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The safety of recombinant human hyaluronidase PH20 in nonclinical models: An overview of toxicology, pharmacology, and impact of anti-PH20 antibodies

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ABSTRACT

Hyaluronan (HA) is a glycosaminoglycan that forms a gel-like barrier in the subcutaneous (SC) space, limiting bulk fluid flow and the dispersion of SC-administered therapeutics. Recombinant human hyaluronidase PH20 (rHuPH20) facilitates the rapid delivery of co-administered therapeutics by depolymerizing HA in the SC space. Administration of rHuPH20 can induce the formation of anti-rHuPH20 antibodies, or anti-drug antibodies (ADAs), with the potential to bind endogenous PH20 hyaluronidase in the adult testes and epididymis. Using a variety of relevant animal models and multiple dose regimens of rHuPH20 across the full spectrum of animal development, we demonstrated that rHuPH20 administration resulted in the formation of ADAs. Although these ADAs can bind both the recombinant rHuPH20 enzyme and recombinant versions of animal model-specific hyaluronidases, they had no impact on fertility parameters (as measured by sperm concentration and motility, litter size, and litter viability) or fetal development. We present the result of our nonclinical studies in order of the developmental lifecycle, beginning with adults. Toxicology studies that extend beyond the standard package are also presented.

These studies demonstrate the favorable safety profile of rHuPH20 and ADAs in nonclinical models. Additionally, we identified substantial safety margins for clinically relevant doses of rHuPH20.

1. Introduction

Subcutaneous (SC) administration entails delivery of therapeutics to the skin interstitium, followed by local dispersion and eventual absorption into systemic circulation [1]. SC administration has several advantages over intravenous (IV) administration, including reduced administration time, the possibility for self-administration, wider choice of injection sites, a reduced risk of systemic infection, lower administration costs, a beneficial pharmacokinetic profile with a lower maximum plasma concentration (C_{max}), and fewer infusion-related reactions [2–5]. However, a key barrier to rapid, large-volume SC administration is hyaluronan (HA), one of the naturally occurring glycosaminoglycans in the extracellular, pericellular, and intracellular matrices and a key component of the skin [6–8]. HA is hydrophilic and forms a gel-like substance with water that resists bulk fluid flow through the SC space, which limits drug delivery, dispersion, and absorption [6,8–10].

Recombinant human hyaluronidase PH20 (rHuPH20) is a glycosylated, single-chain protein that enzymatically depolymerizes HA [11]. rHuPH20 acts transiently and locally in the SC space. rHuPH20 facilitates effective SC delivery of co-administered therapeutic agents and overcomes the volume and time limitations of traditional SC delivery and dispersion [8,11–15]. In nonclinical studies, rHuPH20 demonstrated rapid clearance and low systemic exposure when administered intradermally in mice [16], and rHuPH20 co-formulated with immunoglobulin G (IgG) solutions results in increased dispersion, reduced swelling, and shorter injection times in a miniature pig model [17,18].

In humans, rHuPH20 shows rapid distribution and clearance when administered intravenously in healthy individuals and it has not been detected systemically at doses used for SC administration [19,20]. Clinical studies demonstrate that SC-administered rHuPH20 has been generally well tolerated across diverse study populations with the most

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frequent adverse events involving mild injection site reactions during SC administration of large volumes of fluids [14,21]. Furthermore, numerous clinical studies have demonstrated the tolerability and safety of rHuPH20 in co-formulated therapeutics, including in long-term use scenarios (reviewed in Locke *et al* [8]), and in pregnant people and their infants [22].

Currently, SC administration of rHuPH20 is approved for use as a hyaluronidase human injection (HYLENEX®) to achieve hydration during SC fluid administration, increase the dispersion and absorption of other injected drugs, and in SC urography for improving resorption of radiopaque agents [21]. Approved co-formulations include the anti-cancer therapies trastuzumab (Herceptin® SC) [23,24], pertuzumab plus trastuzumab (PHESGO®) [25,26], rituximab (RITUXAN HYCELA®/RITUXAN® SC/MabThera® SC) [27,28], daratumumab (DARZALEX/DARZALEX Faspro®) [29,30], and atezolizumab (Tecentriq® SC) [31]. Additionally, rHuPH20 is approved in a co-formulated product for the treatment of generalized myasthenia gravis (VYVGART® HYTRULO) [32], and for sequential administration with human immunoglobin (HYQVIA®) for the treatment of primary immunodeficiency [33].

Administration of recombinant proteins has the potential to induce immunogenicity, which can contribute to adverse events. Anti-drug antibodies (ADAs) that bind endogenous counterparts with or without neutralization have the potential to cause severe side effects [34–36]. Because rHuPH20 has 100% homology to endogenous HuPH20 (minus the C-terminal glycosylphosphatidylinositol [GPI]-anchor), as well as more limited homology to other human hyaluronidases, rHuPH20-reactive ADAs have the potential to bind endogenous human hyaluronidases [37,38]. Notably, one analysis of 18 different clinical trials revealed that administration of rHuPH20 induced treatment-emergent ADAs in 1% to 45% of treated participants [37]. However, there has been no observed association between the presence of ADAs and adverse events from >20 completed studies (>2500 participants), and confirmed neutralizing antibodies are uncommon (<0.5%) [14,37,38].

Endogenous PH20 is expressed in the adult testes and epididymis, making fertility an important aspect to consider when studying anti-rHuPH20 antibodies [37,39,40]. Pre-existing rHuPH20-reactive antibodies have been detected at baseline in 3 to 13% of participants in clinical studies despite no prior exposure to rHuPH20 [37], and though the cause of these ADAs was unknown, their presence did not affect fertility [41]. Additionally, previous nonclinical studies attempted to achieve contraception by immunizing against PH20 across multiple animal species [42–44]. Despite the successful generation of PH20-reactive antibodies, these studies failed to demonstrate disruption of fertility or pregnancy [42–44]. As rHuPH20 is administered both in co-formulated products and in sequential doses over extended timeframes (months to years for the oncology therapies and chronic lifetime administration for HYQVIA®), we conducted nonclinical studies to evaluate the potential impact of ADAs in dosing regimens that reflect the real-world use of rHuPH20 [21,23–33].

Here, we report the nonclinical studies designed to evaluate the pharmacokinetic, pharmacodynamic, and toxicity profiles of rHuPH20 and rHuPH20-induced ADAs. In order to further investigate anti-rHuPH20 ADAs beyond the Good Laboratory Practices (GLP) standard toxicology package, we extended the toxicity studies to multiple species and across the full reproductive and developmental lifecycle. These extensive toxicology studies highlight the favorable nonclinical safety profile of rHuPH20 and ADAs, particularly in repeat-dosing and long-term use scenarios. The safety margins generated by these data relative to the doses of rHuPH20 in currently approved products with rHuPH20 are presented. The data collected in these comprehensive nonclinical studies were included in the HYQVIA® submission to the US Food and Drug Administration and the European Medicines Association [8].

2. Materials and methods

2.1. Overview of animal models

Several animal models were used, as indicated in Fig. 1 and Table 1, to study rHuPH20 and ADAs in the context of general toxicity, reproduction, and development. Due to the presence and identical structure of the HA substrate across species, rats, monkeys, mice, and rabbits were considered pharmacologically relevant and chosen for evaluating the effects of rHuPH20. ADAs generated in each model were assessed for cross-reactivity to species-specific recombinant versions of PH20 (or hyaluronidase enzymes [Hyals], as appropriate) to verify the appropriateness of the model in assessing the potential impact of ADAs on endogenous hyaluronidase function. We selected a range rHuPH20 doses to enable identifying a maximum no-observed-adverse-effect-level (NOAEL) in excess of standard human dose equivalents (see Table 2) and induce persistent levels of ADAs. Previous pharmacology and pharmacokinetic studies helped inform the chosen rHuPH20 dose levels.

All animals were housed in compliance with the National Research Council's "Guide for the Care and Use of Laboratory Animals" [45] and all studies were performed following detailed written protocols approved by the Institutional Animal Care and Use Committee (IACUC; Halozyme report numbers available in Table 1) of the conducting laboratory. Studies involving rabbits or mice were performed at Charles River Laboratories, Inc. (Horsham, PA, USA), and studies with monkeys were performed at SNBL USA, Ltd. (Everett, WA, USA). Local skin reactions in rats were studied at a Halozyme testing facility (San Diego, CA, USA) and the pathology report was performed by Nova Pathology, PC (Oak Harbor, WA, USA). A brief description of each animal model and study is given below, and all dosing information is listed in Table 1. Further experimental details are available in the supplemental materials.

2.2. Toxicology studies

2.2.1. Assessment of local skin reactions in Sprague Dawley rats

Local skin reactions were assessed after intradermal (ID) administration of rHuPH20 to Sprague Dawley rats. The ID route of administration in rats was selected because it is a close approximation to the SC space in humans [11] and facilitated visualization of potential skin reactions.

2.2.2. Cynomolgus monkeys: Nonpivotal 7-day repeat-dose and pivotal 39-week repeat-dose toxicity studies

Cynomolgus monkeys were assessed in repeat-dose toxicity studies because this species is closely related to humans, both phylogenetically and physiologically, and is commonly used for nonclinical toxicity evaluations [46]. Additionally, cynomolgus monkeys share a high degree of shared PH20 sequence identity with humans [47]. Male and female adult cynomolgus monkeys were assigned to different dosages of rHuPH20 as described in Table 1, then observed for signs of toxicity. Pharmacokinetic parameters were also measured throughout each study. We performed an immunohistochemical analysis to evaluate the potential deposition of IgG molecules in the testis, epididymis, and enteric nerve plexus (ENP) of cynomolgus monkeys in the 39-week study.

2.2.3. Pivotal mouse embryo-fetal development study in Crl:CD1(ICR) mice

The effects of rHuPH20 and ADAs on fertility, embryo-fetal development, and offspring maturation were assessed in Crl:CD1(ICR) mice because this species is a well-established model for reproductive and developmental toxins. Additionally, previous studies show that rHuPH20 is pharmacologically active and immunogenic in the species and strain, and antibodies generated against rHuPH20 have been shown to cross-react with the murine counterpart. This study evaluated the ICH S5 (R2) guideline stages C and D of the reproductive process [48].

Petitioner Merck, Ex. 1190, p. 370
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

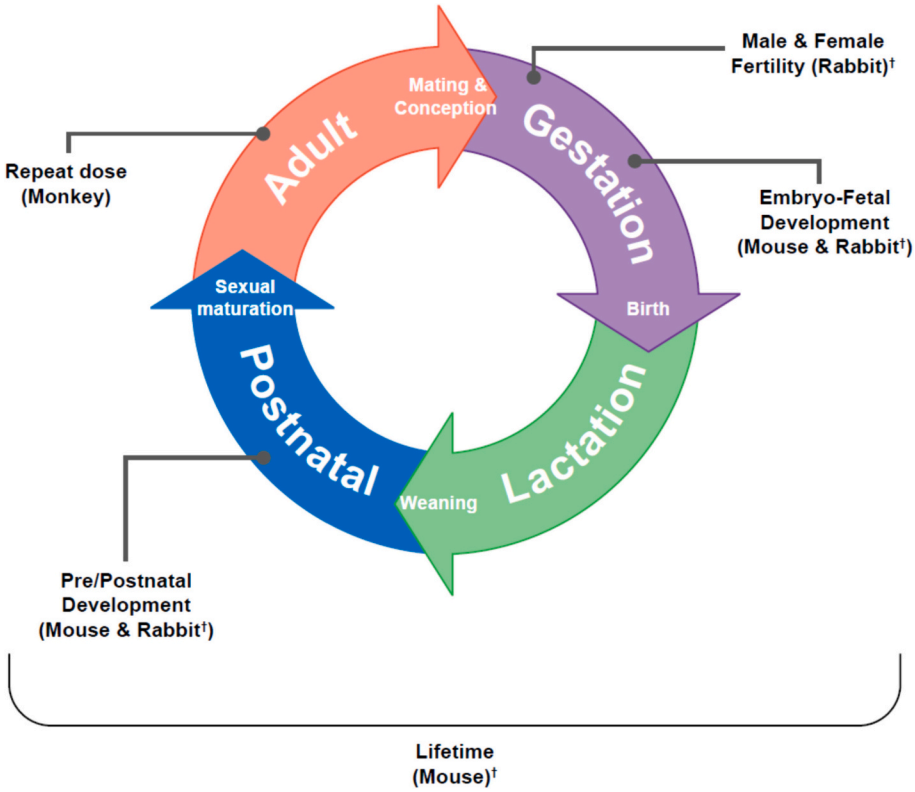


Fig. 1. Overview of all GLP-compliant studies and the correlating life stages. †Indicates extended toxicology studies.

Presumed pregnant female Crl:CD1(ICR) mice were treated as outlined in Table 1.

2.2.4. Pivotal mouse pre/postnatal development (PPND) study in Crl:CD1 (ICR) mice

This study was designed to evaluate ICH S5 (R2) guideline stages C through F of the reproductive process [48], but did not include an evaluation of Cesarean-delivered fetuses (stages C and D) because this had already been evaluated in the pivotal study described above. Because manifestations of effects induced during this period may be delayed, observations were continued through sexual maturity of the first filial (F1) generation mice. rHuPH20 or vehicle control was administered to pregnant Crl:CD1(ICR) female mice as described in Table 1 and further detailed in the supplementary methods.

2.3. Extended toxicology studies

Fertility, embryo-fetal development, and offspring maturation outcomes in the presence of ADAs were assessed in extended toxicology studies in New Zealand White (NZW) rabbits because the test facility had previously demonstrated that this model produces anti-rHuPH20 antibodies, and the timing of placental transfer of maternal IgG in rabbits is similar to that in humans [49]. Furthermore, the rabbit animal model was selected because it is an accepted model species in embryotoxicity studies [48]; this species and strain is sensitive to developmental toxicants.

2.3.1. Effect of ADAs on male fertility and general reproduction in NZW rabbits

Male fertility was examined by analysis of semen parameters and through implantation (mating outcome) by Cesarean-sectioning of nontreated female rabbits mated to ADA-positive male rabbits. This study was designed to evaluate ICH S5 (R2) guideline stage A (adult male reproductive functions, mating behavior, and fertilization) and stage B (paternally mediated effects) of the reproductive process [48].

2.3.2. Effect of ADAs on female fertility and embryo-fetal development in NZW rabbits

Female rabbits were treated as described in Table 1 and the supplementary methods, then mated to nontreated males on day of study (DS) 44, also designated as day of gestation (DG) 0. Fertility and ADA parameters were assessed.

2.3.3. Pivotal mouse lifetime study in Crl:CD1(ICR) mice

Male and female juvenile Crl:CD1(ICR) mice were assigned to a Juvenile Subset or a Chronic Subset, then treated as described in Table 1 and assessed for toxicity and developmental parameters.

2.4. rHuPH20 activity assay

Enzymatic activity of rHuPH20 was measured using quantitative assays developed and validated by MicroConstants (San Diego, CA, USA), based on protocols provided by Halozyme Therapeutics. For mouse plasma samples, rHuPH20 activity was quantified using a microplate-based chromogenic method. The qualified method for mouse plasma has a quantitation range from 0.00625 to 0.200 U/mL (lower limit of quantitation [LLOQ] to upper limit of quantitation [ULOQ]). Experimental details are available in the supplementary methods.

A modified USP turbidimetric method was adapted to quantify rHuPH20 activity in monkey plasma. Based on the validation results, this method is applicable for measuring hyaluronidase activity in cynomolgus monkey plasma within a range of 10 to 100 U/mL (LLOQ to ULOQ). Experimental details are available in the supplementary methods.

2.5. Anti-rHuPH20 antibody measurements

ADA titers: Antibodies against rHuPH20 in mouse and rabbit plasma

Petitioner Merck, Ex. 1190, p. 371
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Table 1
Overview of the toxicology program for rHuPH20.

