levels of βA-MDA. It is contemplated that by monitoring the levels of βA-MDA in blood, serous fluid and/or CSF of patients undergoing treatment for Alzheimer's disease, determinations regarding the effectiveness of treatment regimes are possible. For example, reduced levels of βA-MDA over time indicate that the treatment used to treat a subject with Alzheimer's disease is effective.

It is contemplated that the present invention will find use in testing subjects such as those who have been previously diagnosed with Alzheimer's disease, those who are suspected of having Alzheimer's disease, and those at risk of developing Alzheimer's disease. For example, patients diagnosed with dementia, in particular, those patients who were previously clinically normal, are suitable subjects. However, it is not intended that the present invention be limited to use with any particular subject or patient types. The methods of the present invention are also useful for detecting early onset Alzheimer's disease and late onset Alzheimer's disease, as well as for detecting sporadic Alzheimer's disease and familial Alzheimer's disease.

The present invention also provides compositions and methods for the detection and quantitation (*i.e.*, measurement) of βA-MDA peptide in the blood, serous fluid and CSF. Standard techniques known in the art are easily adapted to quantitate the levels of circulating βA-MDA peptide in blood, serous fluid and/or CSF samples, including but not limited to, ELISA.

Factors contributing to the success of the ELISA methods of the present invention include their sensitivity, versatility, long reagent shelf-life, ease of preparation of reagents, non-radioactive reagents, and assay speed. Furthermore, in some embodiments, the assay is quantitative. In addition, reagents and equipment designed specifically for use in ELISA protocols are readily available from numerous manufacturers, including Pierce Chemical Company, Bio-Rad, Dynatech Industries, GibcoBRL/Life Technologies, Fisher Scientific, and Promega.

Many ELISA applications and formats have been described. Various sources provide discussion of ELISA chemistry, applications, and detailed protocols (*See e.g.*, Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in *Molecular Biomethods Handbook*, Rapley *et al.* [eds.], pp. 595-617, Humana Press, Inc., Totowa, NJ [1998]; Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; Ausubel *et al.* (eds.),

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Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]; and Laurino *et al.*, *Ann. Clin. Lab Sci.*, 29(3):158-166 [1999]).

In preferred embodiments of the present invention, ELISA methods for quantitation of antigen are provided. In some of these methods, the antigen (*e.g.*, the βA-MDA peptide) is first immobilized on a solid support (*e.g.*, in a microtiter plate well). Detection and quantitation of the immobilized antigen is accomplished by use of an antibody-enzyme conjugate capable of binding to the immobilized antigen and producing a quantifiable signal. In some embodiments, the amount of antigen present is directly proportional to the amount of enzyme reaction product produced after the addition of an appropriate enzyme substrate.

As indicated previously, enzymes commonly used in ELISAs include horseradish peroxidase (HRPO), urease, alkaline phosphatase, glucoamylase and β-galactosidase. Protocols for the preparation of suitable antibody-enzyme conjugates are well known in the art. The present invention provides methods for the preparation of an antibody-enzyme (*i.e.*, HRPO enzyme) conjugate that specifically recognizes the antigen of interest (*i.e.*, βA-MDA peptide) for use in immunoassay (*e.g.*, ELISA) methods for detection of Alzheimer's disease. It is not intended that the present invention be limited to the antibody-enzyme conjugation method provided herein, as those of skill in the art will recognize other methods for antibody-enzyme conjugation that find use with the present invention.

Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme (*e.g.*, HRPO or alkaline phosphatase) and the antibody (*e.g.*, the polyclonal anti-βA-MDA antibody), where neither the antigen-binding site of the antibody nor the active site of the enzyme is functionally altered.

The conjugation of antibody and HRPO is dependent on the generation of aldehyde groups by periodate oxidation of the carbohydrate moieties on HRPO (Nakane and Kawaoi, *J. Histochem. Cytochem.*, 22:1084-1091 [1988]). Combination of these active aldehydes with amino groups on the antibody forms Schiff bases that, upon reduction by sodium borohydride, become stable.

Protocols to make antibody-enzyme conjugates using urease or alkaline phosphatase enzymes are also known in the art (Healey *et al.*, *Clin. Chim. Acta* 134:51-58 [1983]; Voller *et al.*, *Bull. W.H.O.*, 53:55-65 [1976]; and Jeanson *et al.*,

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J. Immunol. Methods 111:261-270 [1988]). For urease conjugation, cross-linking of the urease enzyme (*e.g.*, Urease Type VII, Sigma No. U0376) and antibody using *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS) is achieved through benzoylation of free amino groups on the antibody. This is followed by thiolation of the maleimide moiety of MBS by the cysteine sulfhydryl groups of urease. To prepare an alkaline phosphatase-antibody conjugate, a one-step glutaraldehyde method is the simplest procedure (Voller *et al.*, *Bull. W.H.O.*, 53:55-65 [1976]). This antibody-alkaline phosphatase conjugation protocol uses an enzyme immunoassay grade of the alkaline phosphatase enzyme.

The end product of an ELISA is a signal typically observed as the development of color or fluorescence. Typically, this signal is read (*i.e.*, quantitated) using a suitable spectrocolorimeter (*i.e.*, a spectrophotometer) or spectrofluorometer. The amount of color or fluorescence is directly proportional to the amount of immobilized antigen. In some embodiments of the present invention, the amount of antigen in a sample (*e.g.*, the amount of βA-MDA peptide in a blood or CSF sample) is quantitated by comparing results obtained for the sample with a series of control wells containing known concentrations of the antigen (*i.e.*, a standard concentration curve). A negative control is also included in the assay system.

It is contemplated that any suitable chromogenic or fluorogenic substrates will find use with the enzyme-conjugated antibodies of the present invention. In some embodiments of the present invention, the substrate *p*-nitrophenyl phosphate (NPP) in diethanolamine is the preferred substrate for use in colorimetric ELISA methods, and 4-methylumbelliferyl phosphate (MUP) is the preferred alkaline phosphatase substrate in fluorometric ELISA methods.

The present invention provides various ELISA protocols for the detection and/or quantitation of βA-MDA antigen in a sample. In one embodiment, the present invention provides a "direct ELISA" for the detection of βA-MDA antigen in a sample. In some embodiments, the antigen of interest in a sample (*i.e.*, the βA-MDA peptide) is bound (along with unrelated antigens) to the solid support (*e.g.*, a microtiter plate well). The immobilized antigen is then directly detected by the antigen-specific enzyme-conjugated antibody, also provided by the present invention. Addition of an appropriate detection substrate results in color

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development or fluorescence that is proportional to the amount of antigen present in the well.

In another embodiment, the present invention provides an indirect ELISA for the detection of antigen in a sample. In this embodiment, antigen of interest in a sample is immobilized (along with unrelated antigens) to a solid support (*e.g.*, a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody, then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (*e.g.*, a goat anti-rabbit antibody), which are available from various manufacturers known to one in the art (*e.g.*, Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

In some embodiments, the concentration of sample added to each well is titrated, so as to produce an antigen concentration curve. In other embodiments, the concentration of conjugated antibody is titrated. Indeed, such titrations are typically performed during the initial development of ELISA systems.

In another embodiment, the present invention provides "sandwich ELISA" methods, in which the antigen in a sample is immobilized on the solid support by a "capture antibody" that has been previously bound to the solid support. In general, the sandwich ELISA method is more sensitive than other configurations, and is capable of detecting 0.1-1.0 ng/ml protein antigen. As indicated above, the sandwich ELISA method involves pre-binding the "capture antibody" which recognizes the antigen of interest (*i.e.*, the βA-MDA peptide) to the solid support (*e.g.*, wells of the microtiter plate). In some embodiments, a biotinylated capture antibody is used in conjunction with avidin-coated wells. Test samples and controls are then added to the wells containing the capture antibody. If antigen is present in the samples and/or controls, it is bound by the capture antibody.

In some embodiments, after a washing step, detection of antigen that has been immobilized by the capture antibody is detected directly (*i.e.*, a direct sandwich ELISA). In other embodiments detection of antigen that has been immobilized by the capture antibody is detected indirectly (*i.e.*, an indirect sandwich ELISA). In the direct sandwich ELISA, the captured antigen is detected using an antigen-specific enzyme-conjugated antibody. In the indirect sandwich ELISA, the captured antigen is detected by using an antibody directed against the

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antigen, which is then detected by another enzyme-conjugated antibody which binds the antigen-specific antibody, thus forming an antibody-antigen-antibody-antibody complex. In both the direct and indirect sandwich ELISAs, addition of a suitable detection substrate results in color development or fluorescence that is proportional to the amount of antigen that is present in the well.

In the sandwich ELISA, the capture antibody used is typically different from the second antibody (the "detection antibody"). The choice of the capture antibody is empirical, as some pairwise combinations of capture antibody and detection antibody are more or less effective than other combinations. The same monoclonal antibody must not be used as both the capture antibody and the conjugated detection antibody, since recognition of a single epitope by the capture antibody will preclude the enzyme-conjugated detection antibody from binding to the antigen. However, in some embodiments, two different monoclonal antibodies that recognize different epitopes are used in this assay. In other embodiments, the same polyclonal antibody preparation is used as both the capture antibody and conjugated detection antibody, since multiple epitopes are recognized in the pool of polyclonal antibody species. In some embodiments, the polyclonal anti-βA-MDA antibody provided by the present invention is used as both the capture antibody and the enzyme-conjugated detection antibody in the sandwich ELISA. Alternatively, in other embodiments, it is contemplated that a monoclonal or polyclonal antibody which recognizes an unmodified βA peptide will find use as the capture antibody, and the anti-βA-MDA polyclonal antibody is used as the enzyme-conjugated detection antibody.

Antibodies which recognize unmodified forms of the βA peptide suitable for use in the present invention are readily available from various manufacturers, including Oncogene Research Products (Catalog Nos. PC149, PC150, PC151 and PC152), Santa Cruz Biotechnology (Catalog Nos. sc-5399, sc-5400, sc-7496, sc-7497, sc-7498 and sc-9129), Sigma (Catalog Nos. A5213), Zymed (Catalog Nos. 13-0100 and 71-5800) and Pharmingen/Transduction Laboratories (Catalog Nos. 66471G, 66481G and 66491G). However, it is not intended that the present invention be limited to the antibodies listed here, as other antibodies also find use with the present invention. It is also not intended that the sandwich ELISA of the present invention be limited to any particular sandwich ELISA method.

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Furthermore, it is not intended that the present invention be limited to the direct ELISA and sandwich ELISA protocols particularly described herein, as the art knows well numerous alternative ELISA protocols that also find use in the present invention (*See, e.g.*, Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in *Molecular Biomethods Handbook*, Rapley *et al.* [eds.], pp. 595-617, Humana Press, Inc., Totowa, NJ [1998]; and Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, Ch. 11, John Wiley & Sons, Inc., New York [1994]). Thus, any suitable ELISA method including, but not limited to, competitive ELISAs also find use with the present invention.

In another embodiment, the present invention provides methods for the detection and quantitation of βA-MDA reactive antibodies. Briefly, in some embodiments, variations of indirect ELISAs are used. In preferred embodiments, antigen (*i.e.*, βA-MDA) is first used to coat the wells of a 96-well microtiter plate. The test sample is then added to the antigen-coated wells. If the test sample contains βA-MDA reactive antibodies, these antibodies specifically bind to the purified antigen coating the well. The βA-MDA reactive antibodies are then visualized by the addition of a second detection antibody, where the detection antibody is coupled to an enzyme and is species-specific or isotype-specific for anti-βA-MDA antibody. As with all ELISA methods, appropriate negative and positive controls are included in order to ensure the reliability of the assay results.

It is contemplated that patients with Alzheimer's disease produce βA-MDA-reactive auto-antibodies, and an ELISA to detect βA-MDA reactive antibodies in such samples will find use in the diagnosis of Alzheimer's disease. It is further contemplated that the presence of anti-βA-MDA auto-antibodies in a patient is diagnostic of Alzheimer's disease.

It is also contemplated that the present invention will find use in detection of βA-MDA reactive antibodies in various other settings (*e.g.*, in the screening of monoclonal hybridoma culture supernatants [*i.e.*, conditioned hybridoma culture medium], ascites fluid and/or polyclonal antisera).

The present invention also provides ELISA amplification systems. These embodiments produce at least 10-fold, and more preferably, a 500-fold increase in sensitivity over traditional alkaline phosphatase-based ELISAs. In one preferred embodiment of the ELISA amplification protocol, bound alkaline phosphatase acts

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on an NADPH substrate, whose reaction product initiates a secondary enzymatic reaction resulting in a colored product. Each reaction product from the first reaction initiates many cycles of the second reaction in order to amplify the signal (*See e.g.*, Bio-Rad ELISA Amplification System, Cat. No. 19589-019).

The present invention also provides ELISA kits for the detection of antibodies and/or antigen. In addition, in some embodiments, the kits are customized for various applications. However, it is not intended that the kits of the present invention be limited to any particular format or design. In some embodiments, the kits of the present invention include, but are not limited to, materials for sample collection (*e.g.*, spinal and/or venipuncture needles), tubes (*e.g.*, sample collection tubes and reagent tubes), holders, trays, racks, dishes, plates (*e.g.*, 96-well microtiter plates), instructions to the kit user, solutions or other chemical reagents, and samples to be used for standardization, and/or normalization, as well as positive and negative controls. In particularly preferred embodiments, reagents included in ELISA kits specifically intended for the detection of βA-MDA peptide or anti-βA-MDA antibodies include control βA-MDA peptide, anti-βA-MDA antibody, anti-βA-MDA antibody-enzyme conjugate, 96-well microtiter plates precoated with control βA-MDA peptide, suitable capture antibody, 96-well microtiter plates precoated with a suitable βA-MDA capture antibody, buffers (*e.g.*, coating buffer, blocking buffer, and distilled water), enzyme reaction substrate and premixed enzyme substrate solutions.

It is contemplated that the compositions and methods of the present invention will find use in various settings, including research and clinical diagnostics. For example, the anti-βA-MDA antibody of the present invention also finds use in studies of APP metabolism and in *in situ* hybridization studies of brain tissue sections to observe Alzheimer's disease pathology. In addition, methods to quantitate βA-MDA in samples find use in monitoring and/or determining the effectiveness of Alzheimer's disease treatment, as it is contemplated that decreasing levels of βA-MDA peptide in a subject's samples over time indicates the effectiveness of an Alzheimer's disease treatment. Uses of the compositions and methods provided by the present invention encompass human and non-human subjects and samples from those subjects, and also encompass research as well as

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diagnostic applications. Thus, it is not intended that the present invention be limited to any particular subject and/or application setting.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); cm (centimeters); g (grams); l or L (liters); μg (micrograms); μl (microliters); μm (micrometers); μM (micromolar); μmol (micromoles); mg (milligrams); ml (milliliters); mm (millimeters); mM (millimolar); mmol (millimoles); M (molar); mol (moles); ng (nanograms); nm (nanometers); nmol (nanomoles); N (normal); pmol (picomoles); Aldrich (Sigma/Aldrich, Milwaukee, WI); Amersham (Amersham/Pharmacia Biotech, Piscataway, NJ); Bio-Rad (Bio-Rad Laboratories, Hercules, CA), Boehringer Mannheim (Boehringer Mannheim Corporation, Indianapolis, IN); Dynex (Dynex Technologies, Inc., Chantilly, VA); Fisher Scientific (Fisher Scientific, Pittsburgh, PA), GiboBRL/Life Technologies (GibcoBRL/Life Technologies, Gaithersburg, MD), Oncogene Research Products (Oncogene Research Products, Cambridge, MA); Pharmingen/Transduction Laboratories (Pharmingen/Transduction Laboratories/Becton Dickinson Company, San Diego, CA); Pierce Chemical Company (Pierce Chemical Company, Rockford, IL); Promega (Promega Corporation, Madison, WI); Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Sigma (Sigma Chemical Co., St. Louis, MO); and Zymed (Zymed Laboratories, Inc., South San Francisco, CA).

Brain tissue samples from an Alzheimer's disease patient and from a matched control individual (*i.e.*, without Alzheimer's disease) were obtained from the Alzheimer's Disease Center at University of California, San Diego.

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EXAMPLE 1

In Vitro Modification of Synthetic β-Amyloid Fragment 1-42

β-amyloid peptide fragment 1-42 (SEQ ID NO: 1; Sigma, Catalog No. A9810) was oxidatively modified to form a β-amyloid peptide containing malondialdehyde adduct(s). This modified peptide is herein called βA-MDA. A 100 µl volume of modification buffer (88 µl bis-dimethylacetal (Aldrich), 12 µl of 4 N HCl, 400 µl water, incubated at 37°C for 30 minutes, pH to 7.4 with NaOH) was used to dissolve 1.0 mg of the unmodified β-amyloid peptide fragment. The resulting solution was incubated at 37°C for 3 hours, followed by lyophilization to dryness. βA-MDA was then resuspended and stored at -70°C.

EXAMPLE 2

Generation and Purification of Polyclonal Anti-βA-MDA Antibodies

Antisera against the βA-MDA peptide was raised in rabbits using standard techniques. Briefly, the βA-MDA peptide was conjugated to keyhole limpet hemocyanin (KLH), mixed with an equal volume of Freund's incomplete adjuvant, and used to immunize New Zealand white rabbits by injection at 8-10 subcutaneous dorsal sites. Animals received inoculation boosts at weeks 4, 6 and 8. Bleeds were obtained and tested at weeks 8, 12 and 16 for analysis. When antibody levels plateaued, the rabbits were exsanguinated by cardiac puncture, and the collected sera was stored at -80°C until used.

Antibodies contained within the anti-βA-MDA antisera were purified by ammonium sulfate precipitation and immunoaffinity purification using standard techniques. Briefly, the anti-βA-MDA antiserum was subjected to ammonium sulfate precipitation by the addition of ammonium sulfate to 50% saturation. At this concentration, the IgG fraction was precipitated, while a significant percentage of the contaminating proteins remained in solution. The resulting precipitate was pelleted by centrifugation and washed once in 50% saturated ammonium sulfate solution. The precipitate was pelleted again, then resuspended and dissolved in PBS. The solution was then dialyzed once against PBS.

The PBS solution containing the precipitated IgG fraction was further purified using affinity chromatography. The affinity resin used in this purification was an activated CH-Sepharose coupled to βA-MDA peptide prepared as described

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in Example 1, at a concentration of 10 mg of peptide per 1.0 g of sepharose. The IgG fraction was loaded onto the column and washed with 6 volumes of PBS, followed by a second wash with 6 volumes of PBS containing 1.0 M NaCl. The PBS solution containing the precipitated IgG was loaded onto the column, washed, and the anti-βA-MDA-specific IgG fraction was eluted in 2.0 ml fractions with 100 mM glycine, pH 2.5. Following elution, 200 µl of 1.0 M Tris pH 8.1 was added to the fractions. The concentration of the immunoglobulin was determined using a total protein colorimetric determination (Bio-Rad).

It is not intended that the present invention be limited to the use of this particular protocol for the production and purification of antibodies, as numerous protocols are available and known to those in the art (*See, e.g.*, Sambrook *et al.* (eds.), Molecular Cloning, Cold Spring Harbor Laboratory Press [1989]; Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press [1988]; and Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, Ch. 11, John Wiley & Sons, Inc., New York [1994]). The only criterion for antibody production methods finding use with the invention is that sufficiently purified antibody preparations be produced. As discussed further in Example 3, any method suitable for the production of monoclonal antibodies also finds use with the present invention.

EXAMPLE 3

Generation of Monoclonal Anti-βA-MDA Antibodies

For preparation of monoclonal antibodies directed toward βA-MDA, any technique that provides for the production of monoclonal antibodies will find use with the present invention. This Example provides but one method for the production of such monoclonal antibodies. However, it is not intended that the present invention be limited to this particular method, as any method suitable for the production of anti- βA-MDA peptide antibodies will find use with the present invention.

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A) Inoculation and Recovery of Antibody-Producing Cells

The modified βA-MDA peptide fragment, or a portion thereof, is administered as an antigen to a suitable animal (*e.g.*, rat, mouse or rabbit). The dosage of the antigen administered per animal is typically be between 0.1 and 10 mg when no adjuvant is used, and between 1.0 and 100 μg when an adjuvant is used. As an adjuvant, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), aluminum hydroxide adjuvant or the like are suitable for use. Immunization is by any suitable route and immunization schedule, as known to those in the art. Subsequently, at 1 to 10 days, preferably 3 days, after the final immunization, antibody-producing cells (*e.g.*, spleen, lymph nodes and peripheral blood cells) are collected.

B) Cell Fusion and Formation of Hybridoma Cell Lines

In order to obtain hybridomas which produce the monoclonal antibody, cell fusions between the antibody-producing cells described above and myeloma cells are performed. In particularly preferred embodiments, spleen cells harvested from the animals immunized as described above are used. The fusion partner chosen for production of the monoclonal antibodies is preferably a cell line which has drug selectivity and cannot survive in HAT selective medium (*i.e.*, containing hypoxanthine, aminopterin and thymidine) unless fused to antibody-producing cells. Mouse myeloma cell lines including but not limited to, P3X63Ag.8.U1(P3U1), Sp2/0, NS-1 and other cell lines find use as suitable myeloma cell lines for fusion with antibody-producing cells.

The myeloma cells and the antibody-producing cells described above are fused using any suitable method. For example, 1×10^9 cells/ml of the antibody-producing cells and 1×10^8 cells/ml of the myeloma cells are mixed together in equal volumes in an animal cell culture medium such as serum-free DMEM or RPMI-1640, and reacted in the presence of a cell fusion promoting agent. In some embodiments, polyethylene glycol with an average molecular weight of 1,500 Da is used as the cell fusion promoting agent. Alternatively, the antibody-producing cells and the myeloma cells are fused in a commercial cell fusion apparatus utilizing electric stimulation (*e.g.*, electroporation). These cells are

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plated in appropriate vessels and incubated under suitable conditions, as described below.

C) Selection and Cloning of Hybridoma Lines

Following cell fusion, suitable hybridomas are selected from the cell cultures. As a method for this selection, the resultant cell suspension is appropriately diluted with fetal bovine serum containing RPMI-1640 medium or the like, and then plated on microtiter plates at a density of about 2 x 10^5 cells/well. A selective medium is added to each well, and the cells are cultured in that selective medium. As a result, about 14 days after the start of cultivation in the selective medium, the growing cells develop into antibody-producing hybridomas.

Subsequently, screening is performed in order to determine the presence of the antibody of interest in the culture supernatant of the hybridomas. Hybridoma screening is performed using any suitable methods. For example, a part of the culture supernatant of a well in which a hybridoma is grown is collected and subjected to enzyme immunoassay or radioimmunoassay, as described herein.

The fused cell preparations are cloned using a suitable method (*e.g.*, the limiting dilution method or the like). Finally, the monoclonal antibody-producing hybridoma is established and maintained. In preferred embodiments, aliquots of the monoclonal antibody producing hybridoma are frozen and retained as stock cultures.

D) Recovery and Purification of the Monoclonal Antibody

In some embodiments of the present invention, conventional cell culture methods or the abdominal dropsy formation method are employed for recovering the monoclonal antibody from the a monoclonal antibody-producing hybridomas.

In the cell culture method, the hybridoma is cultured in an animal cell culture medium such as 10% fetal bovine serum-containing RPMI-1640 medium, MEM medium or a serum-free medium under conventional culture conditions (*e.g.*, at 37°C under 5% CO_2) for 2 to 10 days. Then, the monoclonal antibody is then harvested from the culture supernatant, using methods known to those in the art.

In the abdominal dropsy formation method, about 1 X 10^7 cells of the hybridoma are administered into the abdominal cavity of an animal syngeneic to

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the mammal from which the myeloma cells were derived, to thereby greatly propagate the hybridoma. One to two weeks thereafter, the abdominal dropsy or serum is collected.

The antibody obtained is then purified as necessary, using methods known in the art (*e.g.*, protein A/protein G affinity, ammonium sulfate salting out, ion exchange chromatography, gel filtration, affinity chromatography, or using these methods in combination).

The antibodies produced by this method find use in numerous applications, including Western immunoblotting, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation, immunoaffinity purification, and immunohistochemistry.

EXAMPLE 4

Western Immunoblotting Using βA Peptides

βA and βA-MDA peptides were analyzed by Western immunoblotting to determine the reactivity and specificity of the polyclonal anti-βA-MDA antibody produced according to the method described in Example 2. This method utilized a chemiluminescence detection protocol (ECL, Amersham) that was performed according to the manufacturer's instructions. The secondary antibody used for visualization was a goat anti-rabbit IgG-horseradish peroxidase (HRPO)-conjugated antibody (Santa Cruz).

Briefly, the protein concentrations of the synthetic βA peptide fragment 1-42 and the modified βA-MDA peptide (described in Example 1) were determined by a Bradford-based protein assay (Bio-Rad). Following protein quantitation, 1.0 μg samples of each protein were boiled for 5 minutes in Laemmli buffer. Samples were then resolved using SDS-PAGE containing 20% polyacrylamide and electroblotted onto nitrocellulose. Blots were incubated overnight in blocking buffer (phosphate buffered saline [PBS] containing 5% (w/v) nonfat dry milk and 0.1% Tween-20), then incubated for 2 hours with the polyclonal anti-βA-MDA antibody at a 1:1,000 dilution in blocking buffer. Membranes were washed 3 times in washing buffer (PBS containing 0.1% Tween-20) for 15 minutes each wash, then incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) diluted 1:5,000 in blocking buffer, then again washed again 3 times in

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washing buffer for 15 minutes each wash. Membranes were then incubated in chemiluminescent detection substrate (ECL, Amersham) for 1 minute and signal was detected on X-ray film.

The results indicated that the anti-βA-MDA antibody detected the βA-MDA synthetic peptide, but not an unmodified βA peptide. In addition, the preimmune control serum showed no reactivity towards either the βA-MDA peptide nor the unmodified βA peptide.

EXAMPLE 5

Immunoprecipitation of βA from Brain Tissue

Following the determination of the specificity of the purified anti-βA-MDA antibody as described in Example 4, the antibody was tested in experiments to determine whether it was capable of detecting proteins in human brain cortex tissue from an Alzheimer's disease patient and a matched control individual. Post-mortem brain tissue obtained from an Alzheimer's disease patient and a matched control individual was first subjected to immunoprecipitation using a commercially available anti-βA antibody. Western blots were used to analyze these immunoprecipitates, as described in Example 6.

Briefly, 100 mg of brain homogenate were combined with 3.0 ml of ice cold RIPA buffer (PBS containing 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenyl methanesulfonyl fluoride [PMSF], and 0.3 trypsin inhibitor units [TIU] aprotinin) and incubated at 4°C for 10 minutes. This mixture was centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was transferred to a fresh 15 ml conical centrifuge tube on ice. The lysate was precleared by adding 1.0 μg of control rabbit IgG (Sigma) together with 20 μl of suspended Protein A-Agarose beads (Santa Cruz Biotechnologies). This mixture was incubated at 4°C for 30 minutes.

The Protein A-agarose beads were pelleted by centrifugation at 1,000 x g for 5 minutes at 4°C, and the supernatant was removed and placed in a fresh 15 ml conical centrifuge tube on ice. Following protein quantitation (Protein Microassay, Bio-Rad), 500 μg of this total cellular protein supernatant was transferred to a 1.5 ml microcentrifuge tube, to which was added 2 μg of a commercial anti-βA antibody (Transduction Laboratories, Affinity Research Products Catalog No.

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AK6905). This mixture was incubated for 1 hour at 4°C. After this time, 20 µl of suspended Protein A-agarose beads were added to the eppendorf tube, the tube was capped, and incubated at 4°C on either a rocker platform or tube rotating device overnight.

Following this overnight incubation, the Protein A-agarose beads were pelleted by centrifugation at 1,000 x g for 5 minutes at 4°C, and the supernatant was aspirated and discarded. The resulting pellet was washed 4 times with 1 ml RIPA buffer, followed each time by recentrifugation. After the final wash, the supernatant was aspirated and discarded, and the pellet was resuspended in 40 µl of Laemmli buffer.

EXAMPLE 6

Western Immunoblotting Analysis of Human Brain Tissue Immunoprecipitates Using anti-βA-MDA Antibody

Brain tissue anti-βA-MDA immunoprecipitates (prepared as described in Example 5) were analyzed in a Western blot using the protocol provided in Example 4. Briefly, the brain tissue immunoprecipitates resuspended in 40 µl of Laemmli buffer were boiled for 2-3 minutes, and 20 µl aliquots were loaded and resolved on 20% polyacrylamide SDS-PAGE. Unused samples were stored at -20°C.

Following electrophoresis, samples were electroblotted onto nitrocellulose. Blots were incubated overnight in blocking buffer (phosphate buffered saline [PBS] containing 5% (w/v) nonfat dry milk and 0.1% Tween-20), then incubated for 2 hours with the polyclonal anti-βA-MDA antibody (prepared as described in Example 2) at a 1:1,000 dilution in blocking buffer. Membranes were washed 3 times in washing buffer (PBS containing 0.1% Tween-20) for 15 minutes each wash, then incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) diluted 1:5,000 in blocking buffer, then again washed again 3 times in washing buffer for 15 minutes each wash. Membranes were then incubated in chemiluminescent detection substrate (ECL, Amersham) for 1 minute and luminescence signal was detected on X-ray film.

As shown in Figure 1, this Western blot demonstrates that the anti-βA-MDA antibodies detect at least one and possibly multiple proteins in the

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immunoprecipitate from the brain of an Alzheimer's disease patient (lane indicated as "**AD**"). However, no proteins were detected in the brain tissue immunoprecipitate from a disease-free control individual (lane indicated as "**CONTROL**").

The specificity of the primary and secondary antibodies were further confirmed by an anti-β-galactosidase antibody immunoprecipitation of brain tissue from an Alzheimer's disease patient. The immunoprecipitation protocol used was identical to that described in Example 5. This anti-β-galactosidase immunoprecipitate was blotted and probed in parallel with the other two samples in Figure 1 using the same anti-βA-MDA primary antibody and the HRPO-conjugated secondary antibody (lane indicated as "***β*-GAL**"). As shown in the Figure, the anti-βA-MDA primary antibody showed no reactivity towards the anti-β-galactosidase immunoprecipitate from the brain tissue of the Alzheimer's patient.

Thus, the anti-βA-MDA polyclonal antibody produced as described in Example 2 recognizes at least one modified endogenous βA-MDA species present in the brain tissue of individuals with Alzheimer's disease, but not in the brain tissue of unaffected individuals.

EXAMPLE 7

Covalent Conjugation of Antibody and Reporter Enzyme

This Example describes the preparation of an antibody-enzyme conjugate for use in immunoassay (*e.g.*, ELISA) methods for detection of Alzheimer's disease. Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme (*e.g.*, HRPO or alkaline phosphatase) and the antibody (*e.g.*, the polyclonal anti-βA-MDA antibody, or an antibody which recognizes the anti-βA-MDA antibody), where neither the antigen-binding site of the antibody nor the active site of the enzyme is functionally altered. It is not intended that the present invention be limited to the antibody-enzyme conjugation method provided herein, as other methods also find use with the present invention, as known to one skilled in the art.

A detailed method for the synthesis of an HRPO-conjugated antibody of the present invention is provided herein. A purified antibody (*e.g.*, a polyclonal or monoclonal anti-βA-MDA antibody) solution is dialyzed against 100 mM

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phosphate buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 6.8) overnight at 4°C, at an antibody concentration of at least 1.0 mg/ml. A 10 mg/ml solution of HRPO enzyme (Type VI, Sigma No. P8375) is prepared in carbonate buffer (1.36 g sodium carbonate, 7.35 g sodium bicarbonate in 1.0 liter water, pH to 9.2). Then, 250 µl of $NaIO_4$ solution (1.71 mg $NaIO_4$/ml water, made fresh) and 250 µl of HRPO/carbonate solution are mixed together and incubated at room temperature for 2 hours in the dark.

Then, 1.0 ml of the dialyzed 1 mg/ml antibody solution and 0.5 ml of the HRPO/carbonate/$NaIO_4$ solution are placed in a plugged glass wool column, 250 mg Sephadex G-25 is added to this mixture, and incubated for 3 hours at room temperature in the dark.

The antibody-enzyme conjugate is eluted from the column by washing with 750 µl carbonate buffer. To this eluate, 38 µl of $NaBH_4$ solution (0.1 mM NaOH containing 5 mg/ml $NaBH_4$, made fresh) is added, and the mixture is incubated for 30 minutes at room temperature in the dark. After this 30 minute incubation, an additional 112 µl of $NaBH_4$ solution is added, and the mixture is incubated for 60 minutes at room temperature in the dark.

After this incubation, 900 µl of saturated $(NH_4)_2SO_4$ solution is added, and the mixture is incubated at 4°C for 30 minutes, followed by centrifugation at 10,000 x g at 4°C for 15 minutes. The supernatant is discarded, and the pellet is resuspended in 750 µl of TEN buffer (40 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 7.2).

The resuspended pellet is dialyzed against TEN buffer overnight at 4°C. The next day, the antibody-enzyme conjugate is removed, and bovine serum albumin (BSA) added to a final concentration of 20 mg/ml BSA. For storage of the antibody-enzyme conjugate, an equal volume of glycerol is added, and the mixture is placed at -20°C.

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EXAMPLE 8

Enzyme-Linked Immunosorbent Assay (ELISA)

This Example describes experiments useful for the detection and/or quantitation of the levels of βA-MDA peptide in various samples (*e.g.*, samples obtained from patients, as well as research samples). In these experiments, the anti-βA-MDA antibody (prepared as described in Example 2) is used.

A. Direct ELISA Method

As discussed previously, in some embodiments, the direct ELISA method typically involves binding an antigen (*e.g.*, the βA-MDA peptide) directly to microtiter wells, binding of an antigen-specific antibody-enzyme conjugate to the immobilized antigen, followed by detection of that antibody-enzyme conjugate by either colorimetric or fluorometric detection of the enzyme reaction product. Numerous variations of the direct ELISA protocol are known in the art. This Example provides one protocol suitable for use in the present invention.

Briefly, positive control antigen, a negative control antigen, and experimental samples are diluted in coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.6) to a concentration of ≤ 10 μg/ml. In experiments utilizing a pure antigen solution for the coating step, the concentration used is < 2 μg/ml. However, in preferred embodiments, a single optimum concentration, or range of concentrations, of the positive control antigen is determined empirically. First, 50 μl of coating buffer is added to the wells of a suitable 96-well polyvinyl or polystyrene microtiter plate (*e.g.*, Immulon 2™ or Immulon 4™ microtiter plates, Dynex Technologies, Catalog Nos. 011-010-3450 and 011-010-3850, respectively) and tapped to ensure that the solution is evenly distributed in the well. Then, 100 μl of positive control antigen, negative control antigen or experimental sample are added to the appropriate wells. Serial dilutions of the control antigens and/or experimental samples are then made within the rows of the plate. For each sample, 8-10 two-fold or three-fold serial dilutions are made, in order to ensure that at least one of the subsequent absorbance values is within the linear range of the assay. A standard dilution series for the control antigen ranges from 0.1 to 1000 ng/ml. Duplicate or triplicate 96-well plates are used in order to ensure reproducibility.

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The plates are covered and incubated overnight at room temperature or for 2 hours at 37°C.

Following incubation, the antigen solutions in the microtiter plate wells are decanted, and the wells are rinsed three times with distilled water. Then, 200 µl of blocking buffer (phosphate buffered saline [PBS] containing 1 mM EDTA, 0.05% (w/v) Tween-20, 0.25% (v/v) bovine serum albumin [BSA], and 0.05% (w/v) sodium azide [NaN_3]) are added to each well. However, in experiments utilizing an HRPO-conjugated antibody in the ELISA detection step, sodium azide is not used in this or any subsequent buffer. The plates containing blocking buffer are incubated at room temperature for 30-60 minutes. The blocking buffer is decanted, and 50 µl of fresh blocking buffer is added to each well. Then, 100 µl of diluted antibody-enzyme conjugate (diluted in blocking buffer) is added to each well. An appropriate dilution of the antibody-enzyme conjugate must be empirically determined, however, a dilution 1:100 is commonly used, resulting in a concentration of antibody-enzyme conjugate between 25-400 ng/ml. However, in some embodiments, other concentrations are used. The plates are covered and incubated under conditions determined to be optimal for the test system (*e.g.*, 30-60 minutes at 37°C).

Following the incubation, the wells are decanted, then washed three times with distilled water. Each well is then filled with blocking buffer, incubated for 10 minutes at room temperature, then washed 3 more times with distilled water. The wells are then decanted, inverted and patted dry.

Following the wash steps, 50-75 µl of the appropriate enzyme substrate (in blocking buffer) are added to each well on the plate and incubated for approximately 1 hour at room temperature. When using alkaline phosphatase systems, either 4-methylumbelliferyl phosphate (MUP) or *p*-nitrophenyl phosphate (NPP) substrate solutions are used to achieve either fluorescence or color development, respectively (MUP substrate solution: 0.2 mM MUP [Sigma Catalog No. M8883], 50 mM Na_2CO_3, 0.05 mM $MgCl_2$, store at room temperature; NPP substrate solution: 3 mM NPP [Sigma Catalog No. 104-0], 50 mM Na_2CO_3, 0.05 mM $MgCl_2$, store at 4°C). Premade substrate solutions are commercially available (*e.g.*, Bio-Rad ELISA Soluble Substrate Systems, Catalog Nos. 172-1063, 1064, 1066, 1067 and 1068). To assay low levels of enzyme activity, the plate is

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incubated for several hours with the appropriate substrate solution. The enzymatic reactions on the plate are stopped by adding 25 µl of 500 mM NaOH to the wells.

The enzymatic reaction in each well is monitored and/or quantitated visually or in an appropriate microtiter plate reader (*e.g.*, Bio-Rad Benchmark Microplate Reader, Catalog No. 170-6850). For example, hydrolysis of the NPP substrate by alkaline phosphatase results in the appearance of a yellow reaction product, which is monitored by absorbance at a 405 nm wavelength. Hydrolysis of the MUP substrate results in visible illumination with use of a long wavelength UV lamp, and is quantitated in a microtiter plate spectrofluorometer using a 365 nm wavelength excitation filter and a 450 nm wavelength emission filter (*See, e.g.*, Dynex Technologies, MFX™ Microplate Fluorometer). The absorbance versus the known antigen concentrations are plotted, and a standard curve that reads within the linear range of the spectrophotometer or spectrofluorometer is prepared. To determine the antigen concentration of the experimental samples, the absorbance of the experimental samples is compared to the absorbance values of the standard curve.

In view of numerous alternative protocols for a direct ELISA known in the art, it is not intended that the direct ELISA of the present invention be limited to this or any particular direct ELISA method.

B. Sandwich ELISA Method

In one embodiment of the present invention, the sandwich ELISA method involves first binding a "capture antibody" which recognizes the antigen of interest (*i.e.*, the βA-MDA peptide) to the wells of a microtiter plate. Samples containing control concentrations of antigen or experimental samples are then added to the wells containing antibody, where the antigen binds to the antibody present in the wells. After a washing step, a second antibody specific for the antigen and containing a conjugated enzyme is added to the wells, where it specifically binds the bound antigen. After a washing step, the amount of second antibody, and thus the amount of antigen in the well, is quantitated by measuring activity of the conjugated enzyme. As in the direct ELISA, the amount of antigen in an experimental sample (*e.g.*, the amount of βA-MDA peptide in a blood or CSF sample) is proportional to the color or fluorescence intensity in the well, and is

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quantitated by comparing the color or fluorescence value to the values for known concentrations of the antigen in the same assay (*i.e.*, a standard concentration curve).

The anti-βA-MDA antibody (*e.g.*, the antibody produced according to Example 2) is suitable for use as both the capture antibody, as well as the conjugated detection antibody. However, commercially available antibodies (including, for example, antibodies from Oncogene Research Products [Catalog numbers PC149, PC150, PC151 and PC152], Santa Cruz Biotechnology [Catalog numbers sc-5399, sc-5400, sc-7496, sc-7497, sc-7498 and sc-9129], Sigma [Catalog number A5213], Zymed [Catalog numbers 13-0100 and 71-5800] and Pharmingen [Catalog numbers 66471G, 66481G and 66491G]) capable of recognizing the unmodified form of the βA peptide are also suitable for use as the capture antibody.

A sandwich ELISA protocol suitable for use with the present invention is provided below. A purified βA-specific antibody (the "capture antibody") is diluted to a concentration of 0.2 to 10 μg/ml in PBSN (PBS containing 0.05% sodium azide [NaN_3]). However, in experiments using HRPO-conjugated antibody, sodium azide is not used in this or any subsequent steps. The wells of a 96-well microtiter plate are coated with the capture antibody by dispensing 50 μl aliquots of the antibody to the wells. Simultaneously, duplicate or triplicate plates are made and analyzed in parallel to ensure reproducibility. The plate is tapped or shaken to ensure that the antibody solution is evenly distributed over the bottom of each well. The plates are covered and incubated overnight at room temperature or for 2 hours at 37°C.

Following incubation, the antibody-containing solution in the plate wells is decanted. The wells are rinsed a total of three times with distilled water. After washing, each well is filled with blocking buffer (PBS containing 0.05% (w/v) Tween-20, 0.25% (w/v) bovine serum albumin [BSA], 1 mM EDTA and 0.05% (w/v) sodium azide [NaN_3]) and incubated for 30 minutes at room temperature. Following the incubation, the plate is rinsed with water three times, inverted, shaken and patted dry.

Following the blocking step, a serial dilution of purified antigen (*e.g.*, the βA-MDA peptide) is made by successive three-fold dilutions in blocking buffer,

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resulting in a range of antigen concentrations (*e.g.*, from 0.1 to 1000 ng/ml). Serial dilutions of the test samples are also made using blocking buffer. Ideally, dilutions of the experimental samples result in the samples containing between 1 and 100 ng/ml of specific antigen. Then, 50 µl aliquots of the standard antigen dilutions and the diluted experimental samples are placed in the wells of the 96-well microtiter plate, and incubated for at least two hours at room temperature. An antigen serving as a negative control (*e.g.*, an unmodified βA peptide) is also be included.

Following incubation, the plate is rinsed three times with distilled water, and each well is then filled with blocking buffer and incubated for 10 minutes at room temperature. The plate is then rinsed again three times in water, inverted, shaken and patted dry.

Then, 50 µl of specific antibody-enzyme conjugate are added to each well and incubated for 2 hours at room temperature. Reagents and methods for the detection and quantitation of the enzymatic reaction products are identical to those described in the direct ELISA.

Numerous variations of this sandwich ELISA protocol are suitable for use in the present invention. It is not intended that the sandwich ELISA of the present invention be limited to this or any particular sandwich ELISA method.

EXAMPLE 9

Analysis of βA-MDA from Blood and Cerebrospinal Fluid

As discussed above, endogenous unmodified βA is found in blood and cerebrospinal fluid (CSF). This Example describes methods to detect βA-MDA in the blood, serous fluid and CSF of a subject. The quantity of βA-MDA present in the samples is indicative of the severity of Alzheimer's disease, and is predictive for disease outcome (prognosis). In addition, by periodically testing the level of βA-MDA in the blood, serous fluid or CSF of a patient being treated for Alzheimer's disease, the efficacy of a particular treatment regime is monitored.

Blood samples (approximately 0.1 ml) are collected in standard clinical collection tubes containing an anticoagulant, such as heparin or EDTA. Blood plasma is separated by centrifugation at 1,000 x g for 10 minutes at 4°C within 30 minutes following sample collection. The blood and blood plasma samples are

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typically stored at 4°C. CSF is collected using standard procedures. CSF samples (approximately 0.1 ml) are typically stored at 4°C. These samples are then tested using the methods described herein (*e.g.*, by Western blot [Example 4] and/or by ELISA [Example 8]).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in immunology, cell biology, neuroscience, medicine, chemistry, and molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

What is claimed is:

1. An isolated and modified β-amyloid peptide comprising at least one malondialdehyde adduct.

2. The modified β-amyloid peptide of Claim 1, wherein said modified peptide comprises the amino acid sequence of SEQ ID NO:1.

3. The modified β-amyloid peptide of Claim 1, wherein said at least one malondialdehyde adduct comprises at least one malondialdehyde lysine adduct.

4. A purified antibody directed against the modified β-amyloid peptide of Claim 1.

5. A composition comprising the antibody of Claim 4.

6. A method for detecting at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising the steps of:

a) providing:

i) a sample suspected of containing at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, and

ii) the antibody of Claim 4;

b) contacting said sample with said antibody under conditions such that said antibody binds to said modified β-amyloid peptide comprising at least one malondialdehyde adduct, to form an antigen-antibody complex; and

c) detecting the presence of said antigen-antibody complex.

7. The method of Claim 6, wherein said sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid.

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8. The method of Claim 6, wherein said sample is from a subject.

9. The method of Claim 8, wherein said subject is a human.

10. The method of Claim 8, wherein said subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease.

11. The method of Claim 8, further comprising the step of diagnosing Alzheimer's disease.

12. The method of Claim 11, wherein said Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease.

13. The method of Claim 6, wherein said method comprises an enzyme-linked immunosorbent assay.

14. The method of Claim 13, wherein said enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays.

15. The method of Claim 6, wherein said antibody further comprises a conjugated enzyme, wherein said conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases.

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16. The method of Claim 13, further comprising the step of quantitating said at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct in said sample.

17. The method of Claim 16, further comprising the step of monitoring the efficacy of treatment of Alzheimer's disease.

18. The method of Claim 13, wherein said enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

19. The method of Claim 13, further providing at least one capture antibody.

20. The method of Claim 13, further providing at least one detection antibody wherein said detection antibody is directed against said antibody directed against said modified β-amyloid peptide comprising at least one malondialdehyde adduct.

21. The method of Claim 20, wherein said detection antibody further comprises a conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase.

22. A kit for the detection of at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising an immobilized support and the antibody of Claim 4.

23. The kit of Claim 22, wherein said kit comprises an enzyme-linked immunosorbent assay kit.

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24. The kit of Claim 23, wherein said kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, an antibody-enzyme conjugate directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct, at least one capture antibody, 96-well microtiter plates precoated with said at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least one alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

25. A method for detecting at least one antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising:

a) providing:

i) a sample suspected of containing at least one antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct, and

ii) the modified β-amyloid peptide of Claim 1, and

iii) a detection antibody;

b) contacting said sample with said modified β-amyloid peptide, under conditions such that said antibody directed against a modified β-amyloid peptide comprising at least one malondialdehyde adduct binds to said modified β-amyloid peptide to form an antigen-antibody complex;

c) contacting said antigen-antibody complex with said detection antibody, under conditions such that said detection antibody binds to said complex; and

d) detecting said binding of said detection antibody to said antigen-antibody complex.

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26. The method of Claim 25, wherein said sample is selected from the group of samples consisting of blood, serous fluid, plasma, serum, cerebrospinal fluid, hybridoma conditioned culture medium, ascites fluid, and polyclonal antiserum.

27. The method of Claim 25, wherein said sample is from a subject.

28. The method of Claim 27, wherein said subject is human.

29. The method of Claim 27, wherein said subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease.

30. The method of Claim 27, further comprising diagnosing Alzheimer's disease in said subject.

31. The method of Claim 30, wherein said Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease, and sporadic Alzheimer's disease.

32. The method of Claim 25, wherein said method comprises an enzyme-linked immunosorbent assay.

33. The method of Claim 32, wherein said enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays.

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34. The method of Claim 25, wherein said detection antibody further comprises a conjugated enzyme, wherein said conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases.

35. The method of Claim 32, wherein said enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

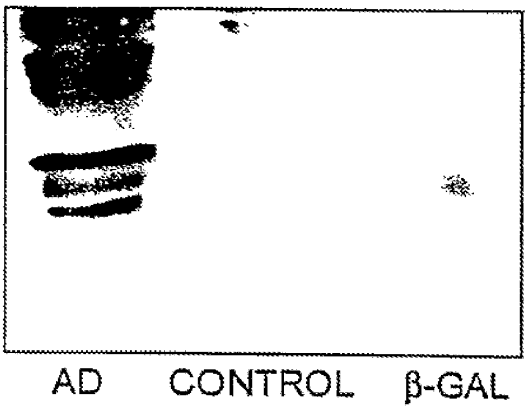
36. A kit for the detection of at least one antibody directed against at least one modified β-amyloid peptide comprising at least one malondialdehyde adduct, comprising an immobilized support, at least one purified and modified β-amyloid peptide comprising at least one malondialdehyde adduct, and at least one detection antibody.

37. The kit of Claim 36, wherein said kit is an enzyme-linked immunosorbent assay kit.

38. The kit of Claim 40, wherein said kit comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one purified antibody directed against at least one β-amyloid peptide comprising at least one malondialdehyde adduct, at least one 96-well microtiter plate precoated with said at least one β-amyloide peptide comprising at least one malondialdehyde adduct, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme reaction substrate solution, and at least one alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

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FIGURE 1



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(54) Title: BETA-SECRETASE TRANSGENIC ORGANISMS, ANTI-BETA-SECRETASE ANTIBODIES, AND METHODS OF USE THEREOF

(57) Abstract: Transgenic non-human animals, including, for example, transgenic rodents and transgenic non-human mammalian cells, which harbor a transgene that eliminates the expression of the β-secretase, BACE1, are provided. In addition, antibodies specific for BACE1 are provided. Also provided are methods of diagnosing a neurodegenerative disease, including Alzheimer's disease, and methods of identifying agents that modulate or treat Alzheimer's disease, and methods of identifying agents that modulate or treat Alzheimer's disease and related pathology.

BETA-SECRETASE TRANSGENIC ORGANISMS, ANTI-BETA-SECRETASE ANTIBODIES, AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to neurological diseases, and more specifically to transgenic non-human organism in which the beta-secretase, BACE1, gene is disrupted and which provide a model system for Alzheimer's disease and related disorders, to antibodies specific for BACE1, and to methods of using such transgenic organisms and antibodies.

BACKGROUND INFORMATION

The amyloidoses are a group of pathological conditions in which normally soluble proteins polymerize to form insoluble amyloid fibrils and amyloid deposits. More than 15 proteins form amyloid fibrils, resulting in diverse clinical conditions. Amyloidoses are usually classified into systemic amyloidoses and localized amyloidoses. Major systemic amyloidoses include AL amyloidosis, amyloid A amyloidosis, and familial transthyretin amyloidosis; the corresponding amyloid proteins associated with these amyloidoses are AL amyloid, amyloid A protein, and transthyretin, respectively. Prominent localized amyloidoses include Alzheimer's disease, prion diseases, and type II diabetes; the corresponding amyloid proteins in these diseases are amyloid β peptide, scrapie prion protein, and human amylin, respectively (Sipe, Ann. Rev. Biochem. 61:947-975, 1992).

Amyloid fibrils, regardless of the amyloid protein from which they are formed, have a cytotoxic effect on various cell types including primary cultured hippocampal neurons (Yankner et al., Science 250:279-282, 1990), pancreatic islet β cells (Lorenzo et al. Nature 368:756-760, 1994) and clonal cell lines (Behl et al., Biochem Biophys. Res. Commun. 186:944-952, 1992; O'Brien et al., Am. J. Pathol. 147:609-616, 1995). In fact, only amyloid proteins in fibrillar form are cytotoxic (Pike et al., Brain Res. 563:311-314, 1991; Lorenzo and Yankner, Proc. Natl. Acad. Sci. 91:12243-12247, 1994). It is likely that the cytotoxic effect of fibrils is mediated by a common mechanism (Lorenzo and Yankner, *supra*; Schubert et al., Proc. Natl. Acad. Sci., USA

92:1989-1993, 1995). Modulation of amyloid protein aggregation is one means of blocking or reducing amyloid toxicity.

