recited CNP variants. As acknowledged by the examiner, Bullens does not refer to this patient population.

To establish *prima facie* obviousness, the Examiner must show that all the elements of the claim are taught or suggested in the prior art (MPEP 2143.03 and Examination Guidelines for Determining Obviousness, Section III.A.1, Fed Reg., Vol 72, No. 195, Pages 57526-35, October 10, 2007), and if all the elements of the claim are described in the prior art, the combination of elements must yield predictable results to render a clamed invention obvious. Further, it should be demonstrated that the prior art reference(s) provide a teaching, suggestion or motivation to combine the references, and/or there is a reasonable expectation of success that combining the elements in the art would provide the invention as claimed (MPEP § 2142 and Federal Register Examination Guidelines for Determining Obviousness, Section III.G, Fed Reg., Vol 72, No. 195, 2007).

Applicants submit that the Examiner has not established a *prima facie* case of obviousness as not all elements of the claims are disclosed or suggested in the prior art, and

the art provides no reasonable expectation of success at arriving at the present invention. Further, the specification provides unexpected benefits of the claimed invention.

Treatment of skeletal dysplasia using CNP has been described as effective for children greater than 5 years old. See e.g., Bullens. However, although Wendt generally discloses that younger children suffering from achondroplasia *could* benefit from treatment with CNP, treatment of the patient population in the claims had heretofore not been disclosed and the treatment effect could not have been predicted in this patient population in view of Bullens and Wendt.

The efficacy of the treatment in the selected patient population, and the recited doses in the dependent claims, would not have been obvious to one of skill in the art based on the disclosures in the cited art. The specification provides initial results from a Phase 2 clinical trial of CNP therapy in patients less than 2 years old. The specification describes that administration of CNP to patients less than 2 years old shows improves annualized growth velocity and overall Z score, which is a measure of height deficiency, compared to normal growth charts. This beneficial effect could not have been predicted from the combination of Bullens and Wendt, which only disclose the effects of CNP therapy in children over 5 years old.

Results of the trial were also published in a peer reviewed journal, see Savarirayan et al., *Vosoritide therapy in children with achondroplasia aged 3-59 months: a multinational, randomised, double-blind, placebo-controlled, phase 2 trial.* Lancet Child Adolesc Health. 2024 Jan;8(1):40-50. Epub 2023 Nov 18. Savarirayan confirms the results set out in the specification and discloses that the doses ranges were still safe and effective in younger patients as well as older children. Further, in view of the results presented in the specification and subsequently published in Savarirayan (*supra)* use of the CNP variant vosoritide was recently approved by the FDA in October 2023 to treat children *less than* 5 years old, and given a positive opinion by the European Medicines Authority in September 2023 for treatment of achondroplasia in *children 4 months and older.*

Nothing in the cited art would have provided a reasonable expectation of success that the same or higher dosage of CNP would have been effective in children under the age of 2. However, the disclosure in the specification, and confirmation in after-filed publications, demonstrate efficacy of the claimed treatment. These surprising therapeutic

benefits would not have been predicted from the cited art. As such, the rejection of the claims as obvious should be withdrawn.

VI. Conclusion

Applicants submit that the application is in condition for allowance and respectfully request notification of the same.

Dated: August 1, 2024

Respectfully submitted,

Electronic signature: /Katherine L. Nevillew/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
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Chicago, Illinois 60606-6357
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Attorney for Applicant

Examiner-Initiated Interview Summary	**Application No.** 17/811,748	**Applicant(s)** Day et al.		
	Examiner JULIE HA	**Art Unit** 1654	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
JULIE HA	Primary Examiner	Telephonic
KATE NEVILLE	Attorney	

Date of Interview: 17 October 2024

Issues Discussed:

Proposed Amendment(s)

The Examiner telephoned the Applicant's representative, Kate Neville, to discuss Examiner's amendment to get the application in condition for allowance on October 7, 2024. Ms. Neville telephoned the Examiner back on October 8, 2024, and the amendment to the claims were discussed. The Examiner requested canceling claims 2-7, drawn to nonelected inventions. The Examiner further requested amending claim 1 to limit the lower age limit of the patient and amending claim 13 to further limit amended claim 1. Ms. Neville indicated that she will discuss the proposed Examiner's amendment with the Applicant and will let the Examiner know as soon as possible. On October 11, 2024, Ms. Neville telephoned the Examiner and authorized the cancellation of claims 2-7. However, claim 1 amendment was further discussed. If claim 1 amendment is accepted, no further amendment is required to claim 13. Ms. Neville will discuss the newly proposed amendment with the Applicant and will get back to the Examiner as soon as possible. Ms. Neville telephoned the Examiner on October 17, 2024, and authorized the Examiner's amendment to claim 1 and canceling claims 2-7.

/JULIE HA/ Primary Examiner, Art Unit 1654	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	**Art Unit**
	JULIE HA	1654

CPC						
Symbol					**Type**	**Version**
A61K	/	38	/	2242	F	2013-01-01
A61K	/	9	/	0019	I	2013-01-01
A61K	/	38	/	1709	I	2013-01-01
A61K	/	47	/	10	I	2013-01-01
A61K	/	47	/	12	I	2013-01-01
A61K	/	47	/	183	I	2013-01-01
A61K	/	9	/	19	A	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

Use images from artifact folder? ◉ Yes ○ No

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	14	
/JULIE HA/ Primary Examiner, Art Unit 1654	17 October 2024	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	**Art Unit**
	JULIE HA	1654

INTERNATIONAL CLASSIFICATION				
CLAIMED				
A61K	/	38	/	22
A61K	/	38	/	00
A61K	/	47	/	10
A61K	/	47	/	12
A61K	/	9	/	19
A61P	/	19	/	08
C07K	/	7	/	00
C07K	/	14	/	00

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

Use images from artifact folder? (•) Yes ( ) No

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 14	
/JULIE HA/ Primary Examiner, Art Unit 1654 (Primary Examiner)	17 October 2024 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	**Art Unit**
	JULIE HA	1654

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	10	16												
2	8	11	17												
3	9	12	18												
4	10	13	19												
5	11	14	20												
6	12														
7	13														
8	14														
9	15														

Use images from artifact folder? ◉ Yes ○ No

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 14	
/JULIE HA/ Primary Examiner, Art Unit 1654 (Primary Examiner) 17 October 2024 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	Art Unit
	JULIE HA	1654