Study type	Test system	Report numbers	Doses tested, mg/kg/dose method and frequency of administration	Duration of study
Standard toxicology package				
Local skin reactions	Rat/Sprague Dawley (n = 6 per test group; 48 total)	R09090	500 U; 1500 U (10,000 U/mL) 1 ID administration	48–120 h
7-day repeat-dose toxicity and toxicokinetics Pivotal	Monkey/ Cynomolgus (n = 2/sex/ dose)	R08056	5 (550,000 U/kg) IV or SC once daily	7 days
39-week repeat-dose toxicity and toxicokinetics Pivotal	Monkey/ Cynomolgus (n = 6/sex/ dose)	R09050	0.02; 0.2; 2.0 (2200; 22,000; 220,000 U/kg) SC once weekly	39 weeks
Embryo-fetal development Pivotal	Mouse/ Crl: CD1(ICR) (Main: n = 25/ dose) (TK: n = 36/ dose)	R08176	3; 9; 18 (330,000; 990,000; 1,980,000 U/kg) SC once daily DG 6 to 15	18 days
Pre/postnatal development Pivotal	Mouse/ Crl: CD1(ICR) (n = 25 females/dose)	R09058	3; 6; 9 (330,000; 660,000; 990,000 U/kg) SC once daily DG 6 to DL 20 or DG 22	21 days
Extended toxicology studies				
Effects of ADA on male fertility	Rabbit/NZW (n = 22 males/ dose)	12208	0.76 SC days 1, 8, 15, 22, 29, 43, and 57	85 days
Effects of ADA on female fertility, embryo-fetal development and PPND assessments	Rabbit/NZW (n = 44 females/dose)	12195	0.76 SC days 1, 8, 15, 22, 29, 42, and 59 (DG15)	262 days
Lifetime repeat-dose toxicity study in juvenile mice Pivotal	Mouse/ Crl: CD1(ICR) (Juvenile Subset: n = 30/sex/dose) (Chronic Subset: n = 25/sex/dose)	15028	1 (110,000 U/kg) SC once daily PND 7–30, then weekly until PND 129 (Juvenile Subset) or PND 241 (Chronic Subset)	34 weeks

All studies followed Good Laboratory Practice, except R09090, which was conducted for research and development purposes and was therefore not regulated by GLP. Abbreviations: ADA = anti-drug antibody; DG = day of (presumed) gestation; DL = day of lactation; GLP = Good Laboratory Practices; ID = intradermal; IV = intravenous; NZW = New Zealand White; PND = postnatal day; PPND = pre/postnatal development; SC = subcutaneous; TK = toxicokinetic.

were detected using a proprietary Halozyme electrochemiluminescence (ECL) immunoassay with biotin and ruthenium-labeled rHuPH20 following a multi-tiered approach: 1) an initial screen, 2) a confirmatory test, and 3) the determination of the relative titer for only those samples considered positive after the confirmatory step. This method is described in Rosengren *et al*, 2015 [38], and in further detail in the supplementary methods.

ADA cross-reactivity: Anti-rHuPH20 cross-reactivity was measured using sandwich format ECL assays as described in the supplementary methods.

Neutralizing ADAs: A modified USP turbidimetric method was qualified for the determination of neutralizing ADAs in mouse, rabbit, and monkey plasma. The method has been previously described (Rosengren *et al*, 2015 [38]), and further experimental details are provided in the supplementary methods.

3. Results

The animal studies are listed below in order of the developmental cycle (as outlined in Fig. 1), starting with adults. Studies that are part of the standard toxicology package are described first, followed by extended toxicology studies.

3.1. Assessment of local skin reactions in Sprague Dawley rats

To evaluate local tolerance of rHuPH20, we performed histologic evaluations following ID administration of rHuPH20 to Sprague Dawley rats (Supplemental Fig. 1). A few sporadic and minimal findings were observed and attributed to the injection procedure, but no meaningful rHuPH20-related findings were identified. More information is available in the supplemental results.

3.2. Nonpivotal monkey 7-day repeat-dose toxicity study in cynomolgus monkeys

The toxicokinetics (TK) and potential toxicity of rHuPH20 were evaluated in adult cynomolgus monkeys following either IV bolus or SC administration of rHuPH20 once daily for 7 consecutive days (Supplemental Fig. 2). The median time to C_{max} (T_{max}) after SC administration was 1 h. For both IV and SC administration routes, a modest decrease in exposure was observed after 7 daily doses. The systemic exposure of rHuPH20 was substantially lower after SC *versus* IV administration. No rHuPH20-related changes were observed for either the IV or SC groups in clinical observations, food consumption, body weight, ophthalmology, urinalysis, hematology, clinical chemistry, coagulation parameters, or histopathology (data not shown). For both the IV and SC administration routes, a NOAEL of 5 mg/kg/dose was assigned to rHuPH20.

3.3. Pivotal monkey 39-week repeat-dose toxicity study in cynomolgus monkeys

Following the 7-day repeat dosing study in cynomolgus monkeys, we expanded our analysis of rHuPH20 administration to an extended repeat-dose study in the same animal model. Adult cynomolgus monkey were administered rHuPH20 by SC injection once weekly for 39 weeks, followed by a 4-week recovery period. Animals were observed for toxicity, TK, ADA incidence, and fertility parameters throughout the study period (Fig. 2A).

There were no unscheduled deaths and no rHuPH20-related changes identified in mortality, clinical observations, food consumption, body weights, electrocardiographic examinations, heart rate, blood pressure, respiratory rate, ophthalmology, clinical and anatomic pathology, or organ weights (data not shown). Histopathology of the injection site revealed rHuPH20-related but reversible and nonadverse, minimal SC perivascular lymphoplasmacytic infiltration (Supplemental Fig. 3A), which is further described in the supplemental results.

TK analysis of plasma samples demonstrated limited systemic exposure in animals receiving rHuPH20 at 2 mg/kg/dose and limited or no detectable hyaluronidase concentrations in animals receiving 0.02 or 0.2 mg/kg/dose (levels at or below the assay limit) (Fig. 2B). Plasma samples from males administered 2 mg/kg/dose showed increasing rHuPH20 concentrations from day 1 to 85 (mean C_{max} = 12.5 U/mL and 28.8 U/mL, respectively), followed by a decrease observed on day 183 (C_{max} = 12.0 U/mL) and no detectable rHuPH20 by day 267 (Fig. 2B). However, rHuPH20 concentrations were higher and remained detectable in females throughout the study period, with mean C_{max} values of 20.8 U/mL, 64.7 U/mL, 64.2 U/mL, and 10.0 U/mL on days 1, 85, 183,

Petitioner Merck, Ex. 1190, p. 372
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Table 2
Safety Margins for a Given rHuPH20 Dose Based on NOAELs from rHuPH20 Toxicity Studies.

Species/strain	Study/route of administration/ frequency	NOAEL mg/kg (U/kg)	HED mg/kg[a] (U/kg)	Duration of study	Safety margin[b]				
					150 U	10,000 U	20,000 U	30,000 U	40,000 U
Monkey/ Cynomolgus	7-day repeat-dose toxicity[c] IV or SC, once daily	5 (IV and SC) (550,000)	1.61 (177,419)	7 days	82,796	1242	621	414	310
	39-week repeat-dose toxicity[d] SC, once weekly	2 (220,000)	0.65 (70,968)	39 weeks	33,118	497	248	166	124
Mouse/ Crl:CD1 (ICR)	Embryo-fetal development SC, once daily[e]	3 (330,000)	0.24 (26,829)	18 days	12,520	188	94	63	47
		18 (1,980,000)	1.46 (160,976)	18 days	75,122	1127	563	376	282
	Pre/postnatal development SC, once daily[f]	9 (990,000)	0.73 (80,488)	21 days	37,561	563	282	188	141
	Lifetime repeat-dose toxicity study in juvenile mice, SC once daily[g]	1 (110,000)	0.081 (8943)	34 weeks	4173	63	31	21	16

Abbreviations: HED = human equivalent dose; IV = intravenous; NOAEL = no-observed-adverse-effect-level; SC = subcutaneous.
[a] An HED based on allometric scaling was determined by dividing the NOAEL by a scaling factor of 3.1 for cynomolgus monkeys or 12.3 for mice [50].
[b] The HED (U/kg) was multiplied by 70 kg (patient body weight) to determine total U, then divided by the human dose of rHuPH20 to determine the safety margin.
[c] Supplemental Fig. 2. There were no adverse effects, and the NOAEL was the highest dose tested.
[d] Fig. 2. There were no adverse effects, and the NOAEL was the highest dose tested.
[e] Fig. 3. rHuPH20 was embryo-fetotoxic at ≥9 mg/kg/dose as demonstrated by increased resorptions, decreased litter size, and decreased fetal weight. There were no maternal adverse effects at the highest dose tested, 18 mg/kg/dose.
[f] Fig. 4. There were no adverse effects, and the NOAEL was the highest dose tested.
[g] Fig. 7.

and 267, respectively (Fig. 2B).

Decreasing rHuPH20 exposure throughout the study period was correlated with the development of neutralizing ADA titers. ADAs were detected in all rHuPH20 dose groups 6 weeks after the first dose, and rHuPH20 neutralizing activity was detectable 13 weeks after the first dose (Fig. 2B). Both rHuPH20 neutralizing activity and total ADA titers correlated with the SC dose administered and generally increased over time with repeated rHuPH20 administration. The development of neutralizing ADAs was associated with a marked decrease in rHuPH20 exposure after repeated SC administration. Despite the presence of neutralizing ADAs, plasma rHuPH20 concentrations were measurable following weekly repeat administration of 2.0 mg/kg/dose rHuPH20 for 6 months in males and 9 months in females (Fig. 2B). The presence of neutralizing ADAs was not correlated with any adverse effects, and titers decreased by approximately 50% from peak levels following the 4-week recovery period.

Fertility parameters were measured to investigate any potential reproductive impact of rHuPH20 exposure or the resulting ADAs. The development of ADAs did not negatively alter sperm concentration and motility in males or menstrual cycle in females (Fig. 2C, D). We then investigated if the anti-rHuPH20 antibodies deposited in monkey testis, epididymis, or colon ENP *via* immunohistochemistry analysis of *ex vivo* tissues (Supplemental Fig. 4). No IgG staining was observed in approximately 99% of >550 total ENPs section and >99% of 4000 examined epididymal lumen profiles. These studies indicate that the ENP and epididymal lumen demonstrate an immunologic blood barrier, as also observed in the testis tissue.

3.4. Pivotal embryo-fetal development study in Crl:CD1(ICR) mice

Our studies in cynomolgus monkeys indicated that rHuPH20 exposure and the resulting development of neutralizing ADAs did not negatively alter fertility parameters in adults. Therefore, we extended our studies to analyze rHuPH20 and ADAs in the context of embryo-fetal development in a mouse model during gestation, from implantation to closure of the hard palate (Fig. 3A). Presumed pregnant mice were treated with rHuPH20 at varying doses (3, 9, or 18 mg/kg/dose), and divided into either a TK analysis group that was studied for rHuPH20 exposure and ADAs, or a fertility study group that was observed for fetal development (Fig. 3A). Ultimately, these studies demonstrated a maternal NOAEL of 18 mg/kg, but the developmental NOAEL was 3 mg/kg due to significant reductions in fetal body weight at higher doses.

There was no evidence of maternal toxicity at any rHuPH20 dosage. Maternal body weights and weight gains were unaffected by rHuPH20, and no signs of local toxicity were observed (data not shown). Unscheduled deaths occurred in 2/25 main study mice at 9 mg/kg/dose, and 1/25 main study mice and 1/36 TK study mice at 18 mg/kg/dose. These mice did not exhibit any signs of toxicity (*i.e.*, adverse clinical signs, reduced rate of body weight gains, or body weight losses) and the deaths were not dose-dependent; therefore, the deaths were not considered related to rHuPH20. Based on these observations, the maternal NOAEL was determined to be 18 mg/kg/dose.

Blood and plasma samples were collected from the TK study mice on DG 6 and 15. TK analyses indicated dose-dependent increases in rHuPH20 exposure from DG 6 to DG 15 (Fig. 3B). Retrospective analysis of plasma samples demonstrated that all mice developed ADA titers within comparable ranges across all rHuPH20 dose groups by DG 15 (Fig. 3B). The ADAs were predominantly of the isotype IgG, which can cross the placenta during gestation, leading to potential fetal exposure to ADAs.

Though there was no difference in fetal viability across the rHuPH20 dose levels, fetuses in the 9 and 18 mg/kg/dose groups demonstrated a significant rHuPH20-related decrease in body weights (Fig. 3C) and a nonsignificant increase in late resorptions. Notably, there were no rHuPH20-related fetal gross external, soft tissue, or skeletal fetal alterations (malformations or variations) at any dosage (data not shown). Due to the rHuPH20-related impact on fetal body weights, the developmental NOAEL was determined to be 3 mg/kg/dose.

3.5. Pivotal developmental and perinatal/postnatal reproduction toxicity in Crl:CD1(ICR) mice, including a postnatal behavioral/functional evaluation

Upon establishing that administration of rHuPH20 did not adversely affect fetal development during gestation at dosages below 9 mg/kg, we then tested the potential impact of rHuPH20 and ADAs throughout lactation and postnatal development in mice. Parental (F0) female mice were administered daily rHuPH20 at 0, 3, 6, or 9 mg/kg/dose throughout gestation and lactation, and development of the F1 generation was analyzed (Fig. 4A). No rHuPH20-related effects were observed in mortality, clinical observations, or litter observations, and no signs of local toxicity were observed. In F0 maternal mice, deaths occurred in 1/25 at 6 mg/kg/dose and 2/25 mice at 9 mg/kg/dose (data not shown). Due to the absence of signs of toxicity, these deaths were considered

Petitioner Merck, Ex. 1190, p. 373
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