Alzheimer's disease (AD) is a progressive disease known generally as senile dementia. The disease falls into two broad categories - late onset and early onset. Late onset AD, which occurs in old age (65+ years), may be caused by the natural atrophy of the brain occurring at a faster rate and to a more severe degree than normal. Early onset AD is much more infrequent, but shows a pathologically identical dementia with brain atrophy that develops well before the senile period, *e.g.*, between the ages of 35 and 60 years.

Alzheimer's disease is characterized by the presence of numerous amyloid plaques and neurofibrillary tangles (highly insoluble protein aggregates) present in the brains of AD patients, particularly in those regions involved with memory and cognition. The production of β-amyloid peptide, a major constituent of the amyloid plaque, can result due to mutations in the gene encoding amyloid precursor protein, which, when normally processed, will not produce the β-amyloid peptide. It is presently believed that a normal (non-pathogenic) processing of the β-amyloid precursor protein occurs via cleavage by a putative "α-secretase," which cleaves between amino acids 16 and 17 of the protein. It is further believed that pathogenic processing occurs via a putative "β-secretase" at the amino-terminus of the β-amyloid peptide within the precursor protein. Moreover, β-amyloid peptide appears to be toxic to brain neurons, and neuronal cell death is associated with the disease.

β-amyloid peptide (also referred to as A4, βAP, Aβ, or AβP; see, U.S. Pat. No. 4,666,829 and Glenner and Wong (1984) Biochem. Biophys. Res. Commun. 120:1131) is derived from β-amyloid precursor protein (βAPP), which is expressed in differently spliced forms of 695, 751, and 770 amino acids (see, Kang et al., Nature 325:773, 1987; Ponte et al., Nature 331:525, 1988; and Kitaguchi et al., Nature 331:530, 1988). Normal processing of amyloid precursor protein (APP) involves proteolytic cleavage at a site between residues Lys^{16} and Leu^{17} (as numbered where Asp^{597} is residue 1, in Kang et al., *supra*, 1997), near the transmembrane domain,

resulting in the constitutive secretion of an extracellular domain, which retains the remaining portion of the β-amyloid peptide sequence (Esch et al., Science 248:1122-1124, 1990). This pathway appears to be widely conserved among species and present in many cell types (see, Weidemann et al., Cell 57:115-126, 1989; and Oltersdorf et al., J. Biol. Chem. 265:4492-4497, 1990). This normal pathway cleaves within the region of the precursor protein which corresponds to the β-amyloid peptide, thus apparently precluding its formation. Another constitutively secreted form of βAPP has been noted (Robakis et al., Soc. Neurosci., Oct. 26, 1993, Abstract No. 15.4, Anaheim, Calif.), which contains more of the βAPP sequence carboxy terminal to the form described by Esch et al. (*supra*, 1990).

Soluble β-amyloid peptide is produced by healthy cells into culture media (Haass et al., Nature 359:322-325, 1992) and in human and animal CSF (Seubert et al., Nature 359:325-327, 1992). Palmert et al. (Biochem. Biophys. Res. Comm. 165:182-188, 1989) describe three possible cleavage mechanisms for βAPP and present evidence that βAPP cleavage does not occur at methionine^{596} in the production of soluble derivatives of βAPP. U.S. Pat. No. 5,200,339, describes the existence of certain proteolytic factor(s), which putatively are capable of cleaving βAPP at a site near the βAPP amino-terminus.

The *APP* gene is located on human chromosome 21. A locus segregating with familial Alzheimer's disease has been mapped to chromosome 21 (St. George Hyslop et al., Science 235:885, 1987) close to the APP gene. Recombinants between the *APP* gene and the AD locus have been reported (Schellenberg et al., Science 241:1507, 1988; Schellenberg et al., Am. J. Hum. Genetics 48:563, 1991; Schellenberg et al., Am. J. Hum. Genetics 49:511, 1991).

The identification of mutations in the amyloid precursor protein gene that cause familial, early onset Alzheimer's disease is evidence that amyloid metabolism is the central event in the pathogenic process underlying the disease. Four reported disease-causing mutations include, with respect to the 770 isoform, V717I (Goate et al., Nature 349:704, 1991), V717G (Chartier Harlan et al., Nature 353: 844, 1991),

V717F (Murrell et al., Science 254:97, 1991), and with respect to the 695 isoform, a double mutation changing K595N and M596L (Mullan et al., Nature Genet 1:345, 1992; Citron et al., Nature 360:672, 1992) referred to as the Swedish mutation.

The development of experimental models of AD that can be used to further study the underlying biochemical events involved in AD pathogenesis would be highly desirable. Such models could presumably be employed, in one application, to screen for agents that alter the degenerative course of Alzheimer's disease. For example, a model system of AD can be used to screen for environmental factors that induce or accelerate the pathogenesis of AD. An experimental model also can be used to screen for agents that inhibit, prevent, or reverse the progression of AD. Presumably, such models could be employed to develop pharmaceuticals that are effective in preventing, arresting, or reversing AD. It would also be desirable to have a model that can be used as a standard or control for comparison of agents the modulate amyloid deposition or activity. Thus, a need exists for a model system of Alzheimer's disease. The present invention satisfies this need, and provides additional advantages.

SUMMARY OF THE INVENTION

The present invention provides a method for modulating the production of Aβ11-40/42 peptide fragments. The method includes contacting a sample or cell containing a beta-site APP-cleaving enzyme 1 (BACE1) and an amyloid precursor protein (APP) with a BACE1-modulating agent such that production of Aβ11-40/42 is modulated. The contacting can be *in vivo* or *in vitro*.

In another embodiment, the invention provides a method for identifying a compound that inhibits BACE1 expression or activity. The method includes incubating components including the compound, BACE1 polynucleotide or polypeptide, and an APP under conditions sufficient to allow the components to interact, and measuring the production of a BACE1 specific enzymatic product.

Also provided are methods for diagnosing a subject having or at risk of having an Aβ11-40/42 peptide accumulation disease. The method includes measuring the

amount of BACE1 in a biological sample from the subject; and comparing the amount BACE1 with a normal standard value of BACE1, wherein a difference between the measured amount and the normal sample or standard value provides an indication of the diagnosis of Aβ11-40/42. The sample can be, for example, blood, serum, cerebrospinal fluid or central nervous system (CNS) tissue.

In yet another embodiment, the invention provides a method for diagnosing a subject having or at risk of having Alzheimer's disease, including measuring Aβ11-40/42 in a biological sample from the subject; comparing the amount of Aβ11-40/42 with a normal sample or standard value of Aβ11-40/42, wherein a difference between the amount in the normal sample or standard value is indicative of a subject having or at risk of having Alzheimer's disease.

In another embodiment, the invention provides a transgenic non-human animal having a transgene disrupting expression of BACE1, chromosomally integrated into the germ cells of the animal, and have a phenotype of reduced Aβ peptide as compared with a wild-type animal. The invention also provides a method for producing a transgenic non-human animal having a phenotype characterized by reduced expression of BACE1 polypeptide. The method includes introducing at least one transgene into a zygote of an animal, the transgene(s) comprising a DNA construct encoding a selectable marker, transplanting the zygote into a pseudopregnant animal, allowing the zygote to develop to term, and identifying at least one transgenic offspring whose genome comprises a disruption of the endogenous BACE1 polynucleotide sequence by the transgene.

In yet another embodiment, the invention provides a method for identifying an agent that modulates the expression or activity of BACE1. The method includes administering an agent to be tested to an organism; and comparing the phenotype of the organism contacted with the agent with that of a BACE1 knockout organism not contacted with the agent, whereby a phenotype substantially equal to the BACE1 knockout organism is indicative of an agent that modulates BACE1 expression or activity.

The invention also provides a method for screening for an agent, which ameliorates symptoms of Alzheimer's disease. The method includes comparing an effect of an agent on an organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent, wherein the organism has a phenotype associated with Alzheimer's disease and wherein an agent which ameliorates said phenotype is identified by having a substantially equal or superior phenotype of the organism in comparison with the BACE1 knockout organism.

In yet another embodiment, the invention provides a method for screening for an agent, which ameliorates symptoms of Alzheimer's disease. The method includes comparing an effect of an agent on a transgenic organism contacted with the agent with that of a BACE1 knockout organism not contacted with the agent, wherein the transgenic organism is characterized as having a phenotype of impaired performance on memory learning tests or abnormal neuropathology in a cortico-limbic region of the brain and the BACE1 knockout organism has a phenotype of reduced expression of BACE1, wherein the impaired performance and the abnormal neuropathology are in compared with the BACE1 knockout organism, whereby an agent which ameliorates the symptoms is identified by substantially equal or superior performance of the transgenic organism as compared with the BACE1 knockout organism on the memory and learning tests.

The invention also provides a kit useful for the detection of an Aβ11-40/42 accumulation disorder comprising carrier means containing therein one or more containers wherein a first container contains a nucleic acid probe that hybridizes to a nucleic acid sequence BACE1 or an antibody probe specific for BACE1 or Aβ11-40/42.

In yet another embodiment, the invention provides a method for predicting the therapeutic effectiveness of a compound for treating Alzheimer's disease in a subject by measuring the accumulation of Aβ11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide before and after treatment with the

compound, wherein a decrease in accumulation of peptide fragments or a decrease in the level of BACE1 polynucleotide or polypeptide after treatment is indicative of a compound that is effective in treating the disease.

In another embodiment, the invention provides a method for monitoring the progression of Alzheimer's disease by measuring the accumulation of Aβ11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide at a first time point and a second time point, thereby monitoring the progression of the disease.

The invention also provides antibodies that specifically bind BACE1 or a peptide portion of BACE1 corresponding to amino acid residues 46 to 163. The antibodies can be polyclonal antibodies or monoclonal antibodies derived therefrom, and can be in a substantially purified form or can be a component of an antiserum. Also provided are methods of using such anti-BACE1 antibodies to detect BACE1 in a sample, for example, a tissue sample such as a brain tissue sample, or to diagnose a disorder associated with an accumulation of amyloid plaques, for example, Alzheimer's disease. As disclosed herein, BACE1 has the characteristics of a susceptibility factor that contributes to brain-specific Aβ amyloidogenesis. BACE1 was found to be abundant in the brain, and is particularly rich in the hippocampus, including the giant boutons of hippocampal mossy fibers, a region that is critical for learning and memory and especially vulnerable in AD. Whereas high levels of BACE1 coupled with low levels of α-secretase and BACE2 activities were observed in neurons, low levels of BACE1 and high levels of BACE2 and α-secretase activities were detectable in non-neuronal cells. Importantly, while the deletion of BACE1 abolished the secretion and deposition of Aβ, the partial reduction of BACE1 (to 50% of normal level) significantly ameliorated amyloid plaque deposition in a mouse model of Aβ amyloidosis. These results demonstrate that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of Aβ in the central nervous system and indicate that polymorphisms that increase levels of BACE1 can have a role in predisposing an individual to AD. Accordingly, the present invention further relates to methods of determining whether an individual has, or is susceptible

or predisposed to developing, Aβ deposition in the brain, including to an amyloidosis such as AD, and further relates to methods of modulating BACE1 activity in a cell, and to methods of preventing or of amelioriating Aβ deposition in an individual.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a map of the wild-type *BACE1* locus, the targeting vector, and the disrupted *BACE1* allele. The first coding exon of BACE1 is indicated by black box. The targeting vector shows the replacement of the first coding exon and flanking genomic sequences by the neomycin gene (*neo*) and the HSV thymidine kinase gene (*tk*). Arrows indicate the sites within the targeted and wild-type alleles from which PCR primers were chosen for genotyping. Lines below denote expected sizes for *Sac*I-digested fragments detected by a 5'-flanking probe (a 0.45 kb *Hin*dIII/*Pst*I fragment, black bar) from targeted and endogenous *BACE1* alleles. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xba*I.

Figure 1B shows an analysis of genomic DNA from $BACE1^{+/-}$ crosses by Southern blot. The *Sac*I fragments detected for wild-type (8.0 kb) and targeted (5.4 kb) *BACE1* alleles with the 5' probe are indicated.

Figure 1C shows PCR analysis of DNA extracted from embryos using primers indicated in A, the 157 bp or 272 bp fragment is specific to the targeted or endogenous *BACE1* allele, respectively.

Figure 1D shows total protein extracts (30 μg) of wild-type (+/+), heterozygous (+/-), and homozygous BACE1 knockout (-/-) from E16.5 embryos. Embryos were immunoblotted using rabbit polyclonal antisera specific for epitopes in the N-terminal 46-163 amino acids of BACE1, and superoxide dismutase 1 (SOD1).

Figure 2A shows a sequence alignment of Aβ1-42 denoting differences between the human and mouse protein sequences (bolded amino acids). The cleavage sites corresponding to BACE1, α and γ secretases are marked and numbered. The asterisk indicate the start of the transmembrane domain.

Figure 2B shows IP-MS analysis of secreted Aβ peptides from primary cultured cortical neurons derived from wide-type (+/+), heterozygous (+/-), and homozygous BACE1 knockout (-/-) E16.5 embryos using the Ciphergen ProteinChip™ system. Peaks corresponding to mouse Aβ peptides, 17-40, 11-40, 11-42, 1-40 and 1-42 are marked by asterisk. The mass of each peptide is labeled within brackets.

Figure 2C shows a determination of Aβ1-40 and Aβ1-42 levels from conditioned media of $BACE1^{+/+}$ and $BACE1^{-/-}$ neuronal cultures following 4 days of infection with adenovirus expressing humanized APPswe by ELISA. The concentrations of Aβ peptides for each genotype are plotted (pg/ml) as mean +/- standard deviation (n=3).

Figure 2D shows conditioned media from $BACE1^{+/+}$ and $BACE1^{-/-}$ neuronal cell cultures radiolabeled with ^{35}S-methionine after 4 days of infection with recombinant adenovirus expressing humanized APPswe were immunoprecipitated with 4G8, an antisera specific for Aβ peptides.

Figure 2E shows a detergent lysates from $BACE1^{+/+}$ and $BACE1^{-/-}$ neuronal cell cultures radiolabeled with ^{35}S-methionine after 4 days of infection with recombinant adenovirus expressing humanized APPswe. The cells were immunoprecipitated with CT15, an antisera recognizing APP C terminus. BACE1 deficient neurons failed to generate APP β-CTF.

Figures 3A-3D show a gel from neuronal cultures infected with adenovirus. Following 4 days of infection with adenovirus expressing humanized APPswe, $BACE1^{+/+}$(Figure 3A; lanes 1-4) and $BACE1^{-/-}$ (lanes 5-8) neuronal cultures were pulse-labeled for 45 minutes (lanes 1 and 5) with ^{35}S-methionine, then chased in the presence of cold L-methionine for 1 hr (lanes 2 and 6), 2 hr (lanes 3 and 7), and 4 hr (lanes 4 and 8). Full-length APP and CTFs of APP were immunoprecipitated with CT15. Aβ and p3 peptides (Figure 3B), soluble APP derivatives (APP^s)(Figure 3C), or α-secretase-generated APP^s ($APP^{s\alpha}$; Figure 3D), were immunoprecipitated with

4G8, 22C11, or 6E10 antisera, respectively, from conditioned media of the corresponding neuronal cultures (Figure 3A).

Figure 3E is a quantitative analysis of $APP^{s\alpha}$ release. Experiments were performed in duplicate on different days. The $APP^{s\alpha}$ and APP^{s} signals at each point of the pulse-chase experiments were quantified by phospho-imaging.

Figure 4 shows that cultured astrocytes have greater BACE2 and α-secretase activity as compared to neurons. The signal intensities of Aβ1-15, Aβ1-6, Aβ1-19 , and Aβ1-20 in glia and astrocytes are shown normalized with respect to Aβ1-40 (see Example 7).

DETAILED DESCRIPTION OF THE INVENTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder causing dementia in the elderly that is characterized, in part, by the deposition of Aβ-amyloid and by neurofibrillary tangles in a variety of brain region, particularly the hippocampus and cerebral cortex. Endoproteolytic cleavages of APP by α-secretase and β-secretase activities result in the generation of toxic Aβ peptides. Two homologous β-secretases, termed BACE1 and BACE2, have been cloned and shown to be transmembrane aspartyl proteases that cleave APP at the +1 Aβ site. Initial studies indicated that *BACE1* and *BACE2* mRNA are expressed ubiquitously, although BACE2 is expressed at lower levels in brain.

The present invention is based upon the discovery that BACE1-knockout transgenic organisms lacking normal expression of BACE1 have reduced accumulation of APP peptide fragments. The transgenic organisms have led to the discovery that BACE1 is the β-secretase responsible for the Aβ+11 peptide fragment of APP. Accordingly, the invention provides diagnostic methods and compositions useful for detecting AD as well as other BACE1-associated and APP-associated disorders. Based on the discovery of the role of BACE1 in AD, the invention also provides screening assays useful for identifying drugs that can inhibit or prevent Aβ11-40/42 production and, therefore, may be effective for AD treatment.

The term "isolated" or "substantially purified" means altered "by the hand of man" from its natural state; *i.e.*, if a material occurs in nature, reference to the material as being "isolated" means that it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated," as the term is employed herein. Thus, anti-BACE1 an antiserum, which contains anti-BACE1 antibodies and is obtained from an immunized animal such as a rabbit, is removed from its natural state, i.e., the rabbit's circulatory system, and, therefore, is an example of a substantially purified material.

As part of or following isolation, a polynucleotide can be joined to other polynucleotides, such as heterologous DNA molecules, for mutagenesis studies, to form fusion proteins, and for propagation or expression of the polynucleotide in a host. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells in culture or in whole organisms. Such polynucleotides, when introduced into host cells in culture or in whole organisms, still are considered isolated as the term is used herein because they are not in their naturally occurring form or environment. Similarly, the polynucleotides and polypeptides can be present in a composition such as a medium formulation (solutions for introduction of polynucleotides or polypeptides, for example, into cells or compositions or solutions for chemical or enzymatic reactions).

Polynucleotide or nucleic acid sequence refers to a polymeric form of nucleotides. In some instances a polynucleotide refers to a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, the term "isolated" includes, for example, a recombinant DNA molecule, which can be incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which can exist as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The

nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. In addition, the polynucleotide sequence involved in producing a polypeptide chain can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons) depending upon the source of the polynucleotide sequence.

The term "polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. Thus, the term "polynucleotides" includes, for example, single stranded and double stranded DNA, DNA that contains both single stranded and double stranded regions, single stranded and double stranded RNA, and RNA that contains both single stranded and double stranded regions, hybrid molecules comprising DNA and RNA that may be single stranded or, more typically, double stranded or a mixture of single stranded and double stranded regions. In addition, a polynucleotide can contain triple stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions can be from the same molecule or from different molecules. The regions can include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple helical region often is an oligonucleotide.

A polynucleotide or nucleic acid sequence can contain one or more modified bases. Thus, DNA or RNA molecules with backbones modified for stability or for other reasons are considered "polynucleotides" as the term is used herein. Moreover, DNA or RNA molecules comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are considered polynucleotides as the term is used herein.

Polynucleotides can be created that encode a fusion protein and can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is "operatively linked" to another coding sequence when RNA polymerase will transcribe the two coding

sequences into a single mRNA, which can be translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence operatively linked to a coding sequence is positioned such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences.

As used herein, the term "expression control sequence" or "control sequences" refers to a nucleotide sequence that regulates the expression of a polynucleotide to which it is operatively linked. Expression control sequences are operatively linked to a polynucleotide when the expression control sequences control and regulate the transcription and, as appropriate, translation of the polynucleotide. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) at the beginning of a protein-encoding sequence, splice signals for introns, which allow for maintenance of the correct reading frame of a polynucleotide containing introns and, therefore, translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, elements whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

Expression control sequences include promoters, enhancers, silencers, and the like. The term "promoter" is used to refer to a minimal sequence sufficient to direct transcription. Also included are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements generally are positioned the 5' to the coding sequence, though enhancer elements also can have promoter activity, in which case the element can be positioned 3' to a coding region of a polynucleotide. Constitutive and inducible promoters are useful for purposes of the present invention (see e.g., Bitter et al., Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as *pL* of bacteriophage, *plac*, *ptrp*, *ptac* (*ptrp-lac* hybrid promoter) and the like may be used. When cloning in mammalian cell

systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) can be used. Promoters produced by recombinant DNA or synthetic techniques also can be used to provide for transcription of the nucleic acid sequences of the invention.

A polynucleotide of the invention including, for example, a polynucleotide encoding a fusion protein, can be inserted into a recombinant expression vector. A recombinant expression vector generally is a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a desired nucleotide sequences. For example, a recombinant expression vector of the invention includes a polynucleotide sequence encoding a polypeptide having BACE1 activity or a fragment thereof, or encoding an APP fusion product or fragment thereof. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al., Gene 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J. Biol. Chem. 263:3521, 1988), baculovirus-derived vectors for expression in insect cells, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV.

A polynucleotide of the invention can also can be operatively linked to a localization sequence such as a nuclear localization signal, signal peptide, or the like, which can direct the linked molecule to particular cellular sites by fusion to appropriate organellar targeting signals or localized host proteins. For example, a polynucleotide encoding a localization sequence, or signal sequence, can be used as a repressor and thus can be operatively linked at the 5' terminus of a polynucleotide encoding a polypeptide of the invention such that the localization or signal peptide is located at the amino terminal end of a resulting polynucleotide/polypeptide. The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor,

NY, 1989, and Current Protocols in Molecular Biology, M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement)). These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination.

In yeast, a number of vectors containing constitutive or inducible promoters can be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel, et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Grant, et al., "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp.516-544, 1987; Glover, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter, "Heterologous Gene Expression in Yeast," Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684, 1987; and The Molecular Biology of the Yeast Saccharomyces, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II, 1982. A constitutive yeast promoter such as *ADH* or *LEU2* or an inducible promoter such as *GAL* can be used ("Cloning in Yeast," Ch. 3, R. Rothstein In: DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors can be used which promote integration of foreign DNA sequences into the yeast chromosome.

An alternative expression system which could be used to express a BACE (e.g., BACE1) polypeptide of the invention is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign or mutated polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The sequence encoding a protein of the invention may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an *AcNPV* promoter (for example the polyhedrin promoter). Successful insertion of the sequences coding for a protein of the invention will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed (see Smith et al., J. Viol. 46:584, 1983; Smith, U.S. Pat. No. 4,215,051).

A polynucleotide of the invention, which can be contained in a vector, can be used to transform a host cell. By transform or transformation is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. A transformed cell or host cell generally refers to a cell (*e.g.*, prokaryotic or eukaryotic) into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding an APP or BACE polypeptide or a fragment thereof.

Transformation of a host cell with recombinant DNA can be carried out by conventional techniques as are well known to those skilled in the art. Where the host is a prokaryotic cell such as *E. coli*, competent cells that are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host cell is a eukaryotic cell, methods of transfection or transformation with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors, as well as others known in the art, may be used. Eukaryotic cells can also be cotransfected with DNA sequences encoding a BACE1 polypeptide and a second foreign DNA molecule encoding APP, or a selectable marker, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). Typically, a eukaryotic host will be utilized as the host cell. The eukaryotic cell may be a yeast cell (*e.g.*, *Saccharomyces cerevisiae*), an insect cell (*e.g.*, *Drosophila sp.*) or may be a mammalian cell, including a human cell.

Eukaryotic systems, including mammalian expression systems, allow for post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells that possess the cellular machinery for processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used. Such host cell lines may include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression can be engineered. For example, when using adenovirus expression vectors, a polynucleotide encoding a BACE (e.g., BACE1) polypeptide may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a BACE polypeptide or a fragment thereof in infected hosts (see Logan and Shenk, Proc. Natl. Acad. Sci. USA, 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter can be used (see Mackett et al., Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett et al., J. Virol. 49:857-864, 1984; Panicali et al., Proc. Natl. Acad. Sci. USA 79:4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of a BACE gene in host cells (Cone and Mulligan, Proc. Natl. Acad. Sci. USA, 81:6349-6353, 1984). High level expression can also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

For long term, high yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the cDNA encoding an APP, APP fragment or BACE polypeptide controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant vector confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems can be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al., Cell, 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy et al., Cell, 22:817, 1980) genes can be employed in *tk-*, *hgprt-* or *aprt-* cells respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., Proc. Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare et al., Proc. Natl. Acad. Sci. USA 8:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan and Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J. Mol. Biol. 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre *et al.*, Gene 30:147, 1984) genes. Additional selectable markers included *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, Proc. Natl. Acad. Sci. USA 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed., 1987).

The term "primer" as used herein refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated or possible. Synthesis of a primer

extension product that is complementary to a nucleic acid strand is initiated in the presence of nucleoside triphosphates and a polymerase in an appropriate buffer at a suitable temperature. For example, if a polynucleotide sequence is inferred from a polypeptide sequence, a primer generated to synthesize the polynucleotide encoding the polypeptide sequence can be a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One or more of the primers in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved' region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences.

The term "polypeptide" or "protein" refers to a polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being typical. Examples of polypeptides useful in the methods and compositions of the invention include APP (see, for example, Cheler, J. Neurochem. 65(4):1431, 1995, which is incorporated herein by reference), fragments of APP, including Aβ1-40, Aβ1-42, Aβ11-40, and Aβ11-42; and BACE1 (see Vassar et al. Science 286:735, 1999, which is incorporated herein by reference). Accordingly, the polypeptides of the invention are intended to encompass isolated naturally occurring proteins, as well as those generated recombinantly or synthesized using chemical or biological methods.

Polypeptide fragments are also encompassed by the invention. Such fragments can have the same or substantially the same amino acid sequence as a portion of the naturally occurring protein. A polypeptide or peptide having substantially the same sequence means that an amino acid sequence is largely, but not entirely, the same, but retains a functional activity of the sequence to which it is related. In general polypeptides of the invention include peptides, or full length protein, that contains substitutions, deletions, or insertions into the protein backbone, that would still have about 70%, generally about 80%, and particularly about 90% sequence identity to the original (reference) protein over the corresponding portion. A yet greater degree of departure from

homology can be allowed if conservative amino acid substitutions are considered as sharing identity with the substituted amino acid residue.

A polypeptide can be substantially related, but for a conservative variation, such polypeptides being encompassed within the present invention. A conservative variation denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine. A conservative variation also can be due to the use of a substituted amino acid in place of an unsubstituted amino acid, provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, or configuration concerns, one skilled in the art will recognize the need to introduce a modification by deletion, replacement, or addition of one or more amino acid residues. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, cytosine for specific disulfide bridge formation. The modified peptides can be chemically synthesized, or the isolated gene can be site-directed mutagenized, or a synthetic gene can be synthesized and expressed in bacteria, yeast, baculovirus, tissue culture and so on.

The present invention also provides a substantially purified antibody that specifically binds a BACE1 polypeptide or an epitopic determinant thereof, and antigen binding fragments of such antibodies. The antibody can be in the form of an antiserum, which is isolated from an immunized animal, can be in the form of substantially purified polyclonal antibodies, which have been isolated from an antiserum containing anti-BACE1 antibodies, or a can be in the form of a monoclonal antibody. The epitopic determinant of BACE1, to which an antibody of the invention specifically binds, can be any portion of BACE1, including a contiguous amino acid sequence or a two or more sequences of the BACE1 polypeptide that are in proximity in the three dimensional structure of BACE1, provided the epitope is unique to BACE1 such that the antibodies of the invention do not substantially cross-react with an unrelated polypeptide. For example, the epitopic determinant can include a structure formed by a peptide containing amino acid residues 46 to 164 of BACE1 (see Example 2).

The term "specifically binds," when used herein in reference to an antibody, means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about 1×10^{-6}, generally at least about 1×10^{-7}, usually at least about 1×10^{-8}, and particularly at least about 1×10^{-9} or 1×10^{-10} or less. As such, Fab, $F(ab')_2$, Fd and Fv fragments of an antibody that retain specific binding activity for BACE1 or a BACE1 epitope are included within the definition of an antibody. The term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric antibodies, bifunctional antibodies and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., Science 246:1275-1281, 1989). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246, 1993; Ward et al., Nature 341:544-546, 1989; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., Protein Engineering: A

practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995)).

Antibodies of the invention can be prepared as disclosed herein or using other methods as are well known and routine in the art. Where a peptide portion of BACE1 used as an immunogen is non-immunogenic, it can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art (see, for example, by Harlow and Lane, *supra*, 1988). Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in Immunochemical Protocols (Manson, ed., Humana Press 1992), pages 1-5; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in Curr. Protocols Immunol. (1992), section 2.4.1).

Monoclonal antibodies also can be obtained using methods that are well known and routine in the art (Kohler and Milstein, Nature 256:495, 1975; Coligan et al., *supra*, 1992, sections 2.5.1-2.6.7; Harlow and Lane, *supra*, 1988). For example, spleen cells from a mouse immunized with BACE1, or an epitopic fragment thereof, can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using, for example, labeled BACE1 to identify clones that secrete monoclonal antibodies having the appropriate specificity, and hybridomas expressing antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies. Polyclonal antibodies similarly can be isolated, for example, from serum of an immunized animal. Such antibodies, in addition to being useful for performing a method of the invention, also are useful, for example, for preparing standardized kits. A recombinant phage that expresses, for example, a single chain antibody also provides an antibody that can used for preparing standardized kits.

Monoclonal antibodies, for example, can be isolated and purified from hybridoma cultures by a variety of well established techniques, including, for example, affinity chromatography with Protein-A SEPHAROSE gel, size exclusion chromatography, and ion exchange chromatography (Barnes et al., in Meth. Mol. Biol. 10:79-104 (Humana Press 1992); Coligan et al., *supra*, 1992, see sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3). Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known. For example, multiplication *in vitro* can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* can be carried out by injecting cell clones into mammals histocompatible with the parent cells, for example, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals can be primed with a hydrocarbon, for example, an oil such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

An antigen binding fragment of an antibody that specifically binds BACE1 also is considered encompassed within the antibodies of the present invention. An antigen binding fragment of an antibody can be used in a method of the invention, as can an antibody derived from such an antibody, for example, a single chain antibody. An antigen binding fragment of an antibody can be prepared by proteolytic hydrolysis of a particular antibody, or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent,

and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, for example, Goldenberg, U.S. Patent No. 4,036,945 and U.S. Pat. No. 4,331,647; Nisonhoff et al., Arch. Biochem. Biophys. 89:230, 1960; Porter, Biochem. J. 73:119, 1959; Edelman et al., Meth. Enzymol., 1:422 (Academic Press 1967); Coligan et al., *supra*, 1992, see sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light/heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques can also be used, provided the fragments specifically bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of variable heavy (V_H) chains and variable light (V_L) chains, which can be a noncovalent association (Inbar et al., Proc. Natl. Acad. Sci., *USA* 69:2659, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (Sandhu, Crit. Rev. Biotechnol. 12:437, 1992). Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are well known (see, for example, by Whitlow et al., "Methods: A Companion to Methods in Enzymology" 2:97, 1991; Bird et al., Science 242:423-426, 1988; Ladner et al., U.S. Pat. No. 4,946,778; Pack et al., BioTechnology 11:1271-1277, 1993; Sandhu, *supra*, 1992).

Another example of an antigen binding fragment of an antibody is a peptide coding for a single complementarity determining region (CDR). CDR peptides can be obtained by constructing polynucleotides encoding the CDR of an antibody of

interest. Such polynucleotides can be prepared, for example, using the polymerase chain reaction to synthesize a variable region encoded by RNA obtained from antibody-producing cells (see, for example, Larrick et al., Methods: A Companion to Methods in Enzymology 2:106, 1991, which is incorporated herein by reference).

Humanized monoclonal antibodies also can be used in a method or kit of the invention if desired. Humanized monoclonal antibodies can be produced, for example, by transferring nucleotide sequences encoding mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. Methods for cloning murine immunoglobulin variable domains are known (see, for example, Orlandi et al., Proc. Natl. Acad. Sci., *USA* 86:3833, 1989), and for producing humanized monoclonal antibodies are well known (see, for example, Jones et al., Nature 321:522, 1986; Riechmann et al., Nature 332:323, 1988; Verhoeyen et al., Science 239:1534, 1988; Carter et al., Proc. Natl. Acad. Sci., *USA* 89:4285, 1992; Singer et al., J. Immunol. 150:2844, 1993; Sandhu, *supra*, 1992).

Antibodies useful in a method of the invention also can be derived from human antibody fragments, which can be isolated, for example, from a combinatorial immunoglobulin library (see, for example, Barbas et al., Methods: A Companion to Methods in Immunology 2:119, 1991; Winter et al., Ann. Rev. Immunol. 12:433, 1994). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library are commercially available (Stratagene; La Jolla CA). In addition, the antibody can be derived from a human monoclonal antibody, which can be obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge (see, for example, by Green et al., Nature Genet. 7:13, 1994; Lonberg et al., Nature 368:856, 1994; and Taylor et al., Int. Immunol. 6:579, 1994; see, also, Abgenix, Inc.; Fremont CA).

The present invention further provides a method of detecting a BACE1 polypeptide in a sample. Such a method can be performed, for example, by contacting the sample

with an antibody or antigen binding fragment thereof of the invention under conditions that allow specific binding of the antibody to BACE1 or an epitopic determinant thereof, and detecting specific binding of the antibody to a component of the sample. An immunoassay of the invention can be adapted to any format as desired, including, for example, an ELISA, RIA, and the like, or an immunohistochemical method. The sample can be any sample in which it is desired to know whether BACE1 is present, including, for example, cells or a cell extract, or a tissue sample, which can be obtained from a subject using biopsy methods or other methods for collecting a biological sample as are known in the art. In one embodiment, the sample is a brain tissue sample, for example, from a subject that has, or is suspected of having, a disorder associated with an accumulation of amyloid plaques, and the method is performed in an immunohistochemical format. As such, a method of the invention can be useful for detecting, for example, the presence or absence of BACE1 in a region of the brain containing amyloid plaques, and, therefore, can be diagnostic of an amyloidosis such as Alzheimer's disease.

For purposes of a detecting method of the invention, the anti-BACE1 antibody can be detectably labeled using any reagent as disclosed herein as useful for labeling a polypeptide or, where appropriate, a polynucleotide, as well as any other label conveniently and routinely used in the art. Alternatively, a separate reagent that specifically binds to an anti-BACE1 antibody can be detectably labeled, and can be used to detect specific binding of the anti-BACE1 antibody to a component of the sample. Such a reagent can be a second antibody, which can specifically bind an immunoglobulin class of which the anti-BACE1 antibody is a member, for example, an IgG, IgM, IgA or the like, or can be a reagent such as Protein A.

If desired, an antibody or antigen binding fragment of the invention can be immobilized to a solid support. The solid support can be any material that is substantially insoluble under the conditions to which a method of the invention will be performed, i.e., under conditions in which immunoassays generally are performed. In addition, a material is selected as a solid support based on its stability to conditions under which an antibody is to be immobilized to the support. Thus, a solid support

can be composed of glass, silicon, gelatin, agarose, a metal, or a synthetic material such as a plastic or other polymer, for example, polystyrene, polydextran, polypropylene, polyvinyl chloride, polyvinylidene fluoride, polyacrylamide, and the like.

Where the solid support has a hydrophobic surface, an antibody can be immobilized to the support simply by contacting the antibody and the surface such that the antibody is immobilized through a hydrophobic interaction with the surface, as is typical for solid phase immunoassays. A solid support also can be modified to contain reactive groups that facilitate binding of an antibody to the support, thereby immobilizing the antibody. Alternatively, or in addition, the antibody can be modified to facilitate immobilization to the support, for example, by modifying the antibody to contain a member of a specific binding pair, wherein the second member of the binding pair is a component of the support. For example, the antibody can be covalently bound, for example, to a magnetic iron oxide bead, which can be modified to contain reactive amine groups or carboxyl groups (Pierce Chemical Co.) or a member of a specific binding pair such as streptavidin (Dynal Biotech), thereby immobilizing the antibody and also providing a convenient means to isolate the antibody, as well as any BACE1 polypeptide specifically bound thereto by contacting the mixture with a magnet (see, for example, Bodinier et al., Nat. Med. 6:707-710, 2000). Accordingly, a method of detecting BACE1 in a sample can further include a step of isolating BACE1 that is specifically bound by the antibody.

Prior to the present disclosure, the role of BACE1 in the processing of APP and fragments thereof was not understood. Accordingly, the present invention provides an understanding of the role of BACE1 in the processing of APP and in AD. Thus, in one embodiment, the invention provides a method for modulating (*e.g.*, inhibiting) the interaction of a BACE1 polypeptide with its substrate APP (either *in vitro* or *in vivo*) by administering to a cell or to a subject an effective amount of a composition that contains a BACE1 polypeptide, or a biologically functional fragment thereof, or an agent such as an antibody, ribozyme, antisense molecule, or double stranded interfering RNA

molecules that interacts with or inhibits expression or the activity of a BACE1 polypeptide.

As used herein, an "effective amount" of a composition containing a BACE1 polypeptide or a BACE1 polypeptide-modulating agent is an amount that can modulate the normal enzymatic activity or interaction of a BACE1 substrate with a BACE1 polypeptide or protein in a subject or cell. A "normal" amount of BACE1 activity can be determined using methods as disclosed herein and statistical analyses as are well known in the art.

The present invention also provides a method for modulating expression of a BACE1 polypeptide, as well as methods for screening for agents that modulate BACE1 polypeptide gene expression. According to such a method, a cell or subject is contacted with an agent suspected or known to have BACE1 polypeptide expression modulating activity. The change in BACE1 polypeptide gene expression is then measured as compared to a control or standard sample. The control or standard sample can be the baseline expression of the cell or subject prior to contact with the agent. An agent that modulates BACE1 polypeptide gene expression can be a polynucleotide, for example, an antisense molecule, a triplex agent, a ribozyme, or a double stranded interfering RNA that interacts with a BACE1. For example, an antisense molecule can be directed to the structural gene region or to the promoter region of a BACE1 gene. The agent also can be a peptide, peptidomimetic, antibody, or small organic molecule, and can act as an agonist or antagonist of BACE1 activity.

Double stranded interfering RNA molecules are especially useful to inhibit expression of a target gene. For example, double stranded RNA molecules can be injected into a target cell or organism to inhibit expression of a gene and the resultant gene products activity. It has been found that such double stranded RNA molecules are more effective at inhibiting expression than either RNA strand alone. (Fire *et al.*, Nature, 1998, 19:391(6669):806-11).

When a disorder is associated with abnormal expression of a BACE1 polypeptide (*e.g.*, overexpression, or expression of a mutated form of the protein) or as a result of expression of a substrate for the BACE1 polypeptide, a therapeutic approach which directly interferes with the translation of a BACE1 polypeptide is possible. Alternatively, similar methodology may be used to study gene activity. For example, antisense nucleic acid, double stranded interfering RNA or ribozymes could be used to bind to a BACE1 polypeptide mRNA sequence or to cleave it.

Antisense RNA or DNA molecules bind specifically with mRNA expressed from a targeted gene, interrupting the expression of the gene product (protein). The antisense binds to the messenger RNA forming a double stranded molecule which cannot be translated by the cell. Antisense oligonucleotides of about 15-25 nucleotides are preferred since they are easily synthesized and have an inhibitory effect just like antisense RNA molecules. In addition, chemically reactive groups, such as iron-linked ethylenediaminetetraacetic acid (EDTA-Fe) can be attached to an antisense oligonucleotide, causing cleavage of the RNA at the site of hybridization. Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target BACE1 polypeptide producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, Anal. Biochem., 172:289, 1988).

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher et al., Antisense Res. and Dev., 1:227, 1991; Helene, Anticancer Drug Design, 6:569, 1991).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes, tetrahymena-type (Hasselhoff, Nature, 334:585, 1988) and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species and 18-base recognition sequences are preferable to shorter recognition sequences. These and other uses of antisense and ribozymes methods to inhibit the *in vivo* translation of genes are known in the art (*e.g.*, De Mesmaeker et al., Curr. Opin. Struct. Biol., 5:343, 1995; Gewirtz et al., Proc. Natl. Acad. Sci. U.S.A., 93:3161, 1996b; Stein, Chem. and Biol. 3:319, 1996).

Delivery of antisense, triplex agents, ribozymes, competitive inhibitors, double stranded interfering RNA and the like can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system or by injection. Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a polynucleotide sequence of interest into the viral

vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing, for example, an antisense polynucleotide.

Another targeted delivery system for polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley et al., Trends Biochem. Sci., 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al., BioTechniques, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of

divalent cations. Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticuloendothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. In general, the compounds bound to the surface of the targeted delivery system will be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound, such as a receptor.

The agents useful in the method of the invention can be administered, for *in vivo* application, parenterally by injection or by gradual perfusion over time. Administration may be intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or

transdermally. For *in vitro* studies the agents may be added or dissolved in an appropriate biologically acceptable buffer and added to a cell or tissue.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents and inert gases and the like.

It is envisioned that the invention can be used to treat pathologies associated with neurodegenerative diseases and associated disorders, Aβ11-40/42 accumulation diseases such as Alzheimer's disease. Therefore, the present invention encompasses methods for ameliorating a disorder associated with neurodegenerative disorders, including treating a subject having the disorder, at the site of the disorder, with an agent which modulates a BACE1 expression or activity or its interaction with its substrate (*e.g.*, APP). Generally, the terms "treating", "treatment" and the like are used herein to mean affecting a subject, tissue or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for an infection or disease and/or adverse effect attributable to the infection or disease. "Treating" as used herein encompasses any treatment of, or prevention of a disease in an invertebrate, a vertebrate, a mammal, particularly a human, and includes: (a) preventing the disorder from occurring in a subject that may be predisposed to the disorder, but has not yet been diagnosed as having it; (b) inhibiting the disorder, *i.e.*, arresting its development; or (c) relieving or ameliorating the disorder, *i.e.*, cause regression of the disorder. By "Aβ11-40/42 accumulation disease" is meant a disease that is characterized as having an increase in Aβ11-40 and Aβ11-42 peptides over normal

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levels. Such accumulations in APP fragments lead to degenerative diseases that include, for example, Alzheimer's disease.

The present invention provides various compositions that can be administered to an individual and are useful for ameliorating symptoms attributable to a BACE1 or APP processing associated disorder. A composition according to one embodiment of the invention is prepared by formulating an anti-BACE1 antibody, a polypeptide or peptide derivative of a BACE1 polypeptide, a BACE1 polypeptide mimetic, a drug, chemical or combination of chemicals, a BACE1 polypeptide-modulating agent, or a combination thereof into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other physiologically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like (see, for example, Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American Pharmaceutical Association (1975), each of which is incorporated herein by reference). The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art (see Goodman and Gilman, The Pharmacological Basis for Therapeutics 7th ed.).

A composition of the invention generally, but not necessarily, is prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the subject, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by

single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The compositions according to the invention can be administered locally or systemically in a therapeutically effective dose. Amounts effective for this use will depend, in part, on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, for example, in Langer, Science, 249:1527, (1990); Gilman et al. (eds.) (1990). Administration of a composition of the invention can be accomplished by any means known to the skilled artisan, and preferably is administered to a vertebrate organism, particularly a mammal, including a human.

An anti-BACE1 antibody can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. Physiologically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners and elixirs containing inert diluents commonly used in the art, such as purified water.

In another embodiment, the invention provides a method for identifying an agent which interacts with or modulates expression or activity of a BACE1 polypeptide including

incubating components comprising an agent and a BACE1 polypeptide, or a recombinant cell expressing a BACE1 polypeptide, under conditions sufficient to allow the agent to interact and determining the effect of the agent on the expression or activity of the gene or polypeptide, respectively. The effect can be any means by which gene expression or protein activity is modulated, and includes measuring the interaction of the agent with the BACE1 protein by physical means including, for example, fluorescence detection of the binding of a the protein to a substrate or binding agent. Such agents can include, for example, polypeptides, peptidomimetics, chemical compounds, small molecules and biologic agents as described below.

Incubating includes conditions which allow contact between the test agent and a BACE1 polypeptide, a cell expressing a BACE1 polypeptide or nucleic acid encoding a BACE1 polypeptide. Contacting includes in solution and in solid phase. The test agent may optionally be a combinatorial library for screening a plurality of agents. Agents identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki et al., BioTechnology, 3:1008-1012, 1985), oligonucleotide ligation assays (OLAs; Landegren et al., Science, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren et al., Science, 242:229-237, 1988). Thus, the methods of the invention includes combinatorial chemistry methods for identifying chemical agents that bind to or affect BACE1 polypeptide expression or activity.

Areas of investigation are the development of therapeutic treatments. The screening identifies agents that provide modulation of BACE1 polypeptide function in targeted organisms. Of particular interest are screening assays for agents that have a low toxicity or a reduced number of side effects for humans. In particular, since the invention provides for the first time that BACE1 activity is species specific and results in the formation of an Aβ11-40/42 product, detection of the effect of an agent on product formation can be easily assayed and thus the identification of potential therapeutics is provided by the present invention.

The term "agent" as used herein refers to any molecule that can altering or mimic the physiological function or expression of a BACE1 polypeptide. Thus, an agent can be a peptide or polypeptide, a polynucleotide, a polysaccharide, a peptidomimetic, a small organic molecule, or a combination thereof, for example, a nucleoprotein or lipoprotein. Generally, a plurality of assay mixtures are run in parallel with different agents or different concentrations of an agent to obtain a differential response to the various concentrations. Typically, a negative control, *i.e.,* no agent or an amount that produces a result below the level of detection is included and, where available, one or more positive controls is included.

In a further embodiment, the invention provides a method of detecting a BACE1 or APP fragments (*e.g.*, Aβ11-40/42), a BACE1 or APP (*e.g.*, Aβ11-40/42) polypeptide or a BACE1 polynucleotide or diagnosing a BACE1 or APP fragments (*e.g.*, Aβ11-40/42)-related disorder (*e.g.*, AD) in a subject including contacting a sample (*e.g.*, blood, serum, cerebrospinal fluid or a cellular sample, or tissue sample) suspected of containing a BACE1 or APP (*e.g.*, Aβ11-40/42) polypeptide or a BACE1 polynucleotide with a reagent which binds to the polypeptide or polynucleotide (herein after sample). The sample can be or contain a nucleic acid, such as DNA or RNA, or a protein. When the sample contains a nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the sample contains protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Additional labels suitable for binding to an antibody or nucleic acid probe are known in the art or can be ascertained using routine experimentation. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. In addition, the antibodies, polypeptides and polynucleotide sequences of the invention can be used to diagnosis a BACE1 or APP (*e.g.*, Aβ11-40/42)-related disorder.

An antiserum, polyclonal antibody or monoclonal antibody of the invention, which specifically binds a BACE1 or APP (*e.g.*, Aβ11-40/42) polypeptide is useful for the *in vivo* and *in vitro* detection of antigen. The detectably labeled monoclonal antibody is given in a dose, which is diagnostically effective. A diagnostically effective amount is an amount of a detectably labeled monoclonal antibody that is sufficient to enable detection of a BACE1 or APP fragments (*e.g.*, Aβ11-40/42) or a BACE1 or APP (*e.g.*, Aβ11-40/42) polypeptide antigen for which the monoclonal antibodies are specific.

The concentration of a detectably labeled monoclonal antibody administered to a subject should be sufficient such that the binding to those cells, body fluid, or tissue having a BACE1 or APP (*e.g.*, Aβ11-40/42) polypeptide that is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay, which is detectable for a given type of instrument. Another factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope is long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 kev range, which can be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements, which are particularly useful in such techniques, include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

In another embodiment, nucleic acid probes can be used to identify a BACE1 polynucleotide from a sample obtained from a subject. Examples of specimens from which nucleic acid sequence encoding a BACE1 polypeptide can be derived include insect, human, primate, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine, opine and bovine species. Such probes also can be used to identify a polynucleotide encoding aBACE1 polypeptide from a specimen obtained from a subject. Examples of specimens from which nucleic acid sequence encoding a BACE1 polypeptide can be derived include human, primate, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine and bovine species.

Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double stranded DNA. For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. By using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace et al., Nucl. Acid Res. 9:879, 1981).

In an embodiment of the invention, purified nucleic acid fragments containing intervening sequences or oligonucleotide sequences of 10-50 base pairs are radioactively labeled. The labeled preparations are used to probe nucleic acids from a specimen by the Southern hybridization technique. Nucleotide fragments from a specimen, before or after amplification, are separated into fragments of different molecular masses by gel electrophoresis and transferred to filters that bind nucleic acid. After exposure to the labeled probe, which will hybridize to nucleotide fragments containing target nucleic acid sequences, binding of the radioactive probe to target nucleic acid fragments is identified by autoradiography (see Genetic Engineering, 1, ed. Robert Williamson, Academic Press, (1981), 72-81). Alternatively, nucleic acid from the specimen can be bound directly to filters to which the radioactive probe selectively attaches by binding nucleic acids having the sequence of interest. Specific sequences and the degree of binding is quantitated by directly counting the radioactive emissions.

Where the target nucleic acid is not amplified, detection using an appropriate hybridization probe may be performed directly on the separated nucleic acid. In those instances where the target nucleic acid is amplified, detection with the appropriate hybridization probe would be performed after amplification.

For the most part, the probe will be detectably labeled with an atom or inorganic radical, most commonly using radionuclides, but also heavy metals can be used. Conveniently, a radioactive label may be employed. Radioactive labels include ^{32}P, ^{125}I, ^{3}H, ^{14}C, ^{111}In, ^{99}Tc, or the like. Any radioactive label may be employed which provides for an adequate signal and has sufficient half-life. Other labels include ligands, which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels routinely employed in immunoassays can readily be employed in the present assay. The choice of the label will be governed by the effect of the label on the rate of hybridization and binding of the probe to a nucleotide sequence. It will be necessary that the label provide sufficient sensitivity to detect the amount of a nucleotide sequence available for hybridization.

The manner in which the label is bound to the probe will vary depending upon the nature of the label. For a radioactive label, a wide variety of techniques can be employed. Commonly employed is nick translation with an a ^{32}P-dNTP or terminal phosphate hydrolysis with alkaline phosphatase followed by labeling with radioactive ^{32}P employing ^{32}P-NTP and T4 polynucleotide kinase. Alternatively, nucleotides can be synthesized where one or more of the elements present are replaced with a radioactive isotope, for example, the replacement of hydrogen-1 with tritium (H-3). If desired, complementary labeled strands can be used as probes to enhance the concentration of hybridized label.

Standard hybridization techniques for detecting a nucleic acid sequence are known in the art. The particular hybridization technique is not essential to the invention. Other hybridization techniques are described by Gall and Pardue, Proc. Natl. Acad. Sci. 63:378, 1969); and John et al., Nature, 223:582, 1969). As improvements are made in hybridization techniques they can readily be applied in the method of the invention.

The amount of labeled probe present in the hybridization solution will vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excess over stoichiometric concentrations of the probe will be employed to enhance the rate of binding of the probe to the fixed target nucleic acid.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means containing one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. One of the container means may comprise a probe which is or can be detectably labeled. Such probe may be a nucleic acid sequence specific for BACE1; or antibodies specific for BACE1, fragments thereof; or APP or fragments thereof. The kit also can include a container comprising a reporter-means, such as an enzymatic, fluorescent, or radionuclide label to identify the detectably labeled oligonucleotide probe or antibody. Where the kit utilizes nucleic acid hybridization to detect the target nucleic

acid, the kit may also have containers containing nucleotide(s) for amplification of the target nucleic acid sequence.