CPC - Searched*		
Symbol	**Date**	**Examiner**
A61K 38/2242, 38/22, 38/00, 9/0019, 38/1709, 47/10, 47/12, 47/183, 9/19	10/07/2024	JH
A61P 19/08	10/07/2024	JH
C07K 7/00, 14/00	10/07/2024	JH

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**
None	10/07/2024	JH

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**
None		10/07/2024	JH

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

/JULIE HA/ Primary Examiner, Art Unit 1654	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/811,748	Day et al.
	Examiner	Art Unit
	JULIE HA	1654

Search Notes		
Search Notes	**Date**	**Examiner**
Updated STIC: sequence searched: can be accessed via PE2E or SCORE	10/07/2024	JH
Updated PE2E all inventors named searched	10/07/2024	JH
Updated PE2E assignee searched	10/07/2024	JH
Updated PE2E please see attached	10/07/2024	JH
Updated PDP all inventors named searched: please see attached	10/07/2024	JH
Updated GOOGLE search history: please see attached	10/07/2024	JH
Updated CONTINUITY MAP searched: please see attached	10/07/2024	JH
Reviewed STN search from 1/28/2024	10/07/2024	JH
Reviewed filing receipt	10/07/2024	JH
PDP CONTINUITY DATA searched: please see attached	10/07/2024	JH

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
Updated STIC: sequence searched	can be accessed via PE2E or SCORE	10/07/2024	JH
A61K	38/2242, 38/22, 38/00, 9/0019, 38/1709, 47/10, 47/12, 47/183, 9/19	10/07/2024	JH
A61P	19/08	10/07/2024	JH
C07K	7/00, 14/00	10/07/2024	JH

/JULIE HA/ Primary Examiner, Art Unit 1654	

Title: C-TYPE NATRIURETIC PEPTIDE VARIANT... **Application number:** 17/811,748

Status: 71/RESPONSE TO NON-FINAL OFFICE ACTION ENTERED AND FORWARDED T...

Examiner: 82939 / HA,JULIE

Filing date: 07/11/2022

GAU: 1654

Continuity/foreign: YES

IDS flag clearance

Continuity/reexam data

Print preview | Global dossier

Parent data

17811748 filed 07/11/2022 Claims Priority from Provisional Application **63350196** , filed 06/08/2022
17811748 Claims Priority from Provisional Application **63220275** , filed 07/09/2021

Child data

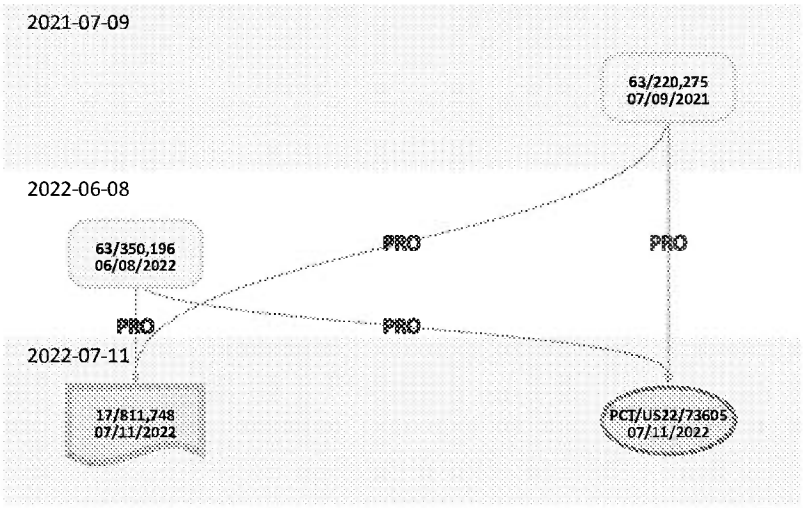
None

Foreign data

No foreign data information

(*) - Request to retrieve electronic copy of foreign priority from participating receiving offices.

Application: 17/811,748 (10/07/2024)
Total: 4 nodes
Filter: No Filters



Web Search History

date, time	web site	search string
10/7/2024 8:30:07 AM	Google	GCFGLKLDRIGSMSGLGC
10/7/2024 8:30:30 AM	Google	PRO-Gly-CNP37
10/7/2024 8:30:49 AM	Google	(PRO-Gly-CNP37) amd (skeletal dysplasia)
10/7/2024 8:31:07 AM	Google	(PRO-Gly-CNP37) amd (skeletal dysplasia) and achondroplasia
10/7/2024 8:33:33 AM	Google	(PRO-Gly-CNP37) amd (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:33:50 AM	Google	(PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC) amd (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:33:56 AM	Google	(PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC) and (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:34:17 AM	Google	(Pro-gly-CNP37) and (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:35:35 AM	Google	GCFGLKLDRIGSMSGLGC and (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:35:54 AM	Google	PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC and (skeletal dysplasia) and achondroplasia and ("2" years old)
10/7/2024 8:36:07 AM	Google	GCFGLKLDRIGSMSGLGC and (skeletal dysplasia) and achondroplasia
10/7/2024 8:36:19 AM	Google	GCFGLKLDRIGSMSGLGC and (skeletal dysplasia)
10/7/2024 8:36:48 AM	Google	GCFGLKLDRIGSNSGLGC and (skeletal dysplasia)

Title: C-TYPE NATRIURETIC PEPTIDE VARIANT... **Application number:** 17/811,748

Status: 71/RESPONSE TO NON-FINAL OFFICE ACTION ENTERED AND FORWARDED TO ...

Examiner: 82939 / HA,JULIE

Filing date: 07/11/2022

GAU: 1654

Continuity/foreign: YES

IDS flag clearance

Inventor information

Inventor name	City	State/country	Inventor oath compliant?
Day, Jonathan	Buckinghamshire	UNITED KINGDOM	N/A
Fisheleva, Elena	Novato	CALIFORNIA, UNITED STATES	N/A

Applicant information

Applicant name	City	State/country
BIOMARIN PHARMACEUTICAL INC.	Novato	CALIFORNIA, UNITED STATES

Assignee information

No assignee information

Application URL: https://opsg-portal.uspto.gov/OPSGPortal/?externalurl=true&General Information=applicantInventor&applicationNo=17811748 (https://opsg-portal.uspto.gov/OPSGPortal/?externalurl=true&General Information=applicantInventor&applicationNo=17811748)