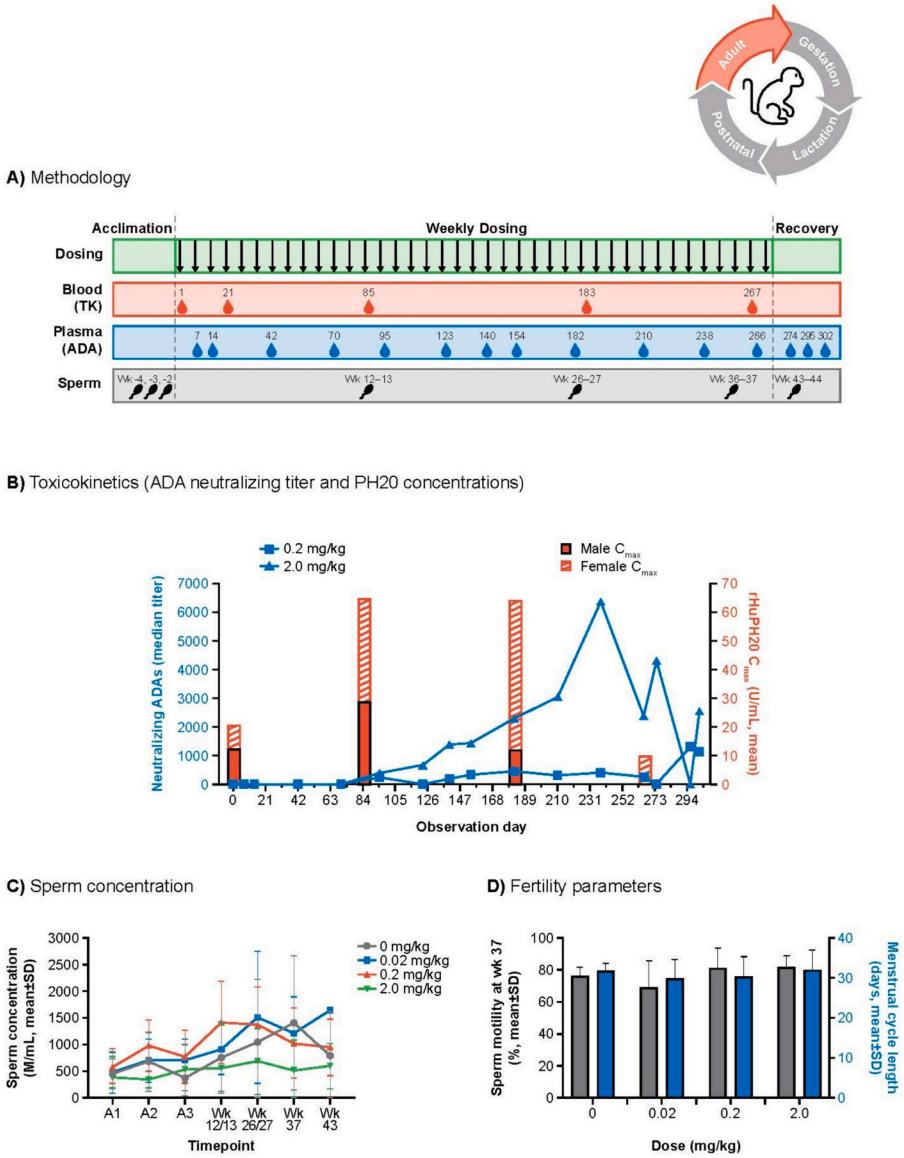


Fig. 2. Impact of weekly administration of rHuPH20 to cynomolgus monkeys for 39 weeks.
A) Methodology. Monkeys were administered rHuPH20 once per week for 39 weeks at a dose of 0, 0.02, 0.2, or 2.0 mg/kg. Blood, plasma, and sperm samples were obtained on the indicated days for toxicokinetic and fertility analysis. B) Toxicokinetics. Neutralizing anti-rHuPH20 antibody titers (left axis, blue) and rHuPH20 activity (right axis, red) are shown. The median neutralizing titer is shown only for the 0.2 mg/kg (squares) and 2.0 mg/kg (triangles) groups because neutralizing ADAs were at or below the limit of detection for the other dose groups. Activity of rHuPH20 is shown as the arithmetic mean maximum concentration of rHuPH20 (C_{max}) in the blood of males (solid red) and females (hatched red). C) Sperm concentration during the acclimation (A1, A2, A3), dosing (Wk12/13, Wk 26/27, Wk 36/37), and recovery (Wk 43) periods of the study for all dose groups. D) Fertility parameters for male and female monkeys. Sperm motility (left axis, grey) is shown for Wk 37. Menstrual cycle length (right axis, hatched red) shows the mean cycle length throughout the study period. Abbreviations: ADA = anti-drug antibodies; rHuPH20 = recombinant human hyaluronidase PH20; SD = standard deviation; TK = toxicokinetic; Wk = week. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unrelated to rHuPH20 and likely related to repeated administration of a human protein. In F1 generation mice, 1 female mouse from the vehicle maternal dose group was found dead on postnatal day (PND) 31. There were no adverse clinical observations or changes in body weight for this mouse (data not shown).

Administration of rHuPH20 to F0 pregnant mice at all doses in this study did not significantly alter the viability index, percent of male pups, or body weights of the F1 generation (Fig. 4B). Additionally, no rHuPH20-related effects were noted in the F1 generation mice in clinical observations, age of sexual maturation, passive avoidance test (for learning, short-term and long-term retention), motor activity test, mating performance, and gross pathologic findings. Cesarean-sectioning observations of pregnant F1 female mice revealed no rHuPH20-related changes to fertility, including corpora lutea, implantations, early and late resorptions, or litter sizes (Fig. 4C). Overall, administration of rHuPH20 to F0 female mice throughout gestation and lactation did not negatively affect the development of the F1 generation, or the F1 generation's ability to produce the F2 generation. Therefore, we concluded that the maternal and F1 dam NOAEL was 9 mg/kg/dose.

3.6. Extended toxicology studies: Impact of anti-rHuPH20 antibodies

The studies performed in the standard toxicology package suggested a favorable safety profile for rHuPH20; however, we considered basic toxicology studies limiting for a comprehensive evaluation of the potential impact of anti-rHuPH20 ADAs in fertility and development. We

Petitioner Merck, Ex. 1190, p. 374
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

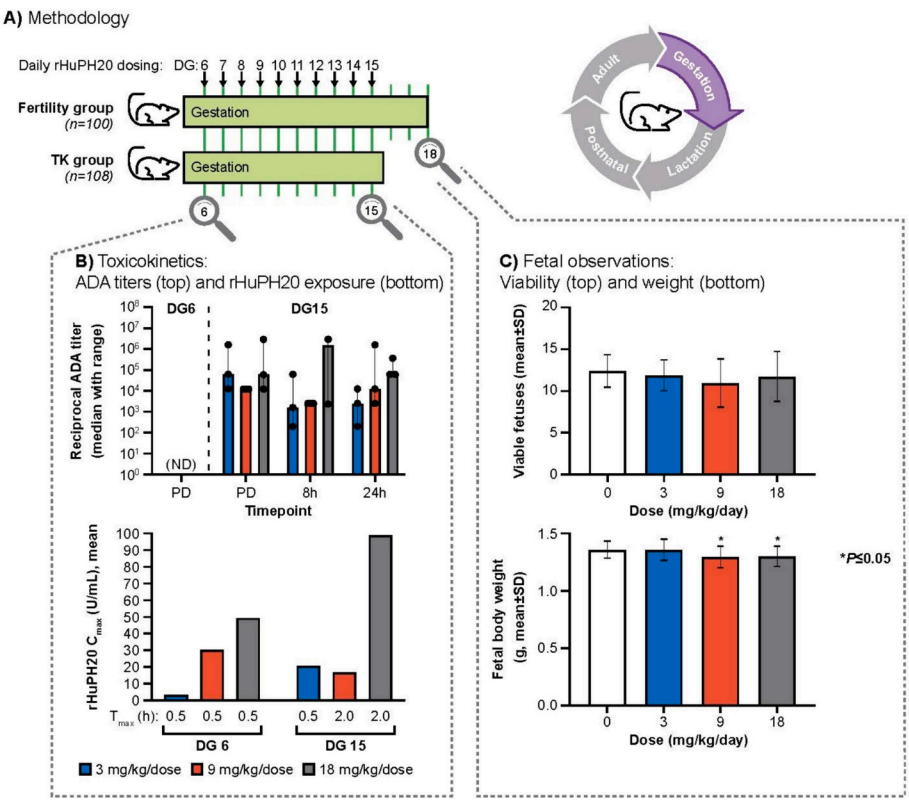


Fig. 3. Embryo-fetal development in mice following daily rHuPH20 administration.
A) Methodology. Presumed pregnant female mice were administered rHuPH20 at 3, 9, or 18 mg/kg/dose from DG 6 through DG 15. Mice in the TK group were analyzed for rHuPH20 exposure and ADAs. The offspring from the fertility group were observed for potential impacts of rHuPH20 on fetal development. B) Toxicokinetics. ADA titers (top) and rHuPH20 exposure (bottom, C_{max}) in mice from the TK group. C) Fetal observations for the C-sectioned offspring of the fertility group on DG 18.
Abbreviations: ADA = anti-drug antibodies; DG = day of (presumed) gestation; g = grams; h = hour; ND = not detected; PD = predose; rHuPH20 = human hyaluronidase PH20; SD = standard deviation; TK = toxicokinetics; U = unit.

therefore expanded our studies to include prospective analyses of ADAs in NZW rabbits, to specifically evaluate male and female fertility parameters as well as embryo-fetal development. This model was chosen because PH20 expression patterns and the timing of placental transfer of maternal IgG in the rabbit is similar to that in humans [49].

3.6.1. *Effect of ADAs on male fertility and general reproduction in NZW rabbits*

Male rabbits were administered SC rHuPH20 at 0.76 mg/kg/dose once per week for 8 weeks, then evaluated for ADAs, sperm parameters, and fertility outcomes (Fig. 5A). All males that were administered rHuPH20 tested positive for ADAs by day 8, with a median reciprocal titer of 1600 (Fig. 5B). Median titers increased to 2,457,600 on day 29 and 6,553,600 on day 85 (last day of study; Fig. 5B). Cross-reactivity to a recombinant form of rabbit PH20 (rRbPH20) was first observed on day 15 in approximately 5% of samples (Fig. 5B); by day 29, all samples were cross-reactive to rRbPH20, and ≥ 90% of samples maintained cross-reactivity throughout the study (Fig. 5B). Most treated males displayed neutralizing activity to both rHuPH20 (82%) and rRbPH20 (60%) 1 day prior to mating (Fig. 5B). There were no rHuPH20-related changes in body weight, body weight gains, or food consumption compared with controls, and no unscheduled deaths attributed to rHuPH20 or ADAs (data not shown). After the fifth dose, erythema was observed at the injection site in the 22 rHuPH20-treated males as follows: Grade 1 (n = 12), Grade 2 (n = 4), and Grade 3 (n = 1; Supplemental Fig. 3B). This resolved within 4 days of each dose and was not considered adverse. Ultimately, weekly SC administration of rHuPH20-induced cross-reactive and neutralizing ADAs to both human and rabbit PH20 in F0 male rabbits, with no evidence of toxicity.

ADA cross-reactivity and neutralizing activity against rabbit PH20 demonstrated the relevance of this model for studying fertility in rabbit. Administration of rHuPH20 and resulting ADAs had no adverse effects on semen evaluations or reproductive outcomes of F0 male rabbits. Sperm motility and concentration were not negatively changed in the treated males compared with the control males (Fig. 5C). Treated males were mated with nontreated females and there were no statistically significant differences in fertility or embryonic assessments, including the number of corpora lutea, implantation sites, or viable embryos (Fig. 5D). These studies illustrate that rHuPH20-administration and resulting ADAs had no impact on clinical observations or the reproductive capacity of F0 males.

3.6.2. *Effect of ADAs on female fertility and embryo-fetal development in NZW rabbits*

Following observations of ADAs in rHuPH20-treated male rabbits, we next studied the potential effects of rHuPH20 and ADAs on maternal F0 rabbits and the resulting F1 generation. rHuPH20 was administered to F0 females before and after mating so that we could study the resulting ADAs throughout mating, fertility, and development of F1 offspring from implantation to closure of the hard palate. Due to the potential for delayed or progressive effects on postnatal development, F1 offspring were additionally monitored for developmental milestones, behavior, activity, and fertility in adulthood.

F0 female rabbits were administered weekly SC rHuPH20 (0.76 mg/kg/dose) or vehicle prior to mating with nontreated males on day 44 of the study. F0 females were treated again after mating to maintain ADA titers throughout gestation (Fig. 6A). Presumed pregnant females were assigned to either a Cesarean-sectioning or natural delivery cohort, and

Petitioner Merck, Ex. 1190, p. 375
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

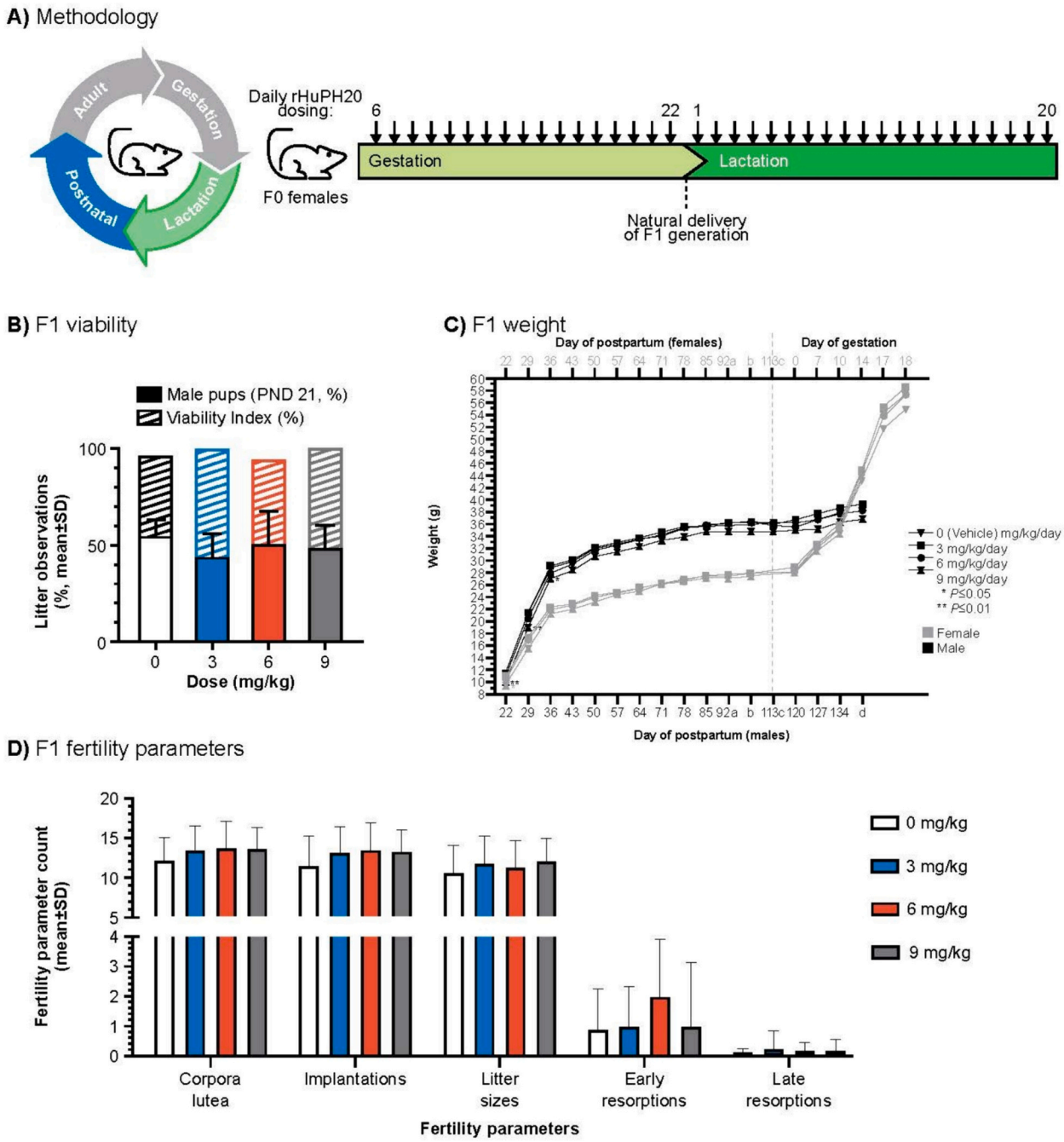


Fig. 4. Pre/postnatal development in mice following daily rHuPH20 administration.
A) Methodology. Presumed pregnant female mice (F0) were administered daily 0 (vehicle control), 3, 6, or 9 mg/kg/dose rHuPH20 starting on DG 6 and continuing through the first 20 days of lactation. B) Assessments of the F1 generation. F1 pups born to F0 rHuPH20-treated mothers were observed for viability (hatched bars) and the percent of male pups per litter, as determined on PND 21 (solid bars). C) Body weight of male (black) and female (grey) F1 mice. a = last weekly value recorded before cohabitation; b = precohabitation body weight; c = first weekly value recorded after cohabitation; d = terminal body weight. D) Fertility assessments of F1 mice. Female mice were mated to control (nontreated) males and examined for the number of corpora lutea, implantations, litter sizes, and resorptions. Abbreviations: DG = day of (presumed) gestation; F0 = parental generation; F1 = first filial generation; rHuPH20 = recombinant human hyaluronidase PH20; SD = standard deviation.

satellite groups (n = 3 per group per interval) were included for ADA analysis of maternal and fetal/pup plasma. Deaths unrelated to rHuPH20 occurred in 2/44 and 1/44 F0 maternal rabbits in the vehicle control and 0.76 mg/kg/dose groups, respectively. The single death in the F0 rHuPH20 group occurred on day 8, and the specific cause could not be determined based on clinical signs; however, the death was likely related to dehydration and/or hypersensitivity to administration of a human protein. No clinical observations or dermal scoring/skin reactions were considered related to rHuPH20.