Various methods to make the transgenic non-human animals of the invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in U.S. Pat. No. 4,873,191.

In another method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Pat No. 5,162,215. If microinjection is to be used with avian species, however, a published procedure by Love et al., (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half hours after the laying of the previously laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

The non-human transgenic animals of the invention can be any vertebrate, including, for example, bovine, porcine, ovine and avian animals. The transgenic non-human animals of the invention are produced by introducing at least one transgene into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for microinjection. The use of zygotes as is target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

The term "transgenic" is used to describe an animal which includes exogenous genetic material within all of its cells. A transgenic animal can be produced by cross-breeding two chimeric animals which include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will be transgenic *i.e.*, animals which include the exogenous genetic material within all of their cells in both alleles, 50% of the resulting animals will include the exogenous genetic material within one allele and 25% will include no exogenous genetic material.

In the microinjection method useful in the practice of the subject invention, the transgene is digested and purified free from any vector DNA, *e.g.*, by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV) , Moloney leukemia virus (MLV), and herpes virus, as well as those from the genes encoding metallothionin, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase. Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can

also be employed. When the animals to be made transgenic are avian, preferred promoters include those for the chicken β-globin gene, chicken lysozyme gene, and avian leukosis virus. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, Proc. Natl. Acad. Sci USA 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan et al. (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner et al., Proc. Natl. Acad. Sci. USA 82: 6927-6931, 1985; Van der Putten et al., Proc. Natl. Acad. Sci USA 82: 6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, et al., EMBO J. 6: 383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al., Nature 298: 623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder may contain various retro viral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner et al., *supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al., Nature 292:154-156, 1981; Bradley et al., Nature 309:255-258, 1984; Gossler et al., Proc. Natl. Acad. Sci USA 83:9065-9069, 1986; and Robertson

et al., Nature 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES ells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal (see Jaenisch, Science 240:1468-1474, 1988).

The transgene can be any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism, i.e., is either stably integrated or as a stable extrachromosomal element, that develops from that cell. Such a transgene can be a gene that is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or can represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence that is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode BACE1 or a selectable marker flanked by regions of sequence having homology to BACE1, and include polynucleotides, which may be expressed in a transgenic non-human animal.

The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" or "knockout" is used herein to refer to the targeted disruption of a gene *in vivo* with substantially complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. Any transgenic technology as discloed herein or otherwise known in the art can be used to produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional (knocked out).

After an embryo has been microinjected, colonized with transfected embryonic stem cells or infected with a retrovirus containing the transgene (except for practice of the

subject invention in avian species which is addressed elsewhere herein) the embryo is implanted into the oviduct of a pseudopregnant female. The consequent progeny are tested for incorporation of the transgene by Southern blot analysis of blood or tissue samples using transgene specific probes. PCR is particularly useful in this regard. Positive progeny (G0) are crossbred to produce offspring (G1) which are analyzed for transgene expression by northern blot analysis of tissue samples.

As used herein, a "heterologous gene" or "heterologous polynucleotide sequence" is defined in relation to the transgenic non-human organism producing or containing such a gene product. A heterologous polypeptide is defined as a polypeptide having an amino acid sequence or an encoding DNA sequence corresponding to that of a heterologous gene not normally found in an organism. The term "heterologous" also is used in reference to two or more polynucleotides or polypeptides, wherein one of the molecules can be considered a reference molecule, and the second or other is compared thereto. As such, a first polynucleotide is considered heterologous to a second polynucleotide, for example, if the two polynucleotides are not normally associated with each other in a cell of an organism.

As used herein, the term "targeting construct" refers to a polynucleotide which comprises: (1) at least one homology region having a sequence that is substantially identical to or substantially complementary to a sequence present in a host cell endogenous gene locus, and (2) a targeting region which becomes integrated into an host cell endogenous gene locus by homologous recombination between a targeting construct homology region and the endogenous gene locus sequence. A transiently incorporated targeting construct is one that is incorporated into the endogenous gene locus and is eliminated from the host genome by selection. A targeting region may comprise a sequence that is substantially homologous to an endogenous gene sequence and/or may comprise a non-homologous sequence, such as a selectable marker (*e.g.*, *neo*, *tk*, *gpt*). The term "targeting construct" does not necessarily indicate that the polynucleotide comprises a gene which becomes integrated into the host genome, nor does it necessarily indicate that the polynucleotide comprises a

complete structural gene sequence. As used in the art, the term "targeting construct" is synonymous with the term "targeting transgene" as used herein.

The term "homology region" as used herein refers to a segment (*i.e.*, a portion) of a targeting construct having a sequence that substantially corresponds to, or is substantially complementary to, a predetermined endogenous gene sequence, which can include sequences flanking said gene. A homology region is generally at least about 100 nucleotides long, preferably at least about 250 to 500 nucleotides long, typically at least about 1000 nucleotides long or longer. Although there is no demonstrated theoretical minimum length for a homology region to mediate homologous recombination, it is believed that homologous recombination efficiency generally increases with the length of the homology region. Similarly, the recombination efficiency increases with the degree of sequence homology between a targeting construct homology region and the endogenous target sequence, with optimal recombination efficiency occurring when a homology region is isogenic with the endogenous target sequence. A homology region does not necessarily denote formation of a base-paired hybrid structure with an endogenous sequence. Endogenous gene sequences that substantially correspond to, or are substantially complementary to, a transgene homology region are referred to herein as "crossover target sequences" or "endogenous target sequences."

As used herein, the term "transcriptional unit" or "transcriptional complex" refers to a polynucleotide sequence that comprises a structural gene (exons), a cis-acting linked regulatory element (*e.g.*, a promoter or enhancer sequence) and other cis-acting sequences necessary for efficient transcription of the structural sequences, distal regulatory elements necessary for appropriate tissue-specific and developmental transcription of the structural sequences, and additional cis sequences important for efficient transcription and translation (*e.g.*, polyadenylation site, mRNA stability controlling sequences).

A correctly targeted construct integrates within or adjacent to an endogenous crossover target sequence, such as a portion of an endogenous BACE1 gene locus.

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For example, a targeting transgene encoding neo and which is flanked by homology regions having substantial identity with endogenous BACE1 gene sequences of the first exon of BACE1 is correctly targeted when the transgene portion is integrated into a chromosomal location so as to replace, for example, the first exon of the endogenous BACE1 gene. It is possible to generate cells having both a correctly targeted transgene(s) and an incorrectly targeted transgene(s). Cells and animals having a correctly targeted transgene(s) and/or an incorrectly targeted transgene(s) may be identified and resolved by PCR and/or Southern blot analysis of genomic DNA.

As used herein, the term "targeting region" refers to a portion of a targeting construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a homology region and an endogenous BACE1 gene sequence. Typically, a targeting region is flanked on each side by a homology region, such that a double crossover recombination between each of the homology regions and their corresponding endogenous BACE1 gene sequences results in replacement of the portion of the endogenous BACE1 gene locus by the targeting region; in such double crossover gene replacement targeting constructs the targeting region can be referred to as a "replacement region". However, some targeting constructs may employ only a single homology region.

As used herein, the term "replacement region" refers to a portion of a targeting construct flanked by homology regions. Upon double crossover homologous recombination between flanking homology regions and their corresponding endogenous BACE1 gene crossover target sequences, the replacement region is integrated into the host cell chromosome between the endogenous crossover target sequences. Replacement regions can be homologous (*e.g.*, have a sequence similar to the endogenous BACE1 gene sequence but having a point mutation or missense mutation), non-homologous (*e.g.*, a *neo* gene expression cassette), or a combination of homologous and non-homologous regions. The replacement region can convert the endogenous BACE1 allele into an mutant BACE1 allele comprising a point mutation

or missense mutation or disrupt the BACE1 allele by integrating a non-homologous transgene at the BACE1 allele.

The terms "functional disruption" or "functionally disrupted" as used herein means that a gene locus comprises at least one mutation or structural alteration such that the functionally disrupted gene is incapable of directing the efficient expression of functional gene product. For example, an endogenous BACE1 gene that has a *neo* gene cassette integrated into an exon of a BACE1 gene, is not capable of encoding a functional protein and is therefore a functionally disrupted BACE1 gene locus. In addition, a targeted mutation in an exon of an endogenous BACE1 gene may result in a mutated endogenous gene that can express a truncated BACE1 protein that is non-functional. Functional disruption can include the complete substitution of a heterologous BACE1 gene locus in place of an endogenous BACE1 locus, so that, for example, a targeting transgene that replaces the entire mouse BACE1 locus with a human BACE1 allele, which may be functional in the mouse, is said to have functionally disrupted the endogenous murine BACE1 locus by displacing it. Preferably, at least one exon which is incorporated into the mRNAs encoding most or all of the BACE1 isoforms are functionally disrupted. Deletion or interruption of essential transcriptional regulatory elements, polyadenylation signal(s), splicing site sequences will also yield a functionally disrupted gene.

Functional disruption of an endogenous BACE1 gene also can be produced by other methods (*e.g.*, antisense polynucleotide gene suppression). The term "structurally disrupted" refers to a targeted gene wherein at least one structural sequence (*e.g.*, an exon sequence) has been altered by homologous gene targeting (*e.g.*, by insertion, deletion, point mutation(s), and/or rearrangement). Typically, BACE1 alleles that are structurally disrupted are consequently functionally disrupted, however BACE1 alleles may also be functionally disrupted without concomitantly being structurally disrupted, *i.e.*, by targeted alteration of a non-exon sequence such as ablation of a promoter. An allele comprising a targeted alteration that interferes with the efficient expression of a functional gene product from the allele is referred to in the art as a "null allele" or "knockout allele".

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As used herein, "isoform", "BACE1", and "BACE1 isoform" refer to a polypeptide that is encoded by at least one exon and includes a sequence as set forth in GenBank Accession No. AF190725 (Vassar et al., Science 286:735, 1999; see, also GenBank Accession No. AAF04142, for BACE1 amino acid sequence). A BACE isoform can be encoded by any BACE allele (or exon thereof) that is associated with a form of Alzheimer's disease or that is not associated with an AD disease phenotype. In some embodiments, the endogenous non-human BACE1 alleles are functionally disrupted so that expression of endogenously encoded BACE1 is suppressed or eliminated. In one variation, an endogenous BACE1 allele is targeted for disruption by homologous recombination.

Gene targeting, which is a method of using homologous recombination to modify a mammalian genome, can be used to introduce changes into cultured cells. By targeting a gene of interest in embryonic stem (ES) cells, these changes can be introduced into the germ lines of laboratory animals to study the effects of the modifications on whole organisms, among other uses. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that has a segment homologous to a target locus and which also comprises an intended sequence modification (*e.g.*, insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted. A common scheme to disrupt gene function by gene targeting in ES cells is to construct a targeting construct which is designed to undergo a homologous recombination with its chromosomal counterpart in the ES cell genome. The targeting constructs are typically arranged so that they insert additional sequences, such as a selectable marker, into coding elements of the target gene, thereby functionally disrupting it. Targeting constructs usually are insertion-type or replacement-type constructs (Hasty et al., Mol. Cell. Biol. 11:4509, 1991).

The invention encompasses methods to produce non-human animals (*e.g.*, non-primate mammals) that have the endogenous BACE1 gene inactivated by gene targeting with a homologous recombination targeting construct. Typically, a non-

human BACE1 gene sequence is used as a basis for producing PCR primers that flank a region that will be used as a homology region in a targeting construct. The PCR primers are then used to amplify, by high fidelity PCR amplification (Mattila et al., Nucleic Acids Res. 19:4967, 1991; Eckert and Kunkel, PCR Methods and Applications 1:17, 1991; U.S. Pat. No. 4,683,202, which are incorporated herein by reference), a genomic sequence from a genomic clone library or from a preparation of genomic DNA, preferably from the strain of non-human animal that is to be targeted with the targeting construct. The amplified DNA is then used as a homology region and/or targeting region. Thus, homology regions for targeting a non-human BACE1 gene may be readily produced on the basis of nucleotide sequence information available in the art and/or by routine cloning (*e.g.*, GenBank Acc. No. AF190725). General principles regarding the construction of targeting constructs and selection methods are reviewed in Bradley et al., Bio/Technology 10:534, 1992, incorporated herein by reference). In addition, to the disruption of endogenous non-human BACE1 genes the transgenic organism may include one or more transgenes encoding for example APP comprising the Swedish mutation.

Targeting constructs can be transferred into pluripotent stem cells, such as murine embryonal stem cells, wherein the targeting constructs homologously recombine with a portion of an endogenous BACE1 gene locus and create mutation(s) (*i.e.*, insertions, deletions, rearrangements, sequence replacements, and/or point mutations) which prevent the functional expression of the endogenous BACE1 gene.

A preferred method of the invention is to delete, by targeted homologous recombination, essential structural elements of the endogenous BACE1 gene. For example, a targeting construct can homologously recombine with an endogenous BACE1 gene and delete a portion spanning substantially all of one or more of the exons to create an exon-depleted allele, typically by inserting a replacement region lacking the corresponding exon(s). Transgenic animals homozygous for the exon-depleted allele (*e.g.*, by breeding of heterozygotes to each other) produce cells which are essentially incapable of expressing a functional endogenous BACE1 polypeptide (preferably incapable of expressing any of the naturally-occurring isoforms).

Similarly, homologous gene targeting can be used, if desired, to functionally disrupt a BACE1 gene by deleting only a portion of an exon.

Targeting constructs can also be used to delete essential regulatory elements of an endogenous BACE1 gene, such as promoters, enhancers, splice sites, polyadenylation sites, and other regulatory sequences, including cis-acting sequences that occur upstream or downstream of the BACE1 structural gene but which participate in endogenous BACE1 gene expression. Deletion of regulatory elements is typically accomplished by inserting, by homologous double crossover recombination, a replacement region lacking the corresponding regulatory element(s).

Another method of the invention is to interrupt essential structural and/or regulatory elements of an endogenous BACE1 gene by targeted insertion of a polynucleotide sequence, and thereby functionally disrupt the endogenous BCE1 gene. For example, a targeting construct can homologously recombine with an endogenous BACE1 gene and insert a non-homologous sequence, such as a *neo* expression cassette, into a structural element (*e.g.*, an exon) and/or regulatory element (*e.g.*, enhancer, promoter, splice site, polyadenylation site) to yield a targeted BCE1 allele having an insertional interruption. The inserted sequence can range in size from about 1 nucleotide (*e.g.*, to produce a frame shift in an exon sequence) to several kilobases or more, as limited by efficiency of homologous gene targeting with targeting constructs having a long non-homologous replacement region. Targeting constructs of the invention can also be employed to replace a portion of an endogenous BACE1 gene with an exogenous sequence (*i.e.*, a portion of a targeting transgene); for example, an exon of a BACE1 gene may be replaced with a substantially identical portion that contains a nonsense or missense mutation.

In one embodiment, inactivation of an endogenous murine BACE1 locus is achieved by targeted disruption of the appropriate gene by homologous recombination in a mouse embryonic stem cell. For inactivation, any targeting construct that produces a genetic alteration in the target BACE1 gene locus resulting in the prevention of effective expression of a functional gene product of that locus may be employed. If

only regulatory elements are targeted, some low-level expression of the targeted gene may occur (*i.e.*, the targeted allele is "leaky"), however the level of expression may be sufficiently low that the leaky targeted allele is functionally disrupted.

In another embodiment of the invention, an endogenous BACE1 gene in a non-human host is functionally disrupted by homologous recombination with a targeting construct that does not comprise a functionally equivalent sequence. In this embodiment, a portion of the targeting construct integrates into an essential structural or regulatory element of the endogenous BACE1 gene locus, thereby functionally disrupting it to generate a null allele. Typically, null alleles are produced by integrating a non-homologous sequence encoding a selectable marker (*e.g.*, a *neo* gene expression cassette) into an essential structural and/or regulatory sequence of a BACE1 gene by homologous recombination of the targeting construct homology regions with endogenous BACE1 gene sequences, although other strategies may be employed.

Generally, a targeting construct is transferred by electroporation or microinjection into a totipotent embryonal stem (ES) cell line, such as the murine AB-1 or CCE lines. The targeting construct homologously recombines with endogenous sequences in or flanking an BACE1 gene locus and functionally disrupts at least one allele of the BACE1 gene. Typically, homologous recombination of the targeting construct with endogenous BACE1 locus sequences results in integration of a non-homologous sequence encoding a selectable marker, such as *neo*, usually in the form of a positive selection cassette. The functionally disrupted allele is termed an BACE1 null allele. ES cells having at least one BACE1 null allele are selected for by propagating the cells in a medium that permits the preferential propagation of cells expressing the selectable marker. Selected ES cells are examined by PCR analysis and/or Southern blot analysis to verify the presence of a correctly targeted BACE1 allele.

Breeding of non-human animals which are heterozygous for a null allele may be performed to produce non-human animals homozygous for said null allele, i.e., "knockout" animals (Donehower et al., Nature 256:215, 1992, which is incorporated herein by reference). In some instances, breeding animals to maintain heterozygosity

may be desired. As described more fully below, the transgenic organisms of the invention have utility as both heterozygous and homozygous BACE1 null alleles. Alternatively, ES cells homozygous for a null allele having an integrated selectable marker can be produced in culture by selection in a medium containing high levels of the selection agent (*e.g.*, G418 or hygromycin). Heterozygosity and/or homozygosity for a correctly targeted null allele can be verified with PCR analysis and/or Southern blot analysis of DNA isolated from an aliquot of a selected ES cell clone and/or from tail biopsies.

If desired, a transgene encoding, for example, a heterologous APP polypeptide comprising the Swedish mutation can be transferred into a non-human host having a BACE1 null allele, preferably into a non-human ES cell that is homozygous for the BACE1 null allele. It is generally advantageous that the transgene comprises a promoter and enhancer which drive expression of structural sequences encoding a functional heterologous Swedish mutation APP gene product. Thus, for example, a knockout mouse homozygous for null alleles at the BACE1 locus can serve as a host for a transgene which encodes and expresses a gene associated with an Alzheimer's disease associated phenotype.

Several gene targeting techniques have been described, including but not limited to co-electroporation, single crossover integration, and double crossover recombination (Bradley et al., BioTechnology 10:534, 1992). The invention can be practiced using essentially any applicable homologous gene targeting strategy known in the art. The configuration of a targeting construct depends upon the specific targeting technique chosen. For example, a targeting construct for single crossover integration targeting need only have a single homology region linked to the targeting region, whereas a double crossover replacement-type targeting construct requires two homology regions, one flanking each side of the replacement region.

For example, in one embodiment a targeting construct comprising, in order: (1) a first homology region having a sequence substantially identical to a sequence within about 3 kilobases upstream (*i.e.*, in the direction opposite to the translational reading frame

of the exons) of an exon of an endogenous BACE1 gene, (2) a replacement region comprising a positive selectable marker (*e.g.*, a pgk promoter driving transcription of a neo gene), (3) a second homology region having a sequence substantially identical to a sequence within about 2 kilobases downstream of said exon of said endogenous BACE1 gene, and (4) a negative selectable marker (*e.g.*, a HSV tk promoter driving transcription of an HSV tk gene). Such a targeting construct is suitable for double crossover replacement recombination which deletes a portion of the endogenous BACE1 locus spanning the desired exon and replaces it with the replacement region having the positive selectable marker. If the deleted exon is essential for expression of a functional BACE1 gene product, the resultant exon-depleted allele is functionally disrupted and is termed a null allele.

Targeting constructs of the invention comprise at least one BACE1 homology region operatively linked to a targeting region. A homology region has a sequence which substantially corresponds to, or is substantially complementary to, an endogenous BACE1 gene sequence of a non-human host animal, and may comprise sequences flanking the BACE1 gene. Although no lower or upper size boundaries for recombinant homology regions for gene targeting have been identified in the art, the typical homology region is believed to be in the range between about 50 base pairs and several tens of kilobases. Thus, targeting constructs are generally at least about 50 to 100 nucleotides long, preferably at least about 250 to 500 nucleotides long, more preferably at least about 1000 to 2000 nucleotides long, or longer. Construct homology regions are generally at least about 50 to 100 bases long, preferably at least about 100 to 500 bases long, and more preferably at least about 750 to 2000 bases long. Homology regions of about 7 to 8 kilobases in length are preferred, with one preferred embodiment having a first homology region of about 7 kilobases flanking one side of a replacement region and a second homology region of about 1 kilobase flanking the other side of said replacement region.

The length of homology (*e.g.*, substantial identity) for a homology region may be selected at the discretion of the practitioner on the basis of the sequence composition and complexity of the endogenous BACE1 gene target sequence(s) and guidance

provided in the art. Targeting constructs have at least one homology region having a sequence that substantially corresponds to, or is substantially complementary to, an endogenous BACE1 gene sequence (*e.g.*, an exon sequence, an enhancer, a promoter, an intronic sequence, or a flanking sequence within about 3-20 kb of a BACE1 gene or BACE1 gene homologue). Such a targeting transgene homology region serves as a template for homologous pairing and recombination with substantially identical endogenous BACE1 gene sequence(s). In targeting constructs, such homology regions typically flank the replacement region, which is a region of the targeting construct that is to undergo replacement with the targeted endogenous BACE1 gene sequence. Thus, a segment of the targeting construct flanked by homology regions can replace a segment of an endogenous BACE1 gene sequence by double crossover homologous recombination. Homology regions and targeting regions are linked together in conventional linear polynucleotide linkage (5' to 3' phosphodiester backbone). Targeting constructs are generally double stranded DNA molecules, most usually linear.

Without wishing to be bound by any particular theory of homologous recombination or gene conversion, it is believed that in such a double crossover replacement recombination, a first homologous recombination (*e.g.*, strand exchange, strand pairing, strand scission, strand ligation) between a first targeting construct homology region and a first endogenous BACE1 gene sequence is accompanied by a second homologous recombination between a second targeting construct homology region and a second endogenous BACE1 gene sequence, thereby resulting in the portion of the targeting construct that was located between the two homology regions replacing the portion of the endogenous BACE1 that was located between the first and second endogenous BACE1 sequences. For this reason, homology regions are generally used in the same orientation (*i.e.*, the upstream direction is the same for each homology region of a transgene to avoid rearrangements). Double crossover replacement recombination thus can be used to delete a portion of an endogenous BACE1 gene and concomitantly transfer a non-homologous portion (*e.g.*, a *neo* gene expression cassette) into the corresponding chromosomal location. Double crossover recombination can also be used to add a non-homologous portion into an endogenous

BACE1 gene without deleting endogenous chromosomal portions. However, double crossover recombination can also be employed simply to delete a portion of an endogenous BACE1 gene sequence without transferring a non-homologous portion into the endogenous BACE1 gene. Upstream and/or downstream from the nonhomologous portion may be a gene which provides for identification of whether a double crossover homologous recombination has occurred; such a gene is typically the HSV tk gene which may be used for negative selection.

The positive selectable marker encodes a selectable marker which affords a means for selecting cells which have integrated targeting transgene sequences. The negative selectable marker encodes a selectable marker which affords a means for selecting cells which do not have an integrated copy of the negative selection expression cassette. Thus, by a combination positive-negative selection protocol, it is possible to select cells that have undergone homologous replacement recombination and incorporated the portion of the transgene between the homology regions (*i.e.*, the replacement region) into a chromosomal location by selecting for the presence of the positive marker and for the absence of the negative marker.

Preferred selectable markers for inclusion in the targeting constructs of the invention encode and express a selectable drug resistance marker and/or a HSV thymidine kinase enzyme. Suitable drug resistance genes include, for example: gpt (xanthine-guanine phosphoribosyltransferase), which can be selected for with mycophenolic acid; neo (neomycin phosphotransferase), which can be selected for with G418 or hygromycin; and DHFR (dihydrofolate reductase), which can be selected for with methotrexate (Mulligan and Berg (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78: 2072; Southern and Berg (1982) J. Mol. Appl. Genet. 1: 327; each of which is incorporated herein by reference).

Selection for correctly targeted recombinants will generally employ at least positive selection, wherein a non-homologous expression cassette encodes and expresses a functional protein (*e.g.*, *neo* or *gpt*) that confers a selectable phenotype to targeted cells harboring the endogenously integrated sequence, so that, by addition of a

selection agent (*e.g.*, G418 or mycophenolic acid) such targeted cells have a growth or survival advantage over cells which do not have an integrated sequence.

It is preferable that selection for correctly targeted homologous recombinants also employ negative selection, so that cells bearing only non-homologous integration of the transgene are selected against. Typically, such negative selection techniques employ an expression cassette encoding the herpes simplex virus thymidine kinase gene (HSV tk) positioned in the transgene so that it integrates only by non-homologous recombination. Such positioning generally as accomplished by linking the HSV tk expression cassette (or other negative selection marker) distal to the recombinant homology regions so that double crossover replacement recombination of the homology regions transfers the positive selection expression cassette to a chromosomal location but does not transfer the HSV tk gene (or other negative selection marker) to a chromosomal location. A nucleoside analog, gancyclovir, which is preferentially toxic to cells expressing HSV tk, can be used as the negative selection agent, as it selects for cells which do not have an integrated HSV tk expression marker. FIAU may also be used as a selective agent to select for cells lacking HSV tk.

Generally, targeting constructs of the invention include: (1) a positive selection marker flanked by two homology regions that are substantially identical to host cell endogenous BACE1 gene sequences, and (2) a distal negative selection marker. However, targeting constructs which include only a positive selection marker can also be used. Typically, a targeting construct will contain a positive selection marker, which includes a *neo* gene linked downstream (*i.e.*, towards the carboxy-terminus of the encoded polypeptide in translational reading frame orientation) of a promoter such as the HSV tk promoter or the pgk promoter.

It is preferred that targeting constructs of the invention have homology regions that are highly homologous to the predetermined target endogenous DNA sequence(s), preferably isogenic (*i.e.*, identical sequence). Isogenic or nearly isogenic sequences may be obtained by genomic cloning or high-fidelity PCR amplification of genomic

DNA from the strain of non-human animals which are the source of the ES cells used in the gene targeting procedure.

For making transgenic non-human animals (which include homologously targeted non-human animals), embryonal stem cells (ES cells) are preferred. The embryonic stem cells described herein can be obtained and manipulated according to published procedures (Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed., IRL Press, Washington, D.C. (1987); Zjilstra et al., Nature 342:435-438 (1989); and Schwartzberg et al., Science 246:799-803 (1989), each of which is incorporated herein by reference). Murine ES cells, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley (1990) Cell 62:1073) essentially as described by Robertson (*supra*, 1987, pages 71-112) can be used for homologous gene targeting. Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) Nature 326: 292-295), the D3 line (Doetschman et al. (1985) J. Embryol. Exp. Morph. 37: 27-45), and the CCE line (Robertson et al. (1986) Nature 323: 445-448).

The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotence of the ES cells (*i.e.*, their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal). The blastocysts containing the injected ES cells are allowed to develop in the uteri of pseudopregnant nonhuman females and are born as chimeric mice. The resultant transgenic mice are chimeric for cells having inactivated endogenous BACE1 loci and are back-crossed and screened for the presence of the correctly targeted transgene(s) by PCR or Southern blot analysis on tail biopsy DNA of offspring so as to identify transgenic mice heterozygous for the inactivated BACE1 locus. By performing the appropriate crosses, it is possible to produce a transgenic non-human animal homozygous for functionally disrupted BACE1 alleles. Such transgenic animals are substantially incapable of making an endogenous BACE gene product.

Non-human animals comprising transgenes which are heterozygous null or homozygous null for BACE1 can be used commercially as controls or standards in the development of AD therapeutics and diagnostics. For example, it is contemplated that the BACE-knockout organisms of the invention can be used as controls in screens for agents having the effect of lowering Aβ production and/or accumulation. Such agents can be developed as pharmaceuticals for treating abnormal APP processing and/or Alzheimer's disease, amongst other neurodegenerative conditions. Other uses include using cells (particularly neuronal cells) derived from the BACE1-knockout organisms for creating protein expression profiles between BACE1-knockout organisms and organisms of identical species having a phenotype associated with Alzheimer's disease.

The effect of test agents on test animals, including transgenic animals, may be measured in various specimens from the test animals. In all cases, it will be necessary to obtain a control value which is characteristic of the level of production of APP and Aβ polypeptide and peptides in animals lacking a phenotype associated with AD. Accordingly, the transgenic animals of the invention (*e.g.*, BACE1 knockout organisms) provide an ideal source of control organisms for studying AD as well as for screening the effects of agents on organisms having an AD-associated phenotype. Once such control level is determined, test compounds can be administered to additional test animals, where deviation from the average control value indicates that the test compound had an effect on the β-secretase activity in the animal. Test substances which are considered positive, *i.e.*, likely to be beneficial in the treatment of Alzheimer's disease or other β-amyloid-related conditions, will be those which are able to reduce the level of ATF-βAPP production, preferably by at least 20%, more preferably by at least 50%, and most preferably by at least 80% or which display a phenotype substantially identical or superior to the phenotype of the BACE1-knockout organisms of the invention.

As used herein, the term "Alzheimer's disease-associated phenotype" includes the appearance in an organism of a progressive formation of insoluble amyloid plaques and vascular deposits of the 4 kDa amyloid β-peptide. In addition, the phenotype can

result in organisms displaying impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

The test agents can be any molecule, compound, or other substance which can be added to the cell culture or administered to the test animal without substantially interfering with cell or animal viability. Suitable test agents may be small molecules, biological polymers, such as polypeptides, polysaccharides, polynucleotides, and the like. The test compounds will typically be administered to transgenic animals at a dosage of from 1 ng/kg to 10 mg/kg, usually from 10 μg/kg to 1 mg/kg.

Test compounds which are able to inhibit secretion or animal production or generate a phenotype substantially identical to the BACE1-knockout organisms of the invention (*e.g.*, having a reduce or negligible amount Aβ1-40, Aβ1-42, Aβ11-40, Aβ11-42 peptides) are considered as candidates for further determinations of the ability to block β-amyloid production in animals and humans. Inhibition of secretion or production indicates that cleavage of βAPP at the amino-terminus of βAP has likely been at least partly blocked, reducing the amount of a processing intermediate available for conversion to β-amyloid peptide.

The present invention further comprises compositions incorporating a compound selected by the above-described method and including a physiologically acceptable carrier. Such compositions, which can be administered to an individual, contain a therapeutic or prophylactic amount of at least one compound identified by the method of the present invention. The carrier can be any compatible, non-toxic substance suitable to deliver the compounds, and the compositions can be administered as discussed above.

Transgenic organisms and/or effects of agents on organisms (*e.g.*, organisms having a phenotype associated with AD) can be screened for presence of the transgene or changes in AD phenotypes in several ways. For example, brain APP protein and RNA expression can be detected and analyzed and the copy number and/or level of expression are determined using methods known to those of skill in the art. The

transgenic animals or organisms displaying a phenotype associated with AD can also be observed for clinical changes. Examples of neurobehavioral disorders for evaluation are poor mating response, agitation, diminished exploratory behavior in a novel setting, inactivity, seizures and premature death.

For a particular strain, organism or transgene, sufficient copies of an APP gene and/or a sufficient level of expression of a coding sequence derived from a particular APP gene which will result in observable clinical and/or behavioral symptoms, together with a measurable biochemical change in relevant brain structures can be determined empirically. Various changes in phenotype are of interest, and include, for example, progressive neurologic disease in the cortico-limbic areas of the brain expressed within a short period of the time from birth; increased levels of an APP gene or gene product above that of BACE1-knockout organisms and the development of a neurologic illness accompanied by premature death; gliosis and intracellular APP/Aβ accretions present in the hippocampus and cerebral cortex; progressive neurologic disease characterized by diminished exploratory/locomotor behavior, impaired performance on memory and learning tests, and diminished 2-deoxyglucose uptake/utilization and hypertrophic gliosis in the cortico-limbic regions of the brain. Such phenotypic characteristics or changes thereof can be used to identify agents which are of interested for further study in the treatment of AD. Such changes can be measurably compared to BACE1-knockout mice as a standard or control organism.

The transgenic animals can also be studied using a species appropriate neurobehavioral test. For example, studies of locomotor/exploratory behavior in mice is a standard means of assessing the neuropsychology (File and Wardill, (1975) Psychopharmacologia (Berl) 44:53-59; Loggi *et al.*, (1991) Pharmacol. Biochem. Behav. 38:817-822). For example, for mice the "corner index" (CI), which is a quick and simple neurobehavioral test to screen animals for evidence of brain pathology, can be used. The CI in transgenic mice which express mutant and wild-type APP is also measured and can be compared to similar behavior in BACE1-knockout mice as a control. A low CI correlates with high mutant APP copy numbers, premature death, and neuropathologic findings. The CI exhibits a dosage dependent relationship to

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APP copy number, which supports the validity of its use in assessing neurobehavioral signs in transgenic mice. The neuropathology of the animals also is evaluated. For rats, the Morris water maze test (see Morris, (1984) J. Neurosci. Meth. 11:47) can be used. A modified version of this test can be used with mice.

Brain regions known to be affected by the syndrome of interest are particularly reviewed for changes. When the disease of interest is Alzheimer's disease, the regions reviewed include the cortico-limbic region, including APP/Aβ excretions, gliosis, changes in glucose uptake and utilization and Aβ plaque formation. However, in strains of animals which are not long-lived, either naturally or when expressing high levels of APP, not all behavioral and/or pathological changes associated with a particular disease may be observed. As an example, transgenic FVB/N mice expressing high levels of APP tend not to develop detectable Aβ plaques, whereas longer lived C57B6/ SJL F1 mice expressing identical transgenes do develop amyloid plaques which are readily detected with thioflavin S and Congo red. Immunologic studies of various brain regions also are used to detect transgene product. Comparing any of the foregoing with BACE1-knockout organisms can provide useful information in identifying novel therapeutic agents and diagnostics.

- The transgenic organisms (*e.g.*, BACE1 knockout organisms) of the invention can be used as controls for tester organisms for agents of interest, *e.g.* antioxidants such as Vitamin E or lazaroids, thought to confer protection against the development of AD. A test organism is treated with the agent of interest, and the neuropathology or behavioral pathology is compared to the BACE1-knockout organisms of the invention, wherein a neuropathology or behavior in the test animal treated with the agent of interest that is substantially similar to or superior to that of the BACE1-knockout organisms is an indication of protection from AD. The indices used preferably are those which can be detected in a live animal, such as changes in performance on learning and memory tests. The effectiveness can be confirmed by effects on pathological changes when the animal dies or is sacrificed.

Careful characterization of the transgenic animals of the invention should lead to elucidation of the pathogenesis of progressive neurologic syndromes such as AD. The sequence of molecular events in BACE1 metabolism leading to disease can be studied. In addition, understanding the role and activity of BACE1 homologues including, for example, BACE2, are provided by the transgenic organisms of the invention. The animals also are useful for studying various proposed mechanisms of pathogenesis, including horizontal transmission of disease. Such knowledge would lead to better forms of treatment for neurologic disorders.

The following examples are provided as a guide for those skilled in the art, and are not to be construed as limiting the invention in any way. All products are used according to manufacturer's instructions, and experiments are conducted under standard conditions, unless otherwise specified.

EXAMPLE 1

Gene Targeting Vector and Embryonic Stem (ES) Cells

To examine the physiological roles of BACE1 and to determine whether BACE1 is the major β-secretase in neurons, mice with targeted inactivation of *BACE1* alleles were developed. A homologous recombination strategy in embryonic stem (ES) cells was used to inactivate the mouse *BACE1* gene. To target the BACE gene in ES cells, BACE genomic clones were isolated from a 129/Sv strain of mouse Lambda FIX II Library (Stratagene, CA) by using a partial mouse BACE cDNA containing the translation initiation codon as probe. In the *BACE1* targeting vector, a 2.0 kb *Bam*HI fragment containing the first coding exon which encode residues 1-87 (including the pro-peptide shown to be important for regulating BACE1 activity and flanking intronic sequences of the *BACE1* gene was replaced with a neomycin-resistance gene (Figure 1A) under the control of the PGK promoter. Introduction of a negative selection marker, the herpes simplex virus thymidine kinase gene, at the 5' end of the construct allowed the use of the positive and negative selection scheme.

The targeting vector was linearized at a unique *Not*I site before transfection into R1 ES cells, which were subjected to double selection. R1 ES cells were transfected

with the linearized *BACE1* targeting vector, and 2 clones (out of 112 screened) were targeted at the *BACE1* locus. Clones were picked and expanded, and DNA was isolated from a portion of the cells and screened by Southern blot analysis. Targeted cells were expanded and injected into C57BL/6J blastocysts to produce highly chimeric male mice that transmitted the targeted *BACE* allele in the germline. $BACE^{+/-}$ mice were intercrossed to obtain the $BACE^{-/-}$ animals. *BACE1*-targeted ES cells were used to generate the $BACE1^{-/-}$ mice. Genotype analyses of the $BACE1^{-/-}$ mice were performed by DNA blotting (Fig. 1B) and PCR methods (Figure 1C). Genotypes were determined by PCR amplification of tail or yolk sac DNA. The primer set (HC69: 5'-AGGCAGCTTTGTGGAGATGGTG (SEQ ID NO:1);
HC70: 5'-CGGGAAATGGAAAGGCTACTCC (SEQ ID NO:2); and
HC77: 5'-TGGATGTGGAATGTGTGCGAG (SEQ ID NO:3)) was used to detect the endogenous and targeted *BACE* alleles.

EXAMPLE 2

Antibody Preparation and Characterization

Antibody Preparation

A synthesized peptide corresponding to the C-terminal 12 residues of mouse BACE coupled to KLH was used to make the anti-peptide antibody (Research Genetics; Huntsville AL). To generate the HIS_6-BACE fusion protein, a DNA fragment corresponding to residues 46 to 163 of BACE was subcloned into pTrcHisA (Invitrogen, San Diego, CA, see, also, GenBank Accession Nos. AF190725 and AAF04142; Vassar et al., Science 286:735, 1999, each of which is incorporated herein by reference). The HIS_6-BACE fusion protein purified by Talon Metal Affinity Resin (Clontech; Palo Alto CA) chromatography was used as antigen for making the anti-fusion protein antibody (Covance Research Products Inc.; Denver PA). BACE anti-peptide and anti-fusion protein antibodies were generated in rabbits, and serum containing the antibodies was collected.

Protein Extraction and Western Blot Analysis

Mouse tissues were dissected out and homogenized in PBS in the presence of protease inhibitors. After extraction with 1% SDS, the lysates were centrifuged at 100,000xg

and supernatants were saved for western blot analyses. For western blot analysis, 50 μg protein was loaded in each lane, separated in a 10% Tris-Glycine gel, and transferred to PVDF membranes for immuno-detection with antibodies specific for BACE1 or superoxide dismutase (SOD1). A 1:3000 dilution of serum containing the anti-BACE antibodies was used as the primary antibody, and specific binding was detected using peroxidase-conjugated Protein A.

Western blot analysis confirmed that the targeting event (Example 1) led to inactivation of the *BACE1* gene. In *BACE1*$^{+/-}$ mice, BACE1 accumulated in brain to approximately 50% of the level of control littermate, whereas the brain of *BACE1*$^{-/-}$ mice showed no detectable level of BACE1 (Figure 1D). Similar results were observed using the antiserum specific to the carboxyl-terminal 13 residues of BACE1. These results confirm the inactivation of *BACE1*.

EXAMPLE 3

Generation of human APP and BACE recombinant adenoviruses

A full-length human *BACE* cDNA was constructed from a near full-length clone isolated from a human fetal brain cDNA library (Origene Technologies Inc., MD) and a 5' cDNA encoding the N-terminal 41 amino acids of BACE obtained by RT-PCR of total RNA from HEK293 cells. Recombinant adenoviruses expressing wild type/mutant human APP or BACE were produced by cloning the full-length wild type/mutant human APP or BACE cDNA, respectively, into the pAd-Track-CMV shuttle vector. Under the control of distinct CMV promoters, this plasmid expresses the human APP or BACE, and in parallel, green fluorescent protein (GFP). The construct was integrated into the adenoviral backbone vector, pAd-Easy-1, by homologous recombination in *E. coli* strain BJ5183. The adenoviral construct was then cleaved with *Pac*I and transfected in a packaging cell line (HEK 293 cells). The titer of the viral stocks was estimated based on the density of GFP-expressing cells.

EXAMPLE 4

Primary cortical cultures and metabolic labeling

Cortical neuronal cultures were established from brains of embryonic day 16.5 fetal mice. The dissected brain cortexes were suspended in HBSS supplemented with 0.25% trypsin and 0.01% DNase I and incubated at 37°C for 10 min. The tissues were then transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and dissociated by repeated trituration. The dispersed cells were collected by centrifugation and plated at approximately 1 x 10^6 cells/well on 6 well cell culture plates (coated with poly-D-lysine) in B27/Neurobasal media (GIBCO/BRL; Gaithersburg MD). Neurons were allowed to mature for 4–7 days in culture before they were used for experiments. Primary neuronal cells cultured for 4 to 7 days were infected with 5 × 10^6 plaque-forming units of adenovirus expressing human APP for 4 days in serum-free medium. For metabolic labeling, neuronal cells were pre-incubated for 30 min in methionine-free DMEM with 1% dialyzed bovine serum and then labeled with 700 μCi/ml of ^{35}S-methionine in methionine-free medium for 5 hr.

For pulse-chase labeling, cells were pulsed for 45 min with methionine-free DMEM containing 1 mCi/ml ^{35}S-methionine. Cells were then chased by washing and incubating in DMEM containing 1% dialyzed fetal bovine serum and 1 mM L-methionine at varying intervals, before the cells were lysed in immunoprecipitation buffer containing detergents and a protease inhibitor cocktail. After metabolic labeling, culture medium and cell extracts were immunoprecipitated and immunoprecipitates were fractionated on either 4%–20% Tris-glycine or 16% Tris-tricine SDS-PAGE. Gels were dried, exposed, and radioactive bands were quantified by phospho-imaging analysis.

To examine the effect of the absence of BACE1 on secretion of Aβ peptides from neurons, primary cortical cultures from control, *BACE1*$^{+/-}$ and *BACE1*$^{-/-}$ embryos were derived from day 16.5 post coitum. The growth rate and morphology of the *BACE1*$^{-/-}$ cultures were identical to those of the *BACE1*$^{+/-}$ or control. Immunoprecipitation-mass spectrometry (IP-MS) analysis of conditioned culture media from control

neurons after 5 days in culture using an antisera (4G8) specific to epitopes between residues 17-28 of Aβ revealed two prominent Aβ species with mass values of 3171 and 4233 corresponding to mouse Aβ11-40 and Aβ1-40 respectively, in addition to several minor species including Aβ11-42 and Aβ1-42 (Figure 2B). While these Aβspecies are similarly observed in conditioned culture media from *BACE1*$^{+/-}$ neurons, secretion of these Aβ species is abolished from *BACE1*$^{-/-}$ neurons except for the Aβ17-40 (p3) fragment (Figure 2B). These data establish that BACE1 is the major β-secretase required for cleavages of βAPP at the +1 and +11 sites of Aβ peptide in embryonic cortical neurons. Because a primary cleavage site for BACE2 is at +19/+20 of Aβ and no Aβ20-40/42 or Aβ21-40/42 was detected, it was inferred therefore that BACE2 plays little role in the cleavage of APP in neurons.

To confirm the unique role of BACE1 in neurons, the processing of APP in control and *BACE1*$^{-/-}$ neuronal cultures following infection with a recombinant adenovirus expressing a humanized APP cDNA (a murine APP cDNA in which the Aβ1-42 region corresponds to the human Aβ1-42) bearing the Swedish variant (APPswe) was examined. Quantitative sandwich ELISA analyses of conditioned media from *BACE1*$^{+/+}$ cultures expressing APPswe showed high levels of Aβ1-40 and Aβ1-42 while undetectable levels of Aβ1-40 and Aβ1-42 were observed from media of *BACE1*$^{-/-}$ cultures expressing APPswe (Figure 2C). Metabolic labeling of control and *BACE1*$^{-/-}$ cortical neurons with ^{35}S-methionine for 5 hours and immunoprecipitation analysis using 4G8 antisera showed the presence of a major band (~4 kDa) corresponding to Aβ and a minor band (~3.2 kDa) corresponding to p3 in control culture (Figure 2D), but, although p3 is readily secreted, no Aβ accumulated in conditioned media from *BACE1*$^{-/-}$ cultures expressing APPswe (Figure 2D). Moreover, immunoprecipitation analysis using CT15, an antibody specific for the carboxyl-terminal 15 residues of APP[12], revealed in *BACE1*$^{-/-}$ detergent lysates the accumulation of full length APP as well as APP β-CTF (Figure 2E); however, this approach failed to detect APP β-CTF in the lysates, which are in control lysates (Figure 2E). Taken together, these results confirm that BACE1 is the primary β-secretase in cortical neurons and infer that BACE2 does not play a significant role in the processing of APP in neuronal cultures.

Since α-secretases and β-secretases compete for the same substrate, we anticipated that in the absence of BACE, APP derivatives produced by the action of α-secretase might be increased. To determine whether the rate of secretion of α-secretase derived APP soluble ectodomain (APPsα) is altered, the processing of APPswe in *BACE1*$^{+/+}$ and *BACE1*$^{-/-}$ cortical neurons was examined. Pulse-chase studies revealed that there is an increase in the rate of secretion of APPsα in *BACE1*$^{-/-}$ neurons as compared to controls (Figures 3C-3E). Furthermore, no accumulation of either β-CTF or Aβ in the *BACE1*$^{-/-}$ neuronal cultures was detected (Figures 3A and 3B). These results establish that BACE1 competes with α-secretase in APP processing and further confirm the view that BACE1 is the major β-secretase in neurons.

Example 5

Mass Spectrometric Analysis

The β-amyloid peptides were captured with 4G8 monoclonal antibody (Senetek; Napa CA) by immunoprecipitation from conditioned media of cultured neurons. After final wash, the immunoprecipitates were rinsed twice with 5 mM HEPES buffer (pH 7.0). A 1 μl sample was spotted on NP-1 series ProteinChip™ array and analyzed by surface-enhanced laser desorption/ionization time of flight MS (Ciphergen Biosystems, Palo Alto CA) in the presence of CHCA matrix solution (Ciphergen Biosystems). External standards were used for calibration.

EXAMPLE 6

Determination of Aβ1-42/43 and Aβ1-40 Levels

Two-site ELISA's that specifically detect the C-terminus of Aβ were performed to measure Aβ levels as suggested by the manufacturer (Biosource International; Camarillo CA). Culture media of neuronal cells infected with adenovirus expressing human APP were collected and analyzed using the quantitative sandwich ELISA to determine both Aβ1-42 and Aβ1-40 levels.

To confirm that BACE1 cleaves APP at both the +1 and +11 sites of Aβ, neuronal cultures infected with adenovirus expressing either humanized wild type APP

(hAPPwt) or its variants (hAPPswe or hAPP717) were examined and the secretion of Aβ peptides from conditioned media as well as the accumulation of both +1 and +11 derived β-CTFs from cell lysates measured. As expected, IP-MS analysis of conditioned media using 4G8 antibody showed that the human and murine Aβ1-40 and Aβ1-42 were secreted, however, the human Aβ11-40 peptide was not secreted into culture media from murine primary neurons infected with adenovirus expressing hAPPwt, although the murine Aβ11-40 was readily detected. Similar results were also observed with murine neurons infected with adenovirus expressing hAPPswe or hAPP717. This apparent discrepancy raised the possibility that the cleavage site at +11 of Aβ is species-specific, *i.e.*, human or murine BACE1 cleaves respectively, human or murine APP at +11 site of Aβ whereas no species selectivity occurs at the +1 site. To test this possibility, the processing of human APP by co-infecting murine neuronal cultures with adenovirus expressing both human BACE1 and hAPPwt or its variants was examined. IP-MS analysis of conditioned media using 4G8 antibody now revealed the secretion of human Aβ11-40 peptide in addition to the murine Aβ11-40 peptide from murine neurons co-expressing human BACE1 and hAPPwt. The human Aβ11-40 peptides are also secreted by primary neurons co-expressing human BACE1 and hAPPswe or hAPP717.

In addition, since human BACE1 cleaves human APP at the +11 site of Aβ, the +11 derived β-CTF was examined to determine whether it accumulated in lysates of neurons co-expressing human BACE1 and human APPwt or its variants. As expected, while β-CTFs are readily immunoprecipitated using the CT15 antibody from control, hAPPwt, hAPPswe or hAPP717 lysates, the +1derived β-CTF is observed only in the hAPPswe lysate. However, when neurons co-expressing human BACE1 and hAPPwt or hAPPswe or hAPP717 there is secreted a peptide corresponding to the +11 derived β-CTF (+11-CTF) in addition to the +1 derived β-CTF. Taken together, these results support the view that the cleavage site at +11 of Aβ is species-specific. To begin to access the determinants that govern this selectivity, the amino acid sequences of Aβ between humans and mice were compared; there is a sequence divergence around the +11 site whereas there is absolute conservation at the +1 site of Aβ (see Figure 2A). Mutagenesis studies will

allow determination of the amino acid residue(s) that confer species specificity at the +11 site of Aβ̃ Although Aβ11-40/42 peptides has been previously observed in neuronal cultures as well as in the brains of cases of AD, the roles of these peptides in the pathogenesis of AD was not understood. Aβ beginning at +11 is a major species in rodents *in vivo* and this peptide is more fibrillogenic and neurotoxic than full length Aβ *in vitro*. Because the finding that the +11 site is a major cleavage site for BACE1, the involvement of Aβ11-40/42 in pathogenesis of AD is important. Aβ11-40/42 plays a critical role in AD, thus antibodies specific to Aβ11-40/42 would prove useful for diagnoses of sporadic AD. The demonstration that the cleavage at +11 is species-specific would infer that the published mutant human APP transgenic models would not be expected to secrete the human Aβ11-40/42 (because murine BACE1 does not cleave at the +11 site) and transgenic mice over-expressing either murine wild type APP or its variants may be instructive in clarifying the pathogenic roles of Aβ11-40/42.

The secretion of Aβpeptides (Aβ1-40/42 as well as Aβ11-40/42) from neurons is abolished in cultures of BACE1-deficient embryonic cortical neurons derived from BACE1- knockout mice. Moreover, while the intracellular β-carboxy terminal fragments of βAPP (β-CTFs) and the corresponding APPsα fragments are not generated in *BACE*$^{-/-}$ neurons, the rate of APPsα secretion is increased in *BACE*$^{-/-}$ neurons as compared to controls. These results establish that BACE1 is the principal neuronal protease required to cleave β-amyloid precursor protein, APP) at +1 and +11 sites of Aβ that generate N-termini of Aβ. In addition, the invention provides for the first time, that while both human and murine BACE1 are capable of cleaving either human or murine βAPP at the +1 site of Aβ, the cleavage at the +11 site is species-specific. Taken together, these results have important implications for the development of novel therapeutic strategies in Alzheimer's disease.