PE2E SEARCH - Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
L36	1	17/811748	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L37	137	Day-Jonathan.in.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L38	2	Fisheleva-Elena.in.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L39	354	(Biomarin Pharmaceutical INC).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L40	354	(("Biomarin") near3 ("Pharmaceutical") near3 ("INC")).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L41	355	(("Biomarin") near3 ("Pharmaceutical")).as,aanm.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L42	27	(("Biomarin") near3 ("Pharmaceutical")).as,aanm. AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L43	2	L2 AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L44	2	L3 AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L45	20	(Pro-Gly-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L46	11	(Pro-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L47	0	(Pro-CNP37) AND (Gly-Pro-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L48	11	(Pro-CNP37) AND (Pro-Gly-CNP37)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L49	10	"9907834".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L50	8	"2009067639".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L51	10	("7276481" OR	(US-PGPUB; USPAT;	ADJ	ON	ON	2024/10/07

		"1994020534" OR "8198242" OR "8598121" OR "7642243" OR "8658373").pn.	FPRS; EPO; JPO; DERWENT)				05:19 AM
L52	0	"1994020534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L53	0	"19940020534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L54	0	"199420534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L55	6	"9420534".pn.	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:19 AM
L56	721013	(A61K38/2242 or A61K9/0019 or A61K38/1709 or A61K47/10 or A61K47/12 or A61K47/183 or A61K9/19 or A61P19/08).cpc.	(US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, TH, TN, TR, TW, UA, VN); FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L57	878186	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc.	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L58	1715	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM

		C07K7/00 OR C07K14/00).cpc. AND ((c-type natriuretic peptide) OR CNP)					
L59	19	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L60	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L61	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37) AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L62	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM

		(PRO-CNP37) AND (PRO-GLY-CNP37) AND ((c-type natriuretic peptide) OR CNP)					
L63	19	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L64	11	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L65	11	(Pro-Gly-CNP37) AND (PRO-CNP37)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L66	11	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L67	11	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia) AND (achondroplasia)	(US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB)	ADJ	ON	ON	2024/10/07 05:19 AM
L68	448387	((A61K38/1709 OR A61K38/2242 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/0019 OR A61K9/19 OR A61P19/08).cpc.)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:29 AM
L69	19	(("DAY; JONATHAN".ine. OR "FISHELEVA; ELENA".ine.))	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:30 AM
L70	0	PGQEHPNARKYKGAN	(US-PGPUB; USPAT;	ADJ	ON	ON	2024/10/07

		KKGLSKGCFGLKDRIG SMSGLGC	FPRS; EPO; JPO; DERWENT)				05:42 AM
L71	65	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:43 AM
L72	40	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:46 AM
L73	2	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 05:47 AM
L74	1	GDLRVDTKSRAAWAR LLQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 06:16 AM
L75	2	GCFGLKLDRIGSMSGL GC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT)	ADJ	ON	ON	2024/10/07 06:17 AM

PE2E SEARCH - Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	British Equivalents	Time Stamp
N1	1	GCFGLKLDRIGSMSGL GC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 06:20 AM
N2	0	GDLRVDTKSRAAWAR LLQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 06:20 AM
N3	1	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia) AND ("2" year)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 06:20 AM
N4	32	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC AND (skeletal dysplasia)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 06:20 AM
N5	56	PGQEHPNARKYKGAN KKGLSKGCFGLKLDRI GSMSGLGC	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 06:20 AM
N6	10	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:20 AM

		AND (achondroplasia)					
N7	10	(Pro-Gly-CNP37) AND (PRO-CNP37) AND (skeletal dysplasia)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:20 AM
N8	10	(Pro-Gly-CNP37) AND (PRO-CNP37)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:20 AM
N9	10	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:20 AM
N10	15	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-GLY-CNP37)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:21 AM
N11	10	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR A61K9/19 OR A61P19/08 OR C07K7/00 OR C07K14/00).cpc. AND (PRO-CNP37) AND (PRO-GLY-CNP37) AND ((c-type natriuretic peptide) OR CNP)	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:21 AM
N12	10	(A61K38/2242 OR A61K38/22 OR A61K38/00 OR A61K9/0019 OR A61K38/1709 OR A61K47/10 OR A61K47/12 OR A61K47/183 OR	(US-PGPUB; USPAT)	ADJ	ON	ON	2024/10/07 07:21 AM

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Bibliographic Data

Application No: 17/811,748

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /JULIE HA/ Examiner's Signature | Initials

Title: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/11/2022	514	1654	30610/57038
RULE			

APPLICANTS

BIOMARIN PHARMACEUTICAL INC., Novato, CA, UNITED STATES

INVENTORS

Jonathan Day, Buckinghamshire, UNITED KINGDOM

Elena Fisheleva,

CONTINUING DATA

This application has PRO of 63350196 06/08/2022

This application has PRO of 63220275 07/09/2021

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

11/22/2022

STATE OR COUNTRY

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233 South Wacker Drive
6300 Willis Tower
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FILING FEE RECEIVED

$1,980

UNITED STATES PATENT AND TRADEMARK OFFICE

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NOTICE OF ALLOWANCE AND FEE(S) DUE

90849 7590 10/29/2024

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233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER	
HA, JULIE	
ART UNIT	PAPER NUMBER
1654	

DATE MAILED: 10/29/2024

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

TITLE OF INVENTION: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	01/29/2025

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

90849 7590 10/29/2024
Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via the USPTO patent electronic filing system or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

TITLE OF INVENTION: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	01/29/2025

EXAMINER	ART UNIT	CLASS-SUBCLASS
HA, JULIE	1654	514-012400

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via the USPTO patent electronic filing system ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 10/29/2024

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

DATE MAILED: 10/29/2024

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. The United States Patent and Trademark Office (USPTO) collects the information in this record under authority of 35 U.S.C. 2. The USPTO's system of records is used to manage all applicant and owner information including name, citizenship, residence, post office address, and other information with respect to inventors and their legal representatives pertaining to the applicant's/ owner's activities in connection with the invention for which a patent is sought or has been granted. The applicable Privacy Act System of Records Notice for the information collected in this form is COMMERCE/PAT-TM-7 Patent Application Files, available in the Federal Register at 78 FR 19243 (March 29, 2013).
https://www.govinfo.gov/content/pkg/FR-2013-03-29/pdf/2013-07341.pdf

Routine uses of the information in this record may include disclosure to:

1) law enforcement, in the event that the system of records indicates a violation or potential violation of law;
2) a federal, state, local, or international agency, in response to its request;
3) a contractor of the USPTO having need for the information in order to perform a contract;
4) the Department of Justice for determination of whether the Freedom of Information Act (FOIA) requires disclosure of the record;
5) a Member of Congress submitting a request involving an individual to whom the record pertains, when the individual has requested the Member's assistance with respect to the subject matter of the record;
6) a court, magistrate, or administrative tribunal, in the course of presenting evidence, including disclosures to opposing counsel in the course of settlement negotiations;
7) the Administrator, General Services Administration (GSA), or their designee, during an inspection of records conducted by GSA under authority of 44 U.S.C. 2904 and 2906, in accordance with the GSA regulations and any other relevant (i.e., GSA or Commerce) directive, where such disclosure shall not be used to make determinations about individuals;
8) another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c));
9) the Office of Personnel Management (OPM) for personnel research purposes; and
10) the Office of Management and Budget (OMB) for legislative coordination and clearance.