ADA titers were detected in the plasma of treated F0 female rabbits starting on day 8 (prior to mating), and titers remained detectable throughout mating and gestation (Fig. 6B). The majority of maternal F0 samples demonstrated neutralizing activity against rHuPH20 prior to mating and during gestation; 83.7% of samples had neutralizing ADAs against rHuPH20 on observation day (OD) 42 and DG 15 (Fig. 6B). ADA titers were also detected in F1 fetal plasma beginning on DG 20,

Petitioner Merck, Ex. 1190, p. 376
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

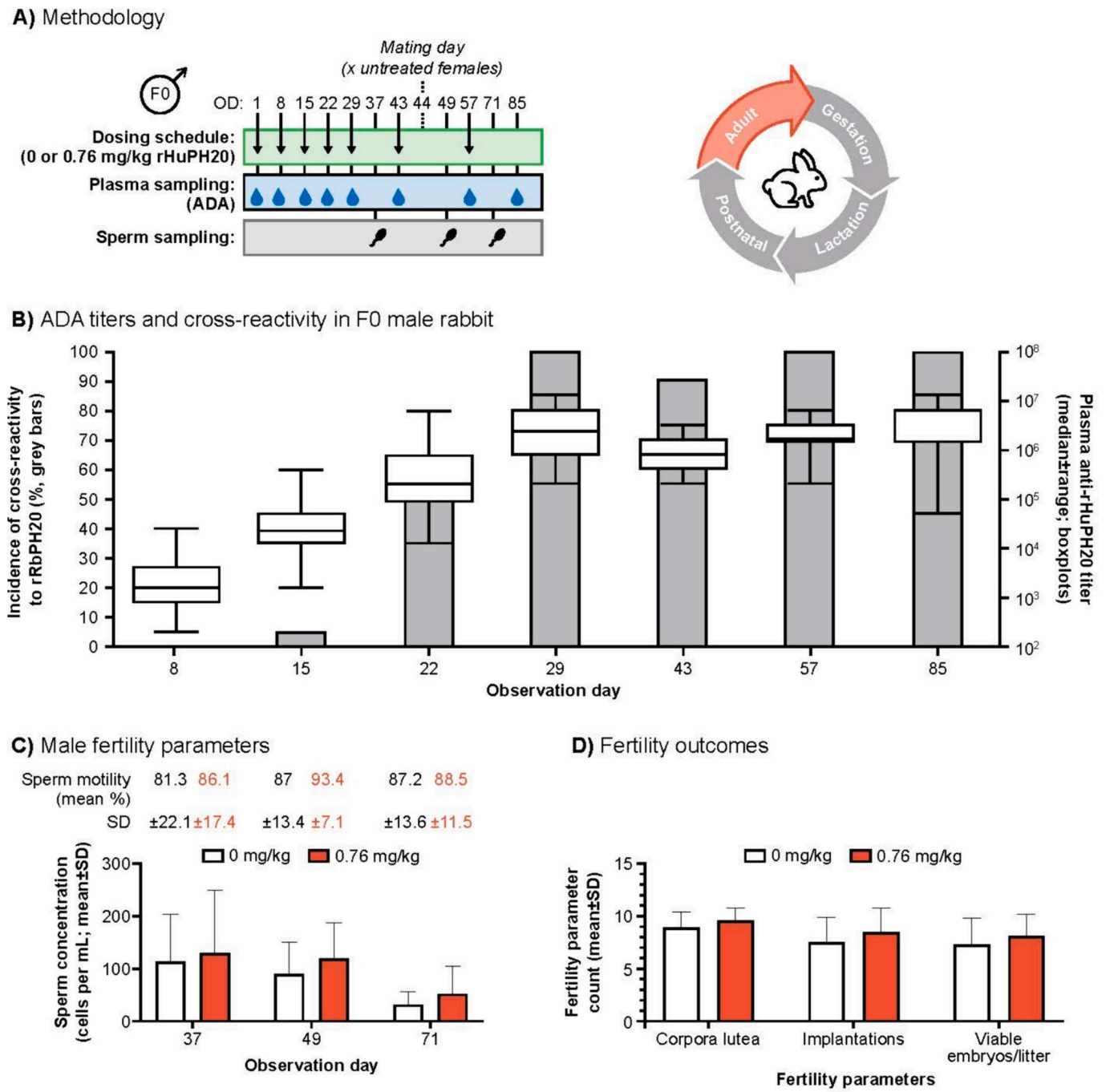
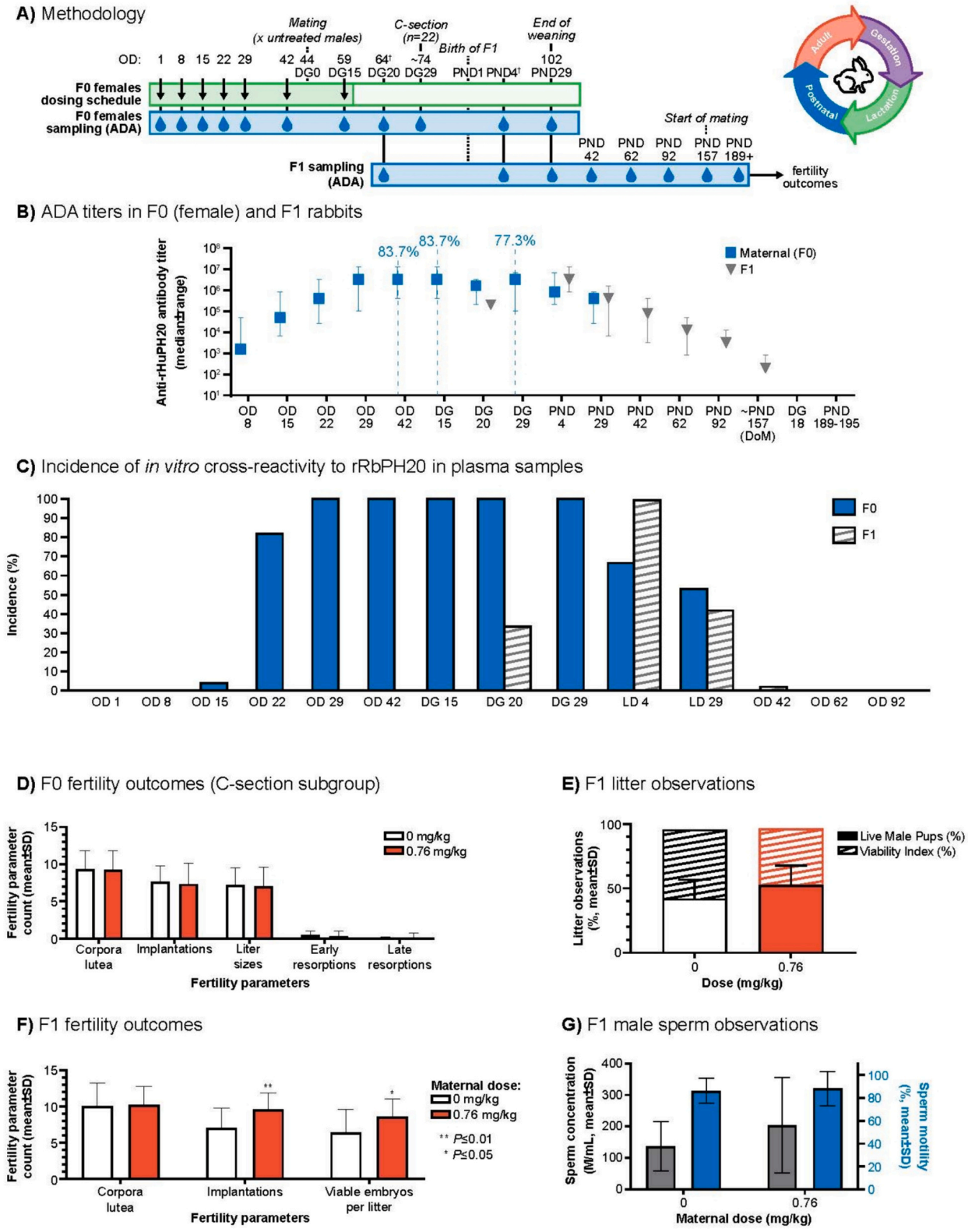


Fig. 5. Impact of rHuPH20 and ADAs on male rabbit fertility (extended toxicology study).
A) Methodology. F0 male mice were administered 0 (vehicle) or 0.76 mg/kg/dose rHuPH20 weekly prior to mating with female rabbits. Plasma and sperm sampling was performed at the indicated timepoints. B) ADA titers (left axis) and neutralizing titers (right axis) in F0 male rabbits. C) Male fertility parameters measured by sperm concentration and motility. D) Fertility outcomes following mating between vehicle or rHuPH20-treated males and nontreated females.
Abbreviations: ADA = anti-drug antibodies; OD = observation day; F0 = parental generation; F1 = first filial generation; rHuPH20 = recombinant human hyaluronidase PH20; SD = standard deviation.

indicating maternal transfer of rHuPH20 antibodies to developing fetuses (Fig. 6B). ADAs from both maternal and F1 samples demonstrated cross-reactivity to rRbPH20 (Fig. 6C), and most maternal samples (≥60%) had neutralizing activity against rRbPH20 throughout the study (Fig. 6C). F1 ADA titers peaked during lactation on PND 4; thereafter, the median titer in F1 rabbits gradually declined through the end of weaning, consistent with decreased maternal antibody transfer. The presence of circulating ADAs in F0 rabbits and transfer to F1 fetuses did not have an effect on F0 female fertility; litter sizes and the number of corpora lutea, implantations, and early and late resorptions were not significantly different between the control and rHuPH20-treated groups (Fig. 6D). Furthermore, there were no significant differences in the viability index or sex ratio of the litters born to treated or control F0 females (Fig. 6E). There were also no significant differences in fetal weights or fetal external, visceral, and skeletal examinations (data not shown).

Though the F1 generation was not directly administered rHuPH20, detectable rHuPH20-reactive ADAs persisted in 50% of the F1

Petitioner Merck, Ex. 1190, p. 377
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042



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Petitioner Merck, Ex. 1190, p. 378
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Fig. 6. Impact of rHuPH20 and ADAs on F0 female rabbit fertility and F1 rabbit development (extended toxicology study).
A) Methodology. F0 female rabbits were administered 0.76 mg/kg/dose rHuPH20 prior to mating with nontreated males. Plasma samples were collected from F0 female rabbits and F1 offspring at the indicated timepoints for ADA analysis. †Indicates satellite animals only; $n = 3$ per interval per generation. B) ADA titers in F0 female (blue square) and F1 offspring (grey triangle) rabbits. Percentages in blue indicate the incidence of rHuPH20-neutralizing ADA activity in maternal plasma samples. C) Incidence of *in vitro* cross-reactivity to rRbPH20 in F0 (blue) and F1 (grey hatched) plasma samples. D) Fertility outcomes for the C-section subgroup of the F0 population after treatment with rHuPH20 (0.76 mg/kg, red) or vehicle control (white). E) Assessments of the F1 generation. F1 pups born to F0 rHuPH20-treated mothers were observed for viability (hatched bars) and the percent of male pups per litter, as determined on PND 29 (solid bars). The Viability Index was calculated as the number of live pups on day 4 postpartum divided by the number of liveborn pups on day 1 postpartum. F) Fertility outcomes for the F1 generation. Nontreated F1 females with rHuPH20-treated ($n = 17$, red) or nontreated ($n = 19$, white) mothers were mated with males and examined for fertility outcomes on day 18 of gestation. G) Sperm observations for F1 generation males. Nontreated F1 males with rHuPH20-treated ($n = 21$, red) or nontreated ($n = 22$, white) mothers were examined for sperm concentration and motility on approximately day 160 postpartum or later. Abbreviations: ADA = anti-drug antibodies; DG = day of (presumed) gestation; DoM = day of mating; F0 = parental generation; F1 = first filial generation; OD = observation day; PND = postnatal day; rHuPH20 = recombinant human hyaluronidase PH20; rRbPH20 = recombinant rabbit PH20; SD = standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

generation into adulthood at the time of mating (~PND 152). To observe any potential influence of ADA exposure on subsequent generations, F1 males were evaluated for sperm parameters and F1 females were mated to males that were not exposed to ADAs. There were no observed negative effects on the ability of the F1 generation to procreate, as evidenced by no detrimental changes in corpora lutea, implantations, or viable embryos per litter (Fig. 6F). Additionally, no negative changes were observed in F1 male sperm parameters (Fig. 6G). Overall, administration of rHuPH20 to F0 female rabbits induced cross-reactive and neutralizing ADAs that were subsequently transferred to F1 fetuses. Administration of rHuPH20 and the resulting ADAs did not affect F0 fertility or development of the F1 generation. Furthermore, exposure to maternally transferred ADAs had no impact on the F1 generation's capacity for reproduction.

3.6.3. Pivotal lifetime study in juvenile Crl:CD1(ICR) mice

The studies described above indicate that F0 adult exposure to rHuPH20 and the resulting rHuPH20-reactive ADAs did not negatively affect F0 fertility parameters or the development of the F1 generation. However, the potential impact on development when rHuPH20 is directly administered to juvenile animals had yet to be explored. We therefore examined the potential toxicity of rHuPH20 and the resulting ADAs when rHuPH20 was administered directly to juvenile mice starting on PND 7 and continuing through adulthood. Mice were assigned to either a Juvenile Subset or Chronic Subset. All mice were subcutaneously administered either vehicle control or rHuPH20 (1 mg/kg/dose) once daily from PND 7 through PND 30, then once weekly thereafter until PND 129 for the Juvenile Subset or PND 241 for the Chronic Subset (Fig. 7A).

Anti-rHuPH20 ADAs were detected in 100% of rHuPH20-administered mice in both subsets by PND 30, and ADA titers persisted throughout the study for each group (Fig. 7B). ADAs demonstrating neutralizing activity to rHuPH20 were detected in ≥75% of rHuPH20-administered mice in both subsets by PND 90, and the incidence of rHuPH20-neutralizing ADAs persisted throughout the end of the study in 97% and 98% of the Juvenile and Chronic subsets, respectively (Fig. 7B). Plasma samples were additionally tested for cross-reactivity to recombinant versions of the murine homolog of PH20, MuPH20, and a closely related murine testicular hyaluronidase, muHyal5. Cross-reactivity to both mouse hyaluronidases was demonstrated in the majority of samples throughout the study, and in all samples by PND 90 (Fig. 7B).

3.6.4. Juvenile Subset

A total of 4 deaths occurred, all of which were considered unrelated to rHuPH20 due to their incidental nature and similar incidence of deaths across groups: 2 females in the control group, and 2 females in the rHuPH20 group. The 2 control females were euthanized due to evidence of injury leading to limited use of hindlimbs. In the rHuPH20-exposed group, 1 female was euthanized due to adverse clinical signs and 1 female was found dead in the absence of clinical signs. All males survived to the study endpoint, PND 129. No rHuPH20-related impacts were evident in clinical observations or effects on body weight, body weight gains, or terminal body weights for males and females in the rHuPH20 group. There were no rHuPH20-related changes in balano-preputial separation in males, vaginal opening in females, neurobehavioral parameters in males and females (passive avoidance, acoustic startle, motor activity, or Morris water maze), or organ weights (data not shown). In addition, there were no rHuPH20-related effects on mating and fertility or sperm parameters (Fig. 7C). Administration of rHuPH20 to the F0 generation did not adversely affect F1 litter parameters such as fetal body weights and fetal sex (Fig. 7D), or fetal external, visceral, or skeletal examinations (data not shown).