While both α-secretase and β-secretase activities represent therapeutic targets for the development of novel protease inhibitors for AD, the discovery of BACE1 and BACE2 now provides the opportunity to determine whether these aspartic proteases are indeed high priority targets. The demonstration that BACE1 is the major

β-secretase in neurons provides excellent rationale for focusing on the design of novel therapeutics to inhibit BACE1 activity in brain as well as using Aβ11-40//42 as novel tools for diagnosing AD. The transgenic organisms of the invention allow for the identification of other important substrates for BACE1 and the evaluation of BACE1 knockout. This information will have significant impact in the design of specific drugs to inhibit BACE1 in the central nervous system. To illustrate this principle, it is instructive to consider the emerging view that the presenilins (PS1 and PS2), which when mutated cause familial AD and which are important for the intramembranous proteolysis of several proteins, including APP and Notch1, may be the putative γ-secretase. Presenilins are involved in the proteolytic processing of Notch1 and are critical for Notch1 functions. PS1 null mice, which die before or at birth, have a developmental defect in patterning of somites; a phenotype resembling that observed in the Notch1 null mice. Recent demonstrations that PS1 co-fractionates with γ-secretase activity, that transition-state analogue inhibitors of γ-secretase can covalently label PS, and that two transmembrane aspartates are required for γ-secretase activity provide support for the view that PS1/2 may possess γ-secretase activity or is a co-factor intimately associated with γ-secretase cleavages. Alternatively, PS1/2 may play a role in trafficking of APP or other molecules. Consistent with the idea that γ-secretase activity is subserved by a multi-subunit catalytic complex is the recent identification of the type 1 transmembrane protein, nicastrin, which interacts with presenilins that are known to modulate both γ-secretase activity and Notch1 function. Thus, the design of therapeutics that inhibit γ-secretase and thus influence Notch1 processing could have in the adult, impact on some cell populations (hematopoietic cell) that utilize Notch1 signaling for cell fate decision. In this case, it would be necessary to try to develop highly selective inhibitors that act principally on γ-secretase activities that cleave APP and have less inhibiting potency on Notch1 cleavage.

The demonstration that BACE1 null mice are viable allows for the development of inhibitors that are brain penetrate (*i.e.*, can cross the blood brain barrier), bind to the active sites (extracellular) of BACE1 to ameliorate β-amyloid deposition, and are without profound adverse effects. BACE1 null mice are valuable for testing whether

the β-amyloid burden can be reduced in mutant APP transgenic models lacking BACE1. Such an outcome would greatly encourage investigators to design novel drugs to inhibit BACE1 activity. The recent report documenting the crystal structure of the protease domain of BACE1 associated with an eight-residue inhibitor provide valuable information towards the development of specific drugs to inhibit BACE1 activity. These compounds can be tested in transgenic mice to determine whether they ameliorate Aβ deposition. If so, these therapeutic can be brought rapidly into clinical trials.

EXAMPLE 7

BACE1 EXPRESSION IN BRAIN AND CORRELATION TO AMYLOIDOSIS

To begin to assess the role of BACE1 as a determinant of selective vulnerability of the brain to Aβ amyloidogenesis, the level and distribution of BACE1 in the CNS and various other tissues was examined in BACE1 knockout mice and wild type littermates. In contrast to the ubiquitous expression of BACE1 mRNA in a variety of tissues, BACE1 protein was abundantly expressed only in the brain and was undetectable in other non-neural tissues, including pancreas, heart, liver and kidney. BACE1 expression also was examined in various regions of the CNS, including the frontal cortex, posterior cortex, cerebellum, hippocampus, olfactory bulb, striatum, thalamus, midbrain, entorhinal cortex, pons, medulla and spinal cord of new born mice (new born mouse brain blot; Chemicon). The accumulation of BACE1 across various regions of the brain was uniform when normalized to the level of β3 tubulin, except that a relatively higher level was observed in the olfactory bulb and a relatively lower level was observed in the frontal cortex and spinal cord.

Although BACE1 protein was expressed at comparable level in most brain regions as shown by western blot, BACE1-specific immunoreactivities were particularly localized in the hippocampus, a region that is critical for learning and memory and is particularly vulnerable in AD. Strong expression was observed in the hilus of dentate gyrus and stratum lucidum of CA3 region (terminal field of mossy fiber pathway); no specific expression was observed in brain sections prepared from BACE1 knockout mice.

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To begin to assess whether the staining pattern observed for BACE1 is pre-synaptic, the staining patterns of several markers were examined. Littermate control and BACE1 knockout mice (or APP and PS1 double transgenic mice, see below) were sacrificed and perfused with 4% PFA, then brain and other organs were paraffin embedded, sectioned, and processed for immunohistochemical analysis using the peroxidase-anti-peroxidase method. Antibodies included those specific for BACE1, synaptophysin, syntaxin, and MAP2, and were used in conjunction with hematoxylin and eosin staining. For BACE1 immunohistochemical analysis, an IgG purified anti-BACE1 fusion protein antibody was applied (1 μg/ml) as primary antibody in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 1% Triton X-100 detergent, after retrieving the antigen by heating the tissue section in a microwave oven.

The BACE1 staining pattern in the hippocampus was remarkably similar to two well characterized pre-synaptic terminal markers, synaptophysin and syntaxin, and was distinct from that of MAP2, which is a postsynaptic marker. Moreover, under higher magnification, BACE1 immunoreactivities were localized to the giant boutons of the mossy fibers that form synapses with the hilar mossy cells and proximal dendrites of CA3 pyramidal cells; the giant boutons also were readily labeled by anti-synaptophysin antisera. These results indicate that BACE1 protein expression is localized to pre-synaptic terminals. Because hippocampal granule cells are continuously undergoing turnover throughout the life of the animal, the particular enrichment of BACE1 protein in these highly plastic cells further indicates that BACE1 can have a role in either synaptic development or plasticity in the brain.

The role of BACE1 in astrocytes was examined with respect to the processing of APP in *BACE1*$^{+/-}$, *BACE1*$^{+/-}$ and *BACE1*$^{-/-}$ astrocyte cultures four days after infection with a recombinant adenovirus expressing a humanized APP cDNA bearing the Swedish variant (APPswe). *BACE1*$^{+/+}$, *BACE1*$^{+/-}$ and *BACE1*$^{-/-}$ neuronal cultures were labeled for 5 hours with ^{35}S-methionine, then full length APP and APP-CTF's were immunoprecipitated with CT15. The signal intensity of APP β-CTF and α-CTF was quantified by phospho-imaging and was normalized against APP full length protein.

Protein blot analysis of *BACE1*$^{-/-}$ detergent lysates revealed the accumulation of full length APP as well as APP α-CTF; APP β-CTF was not detected. To examine the effect of the absence of BACE1 on secretion of Aβ peptides from astrocytes, conditioned media derived from *BACE1*$^{-/-}$ cultures were subjected to ELISA and mass spectrometric analyses. Aβ1-40 and Aβ1-42 were easily detected from *BACE1*$^{+/+}$ and *BACE1*$^{+/-}$ astrocyte cultures by Aβ ELISA assay, but were not detectable from *BACE1*$^{-/-}$ cultures. APP β-CTF from *BACE1*$^{+/-}$ cultures was reduced compared to *BACE1* wild type cultures (n=3; p<o.04, Student's t-test). APP β-CTF was barely detectable in *BACE1*$^{+/-}$ astrocyte cultures infected with the APPswe adenovirus. Quantitative sandwich ELISA analysis of conditioned medium from *BACE1*$^{+/-}$ astrocyte cultures expressing APPswe showed an approximately 50% reduction of levels of Aβ1-40 and Aβ1-42 compared to *BACE1*$^{+/+}$ cultures.

Mass spectrometric profiles of secreted human Aβ 1-19, 1-20, 1-40, and 1-42 from conditioned media of cortical neurons or glial cells infected with adenovirus expressing APPswe were determined by using PS1 Ciphergen ProteinChip™ system coated with 6E10, a monoclonal antibody specific against human Aβ 1-16 revealed identical results as were observed by ELISA. Furthermore, BACE1 deficient astrocytes co-infected with adenoviruses expressing both APPswe and BACE1 restored the ability to secrete Aβ peptides into culture medium. The cultured astrocytes showed stronger BACE2 and α-secretase activities than the cultured neurons (n=4; p<0.001, Student's t-test) These results demonstrate that BACE1, in addition to being the principal β-secretase in neurons, also is the principal β-secretase in cortical astrocytes.

Besides BACE1 and γ-secretase, APP can also be cleaved within the Aβ region by putative "α-secretases" such as TACE and ADAM10 at residue +16, or by BACE2 at residues +19 and +20 of Aβ. Since these three cleavages occur within the Aβ domain, action by BACE2 and α-secretase would preclude the formation of toxic Aβ peptide. In contrast to BACE1, BACE2, TACE and ADAM10 mRNA levels are

relatively low in brain. However, it is not known whether the lower expression level of BACE2 and TACE/ADAM10 correlates with lower α-secretases and BACE2 mediated anti-amyloidogenic activities in neurons.

Using the Ciphergen PS1 ProteinChip™ array coated with 6E10 antibody, the relative amount of BACE2 derived Aβ1-19 and Aβ1-20 and α-secretase derived Aβ1-15 or Aβ1-16 fragments were examined, and normalized to BACE1 derived Aβ1-40. BACE2 mediated cleavages (at +19 and +20 of Aβ) were much higher in cultured astrocytes as compared to neurons (see Figure 4). Similar studies in cultured cell lines, such as fibroblasts and COS-1 cells also revealed high levels of BACE2 or α-secretase activities coupled with low level of BACE1 activity. Because the absence of BACE1 abolished of secretion of BACE2 derived Aβ1-19 and Aβ1-20 peptides, BACE2 primarily cleaves APP at +19 and +20 sites, but not at +1 site of Aβ. These results confirm that the high level of BACE1 coupled with low levels of BACE2 and α-secretase activities in neurons predispose these cells to Aβ amyloidogenesis.

To begin to examine whether BACE1 is a determinant of selective vulnerability of neurons to amyloidogenesis, the relative levels of BACE1 protein or activity in cultured cortical astrocytes as compared to neurons was examined. Protein blot analysis revealed that BACE1 protein level in astrocytes was much lower than that of neurons. This result demonstrates that neurons are the primary source of Aβ and that BACE1 is a major susceptibility factor that predisposes neurons to Aβ amyloidosis in the brain. These results were extended by examining Aβ generation and deposition in a mouse model of amyloidosis lacking BACE1. Since the above results demonstrated that BACE1 was required for the secretion of Aβ peptides by neurons, it was expected that Aβ formation and deposition would be abolished in APP mutant mice lacking BACE1. However, because the absence of BACE1 led to an increase in the α-secretase derived p3 peptide, it also was plausible that p3 could contribute to Aβ deposition in brains of APP mice lacking BACE1. To test this possibility and to determine whether partial reduction of BACE1 ameliorate Aβ deposition, APP/PS1 mutant mice, which express reduced levels of BACE1, were generated. The absence of BACE1 abolished the formation of Aβ1-40 and Aβ1-42 as determined by standard

ELISA methods. Moreover, whereas abundant Aβ deposition was observed in APP/PS1 double transgenic mice, no Aβ plaques were detected in APP/PS1 mice lacking BACE1. BACE1 immunoreactivity also was observed in dystrophic neurites surrounding amyloid plaques in the brains of APP/PS1 double transgenic mice.

Together with the cell culture studies described above, the present results establish that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of Aβ in the central nervous system and indicate the potential therapeutic value of inhibiting BACE1 in efforts to ameliorate Aβ deposition in AD. These results also demonstrate that the anti-BACE1 antibodies are specifically reactive with BACE1, and that the antibodies can be used as a diagnostic reagent to identify regions of selective vulnerability of brain amyloidosis in Alzheimer's disease.

Although both BACE1 and BACE2 are expressed ubiquitously, BACE1 mRNA levels are particularly high in brain and pancreas, whereas the levels of BACE2 mRNA are relatively low in all tissues, except in brain where it is nearly undetectable. While BACE1 was shown to be the principal β-secretase necessary to cleave APP to generate Aβ *in vivo, in vitro* studies indicated that BACE2 was capable of cleaving APP more efficiently at sites within the Aβ domainas compared to the +1 site of Aβ. As BACE1 is the principal β-secretase in neurons and BACE2 serves to limit the secretion of Aβ peptides, the present results indicate that BACE1 is a pro-amyloidogenic enzyme, while BACE2 is an anti-amyloidogenic protease. In this scenario, the relative levels of BACE1 and BACE2 in neurons are determinants of Aβ amyloidosis, and the secretion of Aβ peptides would be expected to be the highest in neurons/brain as compared to other cell types/organs because neurons express high levels of BACE1 coupled with low expression of BACE2. If a high level of BACE1 coupled to a low level of BACE2 is a critical factor that selectively predisposes the brain to Aβ amyloidosis, AD would be predicted to involve the brain rather than heart or pancreas. Seemingly inconsistent with this hypothesis is that very high level of BACE1 mRNA expression is observed in the pancreas. However, some of the pancreatic mRNAs appear to be alternatively spliced to generate a BACE1 isoform that is incapable of cleaving APP Bodendorf et al., J. Biol. Chem. 276:12019-12023,

2001). Thus, our results now demonstrating that while neurons in the brain possess high level of BACE1 coupled with low level of BACE2 activity, astrocytes or fibroblasts showed low level of BACE1 and high BACE2 activity, supports this hypothesis. Importantly, to test in vivo the hypothesis that the abundance of BACE1 is a major determinant of selective vulnerability of neurons to Aβ amyloidosis, we took a genetic approach to reduce the level of BACE1 selectively in neurons/brain of mutant APP/PS1 transgenic mice. As disclosed herein, while the deletion of BACE1 abolished the secretion and deposition of Aβ, the partial reduction of BACE1 (to 50% of normal level) significantly ameliorated amyloid plaque deposition in a mouse model of Aβ amyloidosis. These results demonstrate that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of Aβ in the central nervous system and indicate the potential importance of polymorphisms that can act, for example, to increase levels of BACE1 and thereby predispose individuals to AD.

Although the invention has been described with reference to the certain embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed:

1. A method for modulating the production of Aβ11-40/42 peptide fragments comprising contacting a sample or cell containing a beta-site APP-cleaving enzyme 1 (BACE1) and an amyloid precursor protein (APP) with a BACE1-modulating agent such that production of Aβ11-40/42 is modulated.

2. The method of claim 1, wherein the modulation is inhibition of Aβ11-40/42 peptide formation.

3. The method of claim 1, wherein the contacting is *in vivo*.

4. The method of claim 1, wherein the contacting is *in vitro*.

5. The method of claim 1 wherein the BACE1-modulating agent is an anti-BACE1 antibody or a BACE1 antisense molecule.

6. A method for identifying a compound which inhibits beta-site APP-cleaving enzyme 1 (BACE1) expression or activity comprising:

a) incubating components comprising the compound, BACE1 polynucleotide or polypeptide, and an amyloid precursor protein (APP) under conditions sufficient to allow the components to interact; and

b) measuring the production of a BACE1 specific enzymatic product.

7. The method of claim 6, wherein the compound is a peptide.

8. The method of claim 6, wherein the compound is a small molecule inhibitor.

9. The method of claim 6, wherein the BACE1 polynucleotide or polypeptide is expressed in a cell.

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10. The method of claim 6, wherein the BACE1 specific enzymatic product includes a sequence of Aβ11-40/42.

11. A compound identified by the method of claim 6.

12. The compound of claim 11, in a pharmaceutically acceptable carrier.

13. A method for diagnosing a subject having or at risk of having an Aβ11-40/42 peptide accumulation disease, the method comprising:

measuring the amount of beta-site APP-cleaving enzyme 1 (BACE1) in a biological sample from the subject; and

comparing the amount BACE1 with a normal standard value of BACE1, wherein a difference between the measured amount and the normal sample or standard value provides an indication of the diagnosis of Aβ11-40/42.

14. The method of claim 13, wherein the biological sample is blood, serum, cerebrospinal fluid or central nervous system (CNS) tissue.

15. The method of claim 13, wherein the difference is an increase in BACE1.

16. The method of claim 13, wherein the amount BACE1 is measured by detecting the amount of a polynucleotide encoding BACE1.

17. The method of claim 16, wherein the polynucleotide is mRNA.

18. The method of claim 17, wherein the mRNA is detected by PCR.

19. The method of claim 13, wherein the amount of BACE1 is detected by contacting the sample with an agent that specifically binds to a BACE1 polypeptide.

20. The method of claim 19, wherein the agent is an antibody.

21. The method of claim 20, wherein the antibody is a monoclonal antibody.

22. The method of claim 20, wherein the antibody is a polyclonal antibody.

23. The method of claim 19, wherein the Aβ11-40/42 accumulation disease is Alzheimer's disease.

24. The method of claim 13, further comprising detecting the level of an APP fragment, wherein an increase in the presence of the fragment is indicative of Alzheimer's disease.

25. The method of claim 24, wherein the APP fragment is an Aβ1-40, Aβ1-42, Aβ11-40, or Aβ11-42 fragment.

26. The method of claim 25, wherein the fragments are detected by contacting the sample with an agent the specifically binds to an Aβ1-40, Aβ1-42, Aβ11-40, or Aβ11-42 fragment.

27. The method of claim 26, wherein the agent is an antibody.

28. The method of claim 20 or 27, wherein the antibody is detectably labeled.

29. The method of claim 28, wherein the detectable label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

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30. A method for diagnosing a subject having or at risk of having Alzheimer's disease, the method comprising:

measuring Aβ11-40/42 in a biological sample from the subject; and

comparing the amount of Aβ11-40/42 with a normal sample or standard value of Aβ11-40/42, wherein a difference between the amount in the normal sample or standard value is indicative of a subject having or at risk of having Alzheimer's disease.

31. The method of claim 30, wherein the biological sample is cerebrospinal fluid, central nervous system (CNS) tissue, serum or blood.

32. The method of claim 30, wherein the difference is an increase in Aβ11-40/42 and the increase is indicative of a disposition for Alzheimer's disease.

33. The method of claim 30, wherein the difference is a decrease in Aβ11-40/42.

34. The method of claim 30, wherein the amount of Aβ11-40/42 is detected by contacting the sample with an agent that specifically binds to Aβ11-40/42.

35. The method of claim 34, wherein the agent is an antibody.

36. The method of claim 35, wherein the antibody is a monoclonal antibody.

37. The method of claim 35, wherein the antibody is a polyclonal antibody.

38. The method of claim 34, wherein the agent is an antibody fragment.

39. The method of claim 30, further comprising detecting the level of a BACE1 polypeptide or polynucleotide, wherein an increase in the level of BACE1 is indicative of Alzheimer's disease.

40. The method of claim 35, wherein the antibody is detectably labeled.

41. The method of claim 40, wherein the detectable label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

42. A transgenic non-human animal having a transgene disrupting expression of BACE1, chromsomally integrated into the germ cells of the animal, and have a phenotype of reduced Aβ peptide as compared with a wild-type animal.

43. The transgenic non-human animal of claim 42, wherein the animal is an avian, bovine, ovine, piscine, murine, or porcine species.

44. The transgenic non-human animal of claim 42, wherein the animal is heterozygous or homozygous for the disruption.

45. The transgenic non-human animal of claim 42, wherein the transgene comprises a BACE1 antisense polynucleotide.

46. A method for producing a transgenic non-human animal having a phenotype characterized by reduced expression of BACE1 polypeptide, the method comprising:

(a) introducing at least one transgene into a zygote of an animal, the transgene(s) comprising a DNA construct encoding a selectable marker,

(b) transplanting the zygote into a pseudopregnant animal,

(c) allowing the zygote to develop to term, and

(d) identifying at least one transgenic offspring whose genome comprises a disruption of the endogenous BACE1 polynucleotide sequence by the transgene.

47. The method of claim 46, wherein the introducing of the transgene into the embryo is by introducing an embryonic stem cell containing the transgene into the embryo.

48. The method of claim 46, wherein the transgenic non-human animal is heterozygous or homozygous for the disruption.

49. The method of claim 46, wherein the introducing of the transgene into the embryo is by infecting the embryo with a retrovirus containing the transgene.

50. A method for identifying an agent that modulates the expression or activity of BACE1, said method comprising:

administering an agent to be tested to an organism; and

comparing the phenotype of the organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent, whereby a phenotype substantially equal to the BACE1-knockout organism is indicative of an agent that modulates BACE1 expression or activity.

51. The method of claim 50, wherein the organism is a transgenic organism.

52. The method of claim 51, wherein the transgenic organism is transgenic for overexpression of BACE1; APP expression; Aβ1-40, Aβ1-42, Aβ11-40, Aβ11-42 expression; or a combination thereof.

53. The method of claim 50, wherein the expression of BACE1 is detected by measuring the amount of BACE1 polynucleotide in the organism.

54. The method of claim 53, wherein the BACE1 polynucleotide is RNA or DNA.

55. The method of claim 54, wherein the RNA is mRNA.

56. The method of claim 50, wherein the activity of BACE1 is detected by measuring BACE1 cleavage of APP.

57. The method of claim 50, wherein the phenotype of the organism is associated with Alzheimer's disease.

58. The method of claim 57, wherein the phenotype associated with Alzheimer's disease is characterized as having a phenotype of impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

59. A method for screening for an agent, which ameliorates symptoms of Alzheimer's disease, said method comprising comparing an effect of an agent on an organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent,

wherein the organism has a phenotype associated with Alzheimer's disease, and wherein an agent which ameliorates said phenotype is identified by having a substantially equal or superior phenotype of the organism in comparison with the BACE1-knockout organism.

60. The method of claim 59, wherein the phenotype of the organism is characterized as having a phenotype of impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

61. The method of claim 59, wherein the organism is a transgenic organism.

62. The method of claim 59, wherein the phenotype is measured by assessing an organism's performance on memory and learning tests.

63. The method of claim 59, wherein the phenotype is measured by assessing the neuropathology in a cortico-limbic region of the brain.

64. A method for screening for an agent, which ameliorates symptoms of Alzheimer's disease, said method comprising comparing an effect of an agent on a transgenic organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent,

wherein the transgenic organism is characterized as having a phenotype of impaired performance on memory learning tests or abnormal neuropathology in a cortico-limbic region of the brain and the BACE1-knockout organism has a phenotype of reduced expression of BACE1, and

wherein the impaired performance and the abnormal neuropathology are in compared with the BACE1-knockout organism,

whereby an agent which ameliorates the symptoms is identified by substantially equal or superior performance of the transgenic organism as compared with the BACE1-knockout organism on the memory and learning tests.

65. A kit useful for the detection of an Aβ11-40/42 accumulation disorder comprising carrier means containing therein one or more containers wherein a first container contains a nucleic acid probe that hybridizes to a nucleic acid sequence BACE1 or an antibody probe specific for BACE1 or Aβ11-40/42.

66. The kit of claim 65, wherein the probe comprises a detectable label.

67. The kit of claim 65, wherein the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

68. A method for predicting the therapeutic effectiveness of a compound for treating Alzheimer's disease in a subject comprising measuring the accumulation of AB11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide before and after treatment with the compound,

wherein a decrease in accumulation of peptide fragments or a decrease in the level of BACE1 polynucleotide or polypeptide after treatment is indicative of a compound that is effective in treating the disease.

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69. A method for monitoring the progression of Alzheimer's disease comprising
measuring the accumulation of AB11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide at a first time point and a second time point, thereby monitoring the progression of the disease.

70. An substantially purified antibody that specifically binds a beta-site APP-cleaving enzyme 1 (BACE1) polypeptide or an epitopic determinant thereof.

71. The antibody of claim 70, which is present in an antiserum.

72. The antibody of claim 70, which comprises polyclonal antibodies.

73. The antibody of claim 70, which is a monoclonal antibody.

74. The antibody of claim 70, wherein the epitopic determinant comprises amino acid residues 46 to 164 of BACE1.

75. A method of detecting a beta-site APP-cleaving enzyme 1 (BACE1) polypeptide in a sample, the method comprising contacting the sample with the antibody of claim 70 under conditions that allow specific binding of the antibody to BACE1 or an epitopic determinant thereof, and detecting specific binding of the antibody to a component of the sample.

76. The method of claim 75, wherein the sample is a tissue sample, which is obtained from a subject.

77. The method of claim 76, wherein the tissue sample is a brain tissue sample.

78. The method of claim 76, wherein the subject has or is suspected of having a disorder associated with an accumulation of amyloid plaques.

79. The method of claim 78, wherein the disorder is Alzheimer's disease.

80. The method of claim 75, wherein the antibody comprises a detectable label, and wherein said detecting specific binding comprises detecting the label.

81. The method of claim 75, further comprising contacting the sample with a reagent that specifically binds the antibody, wherein detecting specific binding of the antibody comprises detecting specific binding of the reagent.

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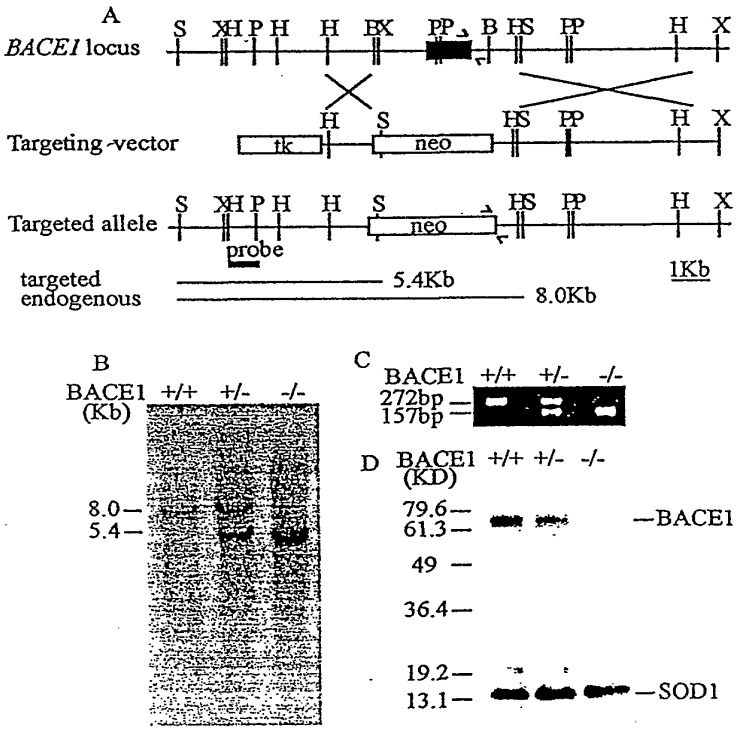

A
BACE1 locus
S XH P H H RX PP B HS PP H X
Targeting vector
H S HS PP H X
tk
neo
Targeted allele
S XH P H H S HS PP H X
neo
probe
1Kb
targeted
5.4Kb
endogenous
8.0Kb
B
BACE1 +/+ +/- -/-
(Kb)
8.0
5.4
C
BACE1 +/+ +/- -/-
272bp
157bp
D BACE1 +/+ +/- -/-
(KD)
79.6
61.3
49
36.4
19.2
13.1
—BACE1
—SOD1

FIGURE 1

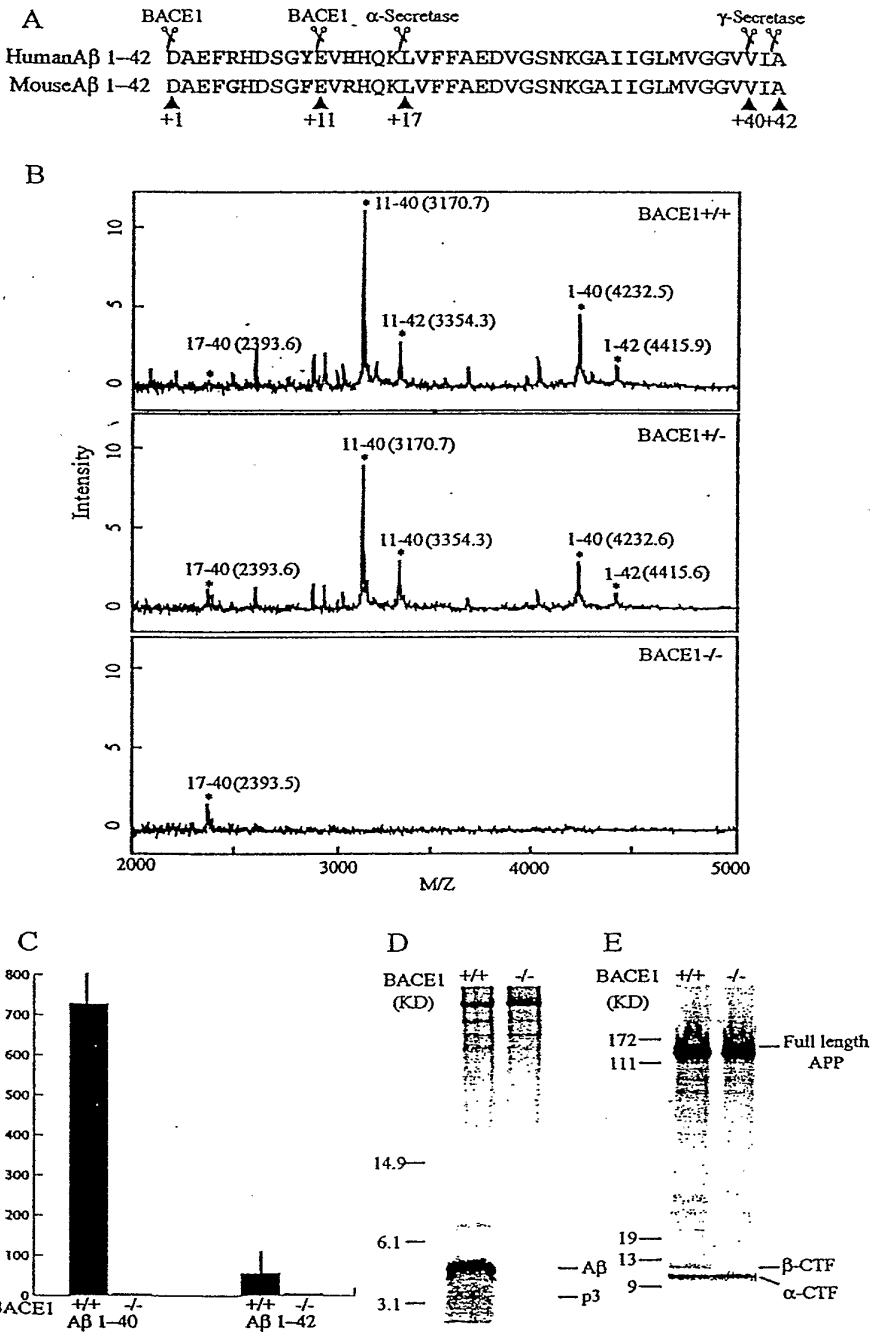

A
BACE1
BACE1
α-Secretase
γ-Secretase
HumanAβ 1–42 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
MouseAβ 1–42 DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
+1
+11
+17
+40
+42
B
11-40 (3170.7)
BACE1+/+
1-40 (4232.5)
11-42 (3354.3)
17-40 (2393.6)
1-42 (4415.9)
BACE1+/-
11-40 (3170.7)
11-40 (3354.3)
1-40 (4232.6)
17-40 (2393.6)
1-42 (4415.6)
BACE1-/-
17-40 (2393.5)
Intensity
M/Z
2000
3000
4000
5000
C
pg/ml
BACE1 +/+ -/- +/+ -/-
Aβ 1–40
Aβ 1–42
D
BACE1 (KD)
+/+ -/-
14.9
6.1
3.1
E
BACE1 (KD)
+/+ -/-
172
111
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13
9
Aβ
p3
β-CTF
α-CTF

FIGURE 2

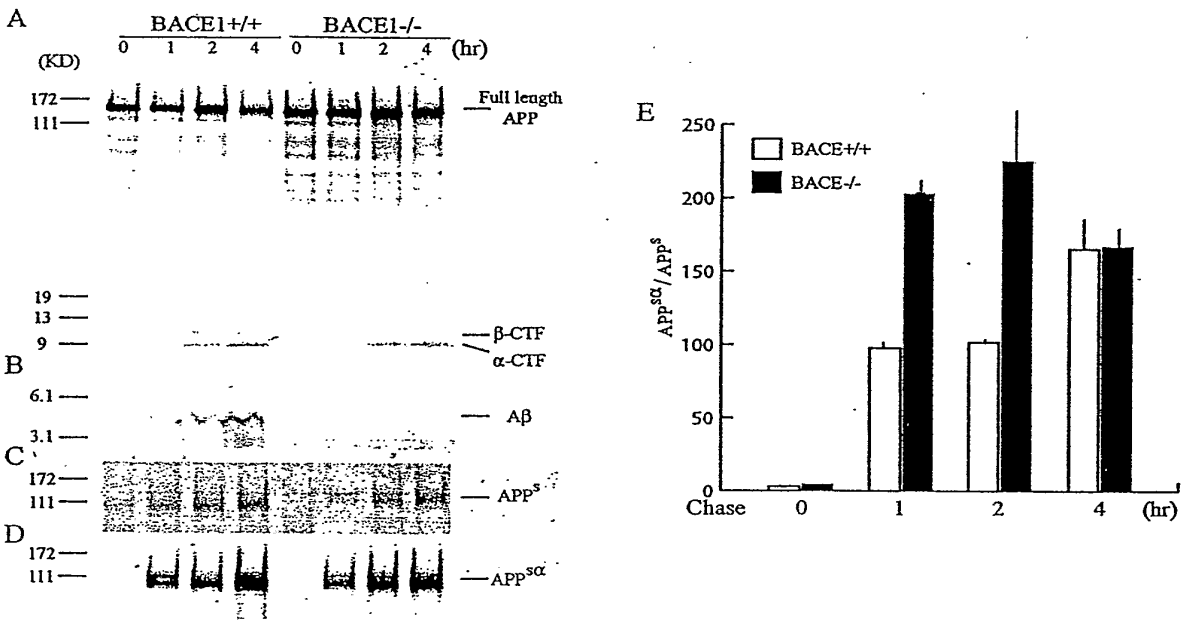

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BACE1+/+
BACE1-/-
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(KD)
172
111
Full length APP
19
13
9
β-CTF
α-CTF
B
6.1
3.1
Aβ
C
172
111
APPs
D
172
111
APPsα
E
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100
50
0
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BACE-/-
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FIGURE 3

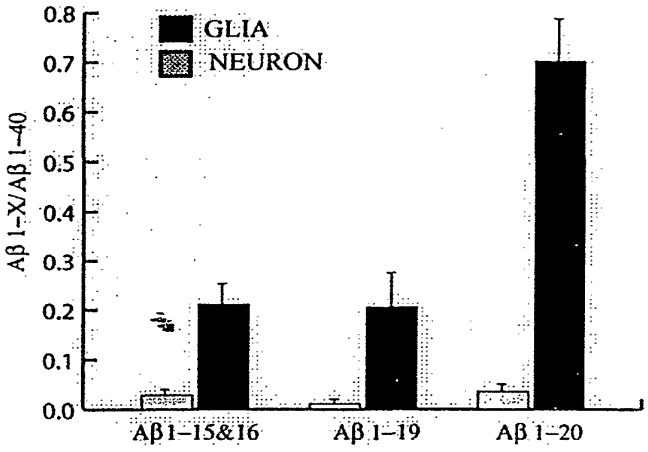

GLIA
NEURON
Aβ 1-X/Aβ 1-40
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Aβ1-15&16
Aβ 1-19
Aβ 1-20

FIGURE 4

SEQUENCE LISTING

<110> THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE
WONG, Philip
CAI, Huaibin
PRICE, Donald

<120> BETA-SECRETASE TRANSGENIC ORGANISMS, ANTI-BETA-SECRETASE ANTIBODIES, AND METHODS OF USE THEREOF

<130> JHU1690WO

<150> US 09/708,096
<151> 2000-11-03

<150> US 60/244,051
<151> 2000-10-27

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(54) Title: METHODS FOR PREDICTING WHETHER SUBJECTS WITH MILD COGNITIVE IMPAIRMENT (MCI) WILL DEVELOP ALZHEIMER'S DISEASE

(57) Abstract: The invention provides methods of diagnosing mild cognitive impairment (MCI) in a patient and methods of predicting cognitive decline in patients who have MCI. The invention also provides methods for predicting if a patient who has MCI will develop Alzheimer's Disease (AD).

METHODS FOR PREDICTING WHETHER SUBJECTS WITH MILD COGNITIVE IMPAIRMENT (MCI) WILL DEVELOP ALZHEIMER'S DISEASE

This application is related to and claims the benefit of U.S. Provisional Application Serial No. 60/424,628 filed November 7, 2002, the disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

The invention relates to methods of diagnosing mild cognitive impairment (MCI) in a patient and predicting the rate of cognitive decline in patients who have MCI. Specifically, the invention relates to methods of predicting if a patient who has MCI will develop Alzheimer's Disease (AD). More specifically, the invention relates to determining if a patient with MCI is likely to develop AD comprising measuring the level of tau phosphorylated at Threonine 231 ($p\text{-}tau_{231}$) in the cerebrospinal fluid (CSF) of the patient.

BACKGROUND OF THE INVENTION

Cross-sectional Alzheimer's Disease (AD) studies typically demonstrate elevated Cerebrospinal fluid (CSF) levels of the microtubule associated protein, tau (Vanmechelen *et al.*, 2001, *Mech. Aging Dev.* 122:2005-2011). Surprisingly, in spite of the well characterized progressive clinical decline and the increasing topography of brain involvement, equal numbers of studies show that the CSF tau levels in AD are progressive (Arai *et al.*, 1997, *JAGS* 45:1228-1231; Blomberg *et al.*, 1996, *Neurosci. Lett.* 214:163-166; Hampel *et al.*, 2001, *Ann. Neurol.* 49:545-546; Kanai *et al.*, 1999, *Neurosci. Lett.* 267:65-68) as are not (Andreasen *et al.*, 1999, *Neurology* 53:1488-1494; Nishimura *et al.*, 1998, *Methods Find. Exp. Clin. Pharmacol.* 20:227-235; Sunderland *et al.*, 1999, *Biol.*

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Psychiatry 46:750-755; Tapiola *et al.*, 2000, *Neurosci. Lett.* 280:119-122). Thus, whether the CSF tau level reflects neuronal damage is unclear from these studies.

Phosphorylation at threonine 231 of tau has been shown to be specific for AD and to precede assembly of paired helical filaments in human brain biopsy tissue (Vincent *et al.*, 1998, *Neurobio. Aging* 19:87-97). AD is the leading cause of dementia, and patients with AD have elevated CSF p-tau$_{231}$ levels compared with control subjects (Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190).

Mild Cognitive Impairment (MCI) is a condition characterized by mild recent memory loss without dementia or significant impairment of other cognitive functions that are beyond what is normal for age or educational background. Many patients with MCI progress to AD. Two recent mild cognitive impairment (MCI) studies using the diagnostically more specific measure of hyperphosphorylated tau (p-tau), reported cross-sectional elevations, but did not observe longitudinal change (Arai *et al.*, 2000, *Exp. Neurol.* 166:201-203; Buerger *et al.*, 2002, *Neurology* 59: 627-629). Since MCI is a major risk factor for Alzheimer's Disease (AD) (Petersen *et al.*, 1995, *JAMA* 273:1274-1278), predicting cognitive decline in patients with MCI is highly desirable to effectively determine which patients might benefit from disease-modifying, anti-dementia drugs. The invention provides methods for predicting which patients are likely to develop AD by measuring the levels of p-tau$_{231}$ in the CSF.

SUMMARY OF THE INVENTION

The invention provides methods of diagnosing mild cognitive impairment (MCI) in a patient and methods of identifying patients who are likely to cognitively deteriorate and develop dementia. Specifically, the invention provides a method of predicting whether a patient will develop Alzheimer's Disease (AD), particularly a patient who has MCI. The invention also

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provides a method for determining the stage of neuronal degeneration in a patient who has AD comprising measuring the level of p-tau$_{231}$ in the CSF of the patient with AD.

In certain aspects, the method of the invention comprises taking a sample of cerebrospinal fluid (CSF) from a patient and measuring the level of phosphorylated tau (p-tau) protein in the CSF, wherein a level of p-tau that is at least about 215 pg/ml is indicative of a patient who will develop or has AD. Preferably, the level is at least about 250 pg/ml. More preferably, the level is at least about 300 pg/ml. Most preferably, the level is at least about 350 pg/ml.

In another aspect, the method further comprises determining the stage of cognitive deterioration of the patient by comparing the level of CSF p-tau with the level of CSF p-tau obtained from at least one control patient, wherein the greater the difference between the level of CSF p-tau from the patient and the level of CSF p-tau from the control patient, the greater the risk of developing AD.

In some aspects, the p-tau can be a tau protein that is phosphorylated at any of amino acids 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, and 422. Preferably, the methods of the invention measure the level of tau phosphorylated at threonine 231 (p-tau$_{231}$), 181, 396, or 404. Most preferably, the methods of the invention measure the level of p-tau$_{231}$.

The invention also provides a method of predicting the rate of neuronal degeneration in a patient comprising the step of determining the level of tau protein phosphorylated at threonine 231 (p-tau$_{231}$) in a sample of cerebrospinal fluid (CSF) taken from the patient, wherein the patient has Alzheimer's Disease.

The invention also provides methods of identifying a patient who is likely to develop Alzheimer's Disease comprising: (a) obtaining a biological sample from a patient; (b) determining the level of phosphorylated tau protein (p-tau) in the biological sample; (c) identifying the patient as being

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likely to develop Alzheimer's Disease if the level of p-tau in the biological sample is about 617 pg/ml or higher.

In addition, the invention provides methods of predicting cognitive decline in a patient, comprising the steps of: (a) obtaining a biological sample from a patient; (b) determining the level of phosphorylated tau protein (p-tau) in the biological sample; (c) identifying the patient as being likely to cognitively decline if the level of p-tau in the biological sample is about 143 pg/ml or higher.

Further, the invention provides methods of diagnosing Alzheimer's Disease in a patient, comprising the steps of: (a) obtaining a biological sample from a patient; (b) determining the level of p-tau protein in the biological sample; and (c) diagnosing the patient as having Alzheimer's Disease if the level of p-tau in the biological sample is about 215 pg/ml or higher.

The invention also provides methods of monitoring cognitive decline in a patient comprising the steps of: (a) determining the level of p-tau protein in a biological sample obtained from the patient; (b) determining the p-tau load in the sample by adjusting the p-tau level using a ventricular volume correction; (c) repeating steps (a) and (b) using a subsequently-collected biological sample obtained from the patient; and (d) comparing the p-tau load determined in step (b) with the amount of the p-tau load determined in step (c) and therefrom monitoring cognitive decline in the patient.

The invention also provides methods of measuring the effectiveness of a pharmaceutical composition as an agent for treating a patient having a condition associated with dementia, comprising the steps of: (a) determining the level of p-tau protein in a biological sample obtained from the a patient; (b) administering an amount of a pharmaceutical composition to the patient; (c) repeating step (a) using a subsequently-collected biological sample obtained from the patient; (d) comparing the level of p-tau protein determined in step (a) with

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the level of p-tau protein determined in step (c), wherein the effectiveness of the pharmaceutical composition is monitored by detecting no change in the level or p-tau or a decrease in the level of p-tau in the subsequently-collected biological sample compared with the biological sample from step (a).

In certain aspects, the methods of the invention can be used to identify patients in need of Alzheimer's therapy. For example, once a patient is identified, by a method of the invention, as having or likely to develop Alzheimer's Disease, or is identified as having MCI, or is identified as being likely to cognitively decline, that patient can be subjected to a therapeutic regime for treating Alzheimer's or other dementias. An Alzheimer's therapy can include, for example, administering an anti-dementia drug to the patient. A non-limiting example of an anti-dementia drug is an anticholinesterase inhibitor (also referred to herein as an anticholinesterase drug).

Also, the invention provides methods of predicting a rate of neuronal degeneration in a patient comprising determining the level of phosphorylated tau (p-tau) protein in a sample of cerebrospinal fluid (CSF) taken from the patient, wherein the patient has Alzheimer's Disease.

In certain aspects, p-tau, as used herein, refers to tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

In other aspects, a biological sample used in methods of the invention is cerebrospinal fluid (CSF).

Specific preferred embodiments of the invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a graph showing the correlation between level of p-tau_{231} in CSF at baseline and annual point loss in MMSE score for 77 patients with mild cognitive impairment (MCI) who were observed during a longitudinal study.

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Figure 2, left panel, shows the right hippocampus (hc) and amygdala (ag) manually outlined in a sagittally oriented volumetric MRI section illustrating the localization of both structures. Figure 2, right panel, shows a view from right anterior-superior on the right hippocampus and amygdala after manual editing. The depicted MRI scan stems from an AD patient. Accordingly the head of the hippocampus and the amygdala show severe atrophy, particularly on the right hand side of the figure.

Figure 3 depicts a graph showing that higher levels of CSF tau proteins [pg/ml] correlate with higher baseline volumes of right and left hippocampus [mm^3] in AD patients.

Figure 4 depicts a graph showing that higher CSF p-tau_{231} levels at baseline [pg/ml] correlate with higher annual rates of right hippocampus atrophy [mm^3/year] derived from a mixed effects regression model in AD patients.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Kohnken *et al.* developed an immunoassay designed to specifically detect tau protein phosphorylated at threonine 231 (p-tau_{231}) that can discriminate between patients with AD and control subjects with high accuracy (*see* Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190). Using the immunoassay, the group also demonstrated that concentrations of p-tau in the cerebrospinal fluid (CSF) correlate with disease progression in individual patients (Hampel *et al.*, 2001, *Ann. Neurol.* 49:545-546), suggesting that p-tau_{231} is a potential biomarker of AD progression. Neuropathologic evidence demonstrated that phosphorylation of tau at threonine 231 occurs very early in AD pathophysiology (Vincent *et al.*, 1998, *Neurobiol. Aging* 19:287-296). As described herein, p-tau_{231} concentrations can also be used to identify individuals who are likely to develop Alzheimer's Disease, particularly individuals who have been previously diagnosed as having mild cognitive impairment (MCI).

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As demonstrated in the Examples, p-tau$_{231}$ levels are elevated in the cerebrospinal fluid (CSF) of patients who have mild cognitive impairment (MCI) compared with healthy control patients (HC). High p-tau$_{231}$ levels at baseline (*i.e.* p-tau$_{231}$ levels at the beginning of the study) correlated with the annual point loss in Mini-Mental State Examination (MMSE) scores in patients with MCI, while no correlation was observed with annual point loss in MMSE and total tau (t-tau) levels, which is a nonspecific marker of neurodegeneration (Blennow *et al.*, 2001, *Mol. Neurobiol* 24:87-97). A subgroup of the MCI patients in the studies described in the Examples converted to Alzheimer's Disease (AD). Consistent with the analysis of rates of cognitive decline, increased levels of p-tau$_{231}$ correlated with conversion to AD, while t-tau levels did not.

The literature shows considerable difficulty in detecting tau concentration changes during the course of AD and in MCI (Arai *et al.*, 2000, *Exp. Neurol.* 166:201-203; Buerger *et al.*, 2002, *Neurology* 59: 627-629). In a review of CSF proteins, Reiber observed a higher ventricular to lumbar level ratio for brain-derived proteins (*e.g.* tau 1.5:1 and S- 100B 18:1) with lower ratios for systemic derived proteins (*e.g.* albumin 1:205) (Reiber, 2001, *Clin. Chim. Acta.* 310:173-186). Amyloid beta (Aβ) levels are higher in lumbar than ventricular CSF (1:2) possibly reflecting its central and peripheral sources. In one embodiment, the invention provides a method for monitoring CSF p-tau levels longitudinally (*i.e.* over a period of time) comprising the step of determining the level of phosphorylated tau protein (p-tau) at various time points and adjusting the level of p-tau at each time point based on the ventricular volume. The level adjustment is referred to herein as a "ventricular volume correction."

The ventricular volume correction is accomplished by measuring the level of p-tau in the CSF of a patient, multiplying the p-tau concentration (pg/ml) by the ventricular volume (ml) and dividing by 1000. Ventricular volume can be

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determined, for example, by acquiring magnetic resonance imaging (MRI) scans from a patient and converting the MRI signal intensities to ventricular volume using an automated protocol as described by Fox and Freeborough (1997, *J. Mag. Reson. Imag.* 7:1069-1075). An adjusted p-tau level is referred to herein as a p-tau load. As demonstrated in Example 3 below, when the adjustment for ventricular volume was made, the p-tau$_{231}$ load showed a significant longitudinal group-by-time interaction. The follow-up tests showed that the p-tau$_{231}$ load increased in patients with MCI, but did not change in the control group. Moreover, regression analyses of the longitudinal data showed that measuring p-tau$_{231}$ load changes significantly improved the prediction of group membership over measuring p-tau$_{231}$ level changes.

The rationale for using the ventricular volume correction is based on the understanding that tau levels are higher in the ventricular CSF than in the lumbar CSF. As demonstrated herein, ventricular volume correction is useful only for the p-tau$_{231}$ measurement. Thus, even though both Aβ40 and p-tau$_{231}$ levels are elevated in MCI, after applying the ventricular volume correction, a longitudinal change (*i.e.* change over time) is observed only for the p-tau load in the MCI group.

In one embodiment, the invention provides a method that comprises using the ventricular volume correction to monitor cognitive decline in a patient. The method comprises the steps of determining the p-tau load, as described above and in the Examples, in a biological sample (preferably CSF) from the patient at various time points. For example, samples can be obtained upon diagnosis of a neurological disorder, such as mild cognitive impairment (MCI) or Alzheimer's Disease (AD), and at various times subsequent to diagnosis, such as every 6 months or annually. Monitoring cognitive decline allows a care provider to determine if a patient is responding well to certain treatments.

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In another embodiment, the level of p-tau can be measured in a patient at various times during treatment for MCI or a condition associated with dementia. For example, levels of p-tau, preferably p-tau_{231}, can be measured at the beginning of treatment with anti-dementia drugs, such as anticholinesterase drugs. Non-limiting examples of anticholinesterase drugs include tacrine, donepezil, rivastigmine, and galantamine. The level of p-tau can then be monitored at various times, such as weekly, monthly, bi-monthly, or any other chosen time periods, during treatment to determine if the drugs are effective. An effective drug will cause the level of p-tau to decrease over time. Alternatively, an effective drug will be identified if the level of p-tau does not increase over time. Non-limiting examples of conditions associated with dementia include, but are not limited to, Alzheimer's Disease (AD), frontotemporal dementia (FTD), vascular dementia, Lewy body dementia, or other neuorological disorders.

In one embodiment, the invention provides a method for identifying a patient who has or is likely to develop AD. In a particular embodiment, the method comprises the steps of taking a biological sample (preferably a sample of CSF) from a patient, determining the p-tau level (preferably p-tau_{231}) in the biological sample, and diagnosing the patient with AD if the p-tau level is about 215 pg/ml or higher. The patient can be an individual who has been diagnosed as having mild cognitive impairment (MCI) or any form of dementia, such as frontotemporal dementia (FTD), vascular dementia, Lewy body dementia, or other neuorological disorders. Alternatively, the patient can be an individual who desires or requires screening for AD but who has not been diagnosed with any neurological disorders, dementias, or MCI.

In another embodiment, a method of the invention comprises the steps of taking a biological sample (preferably a sample of CSF) from a patient, determining the p-tau level (preferably p-tau_{231}) in the biological sample, and identifying the patient as

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being likely to develop AD if the level of p-tau in the sample is about 617 pg/ml or higher. In this embodiment, the level of p-tau indicates a patient who is likely to convert to AD from a more mild neurological disorder. For example, the patient can be diagnosed as having MCI prior to being subjected to a method of the invention.

In yet another embodiment, the invention provides a method of predicting cognitive decline in a patient comprising the steps of taking a biological sample (preferably a sample of CSF) from a patient, determining the p-tau level (preferably p-tau_{231}) in the biological sample, and identifying the patient as being likely to develop AD if the level of p-tau in the sample is about 143 pg/ml or higher. Preferably the patient has been diagnosed as having MCI.