If you do not furnish the information requested on this form, the USPTO may not be able to process and/or examine your submission, which may result in termination of proceedings, abandonment of the application, and/or expiration of the patent.

Notice of Allowability	Application No. 17/811,748	Applicant(s) Day et al.	
	Examiner JULIE HA	Art Unit 1654	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 08/01/2024.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1 and 8-20 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JULIE HA/
Primary Examiner, Art Unit 1654

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Amendment after Non-final office action filed on August 1, 2024 is acknowledged.

3. Claims 1-20 are pending in this application.

Withdrawn Objections and Rejections

4. Objection to the abstract is hereby withdrawn in view of Applicant's amendment to the abstract.

5. Objection to the drawings is hereby withdrawn in view of Applicant's filing Replacement Sheet on August 1, 2024.

6. Rejection of claim 18 is hereby withdrawn in view of Applicant's amendment to the claim.

7. Rejection of claims 12 and 14 under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, is hereby withdrawn in view of Applicant's amendment to the claims.

8. Rejection of claim 15 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, is hereby withdrawn in view of Applicant's amendment to the claim.

9. Rejection of claims 1 and 8-20 under 35 U.S.C. 103 as being unpatentable over Bullens et al (US Patent No. 9907834, filed with IDS) in view of Wendt et al (WO 2009/067639, filed with IDS), is hereby withdrawn in view of Applicant's persuasive arguments and in view of Applicant's amendment to the claims.

EXAMINER'S AMENDMENT

10. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Kate Neville on October 17, 2024.

Claims 1-7 have been amended as follows:

1. (Currently amended) A method of treating skeletal dysplasia in a subject ~~less than or~~ about 0 month to about 2 years old comprising administering to the subject a composition comprising a C-type natriuretic peptide (CNP) variant in an amount effective to treat the skeletal dysplasia in the subject, wherein the CNP variant is selected from the group consisting of:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:1);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 2);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 3);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP53) (SEQ ID NO: 4);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-53(M48N)] (SEQ ID NO: 5);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 6);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 7);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-50) (SEQ ID NO: 8); DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 9);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 10); KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 11);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 12); RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 13); AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 14);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 15); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 16); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 17); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID

NO: 18); LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 19); LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 20);QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 21); EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 22); HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 23); PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 24); NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 25); ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 26); RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 27); KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 28); YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 29); KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 30); GANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-27) (SEQ ID NO: 31); ANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-26) (SEQ ID NO: 32); NKKGLSKGCFGLKLDRIGSMSGLGC (CNP-25) (SEQ ID NO: 33); KKGLSKGCFGLKLDRIGSMSGLGC (CNP-24) (SEQ ID NO: 34); KGLSKGCFGLKLDRIGSMSGLGC (CNP-23) (SEQ ID NO: 35); LSKGCFGLKLDRIGSMSGLGC (CNP-21) (SEQ ID NO: 36); SKGCFGLKLDRIGSMSGLGC (CNP-20) (SEQ ID NO: 37); KGCFGLKLDRIGSMSGLGC (CNP-19) (SEQ ID NO: 38); GCFGLKLDRIGSMSGLGC (CNP-18) (SEQ ID NO: 39); QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP37(M32N) (SEQ ID NO: 40); PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP37) (SEQ ID NO: 41); MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP37) (SEQ ID NO: 42); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP37) (SEQ ID NO: 45); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (HSA-CNP27) (SEQ ID NO: 46); GHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSNSGLGC [HSA-CNP27(M22N)] (SEQ ID NO: 47); PGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-HSA-CNP27) (SEQ ID NO: 48);MGHKSEVAHRFKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-HSA-CNP27) (SEQ ID NO: 49); GANRRGLSRGCFGLKLDRIGSMSGLGC [CNP27(K4,5,9R)] (SEQ ID NO: 50); GANRRGLSRGCFGLKLDRIGSNSGLGC [CNP27(K4,5,9R, M22N)] (SEQ ID NO: 51); PGANRRGLSRGCFGLKLDRIGSMSGLGC [Pro-CNP27(K4,5,9R)] (SEQ ID NO: 52);

MGANRRGLSRGCFGLKLDRIGSMSGLGC [Met-CNP27(K4,5,9R)] (SEQ ID NO: 53); PEG1K-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-CNP27(K4,5,9R)] (SEQ ID NO: 54); PEG1K-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEG1K-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 55); PEG1K-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 56); PEG1K-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEG1K-Met-CNP27(K4,5,9R)] (SEQ ID NO: 57); PEO12-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-CNP27(K4,5,9R)] (SEQ ID NO: 58); PEO12-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO12-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 59); PEO12-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 60); PEO12-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO12-Met-CNP27(K4,5,9R)] (SEQ ID NO: 61); PEO24-GANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-CNP27(K4,5,9R)] (SEQ ID NO: 62); PEO24-GANRRGLSRGCFGLKLDRIGSNSGLGC [PEO24-CNP27(K4,5,9R, M22N)] (SEQ ID NO: 63); PEO24-PGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Pro-CNP27(K4,5,9R)] (SEQ ID NO: 64); and PEO24-MGANRRGLSRGCFGLKLDRIGSMSGLGC [PEO24-Met-CNP27(K4,5,9R)] (SEQ ID NO: 65).

2.-7. (Cancelled)

Claims 8-20, as set forth in the amendment filed on August 1, 2024.

Claims 1 and 8-20 are allowed.