3.6.5. Chronic Subset

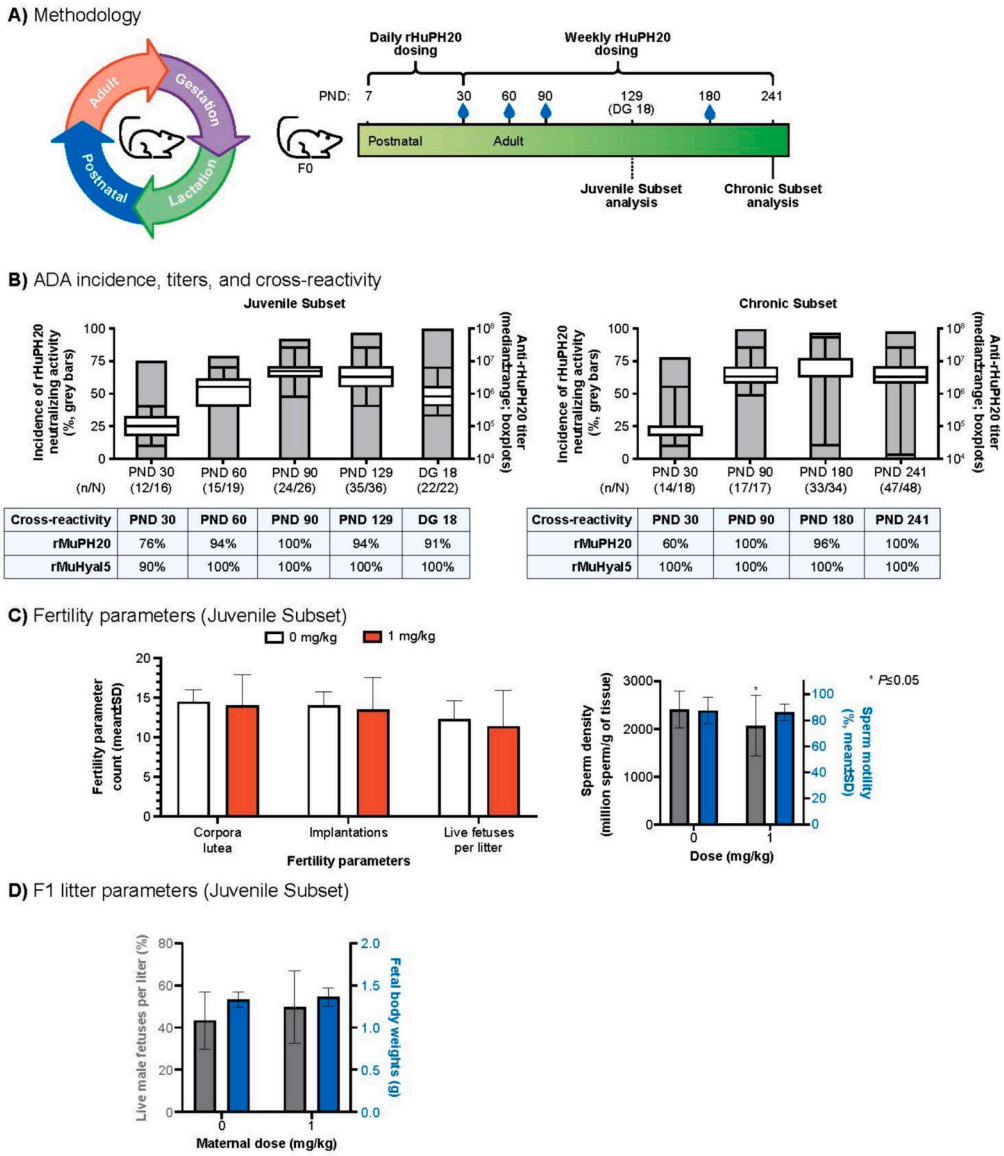
No rHuPH20-related deaths occurred in the Chronic Subset. There were 5 unrelated deaths; 1 control female was found dead in the absence of clinical signs or changes in body weight, 2 control females were euthanized due to adverse clinical signs or moribundity associated with a malignant lymphoma, and 2 males in the 1 mg/kg/dose rHuPH20 group were euthanized due to limited use of the limbs or moribundity associated with a malignant lymphoma. There were no rHuPH20-related clinical observations, effects on body weight, body weight gains, or terminal body weights for mice in the 1 mg/kg/dose rHuPH20 group. In addition, there were no rHuPH20-related changes in any clinical pathology parameters in mice in the 1 mg/kg/dose rHuPH20 group.

Statistically significant increases in absolute spleen weight (22.6%; $P \leq 0.01$), spleen weight relative to body weight (18.1%; $P \leq 0.01$), and spleen weight relative to brain weight (21.0%; $P \leq 0.01$) were observed in females receiving the 1 mg/kg/dose rHuPH20 compared with the control group. Increased spleen weights correlated microscopically with a higher incidence of increased hematopoiesis. These mice did not display any rHuPH20-related hematology changes or bone marrow alterations at PND 160, and organ weights in males administered rHuPH20 were unaffected. Therefore, increased spleen weights were considered a secondary or reactive response to the inflammation at the site of rHuPH20 administration and unrelated to rHuPH20. There were no rHuPH20-related macroscopic findings following necropsy examination in male or female mice. Minimal to moderate injection site reactions were observed in the Chronic Subset and were considered to be nonadverse effects of rHuPH20. These reactions consisted primarily of a mixed cell infiltration within the SC tissues with occasional observations of SC mixed cellular inflammation.

3.7. Safety margins

The NOAELs, human equivalent doses (HEDs), and safety margins from the repeat-dose toxicity, embryo-fetal development, and PPND studies are shown in Table 2. These values are compared with a range of rHuPH20 doses that are used in clinical and commercial products; for example, HYLENEX typically contains 150 U rHuPH20 [21], while co-administered antibody formulations contain 10,000–30,000 U

Petitioner Merck, Ex. 1190, p. 379
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042



Cross-reactivity	PND 30	PND 60	PND 90	PND 129	DG 18
rMuPH20	76%	94%	100%	94%	91%
rMuHyal5	90%	100%	100%	100%	100%

Cross-reactivity	PND 30	PND 90	PND 180	PND 241
rMuPH20	60%	100%	96%	100%
rMuHyal5	100%	100%	100%	100%

Fig. 7. Lifetime study of rHuPH20-specific ADAs in mice.
A) Methodology. F0 mice were administered rHuPH20 daily from PND 7 through 30, then received weekly rHuPH20 until the approximately PND 129 (Juvenile Subset, $n = 30$/sex/dose) or PND 241 (Chronic Subset, $n = 25$/sex/dose). Blue drops indicate days that plasma samples were taken for ADA analysis. Scheduled termination for the Juvenile Subset was PND 129 or DG 18 for pregnant females. B) Incidence of ADAs with neutralizing activity to rHuPH20 (grey bars, left y-axis), anti-rHuPH20 ADA titers (whisker plots, right y-axis), and cross-reactivity to recombinant murine hyaluronidases (shown in tables) were measured for the Juvenile Subset (left) and Chronic Subset (right). The n values for the incidence of rHuPH20-neutralizing ADAs are shown below the relevant day in each graph. C) Fertility parameters in the Juvenile Subset as measured by reproductive outcomes in pregnant mice (left) and sperm measurements in males (right). D) Incidence of male fetuses (grey bars) and fetal body weight (blue bars) in F1 litters from the Juvenile Subset. Abbreviations: ADA = anti-drug antibodies; DG = day of (presumed) gestation; PND = postnatal day; rHuPH20 = recombinant human hyaluronidase PH20; rMuPH20 = recombinant murine PH20; rMuHyal5 = recombinant murine hyaluronidase 5; SD = standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rHuPH20 [24,27,30]. The respective NOAELs determined in the studies described throughout this manuscript are hundreds to thousands of times higher than the doses of rHuPH20 administered to humans.

4. Discussion

4.1. Overview

The comprehensive toxicology and fertility studies conducted in several relevant animal models demonstrate a favorable safety profile of rHuPH20. While rHuPH20 has previously been extensively studied in animal models and researched in clinical trials, reports describing rHuPH20-reactive ADAs warranted further research. Due to the expression of PH20 in the adult testes and epididymis [37,39,40], it was conceivable that rHuPH20-reactive ADAs could bind endogenous PH20 and possibly interfere with fertility. Therefore, understanding the potential consequences of rHuPH20-reactive ADAs on fertility during therapeutically relevant dosing regimens is crucial. Administration of rHuPH20 resulted in the generation of anti-rHuPH20 antibodies in all studied animal models, and these ADAs were also cross-reactive to recombinant versions of species-specific hyaluronidases. In general, rHuPH20 dosing and resulting ADAs had no severe adverse effects on clinical observations, fertility outcomes, or development. rHuPH20-related findings were largely nonadverse [51], minimal, and reversible, and measurable systemic exposure to SC-administered rHuPH20 was only observed at high dosages. Our studies provide the first

Petitioner Merck, Ex. 1190, p. 380
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

substantial nonclinical characterization of the impact of rHuPH20-reactive ADAs on toxicity, fertility, and development.

4.2. Standard toxicology studies

A standard nonclinical toxicity program was performed with SC-administered rHuPH20, including repeat-dose toxicity studies in adult cynomolgus monkeys (once daily for 7 days and once weekly for 39 weeks), as well as embryo-fetal development and PPND studies in Crl:CD1(ICR) mice. Generally, once-daily or once-weekly SC administration of rHuPH20 was well tolerated in adult cynomolgus monkeys up to the highest dosages tested regarding both local tolerance at the site of injection and systemic toxicity. In the 39-week repeat-dose toxicity study, rHuPH20-related changes were limited to the minimal, reversible, nonadverse histopathology effects of SC perivascular lymphoplasmacytic infiltration noted at the injection site. Furthermore, rHuPH20 and ADAs did not impair fertility based on the observed fertility parameters (Fig. 2C, D). These studies indicated that repeat dosing is well tolerated in adult monkeys, with no rHuPH20-related adverse events. Additionally, *ex vivo* analysis of monkey tissues demonstrated that anti-rHuPH20 antibodies were excluded from the ENP, seminiferous tubules, and epididymal lumen compartments. These observations were consistent with previous reports of blood-tissue barriers restricting the immune system/macromolecule access to the myenteric plexus, epididymis, and seminiferous tubules [52–54].

After establishing that repeat rHuPH20 dosing did not result in signs of toxicity or fertility impairment in adults, we next examined how rHuPH20 administration and the resulting ADAs could potentially impact embryo-fetal and pre/postnatal development in mice. Maternal toxicity was not observed at any dose tested, but rHuPH20 was embryo-fetotoxic at doses ≥9 mg/kg/dose of rHuPH20 in mouse embryo-fetal development studies, as evidenced by reduced fetal body weights at these doses (Fig. 3C). However, rHuPH20 did not display overt teratogenic potential at any of the studied doses. Notably, retrospective analysis of ADA levels from this study did not correlate with the dose-dependent increase in resorptions at 9 and 18 mg/kg/dose, suggesting that ADAs were not directly detrimental to embryo-fetal development (Fig. 3B, C). Though the cause of the embryo-fetotoxicity observed at 9 and 18 mg/kg/dose rHuPH20 remains unknown, a disruption or degradation of HA in the developing embryo by rHuPH20 may be a contributing factor. HA is a major component of the cardiac extracellular matrix and is critical for the normal formation of the heart during embryogenesis; loss of HA in the developing heart either by degradation or HA synthase deficiency results in abnormal cardiac development [55,56]. Future studies may identify other aspects of HA disruption during embryogenesis that impair normal fetal development. Doses ≤9 mg/kg/dose did not affect pre/postnatal development in mice (Fig. 4). The maternal (18 mg/kg), pre/postnatal developmental (9 mg/kg), and embryo-developmental (3 mg/kg) NOAEL doses observed in mice studies were 28,800, 14,400, and 4800 times higher (based on U/kg) than the typical monthly human dose with rHuPH20 that is FDA-approved in HYQVIA®, respectively [33]. The NOAEL doses observed throughout these studies were 21 to approximately 400 times (based on allometric scaling) the human dose of 30,000 U, which is the recommended dosage of rHuPH20 with DARZALEX FASPRO™ [30] (Table 2). These values suggest substantial safety margins for therapeutically relevant doses of rHuPH20.

4.3. Extended toxicology studies

Considering the potential long-term consequences of extended rHuPH20 dosing on fetal development into adulthood, we expanded on the standard toxicology package studies to include extended dosing regimens and thorough evaluations on fertility and development. Using a NZW rabbit model, we found that rHuPH20 administration to F0 males does not negatively alter fertility parameters or reproductive capacity (Fig. 5). Furthermore, we demonstrated that rHuPH20-reactive ADAs generated by F0 maternal rabbits are transferred to F1 fetuses (Fig. 6). Despite this exposure to ADAs, F1 fetuses developed normally with no adverse effects on fertility parameters or reproductive capacity in adulthood. The NOAEL dose levels for rabbits (0.76 mg/kg) was 1200 times higher (based on U/kg) than the monthly human dose with HYQVIA® [33]. As part of the extended toxicology studies, we also observed the potential lifetime outcomes of direct rHuPH20 administration to juvenile mice (Fig. 7) and found no negative impacts on clinical observations or fertility in these animals.

rHuPH20 administration was evaluated in multiple experimental protocols and relevant nonclinical models, and a favorable safety profile was demonstrated in each. The wide array of nonclinical studies discussed here supports the potential for safe administration of rHuPH20 in all stages of development and at a variety of subcutaneous dose levels. The wide safety margins provided by the toxicity studies coupled with the nonclinical pharmacology, pharmacokinetic data, and lack of negative effects of anti-rHuPH20 antibodies shown in this study support the safe use of rHuPH20 as a transient, locally acting permeation enhancer that facilitates the SC administration of therapeutics.

5. Conclusion

Based on a variety of relevant animal models, we have demonstrated a favorable nonclinical safety profile of rHuPH20 and ADAs throughout development and adulthood. Administration of rHuPH20 resulted in the generation of ADAs that had no detrimental effects on fertility as measured by sperm concentration and motility, litter size, litter viability, and development. We identified rHuPH20 NOAELs with substantial safety margins compared to clinically relevant doses in humans. Notably, we observed a developmental NOAEL of 3 mg/kg rHuPH20 in mice, as higher doses resulted in statistically significantly reduced fetal body weights and a nonsignificant increase in late resorptions. The exact mechanism of this embryo-fetotoxicity has not yet been determined; however, there were no signs of overt teratogenic potential. Additionally, the developmental NOAEL dose is 4800 times (based on U/kg) more than the typical monthly human dose of rHuPH20 with HYQVIA®. Ultimately, these studies demonstrate that prolonged exposure to rHuPH20 and circulating anti-rHuPH20 antibodies is well tolerated without impacts on reproduction or development.

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Author contributions

RPN was responsible for drafting the paper, revising it critically for intellectual content, and providing final approval of the final draft for publication.

DWK was responsible for the conception, design, and execution of the study, revising the manuscript critically for intellectual content and providing final approval of the final draft for publication.

DCM was responsible for the conception, design, and execution of the study, interpreting the data, revising the manuscript critically for intellectual content, and providing final approval of the final draft for publication.

SPK was responsible for revising the manuscript critically for intellectual content and providing final approval of the final draft for publication.

MJL was responsible for the conception, design, and execution of the study, interpreting the data, revising the manuscript critically for intellectual content, and providing final approval of the final draft for publication.

MAP was responsible for the conception, design, and execution of the study, interpreting the data, drafting the manuscript, revising it

Petitioner Merck, Ex. 1190, p. 381
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

critically for intellectual content, and providing final approval of the final draft for publication.

All authors agree to be accountable for all aspects of the work.

CRediT authorship contribution statement

Ryan P. Nolan: Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation. **David W. Kang:** Writing – review & editing, Investigation, Conceptualization. **Daniel C. Maneval:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Stephen P. Knowles:** Writing – review & editing, Supervision, Resources, Investigation. **Michael J. LaBarre:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Marie A. Printz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Authors are current or former employees and shareholders of Halozyme Therapeutics, Inc. Halozyme, Inc. is not the HALO stock.

Data availability

The studies were conducted by Halozyme Therapeutics, Inc. and the data are held by the company. The datasets supporting the results reported in this article may be provided to researchers upon reasonable request. Such requests can be made by contacting Halozyme Therapeutics, Inc.: 12390 El Camino Real, San Diego, CA 92130, USA; Phone: +1.858.794.8889; Email: publications@halozyme.com.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.07.062.