In another embodiment, a method of the invention comprises the steps of taking a biological sample (preferably a sample of CSF) from a patient, determining the p-tau_{231} level in the biological sample, and associating the p-tau_{231} level with a particular stage of cognitive decline. In another embodiment, the p-tau_{231} level can be adjusted to determine the p-tau_{231} ventricular load by correcting the level for CSF ventricular volume, and the p-tau_{231} load can be associated with a particular stage of cognitive decline.

Any biological sample, such as tissues, cells, or body fluids, can be used in a method of the invention, so long as the sample comprises phosphorylated tau (p-tau) protein. In a preferred embodiment, the biological sample used in any method of the invention is cerebrospinal fluid (CSF). Samples of CSF can be collected by any means commonly used by those skilled in the art. For example, CSF can be collected by lumbar puncture using fluoroscopy to guide a beveled LP needle into the subarachnoid space.

The level of phosphorylated tau proteins (p-tau) can be determined in a method of the invention using, for example, an immunoassay or protein-binding assay. Preferably, p-tau_{231}

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levels are measured as described herein, using a sandwiched enzyme-linked immunosorbent assay (ELISA) to capture tau protein, then detecting phosporylation of tau with a phosphorylated tau specific antibody (*e.g.* CP9, which is an antibody specific for p-tau_{231}) (see Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190).

A ventricular volume correction can be accomplished as described above to determine the p-tau_{231} load. The ventricular volume can be determined, for example, as described in Example 3 below.

In one embodiment, a method of the invention can be used to determine if a patient has MCI or AD, based on the ventricular p-tau_{231} load. In some cases, the patient can be previously diagnosed as having MCI, and the method of the invention can be used to determine the likelihood of the condition to progress to AD. In another embodiment, a patient can be examined at follow-up intervals to assess the progression of MCI.

The methods of the invention can be used alone or in combination with any known diagnostic test(s) and/or marker(s) for diagnosing AD or MCI or for identifying or monitoring cognitive decline, including, but not limited to, mini mental status examination (MMSE), global deterioration scale (GDS) scores, age, amyloid beta, psycohometric test battery (Kluger *et al.* 1999, *J. Geriat. Psychiat. Neurol.* 12:168-179), the Guild Memory Test, the Wechsler adult intelligence scale test, magnetic resonance imaging (MRI), and APOE genotype. Combination with methods of the invention can increase the sensitivity and decrease the number of false positives of known tests and markers for AD, MCI, and cognitive decline.

In one embodiment, the invention provides methods for examining neuronal degeneration in patients having Alzheimer's disease (AD). AD is characterized by progressive loss of specific neuron populations. The temporal sequence of regional neuron loss is not yet well established. Neuropathological and structural imaging evidence, however, suggests that allocortical

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areas, including entorhinal cortex, hippocampus and amygdala, are among the earliest affected brain regions in AD.

In clinical-pathological studies, measurement of hippocampal volume based on magnetic resonance imaging (MRI) accounts for about 80% of variability in hippocampal neuron density in AD. These findings support the notion that *in vivo* measurement of hippocampus volume can serve as an indirect measure for the extent of allocortical neuronal degeneration in AD. Magnetic resonance imaging (MRI) studies showed significant atrophy of the hippocampus even in preclinical stages of AD. Hippocampus atrophy is the most extensively investigated marker of intra-individual structural disease progression in AD in longitudinal MRI studies.

In one embodiment, the invention provides an *in vivo* surrogate measure of AD-related neuronal destruction comprising measuring the level of p-tau$_{231}$ in CSF of patients who have AD. As described in Example 4 below, baseline hippocampus volumes were significantly reduced in AD patients compared to controls ($p < 0.005$). CSF p-tau$_{231}$, but not t-tau, concentrations at baseline correlated with rates of atrophy of left and right hippocampus ($p < 0.02$). In addition, there was a significant positive correlation between tau-proteins and baseline hippocampus volumes ($p < 0.03$). The rates of cognitive decline correlated significantly with rates of hippocampus atrophy ($p < 0.05$).

The following Examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention. The present invention is not to be limited in scope by the exemplified embodiments, which are intended as illustrations of individual aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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EXAMPLES

Example 1

CSF P-tau$_{231}$ correlates with cognitive decline in Mild Cognitive Impairment (MCI) subjects

Seventy-seven subjects having symptoms of mild cognitive impairment (MCI) were selected for a longitudinal study to analyze various biomarkers for MCI. Thirty-nine of the MCI subjects cognitively deteriorated during follow-up (MCI decliners; annual point loss in Mini-Mental State Examination (MMSE) score of <0). MMSE was assessed as described in Folstein *et al.*, 1975, *J. Psyshiatr. Res.* 12:189-198. Twenty-six of the MCI decliners converted to probable Alzheimer's Disease (AD) (NINCDS-ADRDA criteria; McKhann *et al.*, 1984, *Neurology* 34:939-44). For group comparisons, 55 patients with probable AD and 30 healthy control subjects (HC) were also analyzed. Data for patients with AD and HC were collected at baseline.

Patients fasted overnight, and samples of cerebrospinal fluid (CSF) were collected from each patient using fluoroscopy to guide a 22 gauge beveled LP needle. Phosphorylated tau (p-tau) protein levels were measured by an enzyme-linked immunosorbent assays (ELISA) as described in Kohnken *et al.* (Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190) using an anti-p-tau$_{231}$ antibody (Molecular Geriatrics Corporation, Vernon Hills, IL, USA). Levels of t-tau were measured by ELISA using the Innotest hTau kit (art. no. K-1032; Innogenetics, Zwjindrecht, Belgium). Data were reported as means ±SD. The APOE genotype was determined according to standard procedures.

The yearly point loss in MMSE score was modeled with stepwise multiple linear regression to generate a multiple regression model (see Figure 1). The independent variables were baseline p-tau$_{231}$ levels, MMSE score, age, as well as sex, APOE genotype as a two-level variable (0 vs 1/2 ε4 alleles), center, treatment with cognitive enhancers, and time of follow-up. In a second multiple linear regression model, p-tau$_{231}$ was replaced by

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t-tau. To establish cutoff levels for tau proteins to differentiate between MCI decliners and HC, sensitivity and specificity levels were set at ≥80% as recommended by a consensus report from The Ronald and Nancy Reagan Research Institute on Aging Working Group, (1998, *Neurobiol. Aging* 19:109-116).

Characteristics of patients and HC are given in Table 1. p-tau$_{231}$ and t-tau levels were higher ($p < 0.001$) in patients with MCI than in HC (see Table 1). For a subgroup matched for age ($p = 0.27$) of 30 patients with MCI (age, 63.6 ± 4.4 years) and 30 HC, differences in baseline levels of p-tau$_{231}$ (age-matched patients with MCI, 430 ± 436 pg/mL; HC, 78 ± 114 pg/mL) and t-tau (age-matched patients with MCI, 508 ± 243 pg/mL; HC, 247 ± 137 pg/mL) remained stable ($p < 0.001$).

Table 1

Group (no. of subjects)	Mean age in y ± SD* (range)	Females/Males, n	Mean MMSE score ± SD† (range)	Mean CSF p-tau$_{231}$ level, pg/mL ± SD‡	Mean CSF t-tau level, pg/mL ± SD§
AD (55)	71.0 ± 6.7 (53-84)	31/24	18.0 ± 2.9 (13-22)	710 ± 373	721 ± 352
MCI (77)	71.4 ± 7.9 (54-87)	38/39	28.1 ± 1.8 (23-30)	501 ± 400	578 ± 258
HC (30)	61.3 ± 10.2 (44-79)	15/15	29.3 ± 0.7 (27-30)	78 ± 114	247 ± 137

* Differences in age between groups: AD vs HC, $p < 0.001$; MCI vs HC, $p < 0.001$.
† Differences in MMSE score between groups: AD vs HC, $p < 0.001$; AD vs MCI, $p < 0.001$; MCI vs HC, $p < 0.001$.
‡ Differences in CSF p-tau$_{231}$ levels between groups: AD vs HC, $p < 0.001$; AD vs MCI, $p < 0.001$; MCI vs HC, $p < 0.001$.
§ Differences in CSF t-tau levels between groups: AD vs HC, $p < 0.001$; AD vs MCI, $p = 0.037$; MCI vs HC, $p < 0.001$.

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Thirty-nine patients with MCI cognitively deteriorated over time (MCI decliners: p-tau$_{231}$ level, 590 ± 414 pg/mL; t-tau level, 611 ± 260 pg/mL); 38 patients with MCI had no cognitive decline (p-tau231 level, 409 ± 368 pg/mL; t-tau level, 544 ± 254 pg/mL). P-tau$_{231}$ levels were higher ($p = 0.037$) in patients with MCI who converted to AD during follow-up (MCI converters; 617 ± 378 pg/mL) than in nonconverters (442 ± 402 pg/mL). No difference in t-tau levels ($p = 0.20$) between MCI converters (615 ± 212 pg/mL) and nonconverters (559 ± 278 pg/mL) was observed.

Tau protein levels were lower in the MCI group than in patients with AD (p-tau231 level, $p < 0.001$; t-tau level, $p = 0.037$). No difference was found between patients with AD and MCI decliners for p-tau231 levels ($p = 0.13$) and t-tau levels ($p = 0.26$). The limits of the 95% CI for the group difference ranged from -43 pg/mL to 281 pg/mL for p-tau$_{231}$ levels and from -22 pg/mL to 242 pg/mL for t-tau levels.

For the 77 patients with MCI followed longitudinally, the mean cognitive decline ± SD, as measured by point loss in the MMSE score, was -1.7 ± 4.0 points per year.

A significant correlation between CSF p-tau$_{231}$ levels at baseline and the annual point loss in MMSE score in the MCI group was observed, both in the single effect analysis (Spearman $\rho = -0.30$; $p < 0.01$; Figure 1) and after controlling for covariates in the multiple regression model ($\beta = -0.23$; $p = 0.049$). Exclusion of three outliers with a very high annual point loss in MMSE score did not change statistical results (Spearman $\rho = -0.23$; $p = 0.049$). Higher CSF t-tau levels at baseline did not correlate with a higher annual point loss in MMSE score (Spearman $\rho = -0.19$; $p = 0.10$).

In addition, in the multiple regression model, older age at baseline ($\beta = -0.31$; $p < 0.01$) and APOE-ϵ4 carrier status ($\beta = -0.39$; $p < 0.01$) were predictors of cognitive decline. MMSE score at baseline, sex, therapy, time of follow-up, or center

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did not add a significant amount of explanatory power. The model with p-tau231, APOE-ϵ4 carrier status, and age explained 27% of variance of the annual point loss in the MMSE score. When p-tau$_{231}$ was replaced by t-tau in the multiple regression model, only age at baseline ($p < 0.01$) and APOE-ϵ4 carrier status ($p < 0.001$) were predictors of cognitive decline.

The Ronald and Nancy Reagan Research Institute on Aging Working Group concluded in a published consensus report (1998, *Neurobiol. Aging* 19:109-116) that an ideal biomarker for AD should have a sensitivity of ≥80% for detecting AD and a specificity of ≥80% for distinguishing other dementias. At these "a priori" chosen sensitivity and specificity levels of ≥80% (set in accordance with: The Ronald and Nancy Reagan Research Institute on Aging Working Group, 1998, *Neurobiol. Aging* 19:109-116), p-tau$_{231}$ (t-tau) discriminated between MCI decliners and HC with a sensitivity of 82% (82%) and a specificity of 87% (80%). The cutoff level for p-tau$_{231}$ (t-tau) was 143 pg/mL (376 pg/mL).

The data indicated that CSF p-tau$_{231}$ levels were elevated in patients with MCI compared with HC. High p-tau$_{231}$, but not t-tau, levels at baseline correlated with the annual point loss in MMSE scores in patients with MCI. Cognitive decline was chosen as an outcome variable because the objective was to identify subjects who will cognitively deteriorate and develop dementia. A subgroup of the MCI patients in this study converted to AD. Consistent with the analysis of rates of cognitive decline, increased levels of p-tau$_{231}$ correlated with conversion to AD, but t-tau levels did not. Therefore, elevated levels of CSF p-tau$_{231}$ at baseline predict cognitive decline in patients with MCI. The contribution of p-tau$_{231}$ levels to the risk of point loss in the MMSE score for patients with MCI is independent from other established risk factors of cognitive decline.

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Example 2

CSF levels of p-tau$_{231}$ can distinguish AD from other dementias

One-hundred and ninety-two (192) patients with a clinical diagnosis of AD, frontotemporal dementia (FTD), vascular dementia, Lewy body dementia, or other neuorolgical disorder and healthy controls were selected and levels of t-tau and p-tau$_{231}$ in the CSF were determined as described above. The mean CSF levels of p-tau$_{231}$ were significantly elevated in the AD group compared with all other groups. The level of p-tau$_{231}$ discriminated with a sensitivity of 90.2% and a specificity of 80.0% between AD and all non-AD disorders. Moreover, p-tau$_{231}$ levels improved diagnostic accuracy compared with t-tau levels when patients with AD were compared with healthy controls ($P = 0.03$) and demented subjects ($P < 0.001$), particularly those with FTD ($P < 0.001$), but not those with vascular and Lewy body dementias. The cutoff level of p-tau$_{231}$ yielding the best sensitivity and specificity for discriminating patients with AD from those with non-AD disease and healthy controls was about 215 pg/ml. Thus, a p-tau$_{231}$ CSF level of about 215 pg/ml or higher can distinguish AD from non-AD dementias.

Example 3

Ventricular volume of p-tau$_{231}$ increases in patients with mild cognitive impairment

Ten normal elderly volunteers and eight patients (seven MCI and one very mild AD) were subjected at baseline and after 1 year to a screening and diagnostic evaluation that included: medical (history, physical, and laboratory), neurological, psychiatric, and neuropsychological examinations, MRI, and lumbar puncture (LP). The annual evaluations were completed within a 2 month window.

Normal elderly subjects, all volunteers, had global deterioration scale (GDS) scores (assessed as described in Reisberg *et al.*, 1982, *Am. J. Psychiatry* 139:1136-1139) of one or two (differentiated only by subjective reporting of age-

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related memory change). MCI patients, all GDS = 3, showed clinically recognizable memory impairment in the absence of dementia with the cognitive decline corroborated by a close informant. The very mild AD patient had a GDS = 4 and mini mental status examination (MMSE) = 27, as determined using the methods described in Folstein *et al.*, 1975, *J. Psyshiatr. Res.* 12:189-198. At baseline all participants had MMSE scores ≥ 27. Individuals with medical conditions affecting brain structure or function (e.g. stroke, diabetes, head trauma, depression) were excluded.

The psychometric test battery (as described in Kluger *et al.*, 1999, *J. Geriat. Psychiat. Neurol.* 12:168-179) included evaluation of memory (immediate and 10 min delayed recall from the paragraph and the paired associates subtests of the Guild Memory Test) and attention/psychomotor speed (the Digit-Symbol Substitution Test of the Wechsler adult intelligence scale-revised test).

Magnetic resonance imaging (MRI) scans were acquired using a 1.5 T GE Signa imager. A clinical MRI examination covered the entire brain with contiguous 3 mm axial T2- weighted images. The research scan was a 124 sliceT1- weighted Fast-Gradient-Echo acquired in a sagittal orientation as 1.2 mm thick sections (FOV = 25 cm, NEX = 1, matrix = 256 x 128, TR = 35 ms, TE = 9 ms and FA = 60°). Scan signal intensities were normalized with reference to white matter intensity. The baseline and follow-up images were co-registered and reformatted in one sinc interpolation step to a 1.5 mm slice thickness oriented to a standard plane.

A three-dimensional region was constructed to span the baseline and the follow-up ventricular CSF that excluded subarachnoid CSF. Following the technique of Fox and Freeborough (1997, *J. Mag. Reson. Imag.* 7:1069-1075), an automated protocol was applied to the region to segment CSF from surrounding brain tissue at baseline and follow-up. The MRI

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signal intensities were converted to the CSF volume using the partial volume decomposition method.

After fasting overnight, 30 cc of venous blood and 15 cc of clear CSF were collected using fluoroscopy to guide a 22 gauge beveled LP needle. Samples were centrifuged for 10 min at 1500 rpm at 4°C, aliquoted to 0.25 cc polypropylene tubes, and stored at -80°C. All assays were done blind to clinical data and were batch processed.

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect tau phosphorlyated at threonine 231 (p-tau_{231}) in CSF. In this assay, tau was captured with two backbone-directed antibodies, tau-1 and CP-27. The captured tau was then detected by CP9, which is specific for p-tau_{231} (Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190). The detection limit for this assay was 9 pg/ml. The coefficients of variation ranged from 6.0-10.3% (intra-assay) and 11.6-14.4% (inter-assay).

CSF Aβ levels were measured using monoclonal antibody 6E10 (specific to an epitope present on Aβ-16) and to rabbit antisera to Aβ40 and Aβ42, respectively in a double antibody sandwich ELISA (Kohnken *et al.*, 2000, *Neurosci. Lett.* 287:187-190). The detection limit for Aβ40 and Aβ42 was 10 pg/ml. The reproducibility ranged from 8 to 14% (intra-assay) and 10-18% (interassay).

The load of p-tau_{231} (ng) in the CSF was estimated by: multiplying the p-tau_{231} concentration (pg/ml) by the ventricular volume (ml) and dividing by 1000.

Because of high between subject variability for the CSF measures, the Mann-Whitney *U*-test was used to test for cross-sectional differences, and longitudinal group interactions. Significant longitudinal effects were followed up using the Wilcoxon Signed Ranks test to examine within group change. For the other measures, the t-test for independent means was used to examine cross sectional data. Longitudinal effects were evaluated using average group differences between follow-up and

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baseline measures (deltas). Step-wise linear regression models predicting diagnostic group were used to evaluate the unique contribution of the longitudinal dilution correction.

There were no age, education, gender, APOE genotype, or time to follow-up differences found between the normal and MCI groups (see Table 2).

Table 2

Demographic Variables		
Measures	**Groups Normal**	**MCI**
Number of subjects	10	8
Age (years) Baseline	62.5±9.2	69.8±9.2
Male/female	5/5	3/5
ApoEx4/xx	2/8	3/5
Education (years)	16.8±1.7	14.3±3.2
Follow-up (years)	1.3±0.4	1.3±0.2
GDS Baseline**	1.5±0.5	3.1±0.4
GDS Follow-up**	1.6±0.5	3.2±0.5
MMSE Baseline	29.4±0.7	28.5±1.2
MMSE Follow-up*	29.9±0.3	27.1±3.01

*P ≤ 0.05, **P ≤0.01

At both the baseline and the follow-up, both the immediate and delayed paragraph recall were reduced in MCI ($P < 0.05$). No longitudinal change was observed for any neuropsychological measure ($P > 0.05$). One MCI patient converted to very mild AD (GDS = 4, MMSE = 30).

The ventricular volume was approximately 40% greater in MCI at both baseline ($t(16) = -2.1$, $P \leq 0.05$) and at follow-up ($t(16) = -2.2$, $P \leq 0.05$). No longitudinal ventricular changes were observed (see Table 3).

Table 3

Measure	Baseline			Follow-up		
	NL	**MCI**	**%Diff**	**NL**	**MCI**	**%Diff**
VV cc	29.7 ± 9.8	42.3 ± 15.2	42*	32.6 ± 10.3	45.7 ± 14.6	40**
Ptau231 pg/ml	160.0 ± 190.4	534.7 ± 451.8	234*	111.4 ± 158.0	563.9 ± 478.0	406**
Ptau231* VV ng	5.6 ± 9.1	19.7 ± 15.3	252**	4.7 ± 8.6	22.8 ± 17.5	385**+
Aβ 40	9596 ±	12393 ±	29*	8825 ±	11876 ±	35*

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pg/ml	2317	2389		2145	2909	
Aβ 40* VV ng	293.8 ± 128.8	516.1 ± 187.5	76*	285.3 ± 112.0	543.5 ± 241.5	91*
Aβ 42 pg/ml	1015.1 ± 448.3	943.7 ± 486.7	-7	1040.3 ± 413.2	903.7 ± 484.1	-13
Aβ* VV ng	29.9 ± 13.6	41.8 ± 30.3	40	31.8 ± 11.9	42.9 ± 31.1	35

VV, ventricle volume; Ptau, p-tau$_{231}$; Aβ amyloid beta; cc, cubic centimeter

% Diff. = cross-sectional difference in MCI relative to NL, Cross-sectional effects *P≤0.05, **P≤0.01. Longitudinal effects: $^{+}$ = P≤0.05

p-tau$_{231}$ levels were elevated in the MCI group at baseline (U = 14.0, P < 0.05, n = 18) and at follow-up (U = 6.0, P < 0.01, n = 18, see Table 3). Aβ40 levels were also significantly elevated in the MCI group at baseline (U = 13.0, P < 0.05, n = 18) and follow-up (U = 17.0, P < 0.05, n = 18). Aβ42 levels did not differ between the groups. No longitudinal changes were observed for any CSF level measure (P > 0.05).

p-tau$_{231}$ loads were elevated in the MCI group at baseline (U = 11, P < 0.01, n = 18) and at follow-up (U = 6, P = 0.001, n = 18). The Aβ40, but not Aβ42, load was significantly elevated in the MCI group at baseline (U = 15, P < 0.05, n = 18) and follow-up (U = 9, P < 0.01, n = 18). In the longitudinal design, a significant group by time interaction was observed only for the p-tau$_{231}$ load (U = 14.0, P < 0.05, n = 18). Follow-up examination showed a significant p-tau$_{231}$ load increase in the MCI group (Z = -2:1, P < 0.05, n = 8). No longitudinal load effects were observed for the control group.

The longitudinal p-tau load and p-tau level changes were directly compared using two hierarchical linear regression models to predict diagnostic group (reversed orders of entry). Comparing the first entry steps, only the delta p-tau load was related to group membership (R^2 = 0.37, (F[1, 16] = 9.5, P < 0.01). Comparing the second steps, the delta p-tau load

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uniquely increased the variance explained by the delta p-tau level (R^2 change = 0.28, $F[1, 15] = 7.3$, $P < 0.05$).

The cross-sectional data show that two markers of AD pathology (p-tau$_{231}$ and Aβ40) are elevated in MCI. Longitudinally, only the p-tau$_{231}$ increases in MCI, as detected by correcting for the change in ventricle size.

Example 4

p-tau$_{231}$ in the CSF of patient's who have AD

Twenty-two patients with the clinical diagnosis of probable AD according to the NINCDS-ADRDA criteria were selected. For comparison of baseline MRI measures, 21 healthy volunteers were selected. Subjects were recruited from the Department of Psychiatry, Alzheimer Memorial Center and Geriatric Psychiatry Branch, Dementia and Imaging Research Section, Ludwig-Maximilian University Munich, Germany. Subject characteristics are given in Table 4.

Table 4: Subjects' characteristics

Group	Age in years mean [SD][a]/range	f/m[b]	Age at onset mean [SD] /range	MMSE mean [SD][c]/range
Healthy controls (21)	60.8 [± 8.5] 50-79	11/10		29.4 [± 0.7] 28-30
AD patients (22)	67.8 [± 7.9] 53-78	13/9	65 [± 7] 52-74	23.1 [± 4.0] 14-29

a - different between groups, t = 2.8 with 41 df, p < 0.01
b - not different between groups, $\chi^2 = 0.20$ with 1 df, p = 0.66
c - different between groups, Mann-Whitney U = 6.5, p < 0.001;

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Cognitive impairment in the AD patients was assessed using the Mini Mental State Examination (MMSE). Three patients had moderate (10 <= MMSE <20) and 19 patients mild (MMSE >= 20) dementia. Twenty AD patients were studied twice with MRI, 2 AD patients had three MRI scans. In the AD patients, length of observation time ranged between 11.3 and 41.0 months (mean 18.4 months; SD 9.4). The average interscan interval was 17.8 months (SD 9.2), ranging between 11.0 and 41.0 months.

Significant medical co-morbidity in the AD patients and controls was excluded by history, physical and neurological examination, psychiatric evaluation, chest X-ray, ECG, EEG, brain MRI, and laboratory tests (complete blood count, sedimentation rate, electrolytes, glucose, blood urea nitrogen, creatinine, liver-associated enzymes, cholesterol, HDL, triglycerides, antinuclear antibodies, rheumatoid factor, VDRL, HIV, serum B12, folate, thyroid function tests and urine analysis). Two AD patients had mild hypertension, no subject had diabetes. With exception of one patient, all AD patients received state of the art anti-dementive treatment during clinical follow up. Of the 21 treated patients, 15 were treated with an acetylcholine-esterase inhibitor, the remaining 6 patients were treated with other drugs, including Akatinol, Ginkgo biloba or other symptomatic nootropic substances. All subjects or the holders of their Durable Power of Attorney signed consent forms to undergo MRI, lumbar puncture and neuropsychological assessment for clinical investigation and research. The protocol was approved by the Ethical Review Board of the Medical Faculty of the Ludwig-Maximilian University, Munich, Germany.

MRI examinations were performed on a 1.5 T Siemens Magnetom Vision MRI scanner (Siemens, Erlangen, Germany). All subjects were investigated with a volumetric T1 weighted sagittal oriented MRI sequence (TR = 11.6 ms, TE = 4.9 ms, resolution = 0.94 by 0.94 by 1.2 mm).

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Image preprocessing and segmentation was performed at the McConnell Brain Imaging Center of the Montreal Neurological Institute, Montreal, Quebec, Canada. All images were transferred to a Silicon Graphics workstation (Silicon Graphics, Mountain View, CA, USA). Prior to volumetric measurements, all MRI volumes were corrected for image intensity non-uniformities, mapped by linear stereotaxic transformation into coordinates based on Talairach atlas, and resampled onto a 1mm voxel grid. The correction for image intensity has been proven to recover most of the artifacts present in MR images. This preprocessing increases inter- and intra-rater reliability of volumetric measurements and corrects for effects of whole brain atrophy. This is of particular importance when analyzing longitudinal datasets, because reduced measurement reliability at follow up due to greater overall atrophy would lead to systematic non-biological intra-individual effects.

Volumetric analysis was performed with the interactive software package *DISPLAY* developed at the Brain Imaging Center. This program allows simultaneous visualization and segmentation of volumes in coronal, sagittal and horizontal orientations.

The anatomical boundaries used for HC segmentation have been described in detail before. Figure 2 shows the localization and morphology of the hippocampus. In short, the following procedures were employed.

The most posterior part of the HC was defined as the first appearance of gray matter inferiomedial to the trigone of the lateral ventricle (TLV) in the caudorostral extent of the HC. The lateral border in the tail of the HC was the TLV. Medially, the border of the HC was identified by white matter. Moving further anterior, an arbitrary border was defined for the superior and medial border of the HC, in order to differentiate HC gray matter from the gray matter of the Andreas Retzius gyrus, the fasciolar gyrus, and the crus of the fornix. The inferior border of the HC at this point was again identified by white matter.

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In both the HC tail and body, the white matter band at the superolateral level of the HC, the fimbria, was included. The dentate gyrus, located in between the four CA regions in the hippocampal formation, together with the CA regions themselves and the subiculum, were included. The lateral border at this point was identified by the inferior horn of the lateral ventricle. The quadrogeminal cistern defined the superomedial border of the HC.

The most important structures for identification of lateral, anterior and superior borders of the HC head were the uncal recess of the inferior horn of the lateral ventricle and the alveus. Besides the coronal view, the sagittal and horizontal views were employed for identification of the anterior border of the HC head.

Inter-rater reliability was assessed using four raters independently measuring the same set of 5 MRI scans. The coefficient of variation between the raters ranged between 1.8% and 2.2% for right and left hippocampus. The intraclass correlation coefficients ranged between 0.94 and 0.86 for right and left hippocampus. The intra-rater reliability was determined by one rater measuring four times the same set 5 MRI scans within a previously not measured set of MRI scans. The coefficients of variation within rater ranged between 2.2 % and 2.4 % for right and left hippocampus. The intraclass correlation coefficients ranged between 0.91 and 0.94 for right and left hippocampus.

CSF samples were taken by lumbar puncture and processed immediately. Aliquots were stored at -80°C until further examination. The detailed CSF protocol has been described previously.

Tau protein levels were measured using enzyme-linked immunosorbent assays (ELISA) as described above.

Differences in hippocampus and amygdala volumes were compared between groups (AD and controls) using Student's t-test. To control for potential effects of age, between-group

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differences in hippocampus volumes were assessed using multiple regression analysis with volumes as dependent and age and diagnosis as predictor variables.

Rates of atrophy of left and right hippocampus were determined with mixed effect regression models using SAS 8.02 Proc Mixed software (SAS Institute Inc., Cary, NC, USA). In a first step, each volume (right and left hippocampus) was predicted by a mixed effect regression term, incorporating random-effect terms to account for subject and subject by time differences in volumes (random trend model). The random trend model conceptualizes each patient's volume change as following a straight line parameterized by a slope and an intercept. The slope measures the patient's rate of atrophy, the intercept the patient's baseline volume. Individual slopes and intercepts are regarded as a random sample of a normal population with unknown mean and variance. Individual slopes were determined by iterative restricted maximum likelihood estimation, taking into account the information of the entire sample to determine individual rates of change. From these models, we derived individual random regression coefficients of time related change in hippocampus volumes. In a second step, additional terms were included into the random effect model to account for effects of p-tau$_{231}$ levels on baseline volume and on individual rates of change in volume. An additional random trend model was used to determine the individual rates of decline in MMSE score

Spearman's rank correlation and Pearson's product moment correlation were used for correlation analyses.

Hippocampus volumes were significantly reduced in AD patients compared to controls ($p < 0.003$ for all comparisons). These effects remained significant after controlling for age differences between groups using a multiple regression model ($p < 0.05$). Hippocampal volumes are shown in Table 5. In the AD group, levels of p-tau$_{231}$ were 729.6 (SD 404.3) pg/ml, and levels of t-tau were 608.1 (SD 314.6) pg/ml. Annual rates of atrophy

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in the AD patients were about 14% for right and left hippocampus (Table 6).

Table 5: Hippocampus volumes

Region	healthy controls (n = 21)	AD patients (n = 22)	% difference AD vs. controls
left hippocampus	3158.5 [SD 582.4]	2267.2 [SD 640]	-28%*
right hippocampus	3328.0 [SD 556.4]	2262.7 [SD 651]	-32%*

Mean [± SD] volumes of left and right amygdala and hippocampus in mm^3.

* - significant difference between AD patients and controls after controlling for age ($p < 0.001$)

Table 6: Rates of atrophy of hippocampal volumes in AD patients (n = 22)

region	annual %-rates of atrophy
left hippocampus	-13.8 [SD 5.2]*
right hippocampus	-14.3 [SD 5.7]*

Mean [± SD] annual percent rates of atrophy for hippocampal volumes based on random trend model estimates
* - significantly different from zero slope, $p < 0.001$

There was a significant effect of p-tau_{231} and t-tau on baseline volumes of left hippocampus (p-tau_{231}: beta = 0.65, $p < 0.002$; t-tau: beta = 0.50, $p < 0.01$) and right hippocampus (p-tau_{231}: beta = 0.63, $p < 0.001$; t-tau: beta = 0.46, $p < 0.03$), with higher tau protein levels corresponding to higher baseline

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hippocampus volumes (Figure 3). Increased levels of $p\text{-}tau_{231}$ were significantly correlated with higher rates of atrophy of left hippocampus (beta = -0.36, $p < 0.001$) and right hippocampus (beta = -0.31, $p < 0.02$) (Figure 4). T-tau levels were not correlated with rates of atrophy. Average rate of decline of MMSE score was -1.32 (SD 1.05) points per year in AD. Higher rates of MMSE score decline correlated significantly with higher rates of atrophy of left and right hippocampus volumes (rho = 0.52 and 0.47, $p < 0.05$). There was no effect of levels of tau proteins on baseline MMSE scores or on rates of decline in MMSE scores in the AD patients.

There was no effect of age on rate of atrophy in AD or on baseline volumes in AD and healthy controls. There was no effect of baseline MMSE score, age at onset and disease duration on baseline volumes or rates of volume loss in AD patients. Treatment (coded as "no treatment", "acetylcholine-esterase inhibitors", "other drugs") had no effect on rates of MMSE score decline or atrophy in AD. There was no effect of age, age at onset, disease duration or MMSE score at baseline on CSF levels of t-tau and $p\text{-}tau_{231}$ in AD.

Patient and control groups were matched for gender distribution, but were different in age (Table 4).

The data demonstrate a significant effect of baseline levels of tau proteins on baseline volumes of hippocampus, and of $p\text{-}tau_{231}$ on progression of hippocampus atrophy over time. These findings agree with the hypothesis that tau protein levels in CSF may reflect the degree of axonal and neuronal destruction in AD, which is the main determinant of subsequent morphological disease progression as visualized with structural MRI.

Levels of $p\text{-}tau_{231}$ predicted the rate of subsequent hippocampus atrophy, accounting for about 20 % of variability in individual rates of atrophy. Levels of t-tau were not predictive for subsequent rates of atrophy. The degree of neuronal degeneration at a given time period may be highly predictive for the rate of neuronal loss in the subsequent time

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period. The rate of neuronal loss in AD, however, can be assessed *in vivo* by MRI based measurement of atrophy, since it has been shown that hippocampus atrophy in MRI accounts for 80% of variability of neuronal cells numbers in hippocampus in AD. On this basis, the significant correlations between p-tau$_{231}$ levels and rates of hippocampal atrophy suggest that CSF levels of p-tau$_{231}$ can serve as a state marker for the degree of neuronal destruction in AD which in turn determines subsequent rates of neuronal loss and regional atrophy.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

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We claim:

1. A method of predicting whether a patient will develop Alzheimer's Disease comprising:

a) determining the level of phosphorylated tau protein (p-tau) in a sample of cerebrospinal fluid (CSF) taken from the patient;

b) determining the CSF level of p-tau obtained from at least one control patient;

c) comparing the CSF level of p-tau from the patient with the CSF level of p-tau obtained from the at least one control patient, wherein the greater the difference between the CSF level of p-tau from the patient and the CSF level of p-tau from the control patient, the greater the risk of developing AD.

2. The method of claim 1, wherein the patient has mild cognitive impairment (MCI).

3. The method of claim 1, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

4. A method of diagnosing a patient who is likely to develop Alzheimer's Disease (AD) comprising measuring the level of p-tau in the CSF of a patient, wherein a CSF p-tau level of at least about 215 pg/ml in the patient is indicative of a patient who is likely to convert to AD.

5. The method of claim 4, wherein the patient has mild cognitive impairment (MCI).

6. The method of claim 4, wherein the level of p-tau is least about 250 pg/ml.

7. The method of claim 4, wherein the level of p-tau is least about 300 pg/ml.

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8. The method of claim 4, wherein the level of p-tau is least about 350 pg/ml.

9. The method of claim 4, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

10. A method of diagnosing a patient as having mild cognitive impairment (MCI) comprising:

a) determining the level of phosphorylated tau protein in a sample of cerebrospinal fluid (CSF) taken from the patient;

b) determining the CSF level of p-tau obtained from at least one control patient;

c) comparing the CSF level of p-tau from the patient with the CSF level of p-tau obtained from the at least one control patient, wherein the patient is diagnosed as having MCI if the CSF level of p-tau is greater in the patient than in the at least one control patient.

11. The method of claim 10, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

12. A method of predicting the rate of neuronal degeneration in a patient comprising determining the level of phosphorylated tau protein in a sample of cerebrospinal fluid (CSF) taken from the patient, wherein the patient has Alzheimer's Disease.

13. The method of claim 12, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

14. A method of identifying a patient who is likely to develop Alzheimer's Disease comprising:

a) obtaining a biological sample from a patient;

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b) determining the level of phosphorylated tau protein (p-tau) in the biological sample;

c) identifying the patient as being likely to develop Alzheimer's Disease if the level of p-tau in the biological sample is about 617 pg/ml or higher.

15. The method of claim 14, wherein the patient has mild cognitive impairment (MCI).

16. The method of claim 14, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

17. The method of claim 16, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

18. The method of claim 17, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

19. The method of claim 14, wherein the biological sample is cerebrospinal fluid (CSF).

20. The method of claim 14, wherein the level of p-tau is determined using an immunoassay.

21. A method of predicting cognitive decline in a patient, comprising the steps of:

a) obtaining a biological sample from a patient;

b) determining the level of phosphorylated tau protein (p-tau) in the biological sample;

c) identifying the patient as being likely to cognitively decline if the level of p-tau in the biological sample is about 143 pg/ml or higher.

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22. The method of claim 21, wherein the patient has mild cognitive impairment (MCI).

23. The method of claim 21, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

24. The method of claim 23, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

25. The method of claim 24, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

26. The method of claim 21, wherein the biological sample is cerebrospinal fluid (CSF).

27. The method of claim 21, wherein the level of p-tau is determined using an immunoassay.

28. A method of diagnosing Alzheimer's Disease in a patient, comprising the steps of:

a) obtaining a biological sample from a patient;

b) determining the level of p-tau protein in the biological sample; and

c) diagnosing the patient as having Alzheimer's Disease if the level of p-tau in the biological sample is about 215 pg/ml or higher.

29. The method of claim 28, wherein the patient is diagnosed as having Alzheimer's Disease if the p-tau level is about 250 pg/ml or higher.

30. The method of claim 28, wherein the patient is diagnosed as having Alzheimer's Disease if the p-tau level is about 300 pg/ml or higher.

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31. The method of claim 28, wherein the patient is diagnosed as having Alzheimer's Disease if the p-tau level is about 350 pg/ml or higher.

32. The method of claim 28, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

33. The method of claim 32, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

34. The method of claim 33, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

35. The method of claim 28, wherein the biological sample is cerebrospinal fluid (CSF).

36. The method of claim 28, wherein the level of p-tau is determined using an immunoassay.

37. A method of monitoring cognitive decline in a patient comprising the steps of:

a) determining the level of p-tau protein in a biological sample obtained from the patient;

b) determining the p-tau load in the sample by adjusting the p-tau level using a ventricular volume correction;

c) repeating steps (a) and (b) using a subsequently-collected biological sample obtained from the patient; and

d) comparing the p-tau load determined in step (b) with the amount of the p-tau load determined in step (c) and therefrom monitoring cognitive decline in the patient.

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38. The method of claim 37, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

39. The method of claim 38, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

40. The method of claim 39, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

41. The method of claim 37, wherein the biological sample is cerebrospinal fluid (CSF).

42. The method of claim 37, wherein the level of p-tau is determined using an immunoassay.

43. A method of measuring the effectiveness of a pharmaceutical composition as an agent for treating a patient having a condition associated with dementia, comprising the steps of:

a) determining the level of p-tau protein in a biological sample obtained from the a patient;

b) administering an amount of a pharmaceutical composition to the patient;

c) repeating step (a) using a subsequently-collected biological sample obtained from the patient;

d) comparing the level of p-tau protein determined in step (a) with the level of p-tau protein determined in step (c), wherein the effectiveness of the pharmaceutical composition is monitored by detecting no change in the level or p-tau or a decrease in the level of p-tau in the subsequently-collected biological sample compared with the biological sample from step (a).

44. The method of claim 43, wherein the condition is Alzheimer's Disease or Mild Cognitive Impairment.

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45. The method of claim 43, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

46. The method of claim 45, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

47. The method of claim 46, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

48. The method of claim 43, wherein the biological sample is cerebrospinal fluid (CSF).

49. The method of claim 43, wherein the level of p-tau is detemined using an immunoassay.

50. A method of predicting a rate of neuronal degeneration in a patient comprising determining the level of phosphorylated tau (p-tau) protein in a sample of cerebrospinal fluid (CSF) taken from the patient, wherein the patient has Alzheimer's Disease.

51. The method of claim 50, wherein the p-tau is tau protein phosphorylated at amino acid 175, 181, 185, 199, 202, 214, 231, 235, 262, 396, 404, 409, or 422.

52. The method of claim 51, wherein the p-tau is tau protein phosphorylated at amino acid 231, 181, 396, or 404.

53. The method of claim 52, wherein the p-tau is tau protein phosphorylated at threonine 231 ($p\text{-}tau_{231}$).

54. The method of claim 50, wherein the biological sample is cerebrospinal fluid (CSF).

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55. The method of claim 50, wherein the level of p-tau is determined using an immunoassay.

56. A method of treating a subject for Alzheimer's Disease comprising diagnosing the subject using the method of claim 28 and administering Alzheimer's therapy to the subject.

57. The method of claim 56, wherein the Alzheimer's therapy comprises administering an anti-dementia drug to the subject.

58. The method of claim 56, wherein the Alzheimer's therapy comprises administering a cholinesterase inhibitor to the subject.

59. The method of claim 28, further comprising treating the patient identified in step (c) with an anti-dementia drug.

60. The method of claim 59, wherein the anti-dementia drug is a cholinesterase inhibitor.

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1. The method of claim 14, further comprising treating the patient identified in step (c) with an anti-dementia drug.

62. The method of claim 61, wherein the anti-dementia drug is a cholinesterase inhibitor.

63. The method of claim 21, further comprising treating the patient identified in step (c) with an anti-dementia drug.

64. The method of claim 63, wherein the anti-dementia drug is a cholinesterase inhibitor.

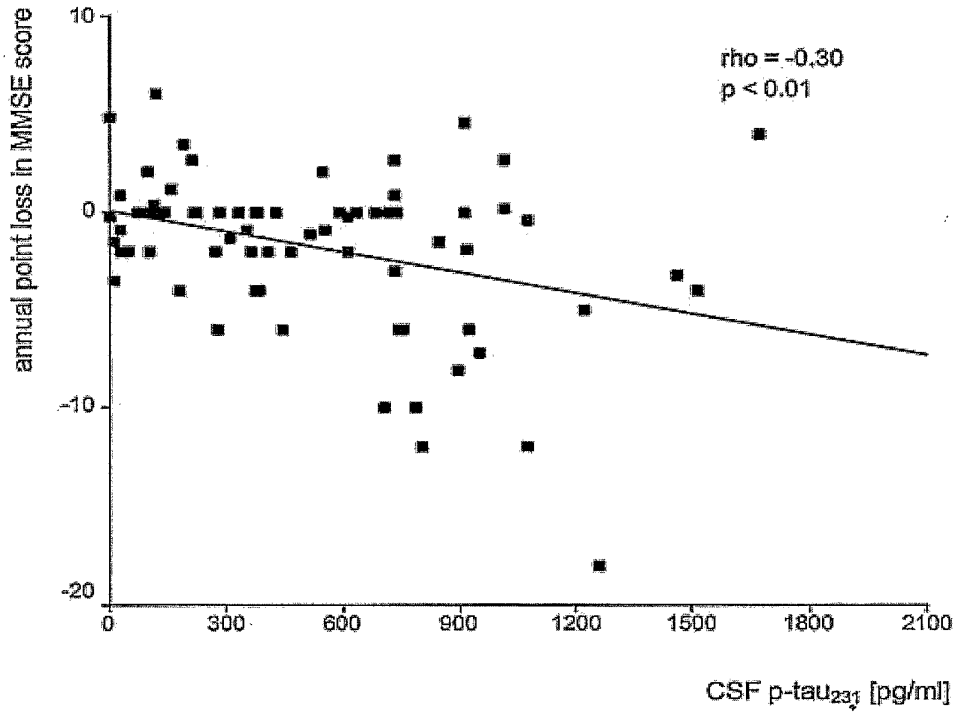
65. A method of treating a patient for cognitive decline comprising diagnosis according to the method of claim 21 and administering an anti-dementia drug to the patient.

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66. The method of claim 65, wherein the anti-dementia drug is a cholinesterase inhibitor.

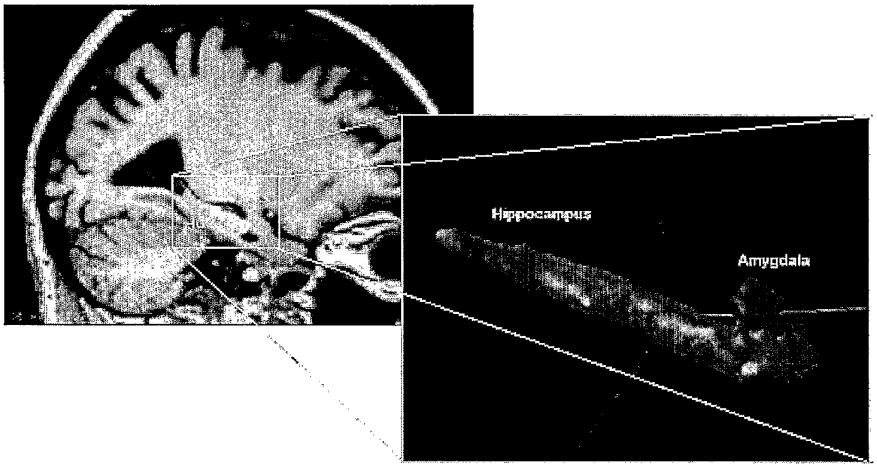
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FIG. 1



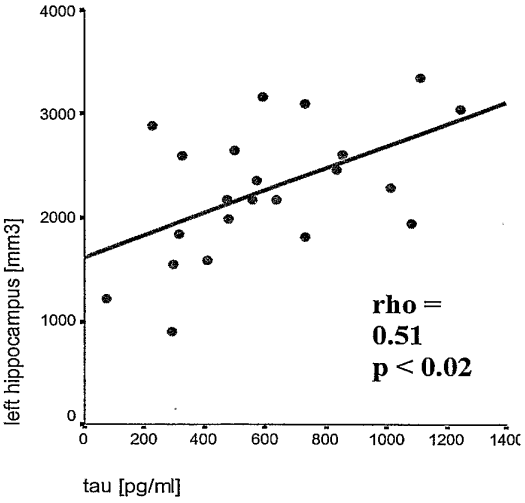
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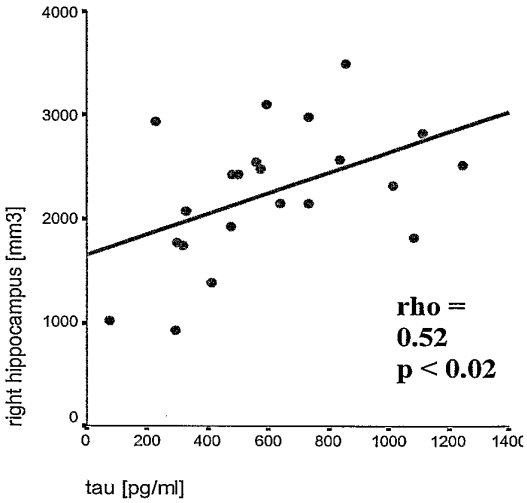
FIG. 2

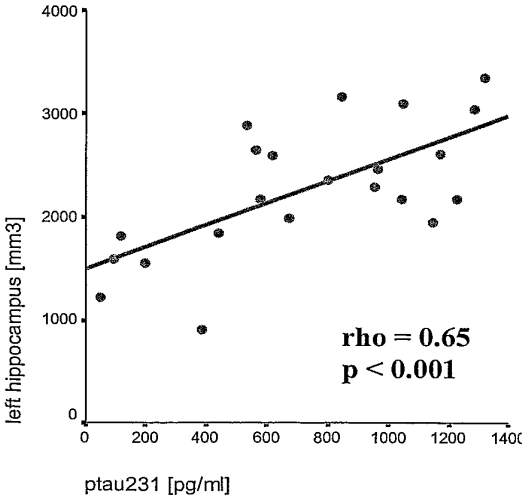


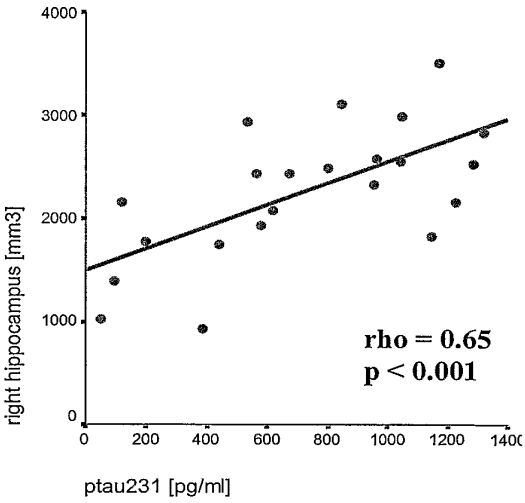
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FIG. 3



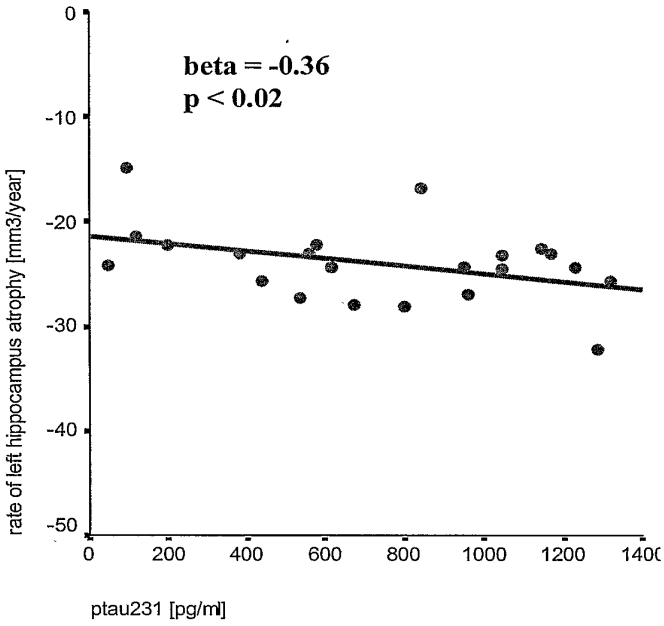


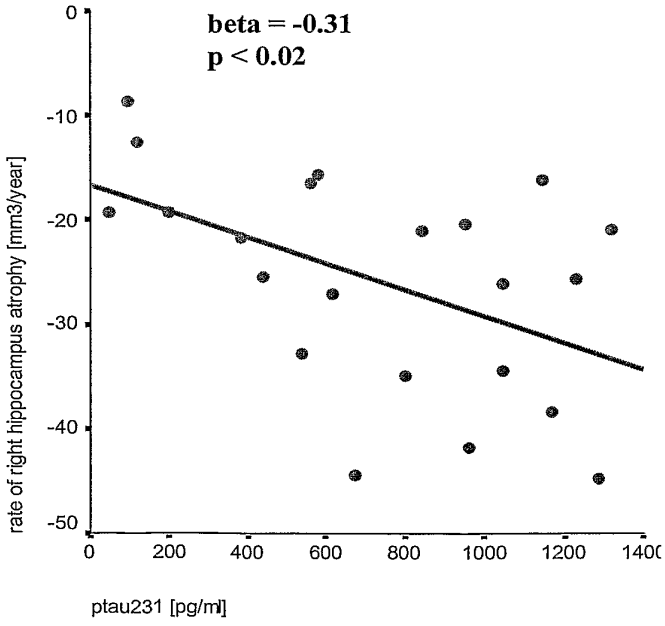




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FIG. 4





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(54) Title: ANTIBODIES SPECIFIC FOR TOXIC AMYLOID BETA PROTEIN OLIGOMERS

(57) Abstract: The present invention provides compositions and methods for diagnosing Alzheimer's disease (AD). In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, and methods of use of these compositions in the diagnosis of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of AD in patients.

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ANTIBODIES SPECIFIC FOR TOXIC AMYLOID BETA PROTEIN OLIGOMERS

This invention was funded, in part, under NIH Grant AG13854. The government may have certain rights in the invention. The present invention claims priority to U.S. Pat. Appln. Ser. No. 60/491,725, filed August 1, 2003, the disclosure of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention provides compositions and methods for diagnosing Alzheimer's disease (AD) and other conditions. In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, and methods of use of these compositions in the diagnosis and treatment of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of AD in patients.