REASONS FOR ALLOWANCE

11. The following is an examiner's statement of reasons for allowance: Claims 1 and 8-20 are free of prior art. As indicated in the previous office action, the closest art is: Bullens et al (US Patent No. 9907834, filed with IDS). Bullens et al teach instant SEQ ID

NO: 1 (see SEQ ID NO: 5) and instant SEQ ID NO: 41 (see SEQ ID NO: 3), for treating patients with skeletal dysplasia (see column 3, lines 25-35). Bullens et al do not teach the patient population wherein the patient is about 0 month to about 2 years of age. In Applicant's response, Applicant has shown the following in the instant specification:

> The efficacy of the treatment in the selected patient population, and the recited doses in the dependent claims, would not have been obvious to one of skill in the art based on the disclosures in the cited art. The specification provides initial results from a Phase 2 clinical trial of CNP therapy in patients less than 2 years old. The specification describes that administration of CNP to patients less than 2 years old shows improves annualized growth velocity and overall Z score, which is a measure of height deficiency, compared to normal growth charts. This beneficial effect could not have been predicted from the combination of Bullens and Wendt, which only disclose the effects of CNP therapy in children over 5 years old.
>
> Results of the trial were also published in a peer reviewed journal, see Savarirayan et al., *Vosoritide therapy in children with achondroplasia aged 3-59 months: a multinational, randomised, double-blind, placebo-controlled, phase 2 trial*. Lancet Child Adolesc Health. 2024 Jan;8(1):40-50. Epub 2023 Nov 18. Savarirayan confirms the results set out in the specification and discloses that the doses ranges were still safe and effective in younger patients as well as older children. Further, in view of the results presented in the specification and subsequently published in Savarirayan (*supra)* use of the CNP variant vosoritide was recently approved by the FDA in October 2023 to treat children *less than* 5 years old, and given a positive opinion by the European Medicines Authority in September 2023 for treatment of achondroplasia in *children 4 months and older*.

. In view of Savarirayan reference provided by the Applicant, one of ordinary skill in the art would not have predicted the results in children from about 0 month old to about 2 years old of instant application.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably

accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

CONCLUSION

Claims 1 and 8-20 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JULIE HA whose telephone number is (571)272-5982. The examiner can normally be reached Monday-Thursday 5:00 am- 6:30 pm EST.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, LIANKO GARYU can be reached on 571-270-7367. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For

additional questions, contact the Electronic Business Center (EBC) at 866-217-9197

(toll-free). If you would like assistance from a USPTO Customer Service

Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JULIE HA/
Primary Examiner, Art Unit 1654

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 11/05/2024

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER
HA, JULIE

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
11/05/2024	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmm.com
mgbdocket@marshallip.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 17811748
Applicant : Day
Filing Date : 07/11/2022
Date Mailed : 11/05/2024

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Naran Hem/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 17811748

IDENTIFICATION OF APPLICATION DEFICIENCIES IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012

[X] Applicant must provide legible text for the following item(s).

- [X] Specification filed 07/11/2022, page(s) 53 - Table 3.
- [ ] Claims filed , claim(s) .
- [ ] Other: .

[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

The response to this notice should contain only amended papers, as the waiver of 37 CFR 1.312 is limited to corrections of documents specifically requested in the notice.

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Jonathan Day et al.	Confirmation No.: 2988
Application No.: 17/811,748	Art Unit: 1654
Filed: July 11, 2022	Examiner: J. Ha
For: C-TYPE NATRIURETIC PEPTIDE VARIANTS TO TREAT SKELETAL DYSPLASIA IN CHILDREN	

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS, NOTICE OF ALLOWANCE MAILED, PURSUANT TO 37 CFR §1.121

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This paper is filed in response to a Notice to File Corrected Application Papers, Notice of Allowance Mailed (hereafter "the Notice") dated November 5, 2024.

Amendments to the Specification begin on page **2** of this paper.

Remarks begin on page **3** of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace Table 3, beginning on page 53 of the specification as filed, with the amended Table 3 set out below:

~~Analysis Population~~	~~Assessment~~	~~Overall~~		~~Cohort 1~~		~~Cohort 2~~		~~Cohort 3~~	
		~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~
~~FAS (~~	~~N~~	~~32~~	~~32~~	~~16~~	~~15~~	~~8~~	~~8~~	~~8~~	~~9~~
	~~Baseline, mean (SD)~~	~~9.60 (1.37)~~	~~11.06 (1.34)~~	~~4.20 (1.78)~~	~~4.74 (1.68)~~	~~10.55 (4.78)~~	~~11.51 (4.66)~~	~~19.45 (2.67)~~	~~21.19 (0.93)~~
	~~LSM change from baseline (95% CI)~~	~~-2.95 (-3.45, -2.45)~~	~~-2.17 (-2.76, -1.58)~~	~~0.89 (0.23, 1.55)~~	~~1.99 (1.31, 2.67)~~	~~-3.00 (-3.86, -2.13)~~	~~-2.36 (-3.22, -1.50)~~	~~-10.14 (-11.48, -8.79)[c]~~	~~-9.34 (-10.78, -7.91)[c]~~
	~~Difference in LSM (95% CI)[a]~~	~~**0.78 (0.02, 1.54)**~~		~~**1.10 (0.13, 2.07)**~~		~~**0.63 (-0.60, 1.87)**~~		~~**0.79 (-1.08, 2.67)[c]**~~	
	~~p-value[b]~~	~~0.0452~~		~~0.0276~~		~~0.2802~~		~~0.4072~~	
~~FAS~~	~~N~~	~~32~~	~~43~~	~~16~~	~~19~~	~~8~~	~~12~~	~~8~~	~~12~~
	~~Baseline, mean (SD)~~	~~9.60 (1.37)~~	~~11.66 (1.19)~~	~~4.20 (1.78)~~	~~5.07 (1.74)~~	~~10.55 (4.78)~~	~~11.66 (4.61)~~	~~19.45 (2.67)~~	~~22.09 (1.07)~~
	~~LSM change from baseline (95% CI)~~	~~-3.32 (-3.81, -2.84)~~	~~-2.41 (-2.88, -1.94)~~	~~0.77 (0.14, 1.40)~~	~~1.78 (1.20, 2.35)~~	~~-3.14 (-3.96, -2.31)~~	~~-2.23 (-2.90, -1.55)~~	~~-10.84 (-12.11, -9.57)[c]~~	~~-9.79 (-10.97, -8.62)[c]~~
	~~Difference in LSM (95% CI)[a]~~	~~**0.92 (0.24, 1.59)**~~		~~**1.00 (0.13, 1.88)**~~		~~**0.91 (-0.17, 1.99)**~~		~~**1.04 (-0.62, 2.71)[c]**~~	
	~~p-value[b]~~	~~0.0075~~		~~0.0256~~		~~0.0932~~		~~0.2200~~	