References

[1] W.F. Richter, S.G. Bhansali, M.E. Morris, Mechanistic determinants of biotherapeutics absorption following SC administration, AAPS J. 14 (3) (2012) 559–570.
[2] B. Bittner, W. Richter, J. Schmidt, Subcutaneous administration of biotherapeutics: an overview of current challenges and opportunities, BioDrugs 32 (5) (2018) 425–440.
[3] S.S. Dychter, D.A. Gold, M.F. Haller, Subcutaneous drug delivery: a route to increased safety, patient satisfaction, and reduced costs, J. Infus. Nurs. 35 (3) (2012) 154–160.
[4] C. Wynne, V. Harvey, C. Schwabe, D. Waaka, C. McIntyre, B. Bittner, Comparison of subcutaneous and intravenous administration of trastuzumab: a phase I/Ib trial in healthy male volunteers and patients with HER2-positive breast cancer, J. Clin. Pharmacol. 53 (2) (2013) 192–201.
[5] E. De Cock, P. Kritikou, M. Sandoval, S. Tao, C. Wiesner, A.M. Carella, et al., Time savings with rituximab subcutaneous injection versus rituximab intravenous infusion: a time and motion study in eight countries, PLoS One 11 (6) (2016) e0157957.
[6] G.I. Frost, Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration, Expert Opin. Drug Deliv. 4 (4) (2007) 427–440.
[7] H.M. Shepard, Breaching the castle walls: hyaluronan depletion as a therapeutic approach to cancer therapy, Front. Oncol. 5 (2015) 192.
[8] K.W. Locke, D.C. Maneval, M.J. LaBarre, ENHANZE® drug delivery technology: a novel approach to subcutaneous administration using recombinant human hyaluronidase PH20, Drug Deliv. 26 (1) (2019) 98–106.
[9] C.C. DuFort, K.E. DelGiorno, S.R. Hingorani, Mounting pressure in the microenvironment: fluids, solids, and cells in pancreatic ductal adenocarcinoma, Gastroenterology 150 (7) (2016) 1545–57 e2.
[10] T.C. Laurent, The ultrastructure and physical-chemical properties of interstitial connective tissue, Pflugers Arch. (1972) S21–S42.
[11] L.H. Bookbinder, A. Hofer, M.F. Haller, M.L. Zepeda, G.A. Keller, J.E. Lim, et al., A recombinant human enzyme for enhanced interstitial transport of therapeutics, J. Control. Release 114 (2) (2006) 230–241.
[12] D.W. Kang, T.A. Nekoroski, M.A. Printz, C.K. Hoh, D.V. Vera, J.F. Skipper, et al., Recombinant human hyaluronidase PH20 (rHuPH20) facilitated subcutaneous delivery of proteins in nonclinical models, Controll. Release Newsl. 30 (6) (2013) 9–11.
[13] E.G. Bywaters, E.J. Holborow, M.K. Keech, Reconstitution of the dermal barrier to dye spread after hyaluronidase injection, Br. Med. J. 2 (4741) (1951) 1178–1183.
[14] S.P. Knowles, M.A. Printz, D.W. Kang, M.J. LaBarre, R.P. Tannenbaum, Safety of recombinant human hyaluronidase PH20 for subcutaneous drug delivery, Expert Opin. Drug Deliv. 18 (11) (2021) 1673–1685.
[15] E.J. Holborow, M.K. Keech, Hyaluronidase skin spreading effect; an analysis of repeated measurements, Br. Med. J. 2 (4741) (1951) 1173–1178.
[16] D.W. Kang, B. Bittner, B.J. Sugarman, M.L. Zepeda, M.A. Printz, Dispersive effects and focused biodistribution of recombinant human hyaluronidase PH20: a locally acting and transiently active permeation enhancer, PLoS One 16 (7) (2021) e0254765.
[17] R.J. Connor, R. Clift, D.W. Kang, Identifying a predictive relationship between maximal flow rate and viscosity for subcutaneous administration of macromolecules with recombinant human hyaluronidase PH20 in a miniature pig model, Drug Deliv. 30 (1) (2023) 2252999.
[18] G.H. Shi, R.J. Connor, D.S. Collins, D.W. Kang, Subcutaneous injection performance in Yucatan miniature pigs with and without human hyaluronidase and auto-injector tolerability in humans, AAPS PharmSciTech 22 (1) (2021) 39.
[19] W.P. Kirschbrown, C. Wynne, M. Kagedal, R. Wada, H. Li, B. Wang, et al., Development of a subcutaneous fixed-dose combination of pertuzumab and trastuzumab: results from the phase Ib dose-finding study, J. Clin. Pharmacol. 59 (5) (2019) 702–716.
[20] M.A. Printz, S.S. Dychter, E.P. DeNoia, R. Harrigan, B.J. Sugarman, M. Zepeda, et al., A phase I study to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of recombinant human hyaluronidase PH20 administered intravenously in healthy volunteers, Curr. Ther. Res. Clin. Exp. 93 (2020) 100604.
[21] Halozyme Therapeutics Inc, Prescribing Information HYLENEX Recombinant (Hyaluronidase Human Injection), Available from, http://hylenex.com/downloads/approved-uspi-lbl301feb2016.pdf, 2016.
[22] M. Borte, S. Raffac, M. Hrubisko, K. Jahnz-Rozyk, E. Garcia, B. McCoy, et al., Long-term safety of facilitated subcutaneous immunoglobulin treatment in pregnant women with primary immunodeficiency diseases: results from a registry study, Immunotherapy 14 (8) (2022) 609–616.
[23] European Medicines Agency, Herceptin Summary of Product Characteristics [updated March 17, 2023. Available from, https://www.ema.europa.eu/en/medicines/human/EPAR/herceptin, 2010.
[24] US Food and Drug Administration, Prescribing Information. HERCEPTIN HYLECTA™ [trastuzumab and hyaluronidase-oysk] Injection, for Subcutaneous Use, Available from, https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/761106s000lbl.pdf, 2019.
[25] European Medicines Agency, PHESGO Summary of Product Characteristics, Available from, https://www.ema.europa.eu/en/documents/product-information/phesgo-epar-product-information_en.pdf, 2021.
[26] Genentech Inc, PHESGO® [package insert]. South San Francisco, CA, 2020.
[27] Genentech Inc, Prescribing Information. RITUXAN HYCELA™, for Subcutaneous Use, Available from, https://www.gene.com/download/pdf/rituxan_hycela_prescribing.pdf, 2020.
[28] European Medicines Agency, MabThera Summary of Product Characteristics, Available from, https://www.ema.europa.eu/en/documents/product-information/mabthera-epar-product-information_en.pdf, 2019.

Petitioner Merck, Ex. 1190, p. 382
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

[29] European Medicines Agency, Darzalex Summary of Product Characteristics, Available from, https://www.ema.europa.eu/documents/product-information/darzalex-epar-product-information_en.pdf, 2018.
[30] Janssen Biotech Inc, Prescribing Information. DARZALEX FASPRO™ (daratumumab and hyaluronidase-fihj) Injection for Subcutaneous Use, Available from, https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/761145s000lbl.pdf, 2020.
[31] UK Electronic Medicines Compendium, Tecentriq 1,875 Mg Solution for Injection [Updated 1 September 2023. Available from: https://www.medicines.org.uk/emc/product/15037/smpc, 2023.
[32] U.S. Food and Drug Administration, VYVGART® HYTRULO (efgartigimod alfa and hyaluronidase-qvfc) Injection, for Subcutaneous Use, Available from, https://www.accessdata.fda.gov/drugsatfda_docs/label/2023/761304s000lbl.pdf, 2023.
[33] Food and Drug Administration, HYQVIA [Immune Globulin Infusion 10% (Human) with Recombinant Human Hyaluronidase] Prescribing Information, Available from, https://www.fda.gov/media/89844/download, 2024.
[34] J. Li, C. Yang, Y. Xia, A. Bertino, J. Glaspy, M. Roberts, D.J. Kuter, Thrombocytopenia caused by the development of antibodies to thrombopoietin, Blood 98 (12) (2001) 3241–3248.
[35] L.C. Lim, Acquired red cell aplasia in association with the use of recombinant erythropoietin in chronic renal failure, Hematology 10 (3) (2005) 255–259.
[36] P. Ragnhammar, M. Wadhwa, Neutralising antibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) in carcinoma patients following GM-CSF combination therapy, Med. Oncol. 13 (3) (1996) 161–166.
[37] M.A. Printz, B.J. Sugarman, R.D. Paladini, M.C. Jorge, Y. Wang, D.W. Kang, et al., Risk factors, hyaluronidase expression, and clinical immunogenicity of recombinant human hyaluronidase PH20, an enzyme enabling subcutaneous drug administration, AAPS J. 24 (6) (2022) 110.
[38] S. Rosengren, S.S. Dychter, M.A. Printz, L. Huang, R.I. Schiff, H.P. Schwarz, et al., Clinical immunogenicity of rHuPH20, a hyaluronidase enabling subcutaneous drug administration, AAPS J. 17 (5) (2015) 1144–1156.
[39] E.A. Evans, H. Zhang, P.A. Martin-DeLeon, SPAM1 (PH-20) protein and mRNA expression in the epididymides of humans and macaques: utilizing laser microdissection/RT-PCR, Reprod. Biol. Endocrinol. 1 (2003) 54.
[40] X. Deng, Y. He, P.A. Martin-Deleon, Mouse Spam1 (PH-20): evidence for its expression in the epididymis and for a new category of spermatogenic-expressed genes, J. Androl. 21 (6) (2000) 822–832.
[41] S. Rosengren, J. Souratha, D. Conway, D.B. Muchmore, B.J. Sugarman, Recombinant human PH20: baseline analysis of the reactive antibody prevalence in the general population using healthy subjects, BioDrugs 32 (1) (2018) 83–89.
[42] C.M. Hardy, G. Clydesdale, K.J. Mobbs, J. Pekin, M.L. Lloyd, C. Sweet, et al., Assessment of contraceptive vaccines based on recombinant mouse sperm protein PH20, Reproduction 127 (3) (2004) 325–334.
[43] M. Pomering, R.C. Jones, M.K. Holland, A.E. Blake, K.W. Beagley, Restricted entry of IgG into male and female rabbit reproductive ducts following immunization with recombinant rabbit PH-20, Am. J. Reprod. Immunol. 47 (3) (2002) 174–182.
[44] D.B. Morton, P.A. McAnulty, The effect on fertility of immunizing female sheep with ram sperm acrosin and hyaluronidase, J. Reprod. Immunol. 1 (1) (1979) 61–73.
[45] National Research Council, Guide for the Care and Use of Laboratory Animals, 7th edition, The National Academies Press, 1996 (125 p).
[46] M.C. Rosso, P. Badino, G. Ferrero, R. Costa, F. Cordero, S. Steidler, Biologic data of Cynomolgus monkeys maintained under laboratory conditions, PLoS One 11 (6) (2016) e0157003.
[47] Y. Lin, L.H. Kimmel, D.G. Myles, P. Primakoff, Molecular cloning of the human and monkey sperm surface protein PH-20, Proc. Natl. Acad. Sci. USA 90 (21) (1993) 10071–10075.
[48] The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertiliy S5(R2), Available from, https://database.ich.org/sites/default/files/S5_R2_Guideline.pdf, 2005.
[49] J.M. DeSesso, A.L. Williams, A. Ahuja, C.J. Bowman, M.E. Hurtt, The placenta, transfer of immunoglobulins, and safety assessment of biopharmaceuticals in pregnancy, Crit. Rev. Toxicol. 42 (3) (2012) 185–210.
[50] U.S. Food and Drug Administration, Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, Available from, https://www.fda.gov/media/72309/download, 2005.
[51] R.W. Lewis, R. Billington, E. Debryune, A. Gamer, B. Lang, F. Carpanini, Recognition of adverse and nonadverse effects in toxicity studies, Toxicol. Pathol. 30 (1) (2002) 66–74.
[52] M.D. Gershon, S. Bursztajn, Properties of the enteric nervous system: limitation of access of intravascular macromolecules to the myenteric plexus and muscularis externa, J. Comp. Neurol. 180 (3) (1978) 467–488.
[53] P. Mital, B.T. Hinton, J.M. Dufour, The blood-testis and blood-epididymis barriers are more than just their tight junctions, Biol. Reprod. 84 (5) (2011) 851–858.
[54] C.Y. Cheng, D.D. Mruk, The blood-testis barrier and its implications for male contraception, Pharmacol. Rev. 64 (1) (2012) 16–64.
[55] H.S. Baldwin, T.R. Lloyd, M. Solursh, Hyaluronate degradation affects ventricular function of the early postlooped embryonic rat heart in situ, Circ. Res. 74 (2) (1994) 244–252.
[56] T.D. Camenisch, A.P. Spicer, T. Brehm-Gibson, J. Biesterfeldt, M.L. Augustine, A. Calabro Jr., et al., Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme, J. Clin. Invest. 106 (3) (2000) 349–360.

Petitioner Merck, Ex. 1190, p. 383
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Supplementary Materials for

The safety of recombinant human hyaluronidase PH20 in nonclinical models: An overview of toxicology, pharmacology, and impact of anti-PH20 antibodies

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Supplementary materials and methods

Assessment of local skin reactions

Sprague Dawley rats received a solution (10 mM histidine and 130 mM NaCl) in the absence (Buffer 1) or presence of human serum albumin (HSA) (1 mg/mL; Buffer 2) and with or without recombinant human hyaluronidase PH20 (rHuPH20; 10,000 U/mL) at a volume of 0.05 or 0.15 mL. Animals were necropsied 48 or 120 h postadministration. Skin biopsies were collected from the injection sites and evaluated histologically for local skin reactions.

Non-pivotal monkey 7-day repeat-dose toxicity study

To evaluate toxicity and toxicokinetics (TK), male and female adult cynomolgus monkeys were given either rHuPH20 (5 mg/kg/dose) or vehicle control (10 mM histidine, 130 mM NaCl, pH 6.0) daily for 7 days (Table 1), administered by subcutaneous (SC) or

Petitioner Merck, Ex. 1190, p. 384
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

intravenous (IV). Each group consisted of 2 males and 2 females. Clinical observations, body weight, food consumption, ophthalmology, hematology, clinical chemistry, coagulation, and urinalysis parameters were assessed. Blood was collected for TK analysis at multiple timepoints on days 1, 2, 7, and 8. For the SC group, blood was collected for TK analysis at multiple timepoints on days 1, 2, 7, and 8. Postmortem examinations included macroscopic observations at necropsy on day 8 and a microscopic examination.

Pivotal monkey 39-week repeat-dose toxicity study

Male and female adult cynomolgus monkeys were assigned to 4 groups (6 animals/sex/group) at dosages of rHuPH20 of 0 (vehicle control of 10 mM histidine, 130 mM NaCl, pH 6.0), 0.02, 0.2, and 2 mg/kg/dose (Table 1). Additionally, 2 animals/sex/group were monitored for a 4-week recovery period following cessation of dose administration to assess the reversibility of any rHuPH20-related changes.

Assessment of toxicity was based on mortality, clinical observations, food consumption, body weights, electrocardiographic examinations, heart rate, blood pressure, respiratory rate, ophthalmology, clinical and anatomic pathology, organ weights, and local injection site evaluations. Specialized fertility endpoints included menstrual monitoring in females and semen analysis (sperm concentration, motility, and morphology) in males. Blood samples were collected for TK analysis (predose and at 0.25, 1, 2, 4, 6, 12, and 16 h postdose on days 1, 21, 85, 183, and 267).

Plasma samples collected on days 7, 14, 42, 70, 95, 123, 140, 154, 182, 210, 238, 266, 274, 295, and 302 were analyzed for binding anti-drug antibodies (ADAs) titer, and neutralizing activity to rHuPH20. Additionally, retrospective analysis was performed

Petitioner Merck, Ex. 1190, p. 385
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

for ADA cross-reactivity to and neutralization of cynomolgus monkey PH20, as well as deposition of immunoglobulin G (IgG) in testes tissue as determined by immunohistochemistry (IHC).