BACKGROUND OF THE INVENTION

AD is the fourth most common cause of death in the United States, next to heart disease, cancer and stroke. It presently afflicts more than four million people, and this number is expected to double during the next forty years with the aging of the population. AD is also the most common cause of chronic dementia, with approximately two million people in the United States suffering from dementia. At present, it is estimated that ten percent of the population older than 65 years of age have mild to severe dementia. This high prevalence, combined with the rate of growth of the elderly segment of the population, make dementia and particularly AD, important current public health problems.

To date, AD is the third most expensive disease in the United States, and costs approximately $100 billion each year. Costs associated with AD include direct medical costs such as nursing home care, direct non-medical costs such as in-home day care, as well as indirect costs such as lost patient and care-giver productivity. Medical treatment may

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have economic benefits by slowing the rate of cognitive decline, delaying institutionalization, reducing care-giver hours, and improving quality of life.

AD is a complex multi-genic neurodegenerative disorder characterized by progressive impairments in memory, behavior, language, and visuo-spatial skills, ending ultimately in death. Hallmark pathologies of AD include granulovascular neuronal degeneration, extracellular neuritic plaques with amyloid β protein deposits, intracellular neurofibrillary tangles and neurofibrillary degeneration, synaptic loss, and extensive neuronal cell death. It is now known that these histopathologic lesions of AD correlate with the dementia observed in many elderly people.

Research on the causes of and treatments for AD has led investigators down numerous avenues. Although many models have been proposed, no single model of AD satisfactorily accounts for all neuropathologic findings; nor do these models of AD satisfactorily account for the requirement of aging for disease onset. Cellular changes, leading to neuronal loss and the underlying etiology of the disease, remain unknown. Proposed causes include environmental factors (Perl, Environmental Health Perspective 63:149 [1985]), metal toxicity (Perl et al., Science 208:297 [1980]), defects in beta-amyloid protein metabolism (Shijo et al., Science 258:126 [1992]; and Kosik, Science 256:780 [1992]), and abnormal calcium homeostasis and/or calcium activated kinases (Mattson et al., J. Neuroscience 12:376 [1992]). The mechanisms of disease progression are equally unclear. Considerable human genetic evidence has implicated alterations in production or processing of the human amyloid precursor protein (APP) in the etiology of the disease. However, intensive research has proven that AD is a multifactorial disease with many different, perhaps overlapping, etiologies.

Early detection and identification of AD facilitate prompt, appropriate treatment and care. However, there is currently no laboratory diagnostic test for AD. Although studies have suggested that calcium imaging measurement in fibroblasts were of potential clinical use in diagnosing AD (Peterson et al., Neurobiology of Aging 9:261 [1988]; and Peterson et al., Proc. Natl. Acad. Sci. USA 83:7999 [1986]), other studies using similar cell lines and techniques have shown no difference in calcium levels in Alzheimer's and normal control fibroblasts (Borden et al., Neurobiology of Aging 13:33 [1991]). Thus, there remains a

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need for diagnostic methods for AD. In particular, reliable and cost-effective methods and compositions are needed to allow reliable diagnosis of AD.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for diagnosing Alzheimer's disease and other conditions. In particular, the present invention finds use with any condition (e.g., including but not limited to neurological conditions) that are directly or indirectly linked to the presence or absence of amyloid β protein assemblies. In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, methods of use of these compositions in the diagnosis and treatment of Alzheimer's disease and other conditions, and methods to monitor treatment and/or disease progression of AD in patients, including methods of screening compounds for diagnostic and therapeutic application. For example, the present invention provides antibodies that may be used for therapeutic use through their ability to specifically bind to particular amyloid β protein assemblies. Following binding, the antibody bound complexes may be targeted with therapeutic compounds that are targeted to the complex or can be degraded and/or cleared by endogenous or exogenous routes. Compounds that find use in treating diseases and conditions can be screened for their ability to target, clear, or otherwise interact with amyloid β protein assemblies (e.g., by recognizing or competing with antibody binding). Thus, the present invention provides diagnostic, therapeutic, and drug screening methods related to biological processes that are linked to the presence or absence of specific amyloid β protein assemblies.

In some embodiments, the present invention provides a composition comprising a purified monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins (although the present invention is not limited to any particular size). In some embodiments, the fibrillar amyloid β protein assemblies comprise more than

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12 amyloid β proteins. In some embodiments, the amyloid β protein is the β1-42 protein. In still further embodiments, the amyloid β protein assemblies are neurotoxic.

The present invention also provides a hybridoma that secretes a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the hybridoma secretes a monoclonal antibody that identifies oligomeric amyloid β proteins comprising the β1-42 protein.

The present invention also provides methods for obtaining and isolating a hybridoma secreting a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies, comprising the steps of: providing spleen cells immunized with an antigen comprising soluble, non-fibrillar oligomeric amyloid β protein assemblies; fusing the immunized cells with myeloma cells under hybridoma-forming conditions; and selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising amyloid β proteins without recognizing fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins.

The present invention further provides a method for producing a monoclonal antibody from a hybridoma secreting a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies, comprising the steps of: culturing the hybridoma in an appropriate medium culture and recovering the monoclonal antibody excreted by the hybridoma, or, alternatively, implanting the hybridoma into the peritoneum of a mouse, and, when ascites have been produced by the animal, recovering the monoclonal antibody then formed from the ascites. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In still further embodiments, the present invention provides a monoclonal antibody produced by this method.

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In some embodiments, the present invention provides a composition comprising a purified monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the amyloid β protein is the β1-42 protein.

The present invention also provides a hybridoma that secretes a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the hybridoma secretes a monoclonal antibody that identifies fibrillar amyloid β proteins comprising the β1-42 protein.

The present invention also provides methods for obtaining and isolating a hybridoma secreting a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies, comprising the steps of: providing spleen cells immunized with an antigen comprising fibrillar amyloid β protein assemblies; fusing the immunized cells with myeloma cells under hybridoma-forming conditions; and selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins.

The present invention further provides a method for producing a monoclonal antibody from a hybridoma secreting a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies, comprising the steps of: culturing the hybridoma in an appropriate medium culture and recovering the monoclonal antibody excreted by the hybridoma, or,

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alternatively, implanting the hybridoma into the peritoneum of a mouse, and, when ascites have been produced by the animal, recovering the monoclonal antibody then formed from the ascites. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In still further embodiments, the present invention provides a monoclonal antibody produced by this method.

The present invention further provides methods for detecting at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising the steps of: providing a sample suspected of containing at least one soluble, non-fibrillar oligomeric amyloid β protein assembly and a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies; contacting the sample with the antibody under conditions such that the antibody binds to the soluble, non-fibrillar oligomeric amyloid β protein assembly, to form an antigen-antibody complex; and detecting the presence of the antigen-antibody complex. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid. In some preferred embodiments, the sample is from a subject. In particularly preferred embodiments, the subject is a human. In further embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In some particularly preferred embodiments, the methods further comprise the step of diagnosing Alzheimer's disease. In additional particularly preferred embodiments, the Alzheimer's disease diagnosed using the methods of the present invention is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease. In some preferred embodiments, the methods further comprise the step of monitoring the efficacy of treatment of Alzheimer's disease.

In some preferred embodiments, the methods comprises an enzyme-linked immunosorbent assay. In particularly preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked

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immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In alternative preferred embodiments, the antibody used in the methods of the present invention further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In some particularly preferred embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system. In alternative preferred embodiments, the methods further comprise at least one capture antibody, while in still further embodiments, the methods further comprise at least one detection antibody wherein the detection antibody is directed against the antibody directed against the soluble, non-fibrillar oligomeric amyloid β protein assemblies. In still further embodiments, the detection antibody further comprises at least one conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase. In still further preferred embodiments, the methods further comprise the step of quantitating the at least one soluble, non-fibrillar oligomeric amyloid β protein assembly in the sample.

The present invention also provides kits for the detection of at least one soluble, non-fibrillar oligomeric amyloid β protein assembly comprising at least one antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit comprises an enzyme-linked immunosorbent assay kit. In still further embodiments, the kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, an antibody-enzyme conjugate directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one capture antibody, 96-well microtiter plates precoated with the at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least one amplifier system. In some preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system. The kits of the present invention may also contain any other useful components, including other antibodies (e.g., for

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detection of multiple different proteins) or other diagnostic reagents, therapeutic agents, instructions, education materials, and the like.

The present invention also provides methods for detecting at least one antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising: a) providing a sample suspected of containing at least one antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly and a detection antibody; b) contacting the sample with the soluble, non-fibrillar oligomeric amyloid β protein assembly, under conditions such that the antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly specifically binds to the soluble, non-fibrillar oligomeric amyloid β protein assembly to form an antigen-antibody complex; c) contacting the antigen-antibody complex with the detection antibody, under conditions such that the detection antibody specifically binds to the complex; and d) detecting the specific binding of the detection antibody to the antigen-antibody complex. In some preferred embodiments, the sample is selected from the group of samples consisting of blood, serous fluid, plasma, serum, cerebrospinal fluid, hybridoma conditioned culture medium, ascites fluid, and polyclonal antiserum. In some particularly preferred embodiments, the sample is from a subject, while in other preferred embodiments, the subject is human. In alternative preferred embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In still further preferred embodiments, the methods further comprise diagnosing Alzheimer's disease in the subject. In some preferred embodiments, the Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease, and sporadic Alzheimer's disease. In preferred embodiments, the method comprises an enzyme-linked immunosorbent assay. In some preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In still further embodiments, the detection antibody further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In

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additional embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

The present invention also provides kits for the detection of at least one antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising at least one soluble, non-fibrillar oligomeric amyloid β protein assembly and at least one detection antibody. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit is an enzyme-linked immunosorbent assay kit. In some preferred embodiments, the kit comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one purified antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one 96-well microtiter plate precoated with at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme reaction substrate solution, and at least one amplifier system. In some particularly preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

The present invention further provides methods for detecting at least one fibrillar amyloid β protein assembly, comprising the steps of: providing a sample suspected of containing at least one fibrillar amyloid β protein assembly and a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies; contacting the sample with the antibody under conditions such that the antibody binds to the fibrillar amyloid β protein assembly to form an antigen-antibody complex; and detecting the presence of the antigen-antibody complex. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid. In some preferred embodiments, the sample is from a subject. In particularly preferred embodiments, the subject is a human. In further embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In

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some particularly preferred embodiments, the methods further comprise the step of diagnosing Alzheimer's disease. In additional particularly preferred embodiments, the Alzheimer's disease diagnosed using the methods of the present invention is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease. In some preferred embodiments, the methods further comprise the step of monitoring the efficacy of treatment of Alzheimer's disease.

In some preferred embodiments, the methods comprises an enzyme-linked immunosorbent assay. In particularly preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In alternative preferred embodiments, the antibody used in the methods of the present invention further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In some particularly preferred embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system. In alternative preferred embodiments, the methods further comprise at least one capture antibody, while in still further embodiments, the methods further comprise at least one detection antibody wherein the detection antibody is directed against the antibody directed against the fibrillar amyloid β protein assemblies. In still further embodiments, the detection antibody further comprises at least one conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase. In still further preferred embodiments, the methods further comprise the step of quantitating the at least fibrillar amyloid β protein assembly in the sample.

The present invention also provides kits for the detection of at least one fibrillar amyloid β protein assembly comprising at least one antibody directed against at least one fibrillar amyloid β protein assembly. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit comprises an enzyme-linked immunosorbent assay kit. In still further embodiments, the kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well

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microtiter plates, instructions, an antibody-enzyme conjugate directed against a fibrillar amyloid β protein assembly, at least one capture antibody, 96-well microtiter plates precoated with the at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least one amplifier system. In some preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts hybridoma screening by antigen/antibody blotting.

FIG. 2 shows an ELISA assay utilizing the monoclonal antibodies 6C3 and 7A2 (labeled "MOAB-2" and MOAB-1" respectively).

FIG. 3 shows Western blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10, and 7A2.

FIG. 4 shows DAB staining and 10x light microscopy with monoclonal antibodies 6C3 and 7A2 in an AD brain.

FIG. 5 shows laser scanning confocal microscopy of AD brain slices using monoclonal antibody 6C3 and polyclonal antibody R1280.

FIG. 6 shows additional Western blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10 and 7A2. Note: "MOAB-1" and "MOAB-2" correspond to 7A2 and 6C3 antibodies, respectively.

FIG. 7 shows dot blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10 and 7A2. Note: "MOAB-1" and "MOAB-2" correspond to 7A2 and 6C3 antibodies, respectively.

Definitions:

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the terms "peptide," "polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent "peptide linkages." In general, a peptide consists of a few amino acids, typically from 2-50 amino acids, and is shorter than

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a protein. The term "polypeptide" encompasses peptides and proteins. In some embodiments, the peptide, polypeptide or protein is synthetic, while in other embodiments, the peptide, polypeptide or protein are recombinant or naturally occurring. A synthetic peptide is a peptide that is produced by artificial means in vitro (i.e., was not produced in vivo).

The terms "sample" and "specimen" are used in their broadest sense and encompass samples or specimens obtained from any source. As used herein, the term "sample" is used to refer to biological samples obtained from animals (including humans), and encompasses fluids, solids, tissues, and gases. In preferred embodiments of this invention, biological samples include cerebrospinal fluid (CSF), serous fluid, urine, saliva, blood, and blood products such as plasma, serum and the like. However, these examples are not to be construed as limiting the types of samples that find use with the present invention.

As used herein, the terms "soluble, non-fibrillar oligomeric amyloid β protein assembly," "oligomeric amyloid β protein assembly" and "oligomeric assembly" all refer to a protein assembly comprised of amyloid β proteins or peptides proteolytically derived from the transmembrane amyloid precursor protein (APP).

As used herein, the terms "fibrillar amyloid β protein assembly" and "fibrillar assembly" refers to a protein assembly comprised of amyloid β proteins or peptides proteolytically derived from the transmembrane amyloid precursor protein (APP).

As used herein, the term "oxidative stress" refers to the cytotoxic effects of oxygen radicals (i.e., superoxide anion, hydroxy radical, and hydrogen peroxide), generated as byproducts of metabolic processes that utilize molecular oxygen (See e.g., Coyle et al., Science 262:689-695 [1993]).

As used herein, the terms "host," "subject" and "patient" refer to any animal, including but not limited to, human and non-human animals (e.g. rodents, arthropods, insects [e.g., Diptera], fish [e.g., zebrafish], non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.), that is studied, analyzed, tested, diagnosed or treated. As used herein, the terms "host," "subject" and "patient" are used interchangeably.

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As used herein, the terms "Alzheimer's disease" and "AD" refer to a neurodegenerative disorder and encompasses familial Alzheimer's disease and sporadic Alzheimer's disease. The term "familial Alzheimer's disease" refers to Alzheimer's disease associated with genetic factors (i.e., demonstrates inheritance) while "sporadic Alzheimer's disease" refers to Alzheimer's disease that is not associated with prior family history of the disease. Symptoms indicative of Alzheimer's disease in human subjects typically include, but are not limited to, mild to severe dementia, progressive impairment of memory (ranging from mild forgetfulness to disorientation and severe memory loss), poor visuo-spatial skills, personality changes, poor impulse control, poor judgement, distrust of others, increased stubbornness, restlessness, poor planning ability, poor decision making, and social withdrawal. In severe cases, patients lose the ability to use language and communicate, and require assistance in personal hygiene, eating and dressing, and are eventually bedridden. Hallmark pathologies within brain tissue include extracellular neuritic β-amyloid plaques, neurofibrillary tangles, neurofibrillary degeneration, granulovascular neuronal degeneration, synaptic loss, and extensive neuronal cell death.

As used herein, the term "early-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring before the age of 65. As used herein, the term "late-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring after the age of 65.

As used herein, the terms "subject having Alzheimer's disease" or "subject displaying symptoms or pathology indicative of Alzheimer's disease" "subjects suspected of displaying symptoms or pathology indicative of Alzheimer's disease" refer to a subject that is identified as having or likely to have Alzheimer's disease based on known Alzheimer's symptoms and pathology.

As used herein, the term "subject at risk of displaying pathology indicative of Alzheimer's disease" refers to a subject identified as being at risk for developing Alzheimer's disease (e.g., because of a familial inheritance pattern of Alzheimer's disease in the subject's family).

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As used herein, the term "lesion" refers to a wound or injury, or to a pathologic change in a tissue. For example, the amyloid plaque lesions observed in the brains of patients having Alzheimer's disease are considered the hallmark pathology characteristic of the disease.

As used herein, the term "antibody" (or "antibodies") refers to any immunoglobulin that binds specifically to an antigenic determinant, and specifically, binds to proteins identical or structurally related to the antigenic determinant that stimulated their production. Thus, antibodies are useful in assays to detect the antigen that stimulated their production. Monoclonal antibodies are derived from a single clone of B lymphocytes (i.e., B cells), and are generally homogeneous in structure and antigen specificity. Polyclonal antibodies originate from many different clones of antibody-producing cells, and thus are heterogenous in their structure and epitope specificity, but all recognize the same antigen. In some embodiments, monoclonal and polyclonal antibodies are used as crude preparations, while in preferred embodiments, these antibodies are purified. For example, in some embodiments, polyclonal antibodies contained in crude antiserum are used. Also, it is intended that the term "antibody" encompass any immunoglobulin (e.g., IgG, IgM, IgA, IgE, IgD, etc.) obtained from any source (e.g., humans, rodents, non-human primates, lagomorphs, caprines, bovines, equines, ovines, etc.).

As used herein, the terms "auto-antibody" or "auto-antibodies" refer to any immunoglobulin that binds specifically to an antigen that is native to the host organism that produced the antibody (i.e., the antigen is directed against "self" antigens). The presence of auto-antibodies is referred to herein as "autoimmunity."

As used herein, the term "antigen" is used in reference to any substance that is capable of being recognized by an antibody. It is intended that this term encompass any antigen and "immunogen" (i.e., a substance that induces the formation of antibodies). Thus, in an immunogenic reaction, antibodies are produced in response to the presence of an antigen or portion of an antigen. The terms "antigen" and "immunogen" are used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. It is intended that the terms antigen and immunogen encompass protein molecules or portions of protein molecules, that contains one or more epitopes. In many cases, antigens are also immunogens, thus the term "antigen" is often used interchangeably

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with the term "immunogen." In some preferred embodiments, immunogenic substances are used as antigens in assays to detect the presence of appropriate antibodies in the serum of an immunized animal.

As used herein, the terms "antigen fragment" and "portion of an antigen" and the like are used in reference to a portion of an antigen. Antigen fragments or portions typically range in size, from a small percentage of the entire antigen to a large percentage, but not 100%, of the antigen. However, in situations where "at least a portion" of an antigen is specified, it is contemplated that the entire antigen is also present (i.e., it is not intended that the sample tested contain only a portion of an antigen). In some embodiments, antigen fragments and/or portions therof, comprise an "epitope" recognized by an antibody, while in other embodiments these fragments and/or portions do not comprise an epitope recognized by an antibody. In addition, in some embodiments, antigen fragments and/or portions are not immunogenic, while in preferred embodiments, the antigen fragments and/or portions are immunogenic.

The terms "antigenic determinant" and "epitope" as used herein refer to that portion of an antigen that makes contact with a particular antibody variable region. When a protein or fragment (or portion) of a protein is used to immunize a host animal, numerous regions of the protein are likely to induce the production of antibodies that bind specifically to a given region or three-dimensional structure on the protein (these regions and/or structures are referred to as "antigenic determinants"). In some settings, antigenic determinants compete with the intact antigen (i.e., the "immunogen" used to elicit the immune response) for binding to an antibody.

The terms "specific binding" and "specifically binding" when used in reference to the interaction between an antibody and an antigen describe an interaction that is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the antigen. In other words, the antibody recognizes and binds to a protein structure unique to the antigen, rather than binding to all proteins in general (i.e., non-specific binding).

As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

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As used herein, the term "adjuvant" is defined as a substance that enhances the immunogenicity of a coadministered antigen. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant--or that the same adjuvant, once used, be used for all subsequent immunizations. The present invention contemplates many adjuvants, including but not limited to, keyhole limpet hemocyanin (KLH), agar beads, aluminum hydroxide or phosphate (alum), Freund's adjuvant (incomplete or complete), Quil A adjuvant and Gerbu adjuvant (Accurate Chemical and Scientific Corporation), and bacterins (i.e., killed preparations of bacterial cells, especially mycoplasma).

As used herein, the terms "purified" and "to purify" and "purification" refers to the removal or reduction of at least one contaminant from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins. Antibodies are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample (i.e., "enrichment" of an antibody).

As used herein, the term "immunoassay" refers to any assay that uses at least one specific antibody for the detection or quantitation of an antigen. Immunoassays include, but are not limited to, Western blots, ELISAs, radio-immunoassays, and immunofluorescence assays. Furthermore, many different ELISA formats are known to those in the art, any of which will find use in the present invention. However, it is not intended that the present invention be limited to these assays. In additional embodiments, other antigen-antibody reactions are used in the present invention, including but not limited to "flocculation" (i.e., a colloidal suspension produced upon the formation of antigen-antibody complexes), "agglutination" (i.e., clumping of cells or other substances upon exposure to antibody), "particle agglutination" (i.e., clumping of particles coated with antigen in the presence of antibody or the clumping of particles coated with antibody in the presence of antigen), "complement fixation" (i.e., the use of complement in an antibody-antigen reaction method), and other methods commonly used in serology, immunology, immunocytochemistry, immunohistochemistry, and related fields.

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The terms "Western blot," "Western immunoblot" "immunoblot" and "Western" refer to the immunological analysis of protein(s), polypeptides or peptides that have been immobilized onto a membrane support. The proteins are first resolved by polyacrylamide gel electrophoresis (i.e., SDS-PAGE) to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to an antibody having reactivity towards an antigen of interest. The binding of the antibody (i.e., the primary antibody) is detected by use of a secondary antibody that specifically binds the primary antibody. The secondary antibody is typically conjugated to an enzyme that permits visualization of the antigen-antibody complex by the production of a colored reaction product or catalyzes a luminescent enzymatic reaction (e.g., the ECL reagent, Amersham).

As used herein, the term "ELISA" refers to enzyme-linked immunosorbent assay (or EIA). Numerous ELISA methods and applications are known in the art, and are described in many references (See, e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, N.J. [1998]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]). In addition, there are numerous commercially available ELISA test systems.

One of the ELISA methods used in the present invention is a "direct ELISA," where an antigen (e.g., an oligomeric or a fibrillar amyloid β protein assembly) in a sample is detected. In one embodiment of the direct ELISA, a sample containing antigen is exposed to a solid (i.e., stationary or immobilized) support (e.g., a microtiter plate well). The antigen within the sample becomes immobilized to the stationary phase, and is detected directly using an enzyme-conjugated antibody specific for the antigen.

In an alternative embodiment, an antibody specific for an antigen is detected in a sample. In this embodiment, a sample containing an antibody (e.g., an anti-oligomeric or an anti-fibrillar assembly antibody) is immobilized to a solid support (e.g., a microtiter plate well). The antigen-specific antibody is subsequently detected using purified antigen and an enzyme-conjugated antibody specific for the antigen.

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In an alternative embodiment, an "indirect ELISA" is used. In one embodiment, an antigen (or antibody) is immobilized to a solid support (e.g., a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody (or antigen), then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (e.g., a goat anti-rabbit antibody), that are available from various manufacturers known to those in the art (e.g., Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

In other embodiments, a "sandwich ELISA" is used, where the antigen is immobilized on a solid support (e.g., a microtiter plate) via an antibody (i.e., a capture antibody) that is immobilized on the solid support and is able to bind the antigen of interest. Following the affixing of a suitable capture antibody to the immobilized phase, a sample is then added to the microtiter plate well, followed by washing. If the antigen of interest is present in the sample, it is bound to the capture antibody present on the support. In some embodiments, a sandwich ELISA is a "direct sandwich" ELISA, where the captured antigen is detected directly by using an enzyme-conjugated antibody directed against the antigen. Alternatively, in other embodiments, a sandwich ELISA is an "indirect sandwich" ELISA, where the captured antigen is detected indirectly by using an antibody directed against the antigen, that is then detected by another enzyme-conjugated antibody that binds the antigen-specific antibody, thus forming an antibody-antigen-antibody-anti- body complex. Suitable reporter reagents are then added to detect the third antibody. Alternatively, in some embodiments, any number of additional antibodies are added as necessary, in order to detect the antigen-antibody complex. In some preferred embodiments, these additional antibodies are labelled or tagged, so as to permit their visualization and/or quantitation.

As used herein, the term "capture antibody" refers to an antibody that is used in a sandwich ELISA to bind (i.e., capture) an antigen in a sample prior to detection of the antigen. For example, in some embodiments, the monoclonal anti-oligomeric or anti-fibrillar assembly antibodies of the present invention serve as a capture antibody when immobilized in a microtiter plate well. This capture antibody binds oligomeric or fibrillar amyloid β protein assembly antigens present in a sample added to the well. In one embodiment of the present invention, biotinylated capture antibodies are used in the present invention in conjunction with avidin-coated solid support. Another antibody (i.e., the

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detection antibody) is then used to bind and detect the antigen-antibody complex, in effect forming a "sandwich" comprised of antibody-antigen-antibody (i.e., a sandwich ELISA).

As used herein, a "detection antibody" is an antibody that carries a means for visualization or quantitation, that is typically a conjugated enzyme moiety that typically yields a colored or fluorescent reaction product following the addition of a suitable substrate. Conjugated enzymes commonly used with detection antibodies in the ELISA include horseradish peroxidase, urease, alkaline phosphatase, glucoamylase and β-galactosidase. In some embodiments, the detection antibody is directed against the antigen of interest, while in other embodiments, the detection antibody is not directed against the antigen of interest. In some embodiments, the detection antibody is an antibody directed against an antibody directed against the antigen of interest. Alternatively, the detection antibody is prepared with a label such as biotin, a fluorescent marker, or a radioisotope, and is detected and/or quantitated using this label.

As used herein, the terms "reporter reagent," "reporter molecule," "detection substrate" and "detection reagent" are used in reference to reagents that permit the detection and/or quantitation of an antibody bound to an antigen. For example, in some embodiments, the reporter reagent is a colorimetric substrate for an enzyme that has been conjugated to an antibody. Addition of a suitable substrate to the antibody-enzyme conjugate results in the production of a colorimetric or fluorimetric signal (e.g., following the binding of the conjugated antibody to the antigen of interest). Other reporter reagents include, but are not limited to, radioactive compounds. This definition also encompasses the use of biotin and avidin-based compounds (e.g., including but not limited to neutravidin and streptavidin) as part of the detection system.

As used herein, the term "signal" is used generally in reference to any detectable process that indicates that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorimetric or colorimetric products/reagents will all find use with the present invention. In various embodiments of the present invention, the signal is assessed qualitatively, while in alternative embodiments, the signal is assessed quantitatively.

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As used herein, the term "amplifier" is used in reference to a system that enhances the signal in a detection method, such as an ELISA (e.g., an alkaline phosphatase amplifier system used in an ELISA).

As used herein, the term "solid support" is used in reference to any solid or stationary material to which reagents such as antibodies, antigens, and other test components are attached. For example, in the ELISA method, the wells of microtiter plates provide solid supports. Other examples of solid supports include microscope slides, coverslips, beads, particles, cell culture flasks, gels, as well as many other suitable items.

As used herein, the term "kit" is used in reference to a combination of reagents and other materials that facilitate sample analysis. In some embodiments, the immunoassay kit of the present invention includes a suitable capture antibody, reporter antibody, antigen, detection reagents and amplifier system. Furthermore, in other embodiments, the kit includes, but is not limited to, components such as apparatus for sample collection, sample tubes, holders, trays, racks, dishes, plates, instructions to the kit user (including, for example, instructions and label as required by regulatory agencies), solutions or other chemical reagents, and samples to be used for standardization, normalization, and/or control samples.

As used herein, the term "in vitro" refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments consist of, but are not limited to, controlled laboratory conditions. The term "in vivo" refers to the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within that natural environment.

DETAILED DESCRIPTION OF THE INVENTION

The cellular processes that underlie the cognitive decline and amnestic dementia associated with AD remain poorly understood. Amyloid β protein is a peptide that is proteolytically derived from the transmembrane amyloid precursor protein (APP). Evidence from numerous studies supports the hypothesis that amyloid β protein accumulation is causally linked to AD. However a causal linkage between pathology, in terms of senile plaques composed primarily of deposited fibrillar amyloid β protein, and symptomology in

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terms of cognitive impairment and dementia, has not been forthcoming. An emerging hypothesis that reconciles this apparent disconnect focuses on small soluble assemblies of amyloid β protein.

Recent experimental evidence has demonstrated that these oligomeric conformations are directly involved in many of the destructive processes that result in neurodegeneration. Oligomeric amyloid β protein assemblies have been isolated from brain, plasma and CSF and soluble amyloid β protein concentrations in brain are correlated with the severity of AD. Furthermore, autosomal dominant mutations in the amyloid precursor protein (APP) and the presenilins (PS) increase the amount of amyloid β1-42, a proteolytic product of APP, and always result in AD. This provides powerful genetic evidence that some form of the amyloid β protein is involved in the disease process.

The role of amyloid β protein in the AD disease process has been put forth as the comprehensive "amyloid hypothesis" (Selkoe, J. Neuorpath. Exp. Neurol., 53: 438 [1991]). In it, it is stated that the production and deposition of amyloid β protein fibrils in plaques induces a neurotoxic event. Presumably, this initial event culminates in the intracellular accumulation of tau polymers as neurofibrillary tangles leading to neuronal dysfunction and death. The amyloid hypothesis gained wide acceptance when initial reports indicated that fibrillar amyloid β protein was cytotoxic *in vitro* (Pike et al., J. Neuroscience, 13: 1676 [1993]; Schenk et al., J. Med. Chem., 38: 4141 [1995]). However, the veracity of the amyloid hypothesis is challenged by a seeming disconnect between aspect of the plaque pathology and AD symptomatology.

In one of the first discoveries challenging this hypothesis, pathologists noted that the correlation between the number, location and distribution of senile plaques (amyloid load) and the degree of dementia as assessed neuropsychologically was poor at best (Swaab et al., Connections, Cognition and Alzheimer's Disease, Springer-Verlag, Berlin/NY [1997]. Second, amyloid deposition in senile plaques is temporarily dissociated from cognitive defects in transgenic mouse models overexpressing APP and PS (Hsai et al., Proc. Natl. Acad. Sci., 96: 3228 [1999]; Holcomb et al., Nature Med., 4: 97 [1998]; Chui et al., Nature Med., 5: 560 [1999]; Moeehars et al., J. Biol. Chem., 274: 6483 [1999]). Finally, several therapeutics designed to block fibril formation have been unsuccessful in delaying AD

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symptoms (Schenk et al., J. Med. Chem., 38: 4141 [1995]; Soto, Mol. Med. Today, 5: 343 [1999]).

On the other hand, several lines of evidence suggest that soluble oligomeric amyloid β protein species, as distinct from large, fibrillar aggregates, do correlate with AD pathology. The concentration of soluble amyloid β protein in the brain is highly correlated with disease severity (Lue et al., Amer. J. Path., 155: 853 [1999]; McLean et al., Ann Neurol., 46: 860 [1999]). Furthermore, results from *in vitro* experiments demonstrate that soluble oligomeric amyloid β proteins not only can readily form, but that these species are highly cytotoxic (Roher et al., J. Biol. Chem., 271: 20631 [1996]; Hartley et al., J. Neurosci., 19: 8876 [1999]; Lambert et al., Proc. Natl. Acad. Sci., 95: 6448 [1998]; Oda et al., Exp. Neurol., 136: 22 [1995]). For example, the process of amyloid β protein oligomerization is enhanced in the media of cells expressing the APP or PS mutations, providing a possible connection between toxic oligomer formation and AD genetics (Podlinsy et al., Biochemistry, 37: 3602 [1998]).

While a compelling argument can be made for the relevance of a toxic, diffusible amyloid β protein oligomer, the presence of this amyloid β protein species has not been demonstrated in normal or AD brain by immunohistochemistry. This is primarily due to a lack of antibodies that can distinguish different conformational forms of the amyloid β protein. Hence, one of the primary limitations to properly dissecting the role of fibrillar versus oligomeric amyloid β protein assemblies has been the lack of conformational-specific antibodies that can distinguish between these two aggregates of the amyloid β protein.

Hence, although it is not clear whether amyloid β protein accumulation causes Alzheimer's disease or is an effect of Alzheimer's disease, considerable evidence has strengthened the view that amyloid β protein accumulation is the causative agent of Alzheimer's disease. However, it is not necessary to understand the cause or effect of amyloid β protein accumulation in Alzheimer's disease in order to practice the present invention, nor is it intended that the present invention be limited to any particular mechanism or mechanisms of disease genesis or toxicity. Indeed, an understanding of any of the mechanisms of pathogenesis are not necessary in order to use the present invention.

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In some embodiments, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies while not reacting with fibrillar amyloid β protein assemblies, and monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies (e.g., as shown in Examples 2 and 3). In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. The present invention, however, is not limited by the number of amyloid β proteins present in the non-fibrillar oligomeric assemblies or fibrillar assemblies. In further embodiments, these antibodies are used to identify soluble, non-fibrillar oligomeric amyloid β protein assemblies or fibrillar amyloid β protein assemblies, respectively. However, it is not intended that the use of these antibodies be limited to identifying oligomeric and filbrillar forms of the amyloid β protein. For example, these antibodies may also be used to inhibit or to precipitate the assembly of amyloid β protein fibrils.

Additionally, the antibodies of the present invention find other uses, including enzyme-linked immunosorbent assays (ELISAs) (e.g., as shown in Example 3), Western blotting (e.g., as shown in Example 4), radioimmunoassays (RIAs), immunofluorescence assays (IFAs), immunoprecipitation, immunohistochemistry (e.g., as shown in Example 5), laser scanning confocal microscopy (e.g., as shown in Example 6) and clinical diagnostic applications using methods known in the art (See e.g., Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Vol. 1-4, John Wiley & Sons, Inc., New York [1994]; and Laurino et al., Ann. Clin. Lab Sci., 29(3):158-166 [1999]).

It is not intended that the production of antibodies of the present invention be limited to any particular method. Indeed, it is contemplated that the antibodies be prepared by any suitable method. Numerous methods for the production and purification of monoclonal antibodies are well known in the art (See e.g., Sambrook et al. (eds.), Molecular Cloning, Cold Spring Harbor Laboratory Press [1989]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, p. 11.4.2-11.15.4, John Wiley & Sons, Inc., New York [1994]; Kohler and Milstein, Nature 256:495-497 [1975]; Kozbor et al., Immunol.

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Today 4:72 [1983]; and Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 [1985]).

In addition, in other embodiments, any suitable amyloid β protein or fragment thereof, is used as an immunogen. (e.g., generation of immnogens is described in Example 1). In some embodiments, the immunogen is native, while in other embodiments, the immunogen is synthetic (i.e., recombinant or produced by in vitro chemical synthesis). Similarly, it is not intended that the present invention be limited to any particular amyloid β protein-derived immunogen, immunization method, immunization schedule, animal species, test protocol for determining antibody production or antibody purification method.

In some embodiments, the monoclonal antibody preparation of the present invention is purified from crude antiserum, hybridoma or cell culture supernatant, ascites fluid, or other starting material using any conventional method. Such purification methods include, but are not limited to, protein A affinity, protein G affinity, ammonium sulfate precipitation, ion exchange chromatography, gel filtration, and immunoaffinity chromatography (See, e.g., Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; and Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]).

Clonal selection of hybridomas is performed by incubating supernatants from each clone in two ELISA wells, one with amyloid β protein oligomers attached and the other with fibrils attached (e.g., described in Example 2). Clonal supernatants from oligomer-immunized mice that are positive on the oligomer-attached plate but negative on the fibril-attached plate are selected for further subcloning. This dual selection protocol is repeated for screening fusion of splenocytes obtained from fibril-immunized mice.

The specificity of antibodies produced from hybridomas during the development of the present invention can be further characterized by antibody/antigen spotting and Western blotting (e.g., as described in Examples 2 and 3, respectively, below).

Experiments conducted during the course of development of the present invention showed an antigen/antibody screen yielding one oligomer-specific antibody. The oligomer-specific antibody (7A2) showed little recognition of fibrils by antigen/antibody blotting

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(e.g., see Example 2 and FIG. 1) and ELISA (e.g., see Example 3 and FIG. 2). By Western analysis, 7A2 detected oligomeric (primarily dimer, tetramer and larger oligomers between approximately 27 and 44 kDa) amyloid β protein assemblies (e.g., see Example 7, FIGS. 6 and 7), whereas 6E10 and 6C3 antibodies detected multiple forms of amyloid β protein1-42 including monomer, trimer, tetramer and oligomers between approximately 27 and 80 kDa. 7A2 oligomer specificity was retained over a wide range of antibody and antigen concentrations, and in the presence of increasing concentrations of fibrillar amyloid β protein1-42 (e.g., see Example 7, FIGS. 6 and 7). In sections from AD brain, little immunoreactivity of 7A2 antibody with fibrillar amyloid protein was detected (e.g., see Example 5 and FIG. 4). 6C3 antibody was used in bright field immunohistochemistry and laser scanning confocal microscopy (LSCM) to detect structures resembling diffuse amyloid plaques not detected by AD polyclonal antibodies in hippocampal sections from the brains of neuropsychologically well-characterized AD patients (e.g., see Example 6 and FIG. 5).

It is known that oligomeric amyloid β protein assemblies are present in human blood and cerebrospinal fluid (CSF) of living subjects. It is contemplated that oligomeric amyloid β protein assemblies are also present in the blood, serous fluid and/or CSF of living subjects. It is contemplated that the presence of oligomeric amyloid β protein assemblies, or the presence of oligomeric assemblies above a threshold level, in these fluids is diagnostic of Alzheimer's disease. Thus, the present invention provides methods and compositions for the diagnosis and prognosis of Alzheimer's disease. Indeed, the present invention provides compositions and methods to analyze disease severity, and the efficacy of Alzheimer's disease therapies. It is contemplated that subjects identified as having higher levels of oligomeric amyloid β protein assemblies (e.g., in blood, serous fluid or CSF) have more advanced Alzheimer's disease than subjects showing lower levels of oligomeric amyloid β protein assemblies. It is contemplated that by monitoring the levels of oligomeric amyloid β protein assemblies in blood, serous fluid and/or CSF of patients undergoing treatment for Alzheimer's disease, determinations regarding the effectiveness of treatment regimes are possible. For example, reduced levels of oligomeric amyloid β protein assemblies over time indicate that the treatment used to treat a subject with Alzheimer's disease is effective.

It is contemplated that the present invention will find use in testing subjects such as those who have been previously diagnosed with Alzheimer's disease, those who are

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suspected of having Alzheimer's disease, and those at risk of developing Alzheimer's disease. For example, patients diagnosed with dementia, in particular, those patients who were previously clinically normal, are suitable subjects. However, it is not intended that the present invention be limited to use with any particular subject or patient types. The methods of the present invention are also useful for detecting early onset Alzheimer's disease and late onset Alzheimer's disease, as well as for detecting sporadic Alzheimer's disease and familial Alzheimer's disease.

The present invention also provides compositions and methods for the detection and quantitation (i.e., measurement) of oligomeric and fibrillar amyloid β protein assemblies in the blood, serous fluid and CSF. Standard techniques known in the art are easily adapted to quantitate the levels of circulating oligomeric and fibrillar amyloid β protein assemblies in blood, serous fluid and/or CSF samples, including but not limited to, ELISA.

Factors contributing to the success of the ELISA methods of the present invention include their sensitivity, versatility, long reagent shelf-life, ease of preparation of reagents, non-radioactive reagents, and assay speed. Furthermore, in some embodiments, the assay is quantitative. In addition, reagents and equipment designed specifically for use in ELISA protocols are readily available from numerous manufacturers, including Pierce Chemical Company, Bio-Rad, Dynatech Industries, GibcoBRL/Life Technologies, Fisher Scientific, and Promega.

Many ELISA applications and formats have been described. Various sources provide discussion of ELISA chemistry, applications, and detailed protocols (See e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, N.J. [1998]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]; and Laurino et al., Ann. Clin. Lab Sci., 29(3):158-166 [1999]).

In preferred embodiments of the present invention, ELISA methods for quantitation of antigen are provided. In some of these methods, the antigen (e.g., oligomeric amyloid β protein) is first immobilized on a solid support (e.g., in a microtiter plate well). Detection

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and quantitation of the immobilized antigen is accomplished by use of an antibody-enzyme conjugate capable of binding to the immobilized antigen and producing a quantifiable signal. In some embodiments, the amount of antigen present is directly proportional to the amount of enzyme reaction product produced after the addition of an appropriate enzyme substrate.

As indicated previously, enzymes commonly used in ELISAs include horseradish peroxidase (HRPO), urease, alkaline phosphatase, glucoamylase and β-galactosidase. Protocols for the preparation of suitable antibody-enzyme conjugates are well known in the art. The present invention provides methods for the preparation of an antibody-enzyme (i.e., HRPO enzyme) conjugate that specifically recognizes the antigen of interest (i.e., oligomeric or fibrillar amyloid β protein assemblies) for use in immunoassay (e.g. ELISA) methods for detection of Alzheimer's disease. It is not intended that the present invention be limited to the antibody-enzyme conjugation method provided herein, as those of skill in the art will recognize other methods for antibody-enzyme conjugation that find use with the present invention.

Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme (e.g., HRPO or alkaline phosphatase) and the antibody (e.g., the monoclonal anti-oligomeric amyloid β protein assembly antibody or the monoclonal anti-fibrillar amyloid β protein assembly antibody), where neither the antigen-binding site of the antibody nor the active site of the enzyme is functionally altered.

The conjugation of antibody and HRPO is dependent on the generation of aldehyde groups by periodate oxidation of the carbohydrate moieties on HRPO (Nakane and Kawaoi, J. Histochem. Cytochem., 22:1084-1091 [1988]). Combination of these active aldehydes with amino groups on the antibody forms Schiff bases that, upon reduction by sodium borohydride, become stable.

Protocols to make antibody-enzyme conjugates using urease or alkaline phosphatase enzymes are also known in the art (Healey et al., Clin. Chim. Acta 134:51-58 [1983]; Voller et al., Bull. W.H.O., 53:55-65 [1976]; and Jeanson et al., J. Immunol. Methods 111:261-270 [1988]). For urease conjugation, cross-linking of the urease enzyme (e.g., Urease Type VII, Sigma No. U0376) and antibody using m-maleimidobenzoyl N-hydroxysuccinimide ester

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(MBS) is achieved through benzoylation of free amino groups on the antibody. This is followed by thiolation of the maleimide moiety of MBS by the cysteine sulfhydryl groups of urease. To prepare an alkaline phosphatase-antibody conjugate, a one-step glutaraldehyde method is the simplest procedure (Voller et al., Bull. W.H.O., 53:55-65 [1976]). This antibody-alkaline phosphatase conjugation protocol uses an enzyme immunoassay grade of the alkaline phosphatase enzyme.

The end product of an ELISA is a signal typically observed as the development of color or fluorescence. Typically, this signal is read (i.e., quantitated) using a suitable spectrocolorimeter (i.e., a spectrophotometer) or spectrofluorometer. The amount of color or fluorescence is directly proportional to the amount of immobilized antigen. In some embodiments of the present invention, the amount of antigen in a sample (e.g., the amount of oligomeric or fibrillar amyloid β protein assemblies in a blood or CSF sample) is quantitated by comparing results obtained for the sample with a series of control wells containing known concentrations of the antigen (i.e., a standard concentration curve). A negative control is also included in the assay system.

It is contemplated that any suitable chromogenic or fluorogenic substrates will find use with the enzyme-conjugated antibodies of the present invention. In some embodiments of the present invention, the substrate p-nitrophenyl phosphate (NPP) in diethanolamine is the preferred substrate for use in calorimetric ELISA methods, and 4-methylumbelliferyl phosphate (MUP) is the preferred alkaline phosphatase substrate in fluorometric ELISA methods.

The present invention provides various ELISA protocols for the detection and/or quantitation of oligomeric or fibrillar amyloid β protein assemblies in a sample. In one embodiment, the present invention provides a "direct ELISA" for the detection of oligomeric or fibrillar amyloid β protein assemblies in a sample. In some embodiments, the antigen of interest in a sample (i.e., the oligomeric or fibrillar amyloid β protein assembly) is bound (along with unrelated antigens) to the solid support (e.g., a microtiter plate well). The immobilized antigen is then directly detected by the antigen-specific enzyme-conjugated antibody, also provided by the present invention. Addition of an appropriate detection substrate results in color development or fluorescence that is proportional to the amount of antigen present in the well.

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In another embodiment, the present invention provides an indirect ELISA for the detection of antigen in a sample. In this embodiment, antigen of interest in a sample is immobilized (along with unrelated antigens) to a solid support (e.g., a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody, then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (e.g., a goat anti-rabbit antibody), which are available from various manufacturers known to one in the art (e.g., Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

In some embodiments, the concentration of sample added to each well is titrated, so as to produce an antigen concentration curve. In other embodiments, the concentration of conjugated antibody is titrated. Indeed, such titrations are typically performed during the initial development of ELISA systems.

In another embodiment, the present invention provides "sandwich ELISA" methods, in which the antigen in a sample is immobilized on the solid support by a "capture antibody" that has been previously bound to the solid support. In general, the sandwich ELISA method is more sensitive than other configurations, and is capable of detecting 0.1-1.0 ng/ml protein antigen. As indicated above, the sandwich ELISA method involves pre-binding the "capture antibody" which recognizes the antigen of interest (i.e., the oligomeric or fibrillar amyloid β protein assemblies) to the solid support (e.g. wells of the microtiter plate). In some embodiments, a biotinylated capture antibody is used in conjunction with avidin-coated wells. Test samples and controls are then added to the wells containing the capture antibody. If antigen is present in the samples and/or controls, it is bound by the capture antibody.

In some embodiments, after a washing step, detection of antigen that has been immobilized by the capture antibody is detected directly (i.e., a direct sandwich ELISA). In other embodiments detection of antigen that has been immobilized by the capture antibody is detected indirectly (i.e., an indirect sandwich ELISA). In the direct sandwich ELISA, the captured antigen is detected using an antigen-specific enzyme-conjugated antibody. In the indirect sandwich ELISA, the captured antigen is detected by using an antibody directed against the antigen, which is then detected by another enzyme-conjugated antibody which binds the antigen-specific antibody, thus forming an antibody-antigen-antibody-- antibody

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complex. In both the direct and indirect sandwich ELISAs, addition of a suitable detection substrate results in color development or fluorescence that is proportional to the amount of antigen that is present in the well.

In the sandwich ELISA, the capture antibody used is typically different from the second antibody (the "detection antibody"). The choice of the capture antibody is empirical, as some pairwise combinations of capture antibody and detection antibody are more or less effective than other combinations. The same monoclonal antibody must not be used as both the capture antibody and the conjugated detection antibody, since recognition of a single epitope by the capture antibody will preclude the enzyme-conjugated detection antibody from binding to the antigen. However, in some embodiments, two different monoclonal antibodies that recognize different epitopes are used in this assay.

Furthermore, it is not intended that the present invention be limited to the direct ELISA and sandwich ELISA protocols particularly described herein, as the art knows well numerous alternative ELISA protocols that also find use in the present invention (See, e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, N.J. [1998]; and Ausubel et al (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]). Thus, any suitable ELISA method including, but not limited to, competitive ELISAs also find use with the present invention.

In another embodiment, the present invention provides methods for the detection and quantitation of oligomeric or fibrillar amyloid β protein assembly reactive antibodies. Briefly, in some embodiments, variations of indirect ELISAs are used. In preferred embodiments, antigens (i.e., oligomeric or fibrillar amyloid β protein assemblies) are first used to coat the wells of a 96-well microtiter plate. The test sample is then added to the antigen-coated wells. If the test sample contains oligomeric or fibrillar amyloid β protein assembly reactive antibodies, these antibodies specifically bind to the purified antigen coating the well. The oligomeric or fibrillar amyloid β protein assembly reactive antibodies are then visualized by the addition of a second detection antibody, where the detection antibody is coupled to an enzyme and is species-specific or isotype-specific for anti-oligomeric or anti-fibrillar amyloid β protein assembly antibody. As with all ELISA

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methods, appropriate negative and positive controls are included in order to ensure the reliability of the assay results.

It is contemplated that patients with Alzheimer's disease produce oligomeric or fibrillar amyloid β protein assembly-reactive auto-antibodies, and an ELISA to detect oligomeric or fibrillar amyloid β protein assembly reactive antibodies in such samples will find use in the diagnosis of Alzheimer's disease. It is further contemplated that the presence of anti-oligomeric or anti-fibrillar amyloid β protein assembly auto-antibodies in a patient is diagnostic of Alzheimer's disease.

It is also contemplated that the present invention will find use in detection of oligomeric or fibrillar amyloid β protein assembly reactive antibodies in various other settings (e.g., in the screening of monoclonal hybridoma culture supernatants [i.e., conditioned hybridoma culture medium], ascites fluid and/or polyclonal antisera).

The present invention also provides ELISA amplification systems. These embodiments produce at least 10-fold, and more preferably, a 500-fold increase in sensitivity over traditional alkaline phosphatase-based ELISAs. In one preferred embodiment of the ELISA amplification protocol, bound alkaline phosphatase acts on an NADPH substrate, whose reaction product initiates a secondary enzymatic reaction resulting in a colored product. Each reaction product from the first reaction initiates many cycles of the second reaction in order to amplify the signal (See e.g., Bio-Rad ELISA Amplification System, Cat. No. 19589-019).

The present invention also provides ELISA kits for the detection of antibodies and/or antigen. In addition, in some embodiments, the kits are customized for various applications. However, it is not intended that the kits of the present invention be limited to any particular format or design. In some embodiments, the kits of the present invention include, but are not limited to, materials for sample collection (e.g., spinal and/or venipuncture needles), tubes (e.g., sample collection tubes and reagent tubes), holders, trays, racks, dishes, plates (e.g., 96-well microtiter plates), instructions to the kit user, solutions or other chemical reagents, and samples to be used for standardization, and/or normalization, as well as positive and negative controls. In particularly preferred embodiments, reagents included in ELISA kits specifically intended for the detection of

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oligomeric or fibrillar amyloid β protein assemblies or anti-oligomeric or anti-fibrillar amyloid β protein assembly antibodies include control oligomeric amyloid β protein assemblies, anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibodies, anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibody-enzyme conjugate, 96-well microtiter plates precoated with control RA-NDA peptide, suitable capture antibody, 96-well microtiter plates precoated with a suitable oligomeric and/or anti-fibrillar amyloid β protein assembly capture antibody, buffers (e.g., coating buffer, blocking buffer, and distilled water), enzyme reaction substrate and premixed enzyme substrate solutions.