Analysis Population	Assessment	Overall		Cohort 1		Cohort 2		Cohort 3	
		Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide	Placebo	Vosoritide
FAS (randomized)	N	32	32	16	15	8	8	8	9
	Baseline, mean (SD)	9.60 (1.37)	11.06 (1.34)	4.20 (1.78)	4.74 (1.68)	10.55 (4.78)	11.51 (4.66)	19.45 (2.67)	21.19 (0.93)
	LSM change from baseline (95% CI)	-2.95 (-3.45, -2.45)	-2.17 (-2.76, -1.58)	0.89 (0.23, 1.55)	1.99 (1.31, 2.67)	-3.00 (-3.86, -2.13)	-2.36 (-3.22, -1.50)	-10.14 (-11.48, -8.79)[c]	-9.34 (-10.78, -7.91)[c]
	Difference in LSM (95% CI)[a]	**0.78 (0.02, 1.54)**		**1.10 (0.13, 2.07)**		**0.63 (-0.60, 1.87)**		**0.79 (-1.08, 2.67)[c]**	
	p-value[b]	0.0452		0.0276		0.2802		0.4072	
FAS	N	32	43	16	19	8	12	8	12
	Baseline, mean (SD)	9.60 (1.37)	11.66 (1.19)	4.20 (1.78)	5.07 (1.74)	10.55 (4.78)	11.66 (4.61)	19.45 (2.67)	22.09 (1.07)
	LSM change from baseline (95% CI)	-3.32 (-3.81, -2.84)	-2.41 (-2.88, -1.94)	0.77 (0.14, 1.40)	1.78 (1.20, 2.35)	-3.14 (-3.96, -2.31)	-2.23 (-2.90, -1.55)	-10.84 (-12.11, -9.57)[c]	-9.79 (-10.97, -8.62)[c]
	Difference in LSM (95% CI)[a]	**0.92 (0.24, 1.59)**		**1.00 (0.13, 1.88)**		**0.91 (-0.17, 1.99)**		**1.04 (-0.62, 2.71)[c]**	
	p-value[b]	0.0075		0.0256		0.0932		0.2200	

REMARKS

This paper is submitted in response to a Notice to File Corrected Application Papers ("Notice") in the above-mentioned application noting that substitute specification is required to fix illegible text in Table 3 on page 53 is due.

Amendment to the Specification

The specification is amended to replace Table 3 as filed to clarify the text. Applicant submits that it appears that the term "FAS (randomized)" was inadvertently cut off in the top of the first column of the Table to read only as "FAS (". Support for the inclusion of the full term is found in Table 2 of the application which refers to the same cohort groups as those analyzed in Table 3. The amendments include no new matter.

No fees are believed due in connection with this filing. If any fees are deemed necessary, the Commissioner is authorized to charge such fees to Deposit Account No. 13-2855, under Order No. 30610/57038.

Dated: December 30, 2024

Respectfully submitted,

Electronic signature: /Katherine L. Neville/
Katherine L. Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 11/05/2024

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER
HA, JULIE

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
11/05/2024	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mgbdocket@marshallip.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 17811748
Applicant : Day
Filing Date : 07/11/2022
Date Mailed : 11/05/2024

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Naran Hem/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 17811748

IDENTIFICATION OF APPLICATION DEFICIENCIES IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012

[X] Applicant must provide legible text for the following item(s).

- [X] Specification filed 07/11/2022, page(s) 53 - Table 3.
- [ ] Claims filed , claim(s) .
- [ ] Other: .

[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

The response to this notice should contain only amended papers, as the waiver of 37 CFR 1.312 is limited to corrections of documents specifically requested in the notice.

UNITED STATES
PATENT AND TRADEMARK OFFICE

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Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**12/30/2024 03:21:05 PM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	68554199	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 4

DOCUMENT		PAGES	DESCRIPTION	SIZE (KB)
Response to Notice to File Corrected Papers 57038.pdf		6	-	134 KB
Response to Notice to File Corrected Papers 57038-N427.pdf	(1-1)	1	Post Allowance Communication - Incoming	13 KB
Response to Notice to File Corrected Papers 57038-SPEC.pdf	(2-2)	1	Specification	29 KB
Response to Notice to File Corrected Papers 57038-REM.pdf	(3-3)	1	Applicant Arguments/Remarks Made in an Amendment	13 KB
Response to Notice to File Corrected Papers 57038-N427.pdf	(4-6)	3	Post Allowance Communication - Incoming	96 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Response to Notice to File Corrected Papers 57038.pdf	5FA5C20A4075129A054602320BC1527170B7DCB6E6AFB8C05 DCB314D95B47D978DB2E788B8912709C64A165BC54952328D D277F317392695C1A02940AE4203EC
Response to Notice to File Corrected Papers 57038-N427.pdf	9A4977CBEA219B426E301D1D431C801DE096D5ADCD3C3FF9 E181E131FBC1DB6D0C21C6A4268B0F24680283456A8421EED 55625CA78176BA81BF6348D29720FAC
Response to Notice to File Corrected Papers 57038-SPEC.pdf	1D7CDD7C013411E23BF7EFCA1D58E03CCDAF8AB1D6EFC32 4342239297C205E476875314F16847A74BDFAABAA9D72B460F 28576291344919C2A174BBA248C6E18
Response to Notice to File Corrected Papers 57038-REM.pdf	050F168CDEA76DC24DD85CBD15016ECABE33D2CA19C8478 A6431EBD003AB6F94CC8C9BC8897E87844CD4871849F5A852 4DC1FB59AB8306588FE25E0118A37136
Response to Notice to File Corrected Papers 57038-N427.pdf	EFAC09F33A22265CDC5A45FA51F08932F6EE62F28C8397230 2104037A8A4076EF448CBC451D231AFB00195E1B5064900C37 8778B6BF33F7AD911425B6AAB4AB7

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 01/08/2025

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
01/08/2025	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mgbdocket@marshallip.com

Response to Rule 312 Communication	Application No.	Applicant(s)	
	17/811,748	Day et al.	
	Examiner	Art Unit	AIA (FITF) Status
	JULIE HA	1654	Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☑ The amendment filed on 02 January 2025 under 37 CFR 1.312 has been considered, and has been:

a) ☐ entered.

b) ☑ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

f) ☐ not entered because the supplemental or corrected Application Data sheet (ADS)

☐ was not accompanied by a petition to accept an unintentionally delayed claim under 37 CFR 1.55 or 27 CFR 1.78;

☐ did not identify the information being changed in accordance with 37 CFR 1.76(c)(2);

☐ was not properly signed in accordance with 37 CFR 1.76(e) (or 37 CFR 1.33(b) for applications filed prior to September 16, 2012).