For these studies, a cynomolgus IgG cocktail was formulated by isolating antibodies from one of the monkeys in the 39-week study. Slides of formalin-fixed paraffin-embedded colon sections for all animals in the study, and Davidson fixed paraffin embedded testis and epididymis sections (5 µm) from all males in the study were obtained from Shin Nippon Biomedical Laboratories (Everett, WA, USA). Sections were de-paraffinized in 3 changes of xylenes for 3 minutes each, then hydrated with alcohol and rinsed in distilled water. To retrieve antigens, sections were heated in citrate buffer (pH 6; Invitrogen, Waltham, MA, USA) to 80°C to 85°C and cooled to ambient temperature. Sections were rinsed and incubated in peroxo-block solution (Invitrogen, Waltham, MA, USA) for 1 to 2 minutes at ambient temperature, rinsed again, then blocked in 10% normal goat serum (Vector Labs, Newark, CA, USA) for 30 minutes at ambient temperature, followed by avidin/biotin blocking (Invitrogen, Waltham, MA, USA) for 10 minutes each. Antigens were visualized by incubating sections with biotinylated goat anti-monkey IgG (Fitzgerald Industries, Acton, MA, USA) or biotinylated goat anti-monkey IgG + cynomolgus IgG cocktail for 1 h at ambient temperature. Sections were detected with HRP-Streptavidin (BD Pharmingen, Franklin Lakes, NJ, USA) for 30 minutes at ambient temperature, developed using the DAB substrate kit (Dako/Agilent, Santa Clara, CA, USA), and counter-stained with hematoxylin (Gill's formulation; Vector Labs, Newark, CA, USA) following manufacturer's instructions. Finally, slides were dehydrated and mounted with cytoseal 60 medium (Thermo Fisher Scientific Inc.,

Petitioner Merck, Ex. 1190, p. 386
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Waltham, MA, USA) and coverslip. Slides were viewed under a bright field microscope; DAB staining was visualized as brown pigment on blue-purple hematoxylin counterstaining.

Pivotal mouse embryo-fetal development study experimental design

This study evaluated the ICH S5 (R2) guideline stages C and D of the reproductive process in female Crl:CD1(ICR). Presumed pregnant female mice received rHuPH20 SC daily from day of gestation (DG) 6 through DG 15 (Table 1). Viability, clinical observations, maternal body weight and body weight changes, food consumption, local tolerance, mating performance, Cesarean-sectioning observations, and fetal parameters were monitored.

A dose-range finding study was performed to determine which rHuPH20 doses to test in the mouse embryo-fetal development studies. Presumed pregnant female mice (8 per dose group) were administered SC rHuPH20 at 0 (vehicle control), 1, 3, 10, and 30 mg/kg once daily from DG 6 through DG 15. Mice administered 30 mg/kg/dose displayed rHuPH20-related decreased maternal body weights and body weight gains, increased resorptions, decreased fetal body weights, and enlarged spleens. Based on these results, we determined that dosages of 0 (vehicle control), 3, 9, and 18 mg/kg/dose of rHuPH20 were recommended for the pivotal embryo-fetal developmental toxicity study in pregnant mice.

One hundred main study mice (25 animals/group) and 108 TK study mice (36 animals/rHuPH20 group) received daily doses of vehicle control (10 mM histidine, 130 mM NaCl, pH 6.5) or rHuPH20 at 3, 9, or 18 mg/kg/dose from DG 6 through DG 15 (Table 1). Blood samples were collected from TK study mice on DG 6 (DG 7 for 24 h

Petitioner Merck, Ex. 1190, p. 387
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

postdose) and on DG 15 (DG 16 for 24 h postdose) at the following timepoints: prior to rHuPH20 administration and at approximately 30 minutes, 2, 4, 8, and 24 h postdose. Toxicokinetic study mice were examined for pregnancy status, and their conceptuses and carcasses were discarded without further evaluation. All surviving main study mice were Cesarean sectioned on DG 18 and examined for the number and distribution of corpora lutea, implantation sites, live and dead fetuses, and early and late resorptions. The fetuses were removed from the uterus and examined individually for sex determination, body weight, gross external alterations, and soft tissue and skeletal alterations. Retrospective analysis of a subset of DG 6 and DG 15 plasma TK samples was performed to measure the presence of ADA.

Pivotal mouse PPND study experimental design

rHuPH20 (3, 6, or 9 mg/kg/dose) or vehicle control (10 mM histidine, 130 mM NaCl, pH 6.5) was administered SC once daily to pregnant Crl:CD1(ICR) female mice (25 females/group) from DG 6 through DG 22 for natural delivery mice that did not deliver a litter, or through day of lactation (DL) 20 for natural delivery mice with a litter (Table 1).

Maternal (F0) mice were examined for clinical observations, local tolerance, abortions, premature deliveries and deaths, litter sizes, and pup viability. Following completion of the 21-day postpartum period, surviving female mice were euthanized and a gross necropsy was performed. The number and distribution of implantation sites were recorded. Mice that did not deliver a litter were euthanized on DG 23 and examined for gross lesions and pregnancy status.

Petitioner Merck, Ex. 1190, p. 388
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Effect of ADAs on male fertility and general reproduction in rabbits

Male rabbits were randomized into 2 groups (22 males/group) and received weekly SC administered doses of vehicle control (10 mM histidine, 130 mM NaCl, pH 6.5) or 0.76 mg/kg rHuPH20 on day of study (DS) 1, 8, 15, 22, 29, and 43 and assessed for fertility parameters.

Males were mated with untreated female rabbits (n=44, 22/group) on DS 44 to assess fertility endpoints. Males received another SC administration of vehicle or rHuPH20 on DS 57 to maintain antibody titers for semen and mating outcome evaluations through to study termination on DS 85. Clinical observations, dermal scoring/local tolerance, body weight and body weight gains, food consumption, mating performance, male reproductive assessments, and ovarian and uterine examinations were assessed. Plasma samples for ADA analyses were collected from males predose on DS 1, 8, 15, 22, 29, 43, 57, and 85 (day of necropsy). ADAs were assessed for titer determination using a validated bridging electrochemiluminescence (ECL) immunoassay, cross-reactivity to rabbit hyaluronidase PH20 (rRbPH20) via direct binding ECL assay, and neutralizing activity to both rHuPH20 and rRbPH20 via turbidimetric methods (described below).

Semen samples were collected from all male rabbits prior to mating on DS 37, following mating on DS 49, and again on DS 71. Sperm were analyzed for motility, count, and morphology using the Hamilton Thorne IVOS for computer-assisted sperm analysis or manual analysis for morphology. Sperm concentration and motility were measured on the day of scheduled euthanasia (approximately day 160 postpartum or later). The sperm concentration was calculated by dividing the sperm count by the

Petitioner Merck, Ex. 1190, p. 389
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

volume in the image area (68.6 x 10^{-6} mL), multiplying by 31 (dilution factor + 1), and multiplying by 10^{-6}. The reproductive tracts of the mated and non-drug-treated females were dissected for ovarian and uterine examinations to assess the fertility of the males on DG 18. The ovaries and uterus were examined for the number and distribution of corpora lutea, implantation sites, placentae, and viable and nonviable embryos.

Effect of ADAs on female fertility and embryo-fetal development in rabbits

Female rabbits were randomized into 2 groups (44 females/group) to receive either weekly SC vehicle control (10 mM histidine, 130 mM NaCl, pH 6.0) or rHuPH20 0.76 mg/kg/dose to induce ADAs in the experimental animals and maintain antibody titers throughout gestation. Females were mated to nontreated males on DS 44, also designated as DG 0. The female rabbits were equally allocated to Cesarean-sectioning or natural delivery cohorts. In addition, a satellite group (3 females/group/interval) was included for ADA analysis of maternal and fetal/pup plasma. Maternal toxicity was assessed based on clinical observations, body weight, food consumption, and local tolerance/dermal scoring (skin grading) throughout the premating, gestation, and lactation periods.

The Cesarean-sectioning phase was evaluated on DG 29 and included maternal assessments of fertility (number of corpora lutea, implantations, live and dead fetuses, early and late resorptions, and pre- and postimplantation loss) and fetal assessments for embryo-fetal development toxicity (fetal viability, weights, sex, and fetal external, visceral, and skeletal examinations). The maternal rabbits in the natural delivery phase were evaluated for parturition (delivery), maternal behavior, and health status, including clinical signs and body weight gain, and were euthanized at the end of lactation. Prior to

Petitioner Merck, Ex. 1190, p. 390
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

weaning, pups were evaluated for survival, clinical signs, body weight, and developmental landmarks. Equal numbers of male and female pups from each litter (1/sex/litter, where possible) were randomly selected at weaning for additional postnatal observations through adulthood, including body weight, food consumption, sexual maturation, neurobehavioral testing, and assessment of fertility and mating outcomes.

ADAs were characterized for specific binding and titer determination using a bridging ECL immunoassay method, cross-reactivity of binding to recombinant rabbit PH20 (rRbPH20) using a sandwich ECL immunoassay method, and neutralization of rHuPH20 and rRbPH20 hyaluronidase activity using turbidimetric methods (described below). Plasma samples for analysis of ADAs were collected: F0 maternal rabbits, at weekly intervals from baseline through DS 29, on DS 42 (prior to mating), and on DG 15 and DG 29, and postnatal day (PND) 4 and PND 29; F0 satellite group dams and fetuses/pups on DG 20 and PND 4; F1 generation on PNDs 29, 42, 62, and 92, on the day of mating (PNDs 152-167), and at study termination (DG 18 for females and PNDs 189-195 for males). Neutralization of rHuPH20 activity was also evaluated in the F0 rabbits on DS 42, DG 15, and DG 29. In vehicle controls, binding ADA testing was performed at baseline on DS 1 and again at DG 29.

Pivotal mouse lifetime study experimental design

Male and female juvenile Crl:CD1(ICR) mice (220 pups/sex) were assigned to a juvenile subset (n=30/sex/group) or a chronic subset (n=25/sex/group) on or before PND 6. Vehicle control (10 mM histidine, 130 mM NaCl, pH 6.5, and sterile water for injection) or rHuPH20 (3, 6, or 9 mg/kg/dose) was administered SC to mice daily from

Petitioner Merck, Ex. 1190, p. 391
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

PND 7 to PND 30 and thereafter administered weekly until PND 129 (after reproductive assessments for the juvenile subset) or PND 241 (chronic subset).

Evaluations included neurobehavioral assessments, reproductive capacity and fetal evaluations, and chronic toxicity through adulthood. Plasma samples were collected for characterization of ADA responses on PNDs 30, 60, 90, 180 (chronic subset only), and/or at scheduled euthanasia. Animals were monitored for viability, clinical observations, body weights, body weight changes, organ weights, macroscopic and microscopic observations, including local tolerance.

Additional endpoints evaluated in the juvenile subset included assessments of postweaning sexual maturation (balano-preputial separation or vaginal patency), neurobehavioral (passive avoidance evaluations, motor activity, acoustic startle habituation, and Morris water maze), mating and fertility, ovarian and uterine parameters, fetal examinations (body weights; sex; and external, visceral, and skeletal abnormalities), and sperm motility, concentration, and morphology. Additional endpoints evaluated in the chronic subset included ophthalmic examinations and assessments for changes in clinical pathology parameters (hematology and clinical chemistry).

rHuPH20 activity assay

Mouse plasma: Biotinylated hyaluronan substrate (bHA; Halozyme Therapeutics, Inc., San Diego, CA, USA) was coated to high-binding 96-well microtiter plates for 24 to 48 hours at 5°C. Controls and plasma samples were diluted and incubated in the bHA-coated plates at 37°C. Plates were washed with 0.05% Tween 20 in phosphate-buffered saline and the remaining uncleaved/bound bHA was detected by streptavidin-linked horseradish peroxidase conjugate (Thermo Fisher Scientific Inc.,

Petitioner Merck, Ex. 1190, p. 392
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Waltham, MA, USA; #21126 or equivalent) at room temperature for 60 minutes, followed by development with the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA; #T0440 or equivalent). The reaction produced a colored solution with an optical density of 450 nm that was inversely proportional to rHuPH20 activity. Samples were measured using the EL-808 microplate reader (Bio-Tek Instruments Inc., Winooski, VT; or equivalent). The qualified method for mouse plasma has a quantitation range from 0.00625 to 0.200 U/mL.

Cynomolgus monkey plasma: Plasma standards and samples were diluted 1:100 in assay diluent and incubated with an hyaluronan (HA) stock solution (Halozyme Therapeutics, Inc.) for approximately 60 minutes at 37°C. Samples were then incubated for 30 minutes at room temperature with acidified horse serum, causing undigested HA to form an insoluble precipitate. The resulting turbidity was measured at 640 nm, and decreased turbidity resulting from enzyme activity on the HA substrate was a measure of rHuPH20 activity. A calibration curve was obtained by fitting the OD640 values of the standards to a 4-parameter logistic curve fit. Decreased turbidity resulting from enzyme activity on the HA substrate was a measure of rHuPH20 activity.

Anti-rHuPH20 antibody measurements

ADA titers: Briefly, plasma samples were diluted 1:50 in 2% pooled naïve CD-1 mouse K3-EDTA plasma in StartingBlock PBS T20 (Thermo Fisher Scientific Inc., Waltham, MA, USA), then incubated overnight at 2°C to 8°C with an equal concentration of biotinylated-rHuPH20 and Sulfo-TAG ruthenium-labeled rHuPH20 (250 ng/mL each; Meso Scale Discovery, Rockville, MD, USA). The resulting complexes were captured on streptavidin-coated plates and detected using ECL Read Buffer in a

Petitioner Merck, Ex. 1190, p. 393
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

SECTOR 4000 instrument (Meso Scale Discovery, Rockville, MD, USA). Samples yielding ECL signal above a predetermined cut-point were considered putative positives for ADAs. Specificity was confirmed using competition with unlabeled rHuPH20. ADA titers were measured by assaying serial dilutions of confirmed positive samples, and titer was defined as the last dilution yielding a positive ECL signal.

ADA cross-reactivity: Recombinant proteins were produced for murine PH20 (rMuPH20, amino acids 1-481), murine Hyal5 (rMuHyal5, amino acids 1-479), and rabbit PH20 (rRbPH20, amino acids 1-489) by stable expression of cDNA in a CHO-S cell line. All recombinant proteins included a FLAG-peptide tag at the C-terminus. Extra purification steps were performed for rMuHyal5 to remove possible mouse antibody leeching. Protein identities were confirmed via tryptic mapping and enzyme function confirmation. Purified recombinant proteins were coated onto standard ECL 96-well plates (Meso Scale Discovery, Rockville, MD, USA) at 5 µg/mL at 2°C to 8°C overnight. Plasma samples were diluted 1:100 in StartingBlock PBS (Thermo Fisher Scientific, Waltham, MA, USA), applied to the coated/blocked plates, and incubated at room temperature with orbital shaking for 1 hour. Mouse cross-reactive antibodies were detected by addition of a mixture of 4 ruthenylated anti-murine IgG antibodies in equal quantities specific for IgG1, IgG2a, IgG2b, and IgG3 (Meso Scale Discovery, Rockville, MD, USA). Rabbit cross-reactive antibodies were detected by addition of biotinylated monoclonal anti-rabbit IgG heavy chain antibody (Abcam, Cambridge, MA, USA), followed by incubation with streptavidin-ruthenium conjugate (Meso Scale Discovery, Rockville, MD, USA). Samples were determined to be positive for cross-reactivity if they

Petitioner Merck, Ex. 1190, p. 394
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

yielded ECL values greater than a cut-point determined from a panel of normal (untreated) mouse or rabbit samples.

Neutralizing ADAs: This assay was based on the formation of an insoluble precipitate when HA binds with acidified serum. When hyaluronidase activity is inhibited (e.g., in the presence of neutralizing antibodies) it can no longer degrade HA, resulting in elevated turbidity; therefore, an increased presence of neutralizing antibodies results in increased turbidity. Plasma samples were diluted and incubated with a constant amount of hyaluronidase, then HA was precipitated by the addition of acidified serum. The resulting turbidity was measured at an optical density of 650 nm. Neutralizing titers were determined by generating a titration curve comparing the sample dilution factor with the turbidimetric value.

Supplementary results

Assessment of local skin reactions

At 48 h postadministration, there were no histologic findings attributable to the histidine buffers, HSA, rHuPH20, or injection volume. A few sporadic, minimal findings, including epidermal ulcers and erosions occurred with histidine buffer alone at 0.05 or 0.15 mL and were related to the ID injection procedure. Inflammation of the dermis along with hyperplasia of the adjacent epidermal epithelium showed a linear pattern that was consistent with a needle tract. Mixed leukocyte and exudative inflammation, not associated with epidermal erosions or ulcers, occurred in animals receiving buffer with HSA (0.05 or 0.15 mL) and buffer with HSA and rHuPH20 at 0.15 mL. At 120 h, there were no histologic findings that were attributable to the buffer, HSA, rHuPH20, or

Petitioner Merck, Ex. 1190, p. 395
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

injection volume. Any sporadic, minimal findings observed at 120 hours were less frequent than at 48 hours and were attributed to the injection procedure (Supplemental Figure 1).