It is contemplated that the compositions and methods of the present invention will find use in various settings, including research and clinical diagnostics. For example, the anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibodies of the present invention also find use in studies of APP metabolism and in *in situ* hybridization studies of brain tissue sections to observe Alzheimer's disease pathology. In addition, methods to quantitate oligomeric and/or fibrillar amyloid β protein assemblies in samples find use in monitoring and/or determining the effectiveness of Alzheimer's disease treatment, as it is contemplated that decreasing levels of oligomeric amyloid β protein assemblies in a subject's samples over time indicates the effectiveness of an Alzheimer's disease treatment. Uses of the compositions and methods provided by the present invention encompass human and non-human subjects and samples from those subjects, and also encompass research as well as diagnostic applications. Thus, it is not intended that the present invention be limited to any particular subject and/or application setting.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: .degree. C. (degrees Centigrade); cm (centimeters); g (grams); l or L (liters); .mu.g (micrograms); .mu.l (microliters); .mu.m (micrometers); .mu.M (micromolar); lmol (micromoles); mg (milligrams); ml (milliliters); mm (millimeters); mM (millimolar); mmol (millimoles); M (molar); mol (moles); ng (nanograms); nm (nanometers); nmol

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(nanomoles); N (normal); pmol (picomoles); Aldrich (Sigma/Aldrich, Milwaukee, Wis.); Amersham (Amersham/Pharmacia Biotech, Piscataway, N.J.); Bio-Rad (Bio-Rad Laboratories, Hercules, Calif.), Boehringer Mannheim (Boehringer Mannheim Corporation, Indianapolis, Ind.); Dynex (Dynex Technologies, Inc., Chantilly, Va.); Fisher Scientific (Fisher Scientific, Pittsburgh, Pa.), GiboBRL/Life Technologies (GibcoBRL/Life Technologies, Gaithersburg, Md.), Oncogene Research Products (Oncogene Research Products, Cambridge, Mass.); Pharmingen/Transduction Laboratories (Pharmingen/Transduction Laboratories/Becton Dickinson Company, San Diego, Calif.); Pierce Chemical Company (Pierce Chemical Company, Rockford, Ill.); Promega (Promega Corporation, Madison, Wis.); Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.); Sigma (Sigma Chemical Co., St. Louis, Mo.); and Zymed (Zymed Laboratories, Inc., South San Francisco, Calif.).

Example 1

Materials and methods.

Generation of fibrillar or oligomeric immunogens for generation of hybridomas. Pretreated stocks of amyloid β protein stored as HFIP films are monomerized in DMSO, then aggregated in dilute acid at low salt (10mM HCL) to produce fibrillar amyloid β protein, or, in cell culture media (phenol-free F12, Gibco BRL) containing physiologic salt and pH levels to produce oligomeric structures. The amyloid β protein fibrils produced under the acidic conditions have diameters that measure approximately ~4nm in z-height and extend for several microns. Oligomers produced in cell culture media range in size from ~2nm in z-height.

Immunizations. Female Balb/c mice are immunized with amyloid β protein oligomers or fibrils produced as described above. The immunogens employed are suspended in Freunds Incomplete Adjuvant at a concentration of 1 μg/ μl. A total of 200 μg is injected subcutaneously every 2 weeks until the serum titer of the mouse is half-maximal at a dilution of $2x10^{-4}$ as judged by ELISA with 50 ng of amyloid β protein oligomers or fibrils attached per well in the solid phase.

Hybridoma production and clonal selection. Once the desired serum titer is attained, immune spleens are removed from the mice, dissociated, and fused with SP2/o myeloma

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cells. The resultant cell suspension is plated in 96 well plates, HAT selected and cultured for 10-14 days to allow clonal growth. Initial clonal selection is performed by incubating supernatants from each clone in two ELISA wells, one with amyloid β protein oligomers attached and the other with fibrils attached. Clonal supernatants from oligomer-immunized mice that are positive on the oligomer-attached plate but negative (or exhibit a two-fold or greater signal diminution) on the fibril-attached plate are selected for further subcloning. This dual selection protocol is repeated for screening fusion of splenocytes obtained from fibril-immunized mice. In this case, clones are selected that bind to the fibrils but not to the oligomers.

Subcloning and antibody production. Mother clones are subcloned 3-4 times to assure monoclonality and allow the hybrids to stabilize. Antibodies are isotyped and the stable clones are adapted to serum-free medium and placed in a bioreactor for antibody expression. Purified, homogenous monoclonal antibodies are then stored at 1 mg/ml in borate buffered saline containing 50% glycerol.

Example 2

Screening of hybridoma supernatants by antigen/antibody blotting.

In order to determine the specificity of the antibodies made by the hybridomas, supernatants of the hybridomas were screened by antigen/antibody blotting. 5 μM amyloid β protein1-42 oligomer or fibril solutions were incubated with Immobilon-P membranes at room temperature for 30 minutes. Following rinsing and blocking, hybridoma supernatant was spotted onto membrane with a 96-pin replicator.

This method identified several hybridomas making antibodies which appeared specific for oligomer amyloid β protein assemblies and not the fibrillar form (FIG. 1). Monoclonal antibodies from the hybridoma 7A2 are oligomer-specific and show little recognition of fibrils by antigen/antibody blotting. In contrast, antibodies from the hybridoma 6C3 do not demonstrate specificity for oligomers or fibrils by antigen/antibody blotting.

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Example 3

ELISA titer: oligomer- versus fibril specificity for 6C3 and 7A2.

Antibodies from hybridomas 7A2 and 6C3 were purified to homogeneity and further characterized in an ELISA assay. Serial dilutions of the purified antibodies were incubated with 25 ng of fibrillar or oligomeric amyloid β protein assemblies in the solid phase. 7A2 antibodies do not recognize fibrils by antigen/antibody blotting (FIG. 1). Additionally, standard ELISA shows that 7A2 antibodies display significant affinity for oligomeric assemblies while not displaying a similar affinity for fibrillar assemblies (FIG. 2). In contrast, although 6C3 does not demonstrate specificity for oligomers or fibrils by antigen/antibody blotting, standard ELISA shows 6C3 antibodies display some preference for oligomeric assemblies over that of fibrillar assemblies (FIG. 2).

Example 4

Western blot analysis using 7A2 and 6C3 antibodies.

To further characterize the 7A2 and 6C3 antibodies, Western blot analysis was performed. Unaggregated (U), oligomeric (O), and fibrillar (F) amyloid β protein1-42 were run on 12% NuPAGE Bis-Tris gels under non-reducing conditions. The blots were probed with purified 6C3 (1:10,000), 6E10 (1:5000), or 7A2 (1:1000) monoclonal antibodies.

The oligomer-specific antibody (7A2) shows little recognition of fibrils by antigen/antibody blotting (FIG. 1) and ELISA (FIG. 2). By Western analysis of SDS-PAGE, 7A2 detects primarily dimer and trimer but no amyloid β protein monomers in unaggregated or oligomeric samples, and little immunoreactivity is detected in the fibril samples (FIG. 3). In contrast, 6C3 demonstrates a slight selectivity for oligomers over fibrils by ELISA (FIG. 2), however no differences are detected in Western blots between unaggregated, oligomer or fibril samples; here it reacts much like other amyloid β protein monoclonal antibodies such as 6E10 and 4G8 (FIG. 3).

Example 5

Bright field immunohistochemistry.

The monoclonal antibodies 7A2 and 6C3 were analyzed on sections of human brain using standard peroxidase-based immunohistochemistry (FIG. 4). Tissue sections from the

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superior parietal lobule were obtained from well-characterized AD patients. The sections were stained with purified 6C3 antibody at a 1:20,000 dilution and with the tissue culture supernatant of 7A2 at a 1:1000 dilution. The magnification is 10x. In sections from AD brain, little 7A2 immunoreactivity with fibrillar amyloid plaques is detected (FIG 4).

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Example 6

Laser scanning confocal microscopy (LSCM) of AD brain slices.

In order to determine whether an antibody specific for soluble oligomers coexists in
10 the same cells, plaques or areas in the neuropil as Thioflavin S-positive plaques, double or triple immunofluorescence assessment using LSCM is useful. Sections from the same brain regions as those studied using the peroxidase procedure above are used. In this procedure, the primary antibodies are each detected with a different fluorochrome (either directly conjugated to the primary antibody or to a species-specific or isoform-specific secondary
15 antibody). The z-sections obtained from a series of confocal images can be stacked and both fluorescence channels combined following pseudo-coloring. These stacks can then be rotated to view the three-dimensional image from a number of angels. For double immunofluorescence confocal microscopy, the co-localization feature of the software Metamorph is used to establish the percent co-localization between two fluorochromes from
20 representative digital images.

A problem frequently encountered in any immunofluorescence studies on aged human brains is the presence of significant amounts of autofluorescent lipofuscin that can be confused with the yellow fluorescence seen during colocalization. In order to minimize
25 this problem, sections are placed in a potassium permanganate solution (0.25% in phosphate buffered saline) for 20 minutes, after which a brown color develops. The sections are washed in phosphate buffered saline for two minutes, and then treated with a solution of 1.0g% potassium metabisulfite and 1.0 g% oxalic acid in phosphate buffered saline until the brown color dissipates, typically occurring in 1-6 minutes. Finally, the sections are washed
30 three times in phosphate buffered saline for two minutes each. Alternatively, Cy 5, a fluorochrome that emits in the infrared range can be used, thereby circumventing the autofluorescence of lipofuscin that does not emit light in the infrared spectrum of light.

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The monoclonal antibody 6C3 and an amyloid β protein polyclonal antibody (R1280) were used to immunohistochemically stain an amyloid plaque and diffuse amyloid β protein deposits in the temporal lobe from an AD patient. 6C3 detected diffuse amyloid plaque-like structures not detected by AD polyclonal antibodies (FIG 5).

Example 7

Western blot and dot blot analysis using 7A2 and 6C3 antibodies

To still further characterize the 7A2 and 6C3 antibodies, Western blot analysis was repeated on unaggregated (U), oligomeric (O), and fibrillar (F) amyloid β protein1-42 as in Example 4. 1100 pMol amyloid β protein1-40 or 200 pMol amyloid β protein1-42 were run on 4-12% BIS-TRIS NuPAGE gels, transferred to PVDF membrane and probed with each respective antibody. In this experiment, the 7A2 antibody only detected oligomeric amyloid β protein1-42 assemblies (primarily dimer, tetramer and larger oligomers between approximately 27 and 44 kDa), whereas 6E10 and 6C3 detected multiple forms of amyloid β protein1-42 including monomer, trimer, tetramer and oligomers between approximately 27 and 80 kDa (FIG. 6). The data presented in Figure 6 accurately reflects the identity of the bands. To further confirm the data, a range of antibody concentrations was hybridized to amyloid β protein1-42 transferred to PVDF. The oligomer specificity of 7A2 was retained over a wide range of antibody concentrations (FIG. 6). Note: "MOAB-1" and "MOAB-2" in Figure 6 correspond to 7A2 and 6C3 antibodies, respectively.

Further confirmation of the specificity of 7A2 for oligomeric amyloid β protein1-42 was achieved using dot blot analysis of different conformations of amyloid β protein immobilized on nitrocellulose. In Figure 7a, 10 pMol of amyloid β protein1-40, unaggregated amyloid β protein1-42, oligomeric amyloid β protein1-42, or fibrillar amyloid β protein1-42 were spotted on nitrocellulose and probed with 6E10, 6C3 or 7A2 antibodies. In Figure 7b, a dilution series of amyloid β protein antigens was probed with 6E10, 6C3 or 7A2. In Figure 7c, different ratios of oligomeric and fibrillar amyloid β protein1-42 were co-applied to nitrocellulose membrane and probed with 6E10, 6C3 or 7A2 antibodies. The dot blot analysis produced results consistent with both Western blot and ELISA results. 7A2 displayed greater affinity for oligomeric amyloid β protein1-42 as compared to amyloid β protein1-40, unaggregated or fibrillar amyloid β protein1-42. This selective high affinity interaction was maintained over a range of antigen concentrations. 6C3 and 6E10 were more immunoreactive to amyloid β protein1-42 than amyloid β protein1-40 but did not

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differentiate between oligomeric or fibrillar assemblies of amyloid β protein1-42. 7A2 also demonstrated specificity for oligomeric species in the presence of increasing concentrations of co-deposited fibrillar amyloid β protein1-42.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

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CLAIMS

What is claimed is:

1. A composition comprising a purified monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies.

2. The composition of Claim 1, wherein said amyloid β protein is the β1-42 protein.

3. A hybridoma that secretes said monoclonal antibody of Claim 1.

4. A method for obtaining and isolating a hybridoma secreting said monoclonal antibody of Claim 1, comprising:

a) providing spleen cells immunized with an antigen comprising soluble, non-fibrillar oligomeric amyloid β protein assemblies, wherein said antigen is recognized by said monoclonal antibody of Claim 1;

b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

c) selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising amyloid β proteins without recognizing fibrillar amyloid β protein assemblies.

5. A composition comprising a purified monoclonal antibody suitable for identification of fibrillar amyloid β protein assemblies that does not identify soluble, non-fibrillar oligomeric amyloid β protein assemblies.

6. The composition of Claim 5, wherein said amyloid β protein is the β1-42 protein.

7. A hybridoma that secretes said monoclonal antibody of Claim 5.

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8. A method for obtaining and isolating a hybridoma secreting said monoclonal antibody of Claim 5, comprising:

a) providing spleen cells immunized with an antigen comprising fibrillar amyloid β protein assemblies, wherein said antigen is recognized by said monoclonal antibody of Claim 5;

b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

c) selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies containing amyloid β proteins without recognizing soluble, non-fibrillar oligomeric amyloid β protein assemblies.

9. A method for detecting at least one amyloid β protein assembly, comprising the steps of:

a) providing

i) a sample from a subject suspected of containing at least one amyloid β protein assembly; and

ii) an antibody that identifies amyloid β protein assemblies;

b) contacting said sample with said antibody under conditions such that said antibody binds to said amyloid β protein assembly, forming an antigen-antibody complex; and

c) detecting the presence of said antigen-antibody complex.

10. The method of Claim 9, wherein said at least one amyloid β protein assembly comprises soluble, non-fibrillar oligomeric amyloid β protein comprising 2-12 amyloid β proteins.

11. The method of Claim 9, wherein said at least one amyloid β protein assembly comprises fibrillar amyloid β protein comprising more than 12 amyloid β proteins.

12. The method of Claim 9, wherein said sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid.

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13. The method of Claim 9, wherein said subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease.

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14. The method of Claim 9, further comprising the step of diagnosing Alzheimer's disease, wherein said Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease.

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15. The method of Claim 9, wherein said detecting at least one amyloid β protein assembly comprises an enzyme-linked immunosorbent assay, wherein said enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich
15 enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays.

16. The method of Claim 9, further comprising the step of quantitating said at least one amyloid β protein assembly in said sample.

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17. The method of Claim 15, wherein said enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

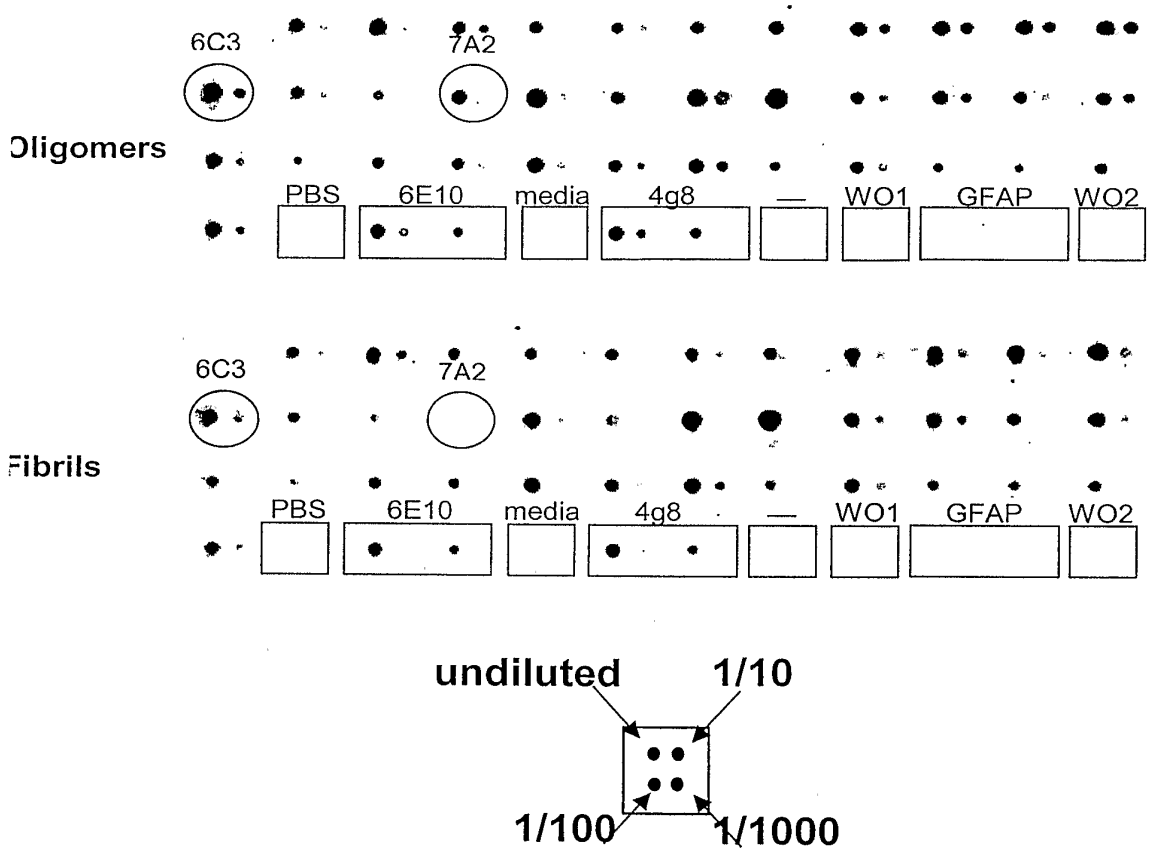
18. The method of Claim 15, further providing at least one capture antibody.

25

19. The method of Claim 18, further providing at least one detection antibody.

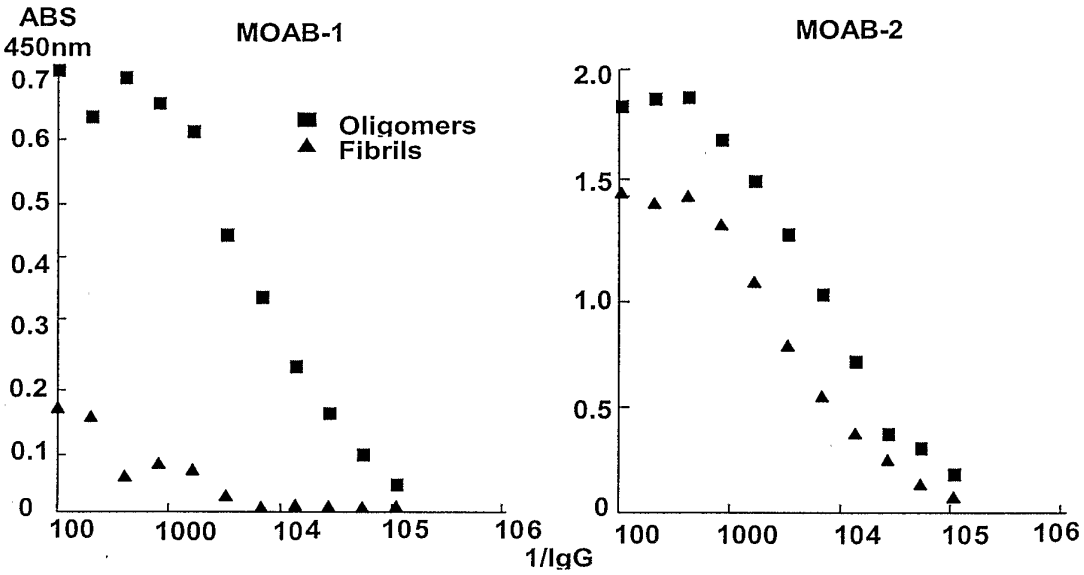
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FIGURE 1



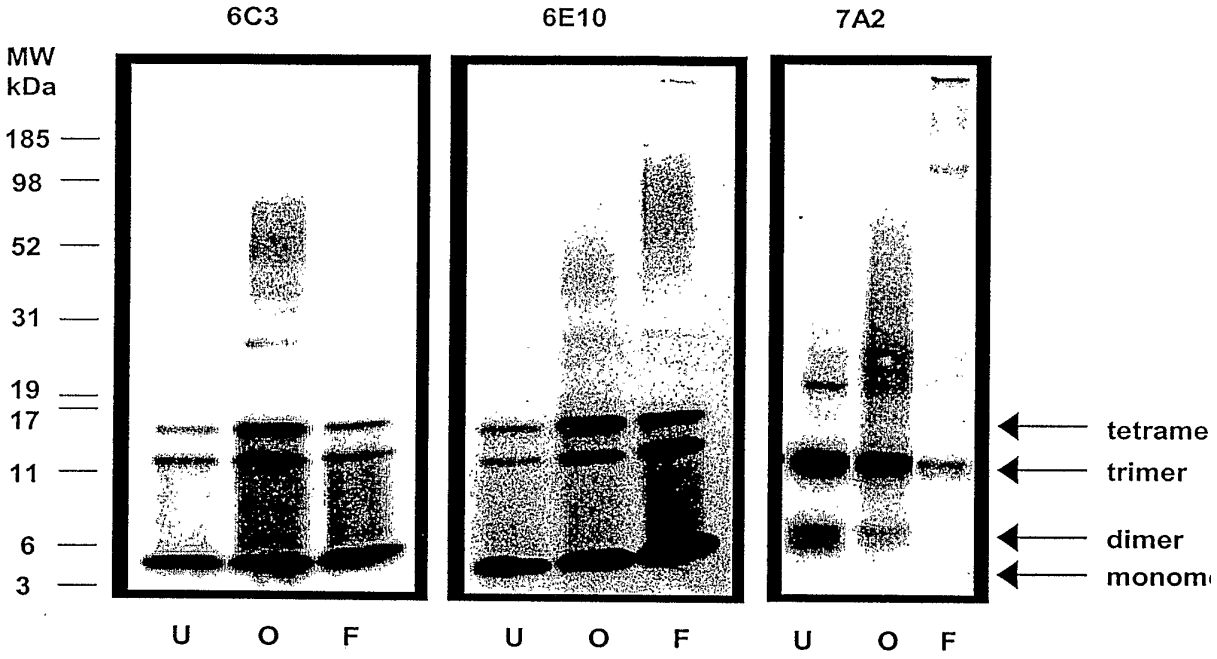
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FIGURE 2



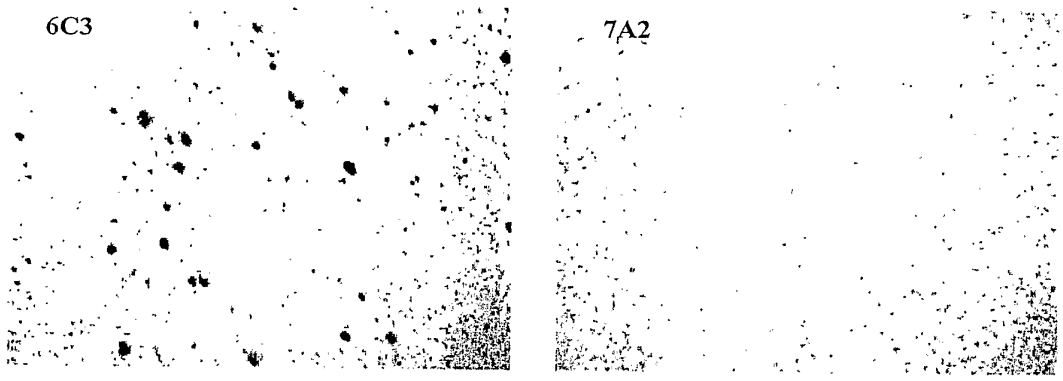
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FIGURE 3



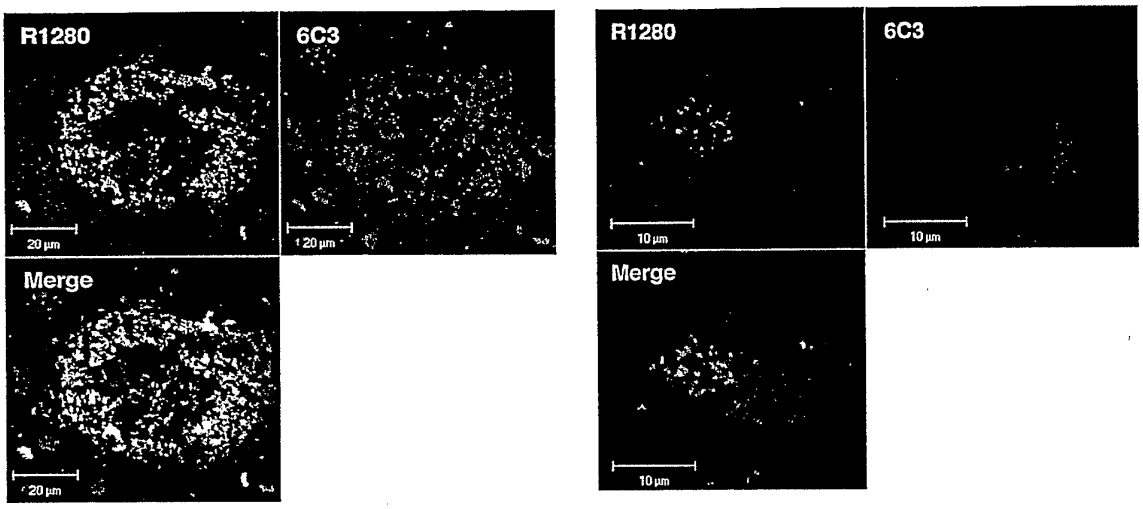
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FIGURE 4



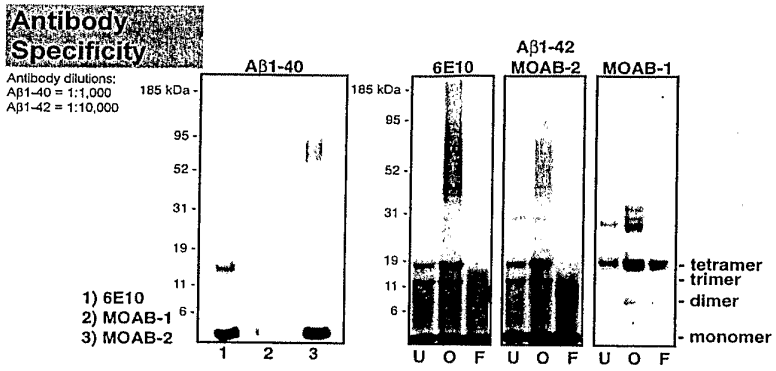
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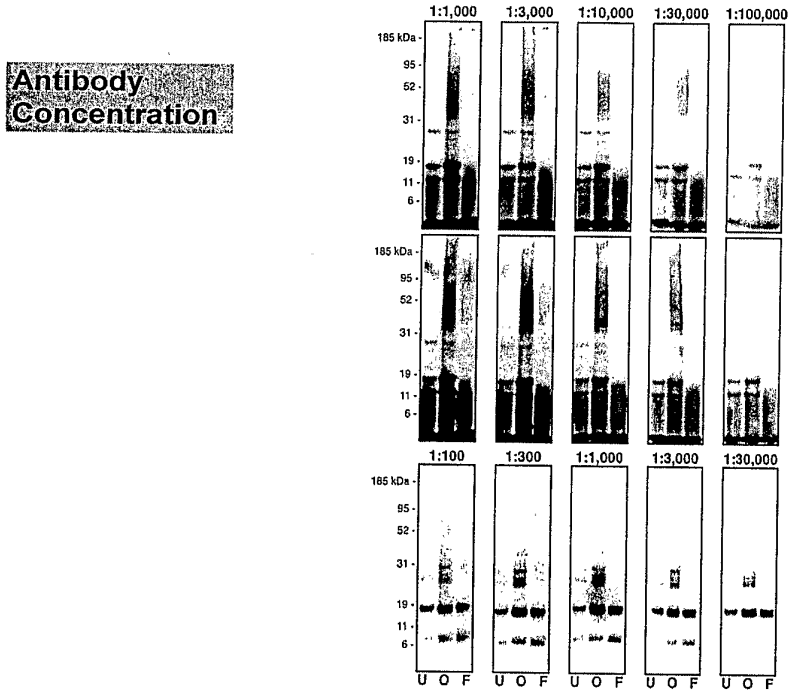
FIGURE 5



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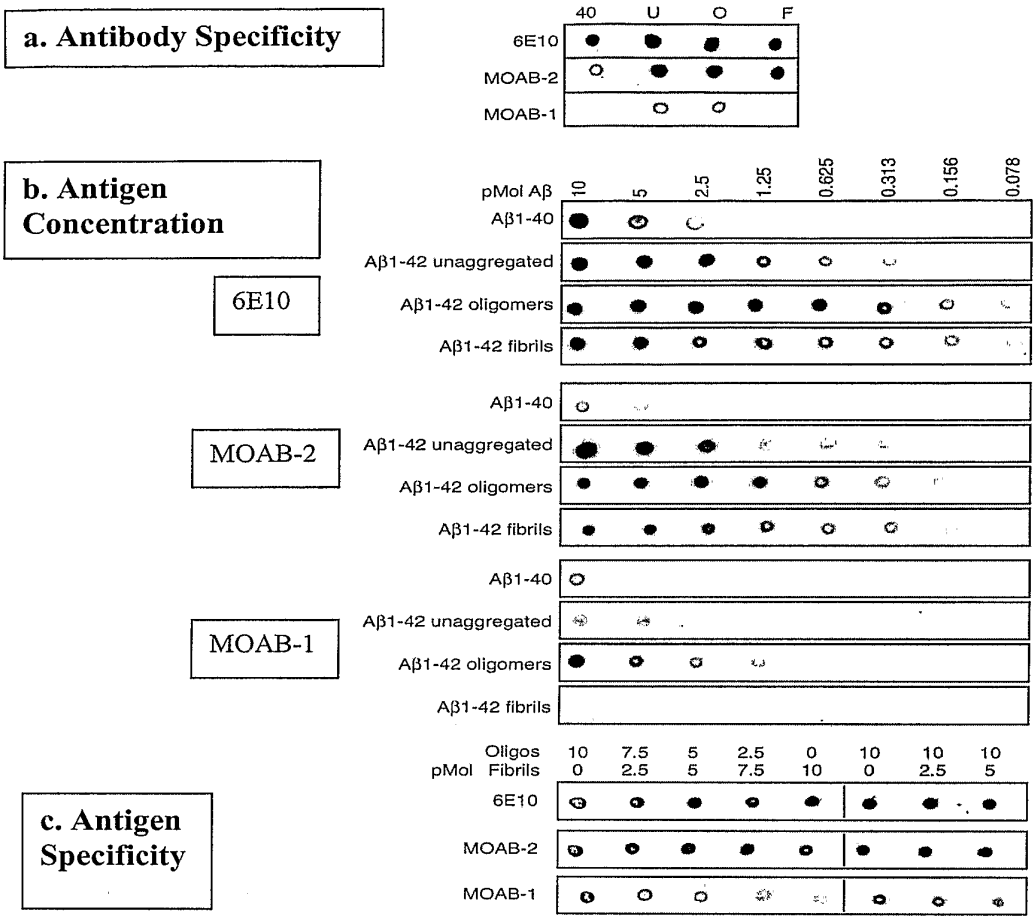
FIGURE 6





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FIGURE 7



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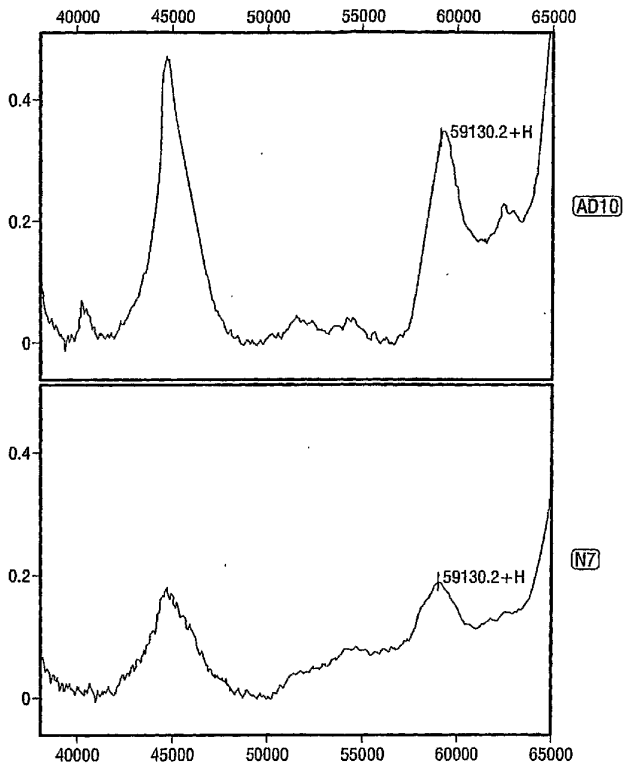
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[Continued on next page]

(54) Title: BIOMARKERS FOR ALZHEIMER'S DISEASE



(57) Abstract: The present invention provides protein-based biomarkers and biomarker combinations that are useful in qualifying Alzheimer's disease status in a patient. In particular, the biomarkers of this invention are useful to classify a subject sample as Alzheimer's or non-Alzheimer's dementia or normal. The biomarkers can be detected by SELDI mass spectrometry. In addition, the invention provides appropriate treatment interventions and methods for measuring response to treatment. Certain biomarkers of the invention may also be suitable for employment as radio-labeled ligands in non-invasive imaging techniques such as Positron Emission Tomography (PET).

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BIOMARKERS FOR ALZHEIMER'S DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States provisional patent application No. 60/518,360, filed November 7, 2003; United States provisional patent application No. 60/526,753, filed December 2, 2003; United States provisional patent application No. 60/546,423, filed February 19, 2004; United States provisional patent application No. 60/547,250, filed February 23, 2004; United States provisional patent application No. 60/558,896, filed April 2, 2004; United States provisional patent application No. 60/572,617, filed May 18, 2004; and United States provisional patent application No. 60/586,503, filed July 8, 2004, the disclosure of each of which is incorporated herein by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Alzheimer's Disease is a progressive neurodegenerative disorder that leads to the death of brain cells that cannot be replaced once lost. The two key neuropathological hallmarks of AD are the presence of senile plaques predominantly comprised of aggregated beta-amyloid protein (Aß) and neurofibrillary tangles (NFTs) formed by the accumulation of hyperphosphorylated tau protein. Currently, the clinical diagnosis of AD requires an evaluation of medical history and physical examination including neurological, neuropsychological and psychiatric assessment, as well as various biological, radiological and electrophysiological tests. Despite the battery of tests, a definitive diagnosis can only be achieved by post-mortem brain examination. Therefore, there is an unmet need for a simple biochemical test that can detect AD at an early stage, monitor progression of the disease, and discriminate between AD, normal, non-AD dementias and other neurological disorders.

[0003] Three different biomarkers in Cerebrospinal Fluid (CSF) have been particularly well researched: neuronal thread protein, tau and derivatives of amyloid precursor protein (APP).

[0004] Neuronal thread protein is known to be overexpressed in brain neurons in AD patients. The company NYMOX has developed a quantitative test for measuring levels of a specific type of neuronal thread protein (AD7c-NTP) in CSF and urine. Quite a number of studies have evaluated CSF-tau as an ante-mortem marker for AD mainly using

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enzyme-linked immunoabsorbent assays (ELISA) as the measurement assay. In most of these studies, total tau has been measured although there is an increasing body of literature also describing the analysis of phosphorylated variants of the same protein involved in the formation of NFTs. ELISAs that can distinguish between the major form of Aß ending at amino acid 40 (Aß40) and the senile plaque forming species ending at position 42 (Aß42) have also been developed and evaluated extensively for CSF analysis. All three assays, either used individually, or in the case of tau and Aß42, in combination, do not have the required sensitivity and specificity values for routine clinical use, particularly for early diagnosis and discrimination between AD and other non-AD dementias. In addition, attempts to measure tau and Aß42 in blood have been met with limited success, further restricting their widespread adoption into clinical practice.

[0005] A wide spectrum of other aberrations, other than NTP, Tau and Aß, has been reported in AD patient CSF. Many of the identified (protein sequence confirmed) CSF markers reported herein have been shown to be either increased or decreased in AD patients versus normal individuals. For example, the protein Ubiquitin is known to complex with hyperphosphorylated Tau during maturation of NFTs in the brains of AD patients (Iqbal *et. al. J Neural Transm Suppl. 53*:169-80 (1998)). Ubiquitin levels in CSF of AD and neurological control groups have been shown to be significantly higher than those of non-neurological aged controls (Wang *et. al. Acta Neuropathol (Berl). 82(1)*:6-12 (1991); Kudo *et. al. Brain Res. 639(1)*:1-7 (1994)).

[0006] The acute phase/inflammatory protein alpha(1)-antichymotrypsin (ACT) is overproduced in the AD brain. ACT also can promote the formation of, and is associated with, neurotoxic amyloid deposits (Potter *et. al. Neurobiol Aging. 22(6)*:923-30 (2001)). The levels of ACT in both serum and CSF are significantly and specifically higher in patients with Alzheimer-type dementia than in control subjects (Matsubara *et. al. Ann Neurol. 28(4)*:561-7(1990)). There is a particularly close association of increases in CSF-ACT with late onset AD (Harigaya *et. al. Intern Med. 34(6)*:481-4 (1995)).

[0007] Chromogranin A (CrA) is the major protein of large dense-core synaptic vesicles and may be of value as a biochemical marker for synaptic function in AD. One report described no difference between the AD, vascular dementia, and age-matched control groups except when comparing a familial subtype (AD Type I) with controls where

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there was a statistically significant elevation of CSF CrA in the diseased individuals (Blennow *et. al. Dementia. 6(6)*:306-11 (1995)).

[0008] Beta-2-Microglobulin (B2M) is an initiator of inflammatory responses modulated by interferons and certain cytokines (Hoekman *et.al. Neth. J. Med. 28:*551–557 (1985)). A proteome analysis of CSF by two-dimensional electrophoresis (2D-gel) has shown a significant increase of B2M in AD patients (Davidsson *et al., Neuroreport, 13*:611-615 (2002)), and more recently these results were confirmed by SELDI analysis (Carrette, O. *et. al., Proteomics, 3:*1486-1494 (2003)).

[0009] Transthyretin (TTR) has been shown to interact with Aß, possibly preventing amyloid formation in biological fluids and in the brain. (Tsuzuki *et al., Neurosci Lett, 10*:171-174 (2000)). One identified TTR isoform was shown to be increased in AD-CSF using 2D gel analysis of a small number of AD and control patients (Davidsson, *supra.*). However, this result conflicts with other reports showing a clear decrease of TTR in CSF from AD patients compared with controls (Serot *et. al. J Neurol Neurosurg Psychiatry. 63(4)*:506-8 (1997); Riisoen *et. al. Acta Neurol Scand. 78(6):*455-9 (1998)). This decrease is also negatively correlated with the senile plaque (SP) abundance (Merched *et. al. FEBS Lett. 425(2):*225-8 (1998)).

[0010] Cystatin C, a cysteine protease inhibitor, has been implicated in the neurodegenerative and repair processes of the nervous system, and the deposition of the same protein together with beta amyloid peptide was found as cerebral amyloid angiopathy (CAA) in different types of dementias (Levy *et.al. J. Neuropathol. Exp. Neurol. 60:*94-104). Full length Cystatin C was found as a CSF marker for AD in a previous SELDI profiling study (Carrette, *supra.).* A relative blood-brain barrier (BBB) dysfunction is associated with AD among very elderly individuals. The CSF/serum albumin ratio can be used as a measure of BBB function. Mean CSF/serum albumin ratio has been reported to be higher in all dementias studied, including AD, than in nondemented individuals (Skoog *et al., Neurology. 50*:966-71 (1998)).

[0011] Transferrin (TF) plays a role in anti-oxidant defense in serum and is also produced in the brain where its role in oxidative stress is unclear. A study on Down's syndrome patients suffering from progressive dementia showed decreased levels of TF when compared to age-matched controls with no neurological disease (Elovaara Acta Neurol Scand. 69(5):302-5(1994)).

[0012] Prior studies evaluating the different biochemical markers in the CSF of dementia patients have employed assay methods, such as ELISA, that use indirect means

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of measuring the analyte of interest. These methods are not capable of discerning the different processed forms of proteins revealed other processes. Furthermore, traditional assay methods have left unexplored avenues of treatment relating to the different processed forms of proteins. Thus, a need exists for methods which diagnosis Alzheimer's disease before significant neuronal loss has occurred, and for therapeutic treatments to prevent progression of the disease.

SUMMARY OF THE INVENTION

[0013] The present invention provides polypeptide-based biomarkers that are differentially present in subjects having Alzheimer's disease versus subjects free of the disease and/or versus subjects suffering from forms of non-Alzheimer's dementia (*e.g.*, LBD, FTD, *etc.*). In addition, the present invention provides methods of using the polypeptide-based biomarkers to qualify Alzheimer's disease in a subject. The present invention also provides methods for identifying Alzheimer's disease therapeutics and treating qualified individuals.

[0014] As such, in one aspect, the present invention provides a method for qualifying Alzheimer's disease status in a subject, the method comprising: (a) measuring at least one biomarker in a biological sample from the subject, wherein the at least one biomarker is selected from the group consisting of the biomarkers of Table I, Table II, Table IV-A and Table IV-B, *supra*; and (b) correlating the measurement with Alzheimer's disease status. In one embodiment, the sample is cerebrospinal fluid (CSF). In another embodiment, the sample is serum. In a preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table II, Table IV-A and Table IV-B. In another preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table IV-B.

[0015] In a preferred embodiment, the at least one biomarker is selected from the biomarkers of Table II or Table IV-A or Table IV-B. In one embodiment, the at least one biomarker is selected from the following biomarkers: M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6

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(Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4. In another preferred embodiment, the method comprises measuring N-acetylated thymosin beta-4. In yet another preferred embodiment, the at least one biomarker is selected from one of the biomarkers in Table II or IV-A or IV-B which is named in Table V.

[0016] In a preferred embodiment, the method comprises additionally measuring one or more of the following biomarkers: M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin), or alternatively, measuring each of these additional biomarkers. In another preferred embodiment, the method comprising measuring at least each of the biomarkers in the following two sets of biomarkers: M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), and M3513.9 (7B2 CT fragment); and M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), M10379.8 (10.3 kDa) and M11725.7 (Beta-2-Microglobulin). In another embodiment, the method comprising measuring at least one biomarker from the set consisting of the following biomarkers: 16207.4 (Pancreatic ribonuclease), 8183.6 (Ubiquitin -4aa from CT), M5227.4, M3806.2, M8955.1, M5263.9, M20839.2, M4320.6 (A-beta 1-40), 7258.2 (Chromogranin B peptide), M6608.9, M5838.3, M23477.4 ((Prostaglandin-D synthase), 7653.2 (Osteopontin CT fragment), M16716.9, M7878.7, 7718.8 (Osteopontin CT fragment phosphor), and M4455.4.

[0017] In one embodiment, the at least one biomarker is measured by capturing the biomarker on an adsorbent of a SELDI probe and detecting the captured biomarkers by laser desorption-ionization mass spectrometry. In certain embodiments, the adsorbent is a cation exchange adsorbent, an anion exchange adsorbent, a metal chelate or a hydrophobic adsorbent. In other embodiments, the adsorbent is a biospecific adsorbent. In another embodiment, the at least one biomarker is measured by immunoassay.

[0018] In another embodiment, the correlating is performed by a software classification algorithm. In certain embodiments, the Alzheimer's disease status is selected from Alzheimer's disease, non-dementia, and non-Alzheimer's dementia. In one embodiment, non-Alzheimer's dementia includes Lewy body dementia (LBD) and frontotemporal dementia (FTD).

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[0019] In yet another embodiment, the method further comprises: (c) managing subject treatment based on the status. If the measurement correlates with Alzheimer's disease, then managing subject treatment comprises administering a choline esterase inhibitor to the subject.

[0020] In a further embodiment, the method further comprises: (d) measuring the at least one biomarker after subject management.

[0021] In another aspect, the present invention provides a method comprising measuring at least one biomarker in a sample from a subject, wherein the at least one biomarker is selected from the group consisting of the biomarkers set forth in Table I, II, IV-A, Table IV-B, or any of the groups of biomarkers discussed above. In one embodiment, the sample is cerebrospinal fluid (CSF). In another embodiment, the sample is serum. In a preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table II. In another preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table IV-A or Table IV-B. In another preferred embodiment, the method comprises measuring N-acetylated thymosin beta-4. In yet another preferred embodiment, the at least one biomarker is selected from one of the biomarkers in Table II, Table IV-A, or Table IV-B which is named in Table V.

[0022] In one embodiment, the at least one biomarker is selected from the following biomarkers: M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4. In another embodiment, the at least one biomarker is selected from the following biomarkers: M11728.3 (β2 microglobulin), M60976.2 (Hemopexin), M11127.8 and M9742.3. In preferred embodiments, the method

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further comprises measuring Cystatin C (M13391). In yet another embodiment, the method further comprises additionally measuring a modified form of Cystatin C (CysC), for example, CysC Δ1-8, a truncated form of CysC missing 8 amino acids from the N-terminus of full-length CysC. In yet other embodiments, the method further comprises measuring at least one of the following additional biomarkers: M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin), or alternatively, measuring each of these additional biomarkers. In another preferred embodiment, the method comprises measuring N-acetylated thymosin beta-4. In yet another preferred embodiment, the at least one biomarker is selected from one of the biomarkers in Table II, Table IV-A, or Table IV-B which is named in Table V.

[0023] In one embodiment, the at least one biomarker is measured by capturing the biomarker on an adsorbent of a SELDI probe and detecting the captured biomarkers by laser desorption-ionization mass spectrometry. In certain embodiments, the adsorbent is a cation exchange adsorbent, an anion exchange adsorbent, a metal chelate or a hydrophobic adsorbent. In other embodiments, the adsorbent is a biospecific adsorbent. In another embodiment, the at least one biomarker is measured by immunoassay.

[0024] In still another aspect, the present invention provides a kit comprising: (a) a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds at least one biomarker from a first group consisting of the biomarkers set forth in Table I, Table II, Table IV-A and Table IV-B; and (b) instructions for using the solid support to detect the at least one biomarker set forth in Table I, Table II, Table IV-A and Table IV-B. In a preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table II. In yet another preferred embodiment, the at least one biomarker is selected from one of the biomarkers in Table II, Table IV-A or Table IV-B which is named in Table V. In another preferred embodiment, the biomarker is N-acetylated thymosin beta-4.

[0025] In one embodiment, the kit provides instructions for using the solid support to detect a biomarker selected from the following biomarkers: M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA

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peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4. In another embodiment, the kit provides instructions for using the solid support to detect a biomarker selected from the following biomarkers: M11728.3 (β2 microglobulin), M60976.2 (Hemopexin), M11127.8 and M9742.3. In preferred embodiments, the kit further comprises instructions for using the solid support to detect Cystatin C (M13391). In yet other embodiments, the kit further comprises instructions for using the solid support to detect at least one of the following additional biomarkers: M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin), or, alternatively, additionally detecting each of these biomarkers. In yet other embodiments, the kit further comprises instructions for using the solid support to detect at least one of the biomarkers in Table II or IV which is named in Table V.

[0026] In another embodiment, the solid support comprising the capture reagent is a SELDI probe. In certain embodiments, the adsorbent is a cation exchange adsorbent, an anion exchange adsorbent, a metal chelate or a hydrophobic adsorbent. In some preferred embodiments, the capture reagent is a cation exchange adsorbent. In other embodiments, the kit additionally comprises (c) an anion exchange chromatography sorbent. In other embodiments, the kit additionally comprises (c) a container containing at least one of the biomarkers of Table I, Table II, Table IV-A or Table IV-B.

[0027] In a further aspect, the present invention provides a kit comprising: (a) a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds at least one biomarker from a first group consisting of the biomarkers set forth in Table I, Table II, Table IV-A, or Table IV-B; and (b) a container comprising at least one of the biomarkers set forth in Table I, Table II, Table IV-A, or Table IV-B. In a preferred embodiment, the at least one biomarker is selected from the group consisting of the biomarkers of Table II, Table IV-A, and Table IV-B. In another preferred embodiment, the at least one biomarker is N-acetylated thymosin beta-4. In yet another preferred embodiment,

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the at least one biomarker is selected from one of the biomarkers in Table II or IV-A or IV-B which is named in Table V.

[0028] In one embodiment, the kit provides instructions for using the solid support to detect a biomarker selected from the following biomarkers: M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4. In preferred embodiments, the kit further comprises instructions for using the solid support to detect Cystatin C (M13391). In yet another embodiment, the kit provides instructions for additionally measuring one of the following biomarkers: In yet other embodiments, the kit further comprises instructions for using the solid support to detect at least one of the following additional biomarkers: M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin), or, alternatively, additionally detecting each of these biomarkers. In yet other embodiments, the kit further comprises instructions for using the solid support to detect at least one of the biomarkers in Table II or Table IV-A or Table IV-B which is named in Table V.

[0029] In another embodiment, the solid support comprising the capture reagent is a SELDI probe. In certain embodiments, the adsorbent is a cation exchange adsorbent, an anion exchange adsorbent, a metal chelate or a hydrophobic adsorbent. In other embodiments, the adsorbent is a biospecific adsorbent. In some embodiments, the capture reagent is a cation exchange adsorbent. In other embodiments, the kit additionally comprises (c) an anion exchange chromatography sorbent.

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[0030] In yet a further aspect, the present invention provides a software product, the software product comprising: (a) code that accesses data attributed to a sample, the data comprising measurement of at least one biomarker in the sample, the biomarker selected from the group consisting of the biomarkers of Table I, Table II, Table IV-A, and Table IV-B; and (b) code that executes a classification algorithm that classifies the Alzheimer's disease status of the sample as a function of the measurement. In a preferred embodiment, the biomarker is selected from the group consisting of the biomarkers of Table IV-A or Table IV-B.

[0031] In one embodiment, the classification algorithm classifies Alzheimer's disease status of the sample as a function of the measurement of a biomarker selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4. In yet another embodiment, the classification algorithm classifies Alzheimer's disease status of the sample as a function of the additional measurement of the one of the following biomarkers: M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin).. In yet other embodiments, the classification algorithm classifies Alzheimer's disease status of the sample as a function of the additional measurement of at least one of the biomarkers in Table II, Table IV-A or Table IV-B. In yet other embodiments, the classification algorithm classifies Alzheimer's disease status of the sample as a function of the additional measurement of at least one of the biomarkers in Table II, Table IV-A or Table IV-B which is named in Table V.

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[0032] In other aspects, the present invention provides purified biomolecules selected from the biomarkers set forth in Table I, Table II, Table IV-A and Table IV-B, and additionally, methods comprising detecting a biomarker set forth in Table I, II, IV-A or Table IV-B by mass spectrometry or immunoassay.

[0033] Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description, examples and claims that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] FIGS. 1A-1Q show mass spectra displaying biomarkers identified according to the techniques described in Example 1. The Figure also provides the mass-to-charge ratio for each biomarker.

[0035] FIG. 2A shows a decision tree for classifying a sample as Alzheimer's or non-Alzheimer's using certain biomarkers of this invention.

[0036] FIG. 3 shows the results of statistical tests used to validate the measurement of β2 microglobulin in CSF as a means for distinguishing between patients suffering from Alzheimer's disease ("AD") versus non-Alzheimer's dementia ("non AD") and versus patients who are not showing symptoms of dementia ("Control"). In particular, the data indicates a statistically significant difference in the average peak intensities of β2 microglobulin between Alzheimer's patients and the control group ("A-C"); between Alzheimer's patients and patients with non-Alzheimer's dementia ("A-N"); and between patients with non-Alzheimer's dementia and the control group ("C-N"). Measurements of peak intensities were performed as described in the Examples, utilizing the Ciphergen H50 ProteinChip.