/JULIE HA/
Primary Examiner, Art Unit 1654

OK TO ENTER: /J.H/

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Jonathan Day et al.	Confirmation No.: 2988
Application No.: 17/811,748	Art Unit: 1654
Filed: July 11, 2022	Examiner: J. Ha
For: C-TYPE NATRIURETIC PEPTIDE VARIANTS TO TREAT SKELETAL DYSPLASIA IN CHILDREN	

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS, NOTICE OF ALLOWANCE MAILED, PURSUANT TO 37 CFR §1.121

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

This paper is filed in response to a Notice to File Corrected Application Papers, Notice of Allowance Mailed (hereafter "the Notice") dated November 5, 2024.

Amendments to the Specification begin on page **2** of this paper.

Remarks begin on page **3** of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace Table 3, beginning on page 53 of the specification as filed, with the amended Table 3 set out below:

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		~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~	~~Placebo~~	~~Vosoritide~~
~~FAS~~	~~N~~	~~32~~	~~32~~	~~16~~	~~15~~	~~8~~	~~8~~	~~8~~	~~9~~
	~~Baseline, mean (SD)~~	~~9.60 (1.37)~~	~~11.06 (1.34)~~	~~4.20 (1.78)~~	~~4.74 (1.68)~~	~~10.55 (4.78)~~	~~11.51 (4.66)~~	~~19.45 (2.67)~~	~~21.19 (0.93)~~
	~~LSM change from baseline (95% CI)~~	~~-2.95 (-3.45, -2.45)~~	~~-2.17 (-2.76, -1.58)~~	~~0.89 (0.23, 1.55)~~	~~1.99 (1.31, 2.67)~~	~~-3.00 (-3.86, -2.13)~~	~~-2.36 (-3.22, -1.50)~~	~~-10.14 (-11.48, -8.79)[c]~~	~~-9.34 (-10.78, -7.91)[c]~~
	~~Difference in LSM (95% CI)[a]~~	~~**0.78 (0.02, 1.54)**~~		~~**1.10 (0.13, 2.07)**~~		~~**0.63 (-0.60, 1.87)**~~		~~**0.79 (-1.08, 2.67)[c]**~~	
	~~p-value[b]~~	~~0.0452~~		~~0.0276~~		~~0.2802~~		~~0.4072~~	
~~FAS~~	~~N~~	~~32~~	~~43~~	~~16~~	~~19~~	~~8~~	~~12~~	~~8~~	~~12~~
	~~Baseline, mean (SD)~~	~~9.60 (1.37)~~	~~11.66 (1.19)~~	~~4.20 (1.78)~~	~~5.07 (1.74)~~	~~10.55 (4.78)~~	~~11.66 (4.61)~~	~~19.45 (2.67)~~	~~22.09 (1.07)~~
	~~LSM change from baseline (95% CI)~~	~~-3.32 (-3.81, -2.84)~~	~~-2.41 (-2.88, -1.94)~~	~~0.77 (0.14, 1.40)~~	~~1.78 (1.20, 2.35)~~	~~-3.14 (-3.96, -2.31)~~	~~-2.23 (-2.90, -1.55)~~	~~-10.84 (-12.11, -9.57)[c]~~	~~-9.79 (-10.97, -8.62)[c]~~
	~~Difference in LSM (95% CI)[a]~~	~~**0.92 (0.24, 1.59)**~~		~~**1.00 (0.13, 1.88)**~~		~~**0.91 (-0.17, 1.99)**~~		~~**1.04 (-0.62, 2.71)[c]**~~	
	~~p-value[b]~~	~~0.0075~~		~~0.0256~~		~~0.0932~~		~~0.2200~~	

Analysis Population	Assessment	Overall		Cohort 1		Cohort 2		Cohort 3	
		Placebo	**Vosoritide**	**Placebo**	**Vosoritide**	**Placebo**	**Vosoritide**	**Placebo**	**Vosoritide**
FAS (randomized)	N	32	32	16	15	8	8	8	9
	Baseline, mean (SD)	9.60 (1.37)	11.06 (1.34)	4.20 (1.78)	4.74 (1.68)	10.55 (4.78)	11.51 (4.66)	19.45 (2.67)	21.19 (0.93)
	LSM change from baseline (95% CI)	-2.95 (-3.45, -2.45)	-2.17 (-2.76, -1.58)	0.89 (0.23, 1.55)	1.99 (1.31, 2.67)	-3.00 (-3.86, -2.13)	-2.36 (-3.22, -1.50)	-10.14 (-11.48, -8.79)[c]	-9.34 (-10.78, -7.91)[c]
	Difference in LSM (95% CI)[a]	**0.78 (0.02, 1.54)**		**1.10 (0.13, 2.07)**		**0.63 (-0.60, 1.87)**		**0.79 (-1.08, 2.67)[c]**	
	p-value[b]	0.0452		0.0276		0.2802		0.4072	
FAS	N	32	43	16	19	8	12	8	12
	Baseline, mean (SD)	9.60 (1.37)	11.66 (1.19)	4.20 (1.78)	5.07 (1.74)	10.55 (4.78)	11.66 (4.61)	19.45 (2.67)	22.09 (1.07)
	LSM change from baseline (95% CI)	-3.32 (-3.81, -2.84)	-2.41 (-2.88, -1.94)	0.77 (0.14, 1.40)	1.78 (1.20, 2.35)	-3.14 (-3.96, -2.31)	-2.23 (-2.90, -1.55)	-10.84 (-12.11, -9.57)[c]	-9.79 (-10.97, -8.62)[c]
	Difference in LSM (95% CI)[a]	**0.92 (0.24, 1.59)**		**1.00 (0.13, 1.88)**		**0.91 (-0.17, 1.99)**		**1.04 (-0.62, 2.71)[c]**	
	p-value[b]	0.0075		0.0256		0.0932		0.2200	

PART B–FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via the USPTO patent electronic filing system.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via the USPTO patent electronic filing system or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

TITLE OF INVENTION: C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1,200.00			$1,200.00	01/29/2025

EXAMINER	ART UNIT	CLASS-SUBCLASS
J. Ha	1654	

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. MARSHALL, GERSTEIN & BORUN LLP
2.
3.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: BIOMARIN PHARMACEUTICAL INC.