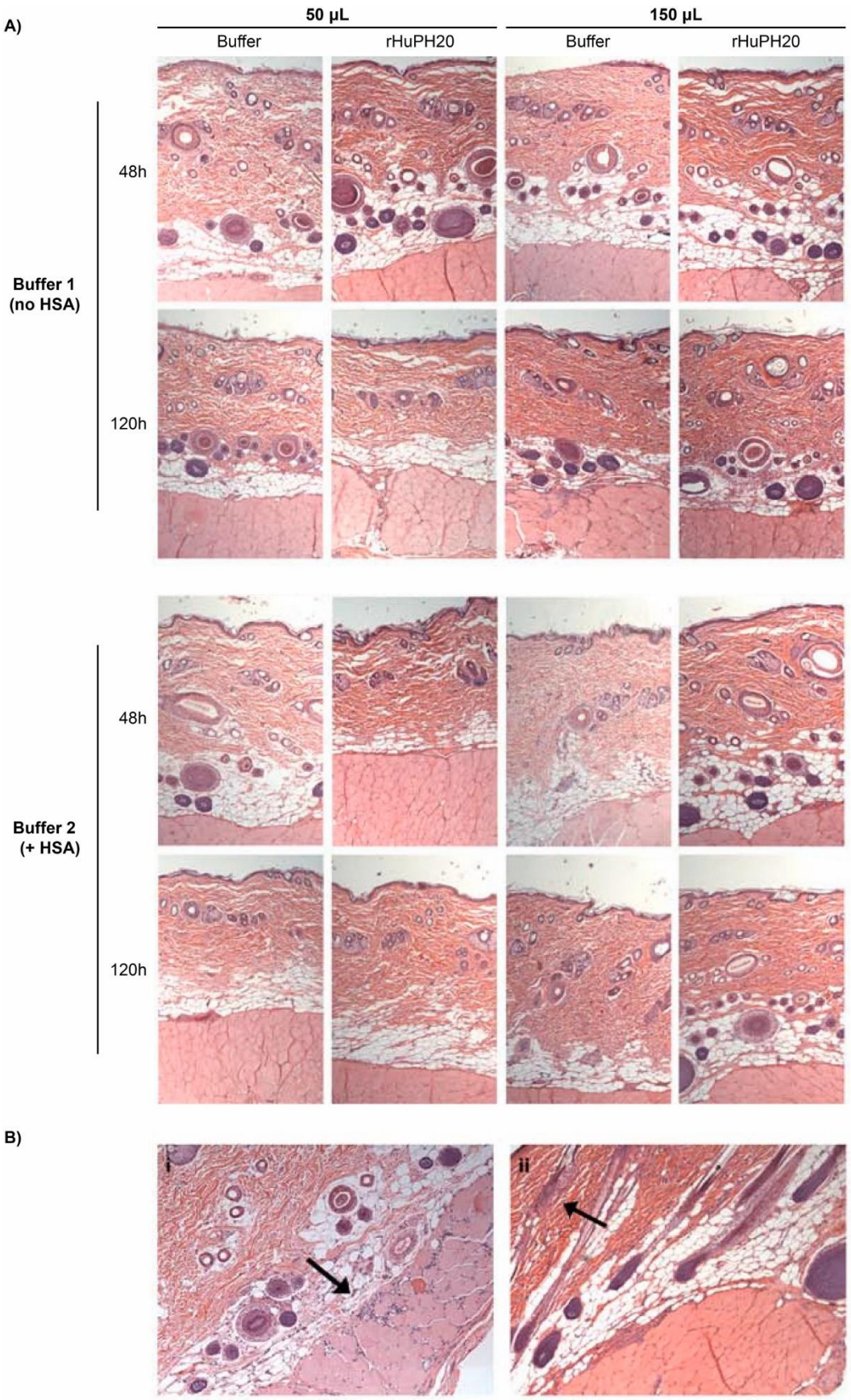
Pivotal monkey 39-week repeat-dose toxicity study

Histopathology of the injection site revealed rHuPH20-related, but reversible and nonadverse, minimal SC perivascular lymphoplasmacytic infiltration (Supplemental Figure 3A). In the terminal necropsy group, this microscopic finding was present in 25% of subjects (1/4 males and 1/4 females) at 0.2 mg/kg/dose and in all subjects (4/4 males and 4/4 females) at 2 mg/kg/dose. Following the 4-week recovery period, minimal SC perivascular lymphoplasmacytic infiltration was present in 1 male that had received rHuPH20 2 mg/kg/dose, demonstrating recovery and reversibility in most of the animals (Supplemental Figure 3A). As the severity of the finding in each case was minimal, the observation was considered nonadverse.

Petitioner Merck, Ex. 1190, p. 396
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Supplementary figures

Petitioner Merck, Ex. 1190, p. 397
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042



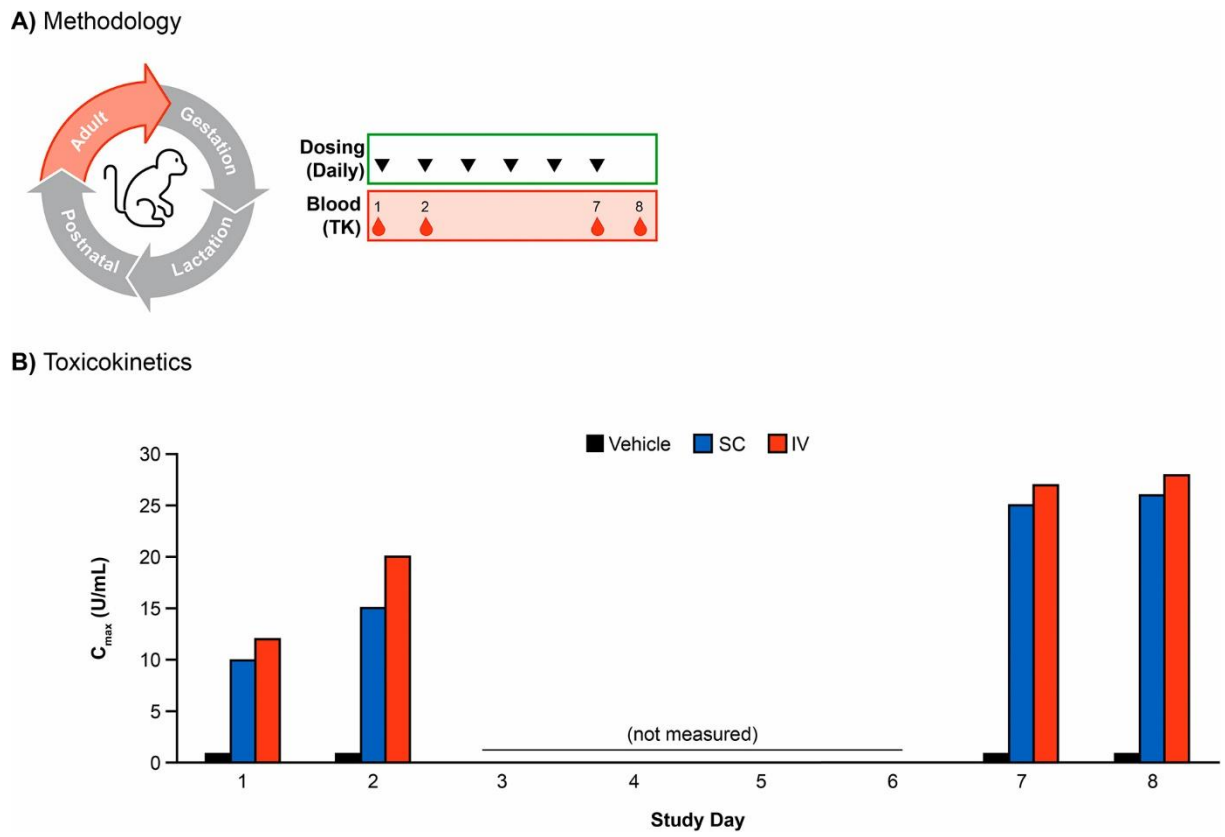
Petitioner Merck, Ex. 1190, p. 398
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Supplemental figure 1. Local skin reactions in Sprague Dawley rats following subcutaneous administration of rHuPH20.

Micrograph showing the epidermal and subcutaneous layers of representative animals per group, per time point (A). No histological findings were attributed to buffer composition, HSA, rHuPH20, or volume of delivery. Representative minimal inflammation, as depicted by focal cellular infiltrate (B, arrows), is attributed to injection procedure, and thus not rHuPH20-related. 50X magnification.

Abbreviations: HSA = human serum albumin; rHuPH20 = recombinant human hyaluronidase PH20.

Petitioner Merck, Ex. 1190, p. 399
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042



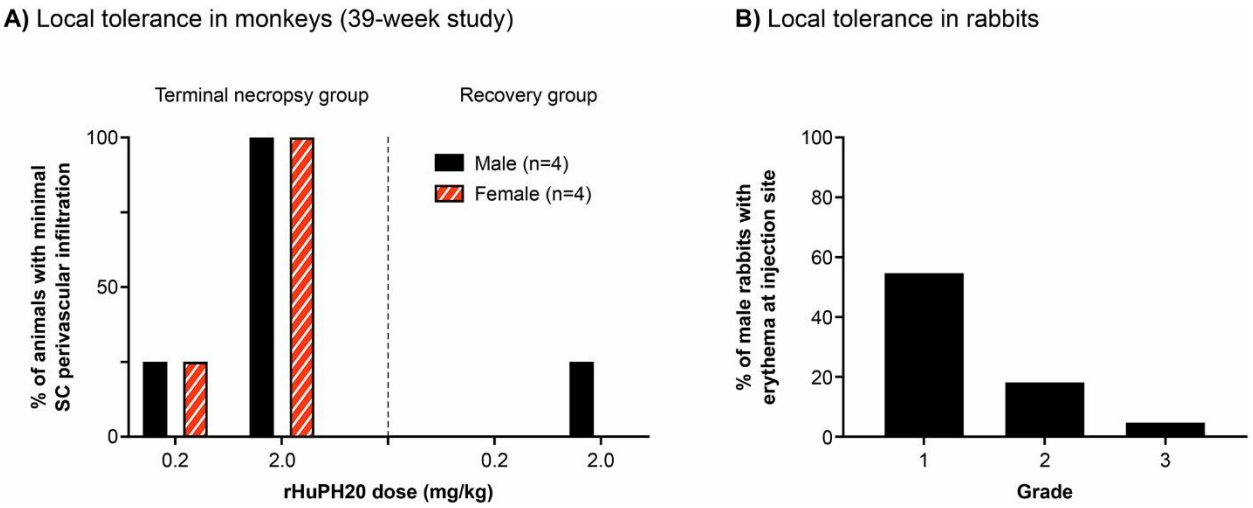
Supplemental figure 2. Toxicology analysis of repeat rHuPH20 doses in adult cynomolgus monkeys.

A) Methodology. Adult monkeys were administered rHuPH20 (5 mg/kg/dose) or vehicle control by SC or IV daily for 7 days. Blood collection timepoints for toxicokinetic (TK) analyses are indicated (days 1, 2, 7, 8). N=2 males and 2 females.

B) TK results for adult monkeys administered rHuPH20 via SC (blue bars) or IV (red bars) compared with vehicle (black bars). C_{max} for each group is shown.

Abbreviations: C_{max} = maximum plasma concentration; IV = intravenous; rHuPH20 = recombinant human hyaluronidase PH20; SC = subcutaneous.

Petitioner Merck, Ex. 1190, p. 400
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

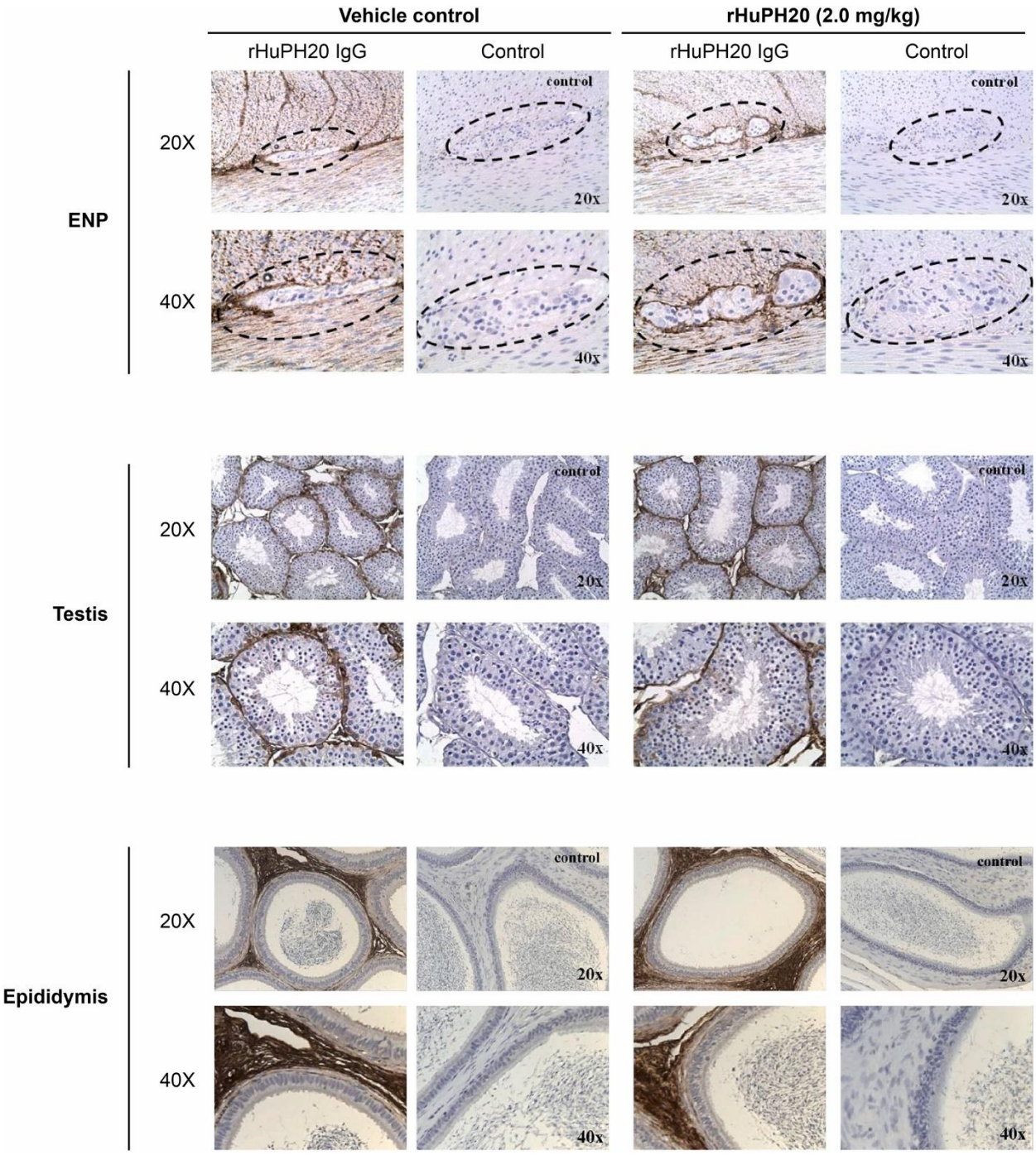


Supplemental figure 3. Local reactions.

Local reaction events are shown for adult cynomolgus monkeys in the 39-week repeat dosing study **(A)** and in male rabbits **(B)**.

Abbreviations: rHuPH20 = recombinant human hyaluronidase PH20; SC = subcutaneous.

Petitioner Merck, Ex. 1190, p. 401
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042



Supplemental figure 4. Immunodetection of IgG in monkey tissue sections.

Abbreviations: ENP = enteric nerve plexus; IgG = immunoglobulin G; rHuPH20 = recombinant human hyaluronidase PH20.

Petitioner Merck, Ex. 1190, p. 402
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042