(B) RESIDENCE: (CITY and STATE or COUNTRY): Novato, California

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required)

4b. Method of Payment (*Please first reapply any previously paid fee shown above*):

☒ Electronic Payment via the USPTO patent electronic filing system ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 13-2855

5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29. NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status. NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature: /Katherine L. Neville/ Date: January 22, 2025

Typed or printed name: Katherine L. Neville Registration No.: 53,379

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**01/22/2025 12:42:40 PM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	68860908	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 1

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
Fee Transmittal -- Part B PTOL-85.pdf	1	Issue Fee Payment (PTO-85B)	38 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Fee Transmittal -- Part B PTOL-85.pdf	49F1E9158428FCA98195639A3B75C7BD29D8BE48B6D34D595 97D468E16308683DFF626FC287DC8226239FBC4B0477E7E24 7741AC37778D27887A0B25810DB949

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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PATENT AND TRADEMARK OFFICE

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Alexandria, VA 22313 - 1450
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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**01/22/2025 12:42:40 PM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	68860908	AUTHORIZED BY	Katherine Neville
CUSTOMER #	90849	FILING DATE	07/11/2022
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Jonathan Day

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 0428	**E20251LC43248922**	**Valeria Rodriguez**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1501	UTILITY ISSUE FEE	1200.00	1	1200.00
			TOTAL AMOUNT:	**$1,200.00**

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New Applications Under 35 U.S.C. 111

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	02/25/2025	12233106	30610/57038	2988

90849 7590 02/05/2025

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Center (https://patentcenter.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional inventors):

Jonathan Day, Buckinghamshire, UNITED KINGDOM;
Elena Fisheleva, Novato, CA;

APPLICANT(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional applicants):

BIOMARIN PHARMACEUTICAL INC., Novato, CA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/811,748	07/11/2022	Jonathan Day	30610/57038	2988

90849 7590 02/25/2025

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
6300 Willis Tower
Chicago, IL 60606

EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
02/25/2025	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

lbigornia@bmrn.com
mgbdocket@marshallip.com

APPLICATION NO.	ISSUE DATE	PATENT NO.
17/811,748	25-Feb-2025	12233106

Marshall, Gerstein & Borun LLP (Biomarin)
233 South Wacker Drive
Chicago, IL 60606

EGRANT NOTIFICATION

Your electronic patent grant (eGrant) is now available, which can be accessed via Patent Center at https://patentcenter.uspto.gov

The electronic patent grant is the official patent grant under 35 U.S.C. 153. For more information, please visit https://www.uspto.gov/electronicgrants

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Alexandria, VA 22313 - 1450
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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**06/05/2025 10:03:39 AM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	12233106
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	70741766	FILING DATE	07/11/2022
CUSTOMER #	90849	FIRST NAMED INVENTOR	Jonathan Day
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Katherine Neville

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
Request for Certificate of Correction 57038.pdf	2	Request for Certificate of Correction	22 KB
Certificate of Correction 57038.pdf	2	Request for Certificate of Correction	29 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Request for Certificate of Correction 57038.pdf	6A1A07C99A1B5C915AF8ECFB455B9EBAE39AC6F2C7990769 B1CF377E8708D97B54B8F0FBE0890AD5377BF9983DA9A45A C581EAA4D8FD6163CD7EFC47ABBEC74A
Certificate of Correction	ADAFDD030DAE84CC11BEF2F31514E84C8BEA85F61B4492F

57038.pdf

C705CC6FC61241359E2FE69AA9213C3BD8185EAE39A4B1D2
2DBB694415AA49C819D1885A2AE068786

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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ELECTRONIC PAYMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
17/811,748	**06/05/2025 10:03:39 AM Z ET**	**30610/57038**

Title of Invention

C-Type Natriuretic Peptide Variants to Treat Skeletal Dysplasia in Children

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	12233106
CONFIRMATION #	2988	FILED BY	Valeria Rodriguez
PATENT CENTER #	70741766	AUTHORIZED BY	Katherine Neville
CUSTOMER #	90849	FILING DATE	07/11/2022
CORRESPONDENCE ADDRESS	-	FIRST NAMED INVENTOR	Jonathan Day

Payment Information

PAYMENT METHOD	PAYMENT TRANSACTION ID	PAYMENT AUTHORIZED BY
CARD / 2617	**E202565A04435416**	**Valeria Rodriguez**

FEE CODE	DESCRIPTION	ITEM PRICE($)	QUANTITY	ITEM TOTAL($)
1811	CERTIFICATE OF CORRECTION	172.00	1	172.00
			TOTAL AMOUNT:	**$172.00**

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage

submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 30610/57038
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of:
Jonathan Day et al.

Patent No.: 12,233,106

Issued: February 25, 2025

For: C-Type Natriuretic Peptide Variants To Treat Skeletal Dysplasia In Children

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 C.F.R. § 1.322

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted the following typographical errors, which should be corrected:

In the Application			In the Patent		Error
On the Cover Page:					
Document			**Field**	**Line**	
Amendments To The Abstract Dated August 01, 2024, Page 2	Under "Amendments To The Specification", Lines 2-3		(57)	1	PTO
In the Claims:					
Amendment	**Claim**	**Line**	**Column**	**Line**	
Notice Of Allowance And Fess Due (PTOL-85) Dated October 29, 2024, Page 4	1	15	63	63	PTO
Notice Of Allowance And Fess Due (PTOL-85) Dated October 29, 2024, Page 5	1	55	66	21	PTO

August 01, 2024, Page 12	19	2	68	25	Applicant
August 01, 2024, Page 12	19	9-10	68	47	Applicant
August 01, 2024, Page 13	20	2	69	2	Applicant

The errors now sought to be corrected are inadvertent typographical errors the correction of which does not involve new matter or require reexamination.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

Please charge our Credit Card in the amount of $172.00 covering the fee set forth in 37 C.F.R. § 1.20(a). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 13-2855, under Order No. 30610/57038.

Dated: June 5, 2025

Respectfully submitted,

By: /Katherine L. Neville/
Katherine Neville
Registration No.: 53,379
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive
6300 Willis Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

PTO/SB/44 (09-07)
Approved for use through 03/31/2026. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.
(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 12,233,106 B2

APPLICATION NO. : 17/811,748

ISSUE DATE : February 25, 2025

INVENTOR(S) : Jonathan Day et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Cover Page:

At field (57), line 1, "treatment" should be -- Treatment --.

In the Claims:

At column 63, line 63, "(SEQ ID NO:)" should be -- (SEQ ID NO:5) --.

At column 66, line 21, "[CNP37 (M32N)" should be -- [CNP37 (M32N)] --.

At column 68, line 25, "consisting of" should be -- consisting of: --.

At column 68, line 47, "[Gly-CNP-37 (M32N)" should be -- [Gly-CNP-37 (M32N)]--.

At column 69, line 2, "consisting of" should be -- consisting of: --.

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