[0037] FIG. 4A-K shows the distribution of peak intensities observed for the various groups and the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed.

[0038] FIG. 5 shows the distribution of peak intensities observed for full-length and truncated ubiquitin biomarkers, and the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed. Results are also presented for comparisons between disease and control groups using age-matched samples and using samples restricted by specific Tau/AB42 cut-off levels.

[0039] FIG. 6 shows the distribution of peak intensities observed for full-length and truncated Cystatin C biomarkers and the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed. Results are also

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presented for comparisons between disease and control groups using age-matched samples and using samples restricted by specific Tau/AB42 cut-off levels.

[0040] FIG. 7 shows the distribution of peak intensities observed for the Thymosin beta-4 biomarker and the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed. Results are also presented for comparisons between disease and control groups using age-matched samples and using samples restricted by specific Tau/AB42 cut-off levels.

[0041] FIG. 8 shows the distribution of peak intensities observed for the neuroendocrine protein 7B2, hemopexin, beta-2-microglobulin and transferrin biomarkers. FIG. 8 also shows the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed.

[0042] FIG. 9 shows mass spectra displaying biomarkers identified according to the techniques described in Example 3, utilizing neat CSF samples.

[0043] FIG. 10 summarizes the mechanistic relationships between a number of the biomarkers identified and described herein.

[0044] FIG. 11 shows the results of statistical tests used to validate the measurement of truncated CysC as a means for distinguishing between patients suffering from Alzheimer's dementia versus non-Alzheimer's dementia. In particular, the Figure shows a statistically significant difference in the average peak intensities of the truncated form of CysC (the 12583.8 Da marker) in Alzheimer's patients versus patients with non-Alzheimer's dementia ("A-N" p-value < 0.0001).

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

I. INTRODUCTION

[0045] A biomarker is an organic biomolecule which is differentially present in a sample taken from a subject of one phenotypic status (*e.g.*, having a disease) as compared with another phenotypic status (*e.g.*, not having the disease). A biomarker is differentially present between different phenotypic statuses if the mean or median expression level of the biomarker in the different groups is calculated to be statistically significant. Common tests for statistical significance include, among others, t-test, ANOVA, Kruskal-Wallis, Wilcoxon, Mann-Whitney and odds ratio. Biomarkers, alone or in combination, provide measures of relative risk that a subject belongs to one phenotypic status or another.

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Therefore, they are useful as markers for disease (diagnostics), therapeutic effectiveness of a drug (theranostics) and drug toxicity.

II. BIOMARKERS FOR ALZHEIMER'S DISEASE

A. Biomarkers

[0046] This invention provides polypeptide-based biomarkers that are differentially present in subjects having Alzheimer's disease versus subjects free of the disease and/or versus subjects suffering from forms of non-Alzheimer's dementia (*e.g.*, LB, FTD, *etc.*). In addition, the present invention provides methods of using the polypeptide-based biomarkers to qualify Alzheimer's disease in a subject. They are characterized by mass-to-charge ratio as determined by mass spectrometry, by the shape of their spectral peak in time-of-flight mass spectrometry and by their binding characteristics to adsorbent surfaces. These characteristics provide one method to determine whether a particular detected biomolecule is a biomarker of this invention. These characteristics represent inherent characteristics of the biomolecules and not process limitations in the manner in which the biomolecules are discriminated. In one aspect, this invention provides these biomarkers in isolated form.

[0047] The biomarkers were discovered using SELDI technology employing ProteinChip arrays from Ciphergen Biosystems, Inc. (Fremont, CA) ("Ciphergen"). CSF samples were collected from subjects diagnosed with Alzheimer's disease and subjects diagnosed as normal (non-demented). In some circumstances, CSF samples were fractionated by anion exchange chromatography (*e.g.*, Example 1). Neat CSF samples may also be used (*e.g.*, Example 3). Samples, either fractionated or neat, were applied to SELDI biochips and spectra of polypeptides in the samples were generated by time-of-flight mass spectrometry on a Ciphergen PBSII mass spectrometer. The spectra thus obtained were analyzed by Ciphergen Express™ Data Manager Software with Biomarker Wizard and Biomarker Pattern Software from Ciphergen Biosystems, Inc. The mass spectra for each group were subjected to scatter plot analysis. A Mann-Whitney test analysis was employed to compare Alzheimer's disease and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p<0.0001$) between the two groups. This method is described in more detail in the Example Section.

[0048] Examples of the discovered biomarkers for qualifying Alzheimer's disease are presented in Tables I, II, IV-A, IV-B and V. The "ProteinChip assay" column refers to chromatographic fraction in which the biomarker is found, the type of biochip to

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which the biomarker binds and the wash conditions, as described in detail in the Examples herein.

[0049] The biomarkers of this invention are characterized by their mass-to-charge ratio as determined by mass spectrometry. The mass-to-charge ratio of each biomarker is provided in the Tables herein. In Table I, for example, the biomarker masses are provided after the "M." Thus, for example, biomarker M2579.3 has a measured mass-to-charge ratio of 2579.3. The mass-to-charge ratios were determined from mass spectra generated on a Ciphergen Biosystems, Inc. PBS II mass spectrometer. This instrument has a mass accuracy of about +/- 0.15 percent (*e.g.*, for a 5,000 Da protein, the error is ± 7.5 Da). Thus, the biomarkers herein which are referred to by a measured apparent mass are not expected to provide precisely the same apparent weight every time their presence is detected in a given sample. Additionally, the PBS II mass spectrometer has a mass resolution of about 400 to 1000 m/dm, where m is mass and dm is the mass spectral peak width at 0.5 peak height. The mass-to-charge ratio of the biomarkers was determined using Biomarker Wizard™ software (Ciphergen Biosystems, Inc.). Biomarker Wizard assigns a mass-to-charge ratio to a biomarker by clustering the mass-to-charge ratios of the same peaks from all the spectra analyzed, as determined by the PBSII, taking the maximum and minimum mass-to-charge-ratio in the cluster, and dividing by two. Accordingly, the masses provided reflect these specifications.

[0050] The biomarkers of this invention are further characterized by the shape of their spectral peak in time-of-flight mass spectrometry. Mass spectra showing peaks representing many of the biomarkers are presented in FIGS. 1 and 9.

[0051] The biomarkers of this invention are further characterized by their binding properties on chromatographic surfaces. The biomarkers of the present invention bind to cation exchange adsorbents (preferably a CM-10 or WCX-2 ProteinChip array (Ciphergen Biosystems, Inc.)), anion exchange adsorbents (preferably a Q-10 ProteinChip array (Ciphergen Biosystems, Inc.)), hydrophobic exchange adsorbents (preferably a H50 ProteinChip array (Ciphergen Biosystems, Inc.)) and/or IMAC adsorbents (preferably an IMAC 3 or IMAC30 ProteinChip array (Ciphergen Biosystems, Inc.)).

[0052] The identities of many of the biomarkers of this invention have been determined and are indicated in the Tables herein. Methods by which these determination were made are also provided, *e.g.*, in the Example Section. For biomarkers whose identify has been determined, the presence of the biomarker can be determined by other methods

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known in the art, for example, by immunoassay, enzymatic activity assay, or by measuring any other detectable property of the biomarker.

[0053] Because the biomarkers of this invention are characterized by mass-to-charge ratio, binding properties and spectral shape, they can be detected by mass spectrometry without knowing their specific identity. However, if desired, biomarkers whose identity is not determined can be identified by, for example, determining the amino acid sequence of the polypeptides. For example, a biomarker can be peptide-mapped with a number of enzymes, such as trypsin or V8 protease, and the molecular weights of the digestion fragments can be used to search databases for sequences that match the molecular weights of the digestion fragments generated by the various enzymes. Alternatively, protein biomarkers can be sequenced using tandem MS technology. In this method, the protein is isolated by, for example, gel electrophoresis. A band containing the biomarker is cut out and the protein is subject to protease digestion. Individual protein fragments are separated by a first mass spectrometer. The fragment is then subjected to collision-induced cooling, which fragments the peptide and produces a polypeptide ladder. A polypeptide ladder is then analyzed by the second mass spectrometer of the tandem MS. The difference in masses of the members of the polypeptide ladder identifies the amino acids in the sequence. An entire protein can be sequenced this way, or a sequence fragment can be subjected to database mining to find identity candidates.

[0054] The preferred biological source for detection of the biomarkers is cerebrospinal fluid ("CSF"). However, in other embodiments, the biomarkers can be detected in serum. Many of the biomarkers of the present invention can be found in both CSF and serum. For instance, it has been discovered that Alzheimer's disease biomarkers Beta-2-microglobulin and Cystatin C (both full-length and variant forms) are found in both CSF and serum.

[0055] The biomarkers of this invention are biomolecules. Accordingly, this invention provides these biomolecules in isolated form. The biomarkers can be isolated from biological fluids, such as CSF or serum. They can be isolated by any method known in the art, based on both their mass and their binding characteristics. For example, a sample comprising the biomolecules can be subject to chromatographic fractionation, as described herein, and subject to further separation by, *e.g.*, acrylamide gel electrophoresis. Knowledge of the identity of the biomarker also allows their isolation by immunoaffinity chromatography.

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B. USE OF MODIFIED FORMS OF A BIOMARKER

[0056] It has been found that proteins frequently exist in a sample in a plurality of different forms characterized by a detectably different mass. These forms can result from either, or both, of pre- and post-translational modification. Pre-translational modified forms include allelic variants, slice variants and RNA editing forms. Post-translationally modified forms include forms resulting from proteolytic cleavage (e.g., fragments of a parent protein), glycosylation, phosphorylation, lipidation, oxidation, methylation, cystinylation, sulphonation and acetylation. The collection of proteins including a specific protein and all modified forms of it is referred to herein as a "protein cluster." The collection of all modified forms of a specific protein, excluding the specific protein, itself, is referred to herein as a "modified protein cluster." Modified forms of any biomarker of this invention (including those set forth in Tables I, II, IV-A, IV-B or V) also may be used, themselves, as biomarkers. In certain cases, the modified forms may exhibit better discriminatory power in diagnosis than the unmodified form of the protein.

[0057] Modified forms of a biomarker including any of those set forth in Tables, including those set forth in Tables I, II, IV-A, IV-B or V, can be initially detected by any methodology that can detect and distinguish the modified form from the biomarker. A preferred method for initial detection involves first capturing the biomarker and modified forms of it, e.g., with biospecific capture reagents, and then detecting the captured proteins by mass spectrometry. More specifically, the proteins are captured using biospecific capture reagents, such as antibodies, aptamers or Affibodies that recognize the biomarker and modified forms of it. This method will also result in the capture of protein interactors that are bound to the proteins or that are otherwise recognized by antibodies and that, themselves, can be biomarkers. Preferably, the biospecific capture reagents are bound to a solid phase. Then, the captured proteins can be detected by SELDI mass spectrometry or by eluting the proteins from the capture reagent and detecting the eluted proteins by traditional MALDI or by SELDI. The use of mass spectrometry is especially attractive because it can distinguish and quantify modified forms of a protein based on mass and without the need for labeling.

[0058] Preferably, the biospecific capture reagent is bound to a solid phase, such as a bead, a plate, a membrane or a chip. Methods of coupling biomolecules, such as antibodies, to a solid phase are well known in the art. They can employ, for example, bifunctional linking agents, or the solid phase can be derivatized with a reactive group, such as an epoxide or an imidizole, that will bind the molecule on contact. Biospecific capture reagents against different target proteins can be mixed in the same place, or they can be

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attached to solid phases in different physical or addressable locations. For example, one can load multiple columns with derivatized beads, each column able to capture a single protein cluster. Alternatively, one can pack a single column with different beads derivatized with capture reagents against a variety of protein clusters, thereby capturing all the analytes in a single place. Accordingly, antibody-derivatized bead-based technologies, such as xMAP technology of Luminex (Austin, TX) can be used to detect the protein clusters. However, the biospecific capture reagents must be specifically directed toward the members of a cluster in order to differentiate them.

[0059] In yet another embodiment, the surfaces of biochips can be derivatized with the capture reagents directed against protein clusters either in the same location or in physically different addressable locations. One advantage of capturing different clusters in different addressable locations is that the analysis becomes simpler.

[0060] After identification of modified forms of a protein and correlation with the clinical parameter of interest, the modified form can be used as a biomarker in any of the methods of this invention. At this point, detection of the modified from can be accomplished by any specific detection methodology including affinity capture followed by mass spectrometry, or traditional immunoassay directed specifically the modified form. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the analytes. Furthermore, if the assay must be designed to specifically distinguish protein and modified forms of protein. This can be done, for example, by employing a sandwich assay in which one antibody captures more than one form and second, distinctly labeled antibodies, specifically bind, and provide distinct detection of, the various forms. Antibodies can be produced by immunizing animals with the biomolecules. This invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays.

III. DETECTION OF BIOMARKERS FOR ALZHEIMER'S DISEASE

[0061] The biomarkers of this invention can be detected by any suitable method. Detection paradigms that can be employed to this end include optical methods, electrochemical methods (voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods, *e.g.*, multipolar resonance spectroscopy. Illustrative of optical methods, in addition to microscopy, both confocal and non-confocal, are detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (*e.g.*, surface plasmon resonance,

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ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry).

[0062] In one embodiment, a sample is analyzed by means of a biochip. Biochips generally comprise solid substrates and have a generally planar surface, to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there.

[0063] "Protein biochip" refers to a biochip adapted for the capture of polypeptides. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems, Inc. (Fremont, CA), Packard BioScience Company (Meriden CT), Zyomyx (Hayward, CA), Phylos (Lexington, MA) and Biacore (Uppsala, Sweden). Examples of such protein biochips are described in the following patents or published patent applications: U.S. Patent No. 6,225,047; PCT International Publication No. WO 99/51773; U.S. Patent No. 6,329,209, PCT International Publication No. WO 00/56934 and U.S. Patent No. 5,242,828.

A. Detection by Mass Spectrometry

[0064] In a preferred embodiment, the biomarkers of this invention are detected by mass spectrometry, a method that employs a mass spectrometer to detect gas phase ions. Examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer and hybrids of these.

[0065] In a further preferred method, the mass spectrometer is a laser desorption/ionization mass spectrometer. In laser desorption/ionization mass spectrometry, the analytes are placed on the surface of a mass spectrometry probe, a device adapted to engage a probe interface of the mass spectrometer and to present an analyte to ionizing energy for ionization and introduction into a mass spectrometer. A laser desorption mass spectrometer employs laser energy, typically from an ultraviolet laser, but also from an infrared laser, to desorb analytes from a surface, to volatilize and ionize them and make them available to the ion optics of the mass spectrometer.

1. SELDI

[0066] A preferred mass spectrometric technique for use in the invention is "Surface Enhanced Laser Desorption and Ionization" or "SELDI," as described, for example, in U.S. Patents No. 5,719,060 and No. 6,225,047, both to Hutchens and Yip. This refers to a method of desorption/ionization gas phase ion spectrometry (*e.g.*, mass spectrometry) in

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which an analyte (here, one or more of the biomarkers) is captured on the surface of a SELDI mass spectrometry probe. There are several versions of SELDI.

[0067] One version of SELDI is called "affinity capture mass spectrometry." It also is called "Surface-Enhanced Affinity Capture" or "SEAC". This version involves the use of probes that have a material on the probe surface that captures analytes through a non-covalent affinity interaction (adsorption) between the material and the analyte. The material is variously called an "adsorbent," a "capture reagent," an "affinity reagent" or a "binding moiety." Such probes can be referred to as "affinity capture probes" and as having an "adsorbent surface." The capture reagent can be any material capable of binding an analyte. The capture reagent may be attached directly to the substrate of the selective surface, or the substrate may have a reactive surface that carries a reactive moiety that is capable of binding the capture reagent, *e.g.*, through a reaction forming a covalent or coordinate covalent bond. Epoxide and carbodiimidizole are useful reactive moieties to covalently bind polypeptide capture reagents such as antibodies or cellular receptors. Nitriloacetic acid and iminodiacetic acid are useful reactive moieties that function as chelating agents to bind metal ions that interact non-covalently with histidine containing peptides. Adsorbents are generally classified as chromatographic adsorbents and biospecific adsorbents.

[0068] "Chromatographic adsorbent" refers to an adsorbent material typically used in chromatography. Chromatographic adsorbents include, for example, ion exchange materials, metal chelators (*e.g.*, nitriloacetic acid or iminodiacetic acid), immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, simple biomolecules (*e.g.*, nucleotides, amino acids, simple sugars and fatty acids) and mixed mode adsorbents (*e.g.*, hydrophobic attraction/electrostatic repulsion adsorbents).

[0069] "Biospecific adsorbent" refers to an adsorbent comprising a biomolecule, *e.g.*, a nucleic acid molecule (*e.g.*, an aptamer), a polypeptide, a polysaccharide, a lipid, a steroid or a conjugate of these (*e.g.*, a glycoprotein, a lipoprotein, a glycolipid, a nucleic acid (*e.g.*, DNA)-protein conjugate). In certain instances, the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Examples of biospecific adsorbents are antibodies, receptor proteins and nucleic acids. Biospecific adsorbents typically have higher specificity for a target analyte than chromatographic adsorbents. Further examples of adsorbents for use in SELDI can be found in U.S. Patent No. 6,225,047. A "bioselective adsorbent" refers to an adsorbent that binds to an analyte with an affinity of at least 10^{-8} M.

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[0070] Protein biochips produced by Ciphergen Biosystems, Inc. comprise surfaces having chromatographic or biospecific adsorbents attached thereto at addressable locations. Ciphergen ProteinChip® arrays include NP20 (hydrophilic); H4 and H50 (hydrophobic); SAX-2, Q-10 and LSAX-30 (anion exchange); WCX-2, CM-10 and LWCX-30 (cation exchange); IMAC-3, IMAC-30 and IMAC 40 (metal chelate); and PS-10, PS-20 (reactive surface with carboimidizole, expoxide) and PG-20 (protein G coupled through carboimidizole). Hydrophobic ProteinChip arrays have isopropyl or nonylphenoxy-poly(ethylene glycol)methacrylate functionalities. Anion exchange ProteinChip arrays have quaternary ammonium functionalities. Cation exchange ProteinChip arrays have carboxylate functionalities. Immobilized metal chelate ProteinChip arrays have have nitriloacetic acid functionalities that adsorb transition metal ions, such as copper, nickel, zinc, and gallium, by chelation. Preactivated ProteinChip arrays have carboimidizole or epoxide functional groups that can react with groups on proteins for covalent binding.

[0071] Such biochips are further described in: U.S. Patent No. 6,579,719 (Hutchens and Yip, "Retentate Chromatography," June 17, 2003); PCT International Publication No. WO 00/66265 (Rich *et al.*, "Probes for a Gas Phase Ion Spectrometer," November 9, 2000); U.S. Patent No. 6,555,813 (Beecher *et al.*, "Sample Holder with Hydrophobic Coating for Gas Phase Mass Spectrometer," April 29, 2003); U.S. Patent Application No. U.S. 2003 0032043 A1 (Pohl and Papanu, "Latex Based Adsorbent Chip," July 16, 2002); and PCT International Publication No. WO 03/040700 (Um *et al.*, "Hydrophobic Surface Chip," May 15, 2003); U.S. Provisional Patent Application No. 60/367,837 (Boschetti *et al.*, "Biochips With Surfaces Coated With Polysaccharide-Based Hydrogels," May 5, 2002) and U.S. Patent Application No. 60/448,467, entitled "Photocrosslinked Hydrogel Surface Coatings" (Huang *et al.*, filed February 21, 2003).

[0072] In general, a probe with an adsorbent surface is contacted with the sample for a period of time sufficient to allow biomarker or biomarkers that may be present in the sample to bind to the adsorbent. After an incubation period, the substrate is washed to remove unbound material. Any suitable washing solutions can be used; preferably, aqueous solutions are employed. The extent to which molecules remain bound can be manipulated by adjusting the stringency of the wash. The elution characteristics of a wash solution can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength, and temperature. Unless the probe has both SEAC and SEND properties (as described herein), an energy absorbing molecule then is applied to the substrate with the bound biomarkers.

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[0073] The biomarkers bound to the substrates are detected in a gas phase ion spectrometer such as a time-of-flight mass spectrometer. The biomarkers are ionized by an ionization source such as a laser, the generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. The detector then translates information of the detected ions into mass-to-charge ratios. Detection of a biomarker typically will involve detection of signal intensity. Thus, both the quantity and mass of the biomarker can be determined.

[0074] Another version of SELDI is Surface-Enhanced Neat Desorption (SEND), which involves the use of probes comprising energy absorbing molecules that are chemically bound to the probe surface ("SEND probe"). The phrase "energy absorbing molecules" (EAM) denotes molecules that are capable of absorbing energy from a laser desorption/ionization source and, thereafter, contribute to desorption and ionization of analyte molecules in contact therewith. The EAM category includes molecules used in MALDI, frequently referred to as "matrix," and is exemplified by cinnamic acid derivatives, sinapinic acid (SPA), cyano-hydroxy-cinnamic acid (CHCA) and dihydroxybenzoic acid, ferulic acid, and hydroxyaceto-phenone derivatives. In certain embodiments, the energy absorbing molecule is incorporated into a linear or cross-linked polymer, *e.g.*, a polymethacrylate. For example, the composition can be a co-polymer of α-cyano-4-methacryloyloxycinnamic acid and acrylate. In another embodiment, the composition is a co-polymer of α-cyano-4-methacryloyloxycinnamic acid, acrylate and 3-(tri-ethoxy)silyl propyl methacrylate. In another embodiment, the composition is a co-polymer of α-cyano-4-methacryloyloxycinnamic acid and octadecylmethacrylate ("C18 SEND"). SEND is further described in U.S. Patent No. 6,124,137 and PCT International Publication No. WO 03/64594 (Kitagawa, "Monomers And Polymers Having Energy Absorbing Moieties Of Use In Desorption/Ionization Of Analytes," August 7, 2003).

[0075] SEAC/SEND is a version of SELDI in which both a capture reagent and an energy absorbing molecule are attached to the sample presenting surface. SEAC/SEND probes therefore allow the capture of analytes through affinity capture and ionization/desorption without the need to apply external matrix. The C18 SEND biochip is a version of SEAC/SEND, comprising a C18 moiety which functions as a capture reagent, and a CHCA moiety which functions as an energy absorbing moiety.

[0076] Another version of SELDI, called Surface-Enhanced Photolabile Attachment and Release (SEPAR), involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a

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photolabile bond in the moiety after exposure to light, *e.g.*, to laser light (*see*, U.S. Patent No. 5,719,060). SEPAR and other forms of SELDI are readily adapted to detecting a biomarker or biomarker profile, pursuant to the present invention.

2. Other mass spectrometry methods

[0077] In another mass spectrometry method, the biomarkers can be first captured on a chromatographic resin having chromatographic properties that bind the biomarkers. In the present example, this could include a variety of methods. For example, one could capture the biomarkers on a cation exchange resin, such as CM Ceramic HyperD F resin, wash the resin, elute the biomarkers and detect by MALDI. Alternatively, this method could be preceded by fractionating the sample on an anion exchange resin before application to the cation exchange resin. In another alternative, one could fractionate on an anion exchange resin and detect by MALDI directly. In yet another method, one could capture the biomarkers on an immuno-chromatographic resin that comprises antibodies that bind the biomarkers, wash the resin to remove unbound material, elute the biomarkers from the resin and detect the eluted biomarkers by MALDI or by SELDI.

3. Data Analysis

[0078] Analysis of analytes by time-of-flight mass spectrometry generates a time-of-flight spectrum. The time-of-flight spectrum ultimately analyzed typically does not represent the signal from a single pulse of ionizing energy against a sample, but rather the sum of signals from a number of pulses. This reduces noise and increases dynamic range. This time-of-flight data is then subject to data processing. In Ciphergen's ProteinChip® software, data processing typically includes TOF-to-M/Z transformation to generate a mass spectrum, baseline subtraction to eliminate instrument offsets and high frequency noise filtering to reduce high frequency noise.

[0079] Data generated by desorption and detection of biomarkers can be analyzed with the use of a programmable digital computer. The computer program analyzes the data to indicate the number of biomarkers detected, and optionally the strength of the signal and the determined molecular mass for each biomarker detected. Data analysis can include steps of determining signal strength of a biomarker and removing data deviating from a predetermined statistical distribution. For example, the observed peaks can be normalized, by calculating the height of each peak relative to some reference. The reference can be background noise generated by the instrument and chemicals such as the energy absorbing molecule which is set at zero in the scale.

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[0080] The computer can transform the resulting data into various formats for display. The standard spectrum can be displayed, but in one useful format only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling biomarkers with nearly identical molecular weights to be more easily seen. In another useful format, two or more spectra are compared, conveniently highlighting unique biomarkers and biomarkers that are up- or down-regulated between samples. Using any of these formats, one can readily determine whether a particular biomarker is present in a sample.

[0081] Analysis generally involves the identification of peaks in the spectrum that represent signal from an analyte. Peak selection can be done visually, but software is available, as part of Ciphergen's ProteinChip® software package, that can automate the detection of peaks. In general, this software functions by identifying signals having a signal-to-noise ratio above a selected threshold and labeling the mass of the peak at the centroid of the peak signal. In one useful application, many spectra are compared to identify identical peaks present in some selected percentage of the mass spectra. One version of this software clusters all peaks appearing in the various spectra within a defined mass range, and assigns a mass (M/Z) to all the peaks that are near the mid-point of the mass (M/Z) cluster.

[0082] Software used to analyze the data can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a peak in a signal that corresponds to a biomarker according to the present invention. The software also can subject the data regarding observed biomarker peaks to classification tree or ANN analysis, to determine whether a biomarker peak or combination of biomarker peaks is present that indicates the status of the particular clinical parameter under examination. Analysis of the data may be "keyed" to a variety of parameters that are obtained, either directly or indirectly, from the mass spectrometric analysis of the sample. These parameters include, but are not limited to, the presence or absence of one or more peaks, the shape of a peak or group of peaks, the height of one or more peaks, the log of the height of one or more peaks, and other arithmetic manipulations of peak height data.

4. General protocol for SELDI detection of biomarkers for Alzheimer's disease

[0083] A preferred protocol for the detection of the biomarkers of this invention is as follows. The sample to be tested is contacted with an affinity capture probe comprising an cation exchange adsorbent (preferably a CM-10 or WCX-2 ProteinChip array (Ciphergen Biosystems, Inc.)), an anion exchange adsorbent (preferably a Q-10 ProteinChip

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array (Ciphergen Biosystems, Inc.)), a hydrophobic exchange adsorbent (preferably a H50 ProteinChip array (Ciphergen Biosystems, Inc.)) and/or an IMAC adsorbent (preferably an IMAC30 ProteinChip array (Ciphergen Biosystems, Inc.)), again as indicated in Tables I and II. The probe is washed with a buffer that will retain the biomarker while washing away unbound molecules. Examples of suitable washes for each biomarker are the buffers identified in the Examples and in Table I. The biomarkers are detected by laser desorption/ionization mass spectrometry.

[0084] In some instances, the sample, *e.g.*, serum, is subject to pre-fractionation before SELDI analysis. This simplifies the sample and improves sensitivity. A preferred method of pre-fractionation involves contacting the sample with an anion exchange chromatographic material, such as Q HyperD (BioSepra, SA). The bound materials are then subject to stepwise pH elution using buffers at pH 9, pH 7, pH 5 and pH 4. (*See*, Example 1 – Buffer list.) (The fractions in which the biomarkers are eluted may be indicated in, *e.g.*, Table I.) Various fractions containing the biomarker are collected. Thereafter, the fractions containing the biomarkers are subjected to SELDI analysis as described above.

[0085] Alternatively, if antibodies that recognize the biomarker are available, for example in the case of β2-microglobulin, cystatin, transferrin, transthyretin, hemopexin, WT ABri/ADan Amyloid Peptide, full length Cystatin C, Cystatin C ΔN1-8, N-terminal fragments of Neurosecretory Protein VGF, Complement 3a des-Arg, C-terminal fragment of Neuroendocrine protein 7B2, and Secretoneurin, these can be attached to the surface of a probe, such as a pre-activated PS10 or PS20 ProteinChip array (Ciphergen Biosystems, Inc.). These antibodies can capture the biomarkers from a sample onto the probe surface. Then the biomarkers can be detected by, *e.g.*, laser desorption/ionization mass spectrometry.

B. Detection by Immunoassay

[0086] In another embodiment, the biomarkers of this invention can be measured by immunoassay. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the biomarkers. Antibodies can be produced by methods well known in the art, *e.g.*, by immunizing animals with the biomarkers. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well known in the art.

[0087] This invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays. In the SELDI-based immunoassay, a biospecific

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capture reagent for the biomarker is attached to the surface of an MS probe, such as a pre-activated ProteinChip array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

IV. DETERMINATION OF SUBJECT ALZHEIMER'S DISEASE STATUS

A. Single Markers

[0088] The biomarkers of the invention can be used in diagnostic tests to assess Alzheimer's disease status in a subject, *e.g.*, to diagnose Alzheimer's disease. The phrase "Alzheimer's disease status" includes distinguishing, *inter alia*, Alzheimer's disease v. non-Alzheimer's disease and, in particular, Alzheimer's disease v. non-Alzheimer's disease normal or Alzheimer's disease v. non-Alzheimer's disease dementia. Based on this status, further procedures may be indicated, including additional diagnostic tests or therapeutic procedures or regimens.

[0089] The power of a diagnostic test to correctly predict status is commonly measured as the sensitivity of the assay, the specificity of the assay or the area under a receiver operated characteristic ("ROC") curve. Sensitivity is the percentage of true positives that are predicted by a test to be positive, while specificity is the percentage of true negatives that are predicted by a test to be negative. An ROC curve provides the sensitivity of a test as a function of 1-specificity. The greater the area under the ROC curve, the more powerful the predictive value of the test. Other useful measures of the utility of a test are positive predictive value and negative predictive value. Positive predictive value is the percentage of actual positives who test as positive. Negative predictive value is the percentage of actual negatives that test as negative.

[0090] The biomarkers of this invention show a statistical difference in different Alzheimer's disease statuses of at least $p \leq 0.05$, $p \leq 10^{-2}$, $p \leq 10^{-3}$, $p \leq 10^{-4}$ or $p \leq 10^{-5}$. Diagnostic tests that use these biomarkers alone or in combination show a sensitivity and specificity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% and about 100%.

[0091] Each biomarker listed in Tables I, II and IV are differentially present in Alzheimer's disease, and, therefore, each is individually useful in aiding in the determination of Alzheimer's disease status. The method involves, first, measuring the selected biomarker in a subject sample using the methods described herein, *e.g.*, capture on a SELDI biochip followed by detection by mass spectrometry and, second, comparing the measurement with a diagnostic amount or cut-off that distinguishes a positive Alzheimer's disease status from a

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negative Alzheimer's disease status. The diagnostic amount represents a measured amount of a biomarker above which or below which a subject is classified as having a particular Alzheimer's disease status. For example, if the biomarker is up-regulated compared to normal during Alzheimer's disease, then a measured amount above the diagnostic cutoff provides a diagnosis of Alzheimer's disease. Alternatively, if the biomarker is down-regulated during Alzheimer's disease, then a measured amount below the diagnostic cutoff provides a diagnosis of Alzheimer's disease.

[0092] Similarly, if the biomarker is up-regulated compared to normal during non-Alzheimer's dementia, then a measured amount above the diagnostic cutoff provides a diagnosis of non-Alzheimer's dementia. Alternatively, if the biomarker is down-regulated during non-Alzheimer's dementia compared to Alzheimer's disease, then a measured amount below the diagnostic cutoff provides a diagnosis of non-Alzheimer's dementia (*i.e.,* a negative diagnosis of Alzheimer's disease).

[0093] As is well understood in the art, by adjusting the particular diagnostic cut-off used in an assay, one can increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician. The particular diagnostic cut-off can be determined, for example, by measuring the amount of the biomarker in a statistically significant number of samples from subjects with the different Alzheimer's disease statuses, as was done here, and drawing the cut-off to suit the diagnostician's desired levels of specificity and sensitivity.

B. Combinations of Markers

[0094] While individual biomarkers are useful diagnostic biomarkers, it has been found that a combination of biomarkers can provide greater predictive value of a particular status than single biomarkers alone. Specifically, the detection of a plurality of biomarkers in a sample can increase the sensitivity and/or specificity of the test.

[0095] The protocols described in the Examples were used to generate mass spectra from patient samples that were diagnosed with Alzheimer's disease and no dementia. The peak masses and heights were abstracted into a discovery data set. This data set was used to train a learning algorithm employing classification and regression tree analysis (CART) (Ciphergen Biomarker Patterns Software™). In particular, CART chose many subsets of the peaks at random. For each subset, CART generated a best or near best decision tree to classify a sample as Alzheimer's disease or non-Alzheimer's disease. Among the many decision trees generated by CART, several had excellent sensitivity and specificity in distinguishing Alzheimer's disease from non-Alzheimer's disease.

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[0096] An exemplar decision tree is presented in FIG. 2. This decision tree uses M11753.4 (β2 microglobulin, therein "B2M"), M60976.2 (Hemopexin, therein "HPX"), M13391 (Cystatin C, therein "CysC"), M11.1K and M9.7K. Accordingly, these biomarkers are recognized as powerful classifiers for Alzheimer's disease when used in combination with each other and, optionally, with other biomarkers. In particular, when used together or in further combination with, for example, M78677.3 (Transferrin), M2432.2 (WT ABri/ADan Amyloid Peptide), M12583.4 (Cystatin C ΔN1-8), M3687.7 (N-terminal fragment of Neurosecretory Protein VGF); M3951.6 (N-terminal fragment of Neurosecretory Protein VGF), M8933.2 (Complement 3a des-Arg), M 3514.5 (C-terminal fragment of Neuroendocrine protein 7B2) and M3680.7 (Secretoneurin), these markers can distinguish Alzheimer's disease from non-Alzheimer's disease with sensitivities of at least 89% and specificities of at least 86%.

[0097] It is also noted that the specifics of a decision tree, in particular the cut-off values used in making branching decisions, depends on the details of the assay used to generate the discovery data set. The data acquisition parameters of the assay that produced the data used in the present analysis is provided in Example 3. In developing a classification algorithm from, for example, a new sample set or a different assay protocol, the operator uses a protocol that detects these biomarkers and keys the learning algorithm to include them.

[0098] In the decision tree set forth in FIG. 2, biomarkers M11753.4 (β2 microglobulin, therein "B2M"), M60976.2 (Hemopexin, therein "HPX"), M13391 (Cystatin C, therein "CysC"), M11.1K and M9.7K. are particularly useful in combination to classify Alzheimer's disease v. non-Alzheimer's disease. This combination is particularly useful in a recursive partitioning process as shown in FIG. 2. Again, the measure of each cut-off depends on the particulars of the assay protocol, of course. In this case, the cut-offs are based on the protocol set forth in Example 3.

[0099] In this decision tree, B2M is the root decision node of the decision tree. Subjects having an amount of this biomarker above the cut-off (*i.e.*, M11753.4<= 1.482) are sent to node 4 based on CysC.

[0100] Subjects having an amount of M13391 below the cut-off (*i.e.*, CysC <= 2.071) are classified as Alzheimer's. Subjects having an amount of M13391 above the cut-off (*i.e.*, CysC <= 2.071) are sent to node 5 based on M9.7K.

[0101] Subjects having an amount of M9.7K below the cut-off (*i.e.*, M9.7K <= 0.293) are classified as normal. Subjects having an amount of M9.7K above the cut-off (*i.e.*, M9.7K <= 0.293) are classified as Alzheimer's.

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[0102] Subjects having an amount of the biomarker B2M below the cut-off (*i.e.*, M11753.4<= 1.482) are sent to node 2 based on HPX.

[0103] Subjects having an amount of HPX above the cut-off (*i.e.*, M60976.2 <= 0.063) are classified as Alzheimer's. Subjects having an amount of HPX below the cut-off (*i.e.*, M60976.2 <= 0.063) are sent to node three based on M11.1K.

[0104] Subjects having an amount of M11.1K below the cut-off (*i.e.*, M11.1K <= 2.659) are classified as normal. Subjects having an amount of M11.1K above the cut-off (*i.e.*, M11.1K <= 2.659) are classified as Alzheimer's.

[0105] As set forth in FIG. 2, this decision tree has a sensitivity of about 89%, and a specificity of about 86%.

C. Determining Risk of Developing Disease

[0106] In one embodiment, this invention provides methods for determining the risk of developing Alzheimer's disease in a subject. Biomarker amounts or patterns are characteristic of various risk states, *e.g.*, high, medium or low. The risk of developing Alzheimer's disease is determined by measuring the relevant biomarker or biomarkers and then either submitting them to a classification algorithm or comparing them with a reference amount and/or pattern of biomarkers that is associated with the particular risk level.

D. Determining Stage of Disease

[0107] In one embodiment, this invention provides methods for determining the stage of Alzheimer's disease in a subject. Each stage of the disease has a characteristic amount of a biomarker or relative amounts of a set of biomarkers (a pattern). The stage of a disease is determined by measuring the relevant biomarker or biomarkers and then either submitting them to a classification algorithm or comparing them with a reference amount and/or pattern of biomarkers that is associated with the particular stage.

E. Determining Course (Progression/Remission) of Disease

[0108] In one embodiment, this invention provides methods for determining the course of Alzheimer's disease in a subject. Disease course refers to changes in disease status over time, including disease progression (worsening) and disease regression (improvement). Over time, the amounts or relative amounts (e.g., the pattern) of the biomarkers changes. For example, the concentrations of biomarkers M9984.6 and M10265.6 (Table IV-B) are increased in samples from Alzheimer's patients, while the concentration of hemopexin is decreased in samples from Alzheimer's patients. Therefore, the trend of these markers, either increased or decreased over time toward diseased or non-diseased indicates the course of the disease. Accordingly, this method involves measuring one or more

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biomarkers in a subject at least two different time points, *e.g.*, a first time and a second time, and comparing the change in amounts, if any. The course of disease is determined based on these comparisons. Similarly, this method is useful for determining the response to treatment. If a treatment is effective, then the biomarkers will trend toward normal, while if treatment is ineffective, the biomarkers will trend toward disease indications.

F. Subject Management

[0109] In certain embodiments of the methods of qualifying Alzheimer's disease status, the methods further comprise managing subject treatment based on the status. Such management includes the actions of the physician or clinician subsequent to determining Alzheimer's disease status. For example, if a physician makes a diagnosis of Alzheimer's disease, then a certain regime of treatment, such as prescription or administration of cholinesterase inhibitors, antiglutamatergic therapy or antioxidants, might follow. Alternatively, a diagnosis of non-Alzheimer's disease or non-Alzheimer's disease dementia might be followed with further testing to determine a specific dementia that might the patient might be suffering from. Also, if the diagnostic test gives an inconclusive result on Alzheimer's disease status, further tests may be called for.

[0110] Additional embodiments of the invention relate to the communication of assay results or diagnoses or both to technicians, physicans or patients, for example. In certain embodiments, computers will be used to communicate assay results or diagnoses or both to interested parties, *e.g.*, physicians and their patients. In some embodiments, the assays will be performed or the assay results analyzed in a country or jurisdiction which differs from the country or jurisdiction to which the results or diagnoses are communicated.

[0111] In a preferred embodiment of the invention, a diagnosis based on the presence or absence in a test subject of any the biomarkers of Tables I, II, IV-A,B or V is communicated to the subject as soon as possible after the diagnosis is obtained. The diagnosis may be communicated to the subject by the subject's treating physician. Alternatively, the diagnosis may be sent to a test subject by email or communicated to the subject by phone. A computer may be used to communicate the diagnosis by email or phone. In certain embodiments, the message containing results of a diagnostic test may be generated and delivered automatically to the subject using a combination of computer hardware and software which will be familiar to artisans skilled in telecommunications. One example of a healthcare-oriented communications system is described in U.S. Patent Number 6,283,761; however, the present invention is not limited to methods which utilize this particular communications system. In certain embodiments of the methods of the invention, all or some

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of the method steps, including the assaying of samples, diagnosing of diseases, and communicating of assay results or diagnoses, may be carried out in diverse (*e.g.*, foreign) jurisdictions.

V. GENERATION OF CLASSIFICATION ALGORITHMS FOR QUALIFYING ALZHEIMER'S DISEASE STATUS

[0112] In some embodiments, data derived from the spectra (*e.g.*, mass spectra or time-of-flight spectra) that are generated using samples such as "known samples" can then be used to "train" a classification model. A "known sample" is a sample that has been pre-classified. The data that are derived from the spectra and are used to form the classification model can be referred to as a "training data set." Once trained, the classification model can recognize patterns in data derived from spectra generated using unknown samples. The classification model can then be used to classify the unknown samples into classes. This can be useful, for example, in predicting whether or not a particular biological sample is associated with a certain biological condition (*e.g.*, diseased versus non-diseased).

[0113] The training data set that is used to form the classification model may comprise raw data or pre-processed data. In some embodiments, raw data can be obtained directly from time-of-flight spectra or mass spectra, and then may be optionally "pre-processed" as described above.

[0114] Classification models can be formed using any suitable statistical classification (or "learning") method that attempts to segregate bodies of data into classes based on objective parameters present in the data. Classification methods may be either supervised or unsupervised. Examples of supervised and unsupervised classification processes are described in Jain, "Statistical Pattern Recognition: A Review", *IEEE Transactions on Pattern Analysis and Machine Intelligence*, Vol. 22, No. 1, January 2000, the teachings of which are incorporated by reference.

[0115] In supervised classification, training data containing examples of known categories are presented to a learning mechanism, which learns one or more sets of relationships that define each of the known classes. New data may then be applied to the learning mechanism, which then classifies the new data using the learned relationships. Examples of supervised classification processes include linear regression processes (*e.g.*, multiple linear regression (MLR), partial least squares (PLS) regression and principal components regression (PCR)), binary decision trees (*e.g.*, recursive partitioning processes such as CART - classification and regression trees), artificial neural networks such as back

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propagation networks, discriminant analyses (*e.g.*, Bayesian classifier or Fischer analysis), logistic classifiers, and support vector classifiers (support vector machines).

[0116] A preferred supervised classification method is a recursive partitioning process. Recursive partitioning processes use recursive partitioning trees to classify spectra derived from unknown samples. Further details about recursive partitioning processes are provided in U.S. Patent Application No. 2002 0138208 A1 to Paulse *et al.*, "Method for analyzing mass spectra."

[0117] In other embodiments, the classification models that are created can be formed using unsupervised learning methods. Unsupervised classification attempts to learn classifications based on similarities in the training data set, without pre-classifying the spectra from which the training data set was derived. Unsupervised learning methods include cluster analyses. A cluster analysis attempts to divide the data into "clusters" or groups that ideally should have members that are very similar to each other, and very dissimilar to members of other clusters. Similarity is then measured using some distance metric, which measures the distance between data items, and clusters together data items that are closer to each other. Clustering techniques include the MacQueen's K-means algorithm and the Kohonen's Self-Organizing Map algorithm.

[0118] Learning algorithms asserted for use in classifying biological information are described, for example, in PCT International Publication No. WO 01/31580 (Barnhill *et al.*, "Methods and devices for identifying patterns in biological systems and methods of use thereof"), U.S. Patent Application No. 2002 0193950 A1 (Gavin *et al.*, "Method or analyzing mass spectra"), U.S. Patent Application No. 2003 0004402 A1 (Hitt *et al.*, "Process for discriminating between biological states based on hidden patterns from biological data"), and U.S. Patent Application No. 2003 0055615 A1 (Zhang and Zhang, "Systems and methods for processing biological expression data").

[0119] The classification models can be formed on and used on any suitable digital computer. Suitable digital computers include micro, mini, or large computers using any standard or specialized operating system, such as a Unix, Windows™ or Linux™ based operating system. The digital computer that is used may be physically separate from the mass spectrometer that is used to create the spectra of interest, or it may be coupled to the mass spectrometer.

[0120] The training data set and the classification models according to embodiments of the invention can be embodied by computer code that is executed or used by a digital computer. The computer code can be stored on any suitable computer readable

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media including optical or magnetic disks, sticks, tapes, etc., and can be written in any suitable computer programming language including C, C++, visual basic, etc.

[0121] The learning algorithms described above are useful both for developing classification algorithms for the biomarkers already discovered, or for finding new biomarkers for Alzheimer's disease. The classification algorithms, in turn, form the base for diagnostic tests by providing diagnostic values (*e.g.*, cut-off points) for biomarkers used singly or in combination.

VI. KITS FOR DETECTION OF BIOMARKERS FOR ALZHEIMER'S DISEASE

[0122] In another aspect, the present invention provides kits for qualifying Alzheimer's disease status, which kits are used to detect biomarkers according to the invention. In one embodiment, the kit comprises a solid support, such as a chip, a microtiter plate or a bead or resin having a capture reagent attached thereon, wherein the capture reagent binds a biomarker of the invention. Thus, for example, the kits of the present invention can comprise mass spectrometry probes for SELDI, such as ProteinChip® arrays. In the case of biospecfic capture reagents, the kit can comprise a solid support with a reactive surface, and a container comprising the biospecific capture reagent.

[0123] The kit can also comprise a washing solution or instructions for making a washing solution, in which the combination of the capture reagent and the washing solution allows capture of the biomarker or biomarkers on the solid support for subsequent detection by, *e.g.*, mass spectrometry. The kit may include more than type of adsorbent, each present on a different solid support.

[0124] In a further embodiment, such a kit can comprise instructions for suitable operational parameters in the form of a label or separate insert. For example, the instructions may inform a consumer about how to collect the sample, how to wash the probe or the particular biomarkers to be detected.

[0125] In yet another embodiment, the kit can comprise one or more containers with biomarker samples, to be used as standard(s) for calibration.

VII. DETERMINING THE QUALITY OF AN IMMUNOASSAY CALIBRATOR

[0126] Calibration of an immunoassay is important for ensuring the quality of results generated in the immunoassay. Calibration generally involves the use of an immunoassay calibrator that contains the target analyte in a prescribed amount or

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concentration. The signal produced by the calibrator in an immunoassay is correlated to the amount of target analyte in the calibrator. This calibration, in turn, is used to correlate the amount of signal measured in a test sample with an amount of target analyte in the test sample. However, the signal generated by the calibrator may not represent the true amount of analyte in the calibrator if, for example, the target analyte in the calibrator is degraded or otherwise modified so as to corrupt the signal.

[0127] For example, in one embodiment, this invention provides a method for determining the quality of a Cystatin immunoassay calibrator. The method involves capturing molecules from a immunoassay calibrator used in an immunoassay against Cystatin with an antibody that captures Cystatin, and specifically measuring the amount of Cystatin or one or more modified forms of Cystatin captured by the antibody. Alternatively, the immunoassay could be directed to measuring a particular modified form of Cystatin and involve the use of antibodies against this form and a calibrator that included this form.

[0128] As mentioned above, once calibrated, an immunoassay capable of accurately measuring levels of modified Cystatin polypeptides, *e.g.*, Cystatin C ΔN1-8, may be used to determine the likelihood that a subject diagnosed with dementia is suffering from Alzheimer's disease as opposed to some form of non-Alzheimer's dementia.

[0129] Similarly, the invention also provides a method for determining the quality of any immunossay calibrator used in an immunassay against any of the biomarkers disclosed herein, comprising the same steps described above with respect to a Cystatin immunoassay calibrator.

VIII. DETERMINING THE QUALITY OF AN ANTIBODY IN AN ANTIBODY REAGENT USED IN AN IMMUNOASSAY

[0130] Immunoassays typically involve the use an immunoassay reagent that comprises an antibody directed against the target analyte. The accuracy of such assays depends upon the integrity and purity of the antibody in the immunoassay reagent. The presence of contaminants in an antibody reagent can interfere with an accurate measurement of the amount of antibody in the antibody reagent. For example, the present invention provides methods for determining the quality of an antibody against an AD biomarker, as used in an immunoassay reagent, by specifically detecting modified, *e.g.*, degraded, forms of the antibody in the reagent.

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[0131] The performance of the assay will be tested alone and in combination with other markers to diagnose and monitor treatment of patients, *e.g.*, dementia patients. Initially, different types of dementia samples including AD and non-AD dementias along with aged normal samples will be analysed to determine assay sensitivity and specificity. The utility of the assay will be determined both for cerebrospinal fluid and matched serum samples. The ultimate objectives are to produce a test that will improve the early diagnosis of AD patients, help with stratification of patients for enrollment in clinical trials and provide a surrogate marker for drug treatment response.

IX. USE OF BIOMARKERS FOR ALZHEIMER'S DISEASE IN SCREENING ASSAYS AND METHODS OF TREATMENT

[0132] The methods of the present invention have other applications as well. For example, the biomarkers can be used to screen for compounds that modulate the expression of the biomarkers *in vitro* or *in vivo*, which compounds in turn may be useful in treating or preventing Alzheimer's disease in patients. In another example, the biomarkers can be used to monitor the response to treatments for Alzheimer's disease. In yet another example, the biomarkers can be used in heredity studies to determine if the subject is at risk for developing Alzheimer's disease.

[0133] Thus, for example, the kits of this invention could include a solid substrate having an cation exchange function, such as a protein biochip (*e.g.*, a Ciphergen WCX2 ProteinChip array, *e.g.*, ProteinChip array) and a sodium acetate buffer for washing the substrate, as well as instructions providing a protocol to measure the biomarkers of this invention on the chip and to use these measurements to diagnose Alzheimer's disease.

[0134] Compounds suitable for therapeutic testing may be screened initially by identifying compounds which interact with one or more biomarkers listed in Tables I, II, IV-A, IV-B or V. By way of example, screening might include recombinantly expressing a biomarker listed in Tables I, II, IV-A, IV-B or V, purifying the biomarker, and affixing the biomarker to a substrate. Test compounds would then be contacted with the substrate, typically in aqueous conditions, and interactions between the test compound and the biomarker are measured, for example, by measuring elution rates as a function of salt concentration. Certain proteins may recognize and cleave one or more biomarkers of Tables I, II, IV-A, IV-B or V, such as Cystatin C, in which case the proteins may be detected by monitoring the digestion of one or more biomarkers in a standard assay, *e.g.*, by gel electrophoresis of the proteins.

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[0135] In a related embodiment, the ability of a test compound to inhibit the activity of one or more of the biomarkers of Tables I, II, IV-A, IV-B, or V may be measured. One of skill in the art will recognize that the techniques used to measure the activity of a particular biomarker will vary depending on the function and properties of the biomarker. For example, an enzymatic activity of a biomarker may be assayed provided that an appropriate substrate is available and provided that the concentration of the substrate or the appearance of the reaction product is readily measurable. The ability of potentially therapeutic test compounds to inhibit or enhance the activity of a given biomarker may be determined by measuring the rates of catalysis in the presence or absence of the test compounds. The ability of a test compound to interfere with a non-enzymatic (*e.g.*, structural) function or activity of one of the biomarkers of Tables I, II, IV-A, IV-B or V may also be measured. For example, the self-assembly of a multi-protein complex which includes one of the biomarkers of Tables I, II, IV-A, IV-B or V may be monitored by spectroscopy in the presence or absence of a test compound. Alternatively, if the biomarker is a non-enzymatic enhancer of transcription, test compounds which interfere with the ability of the biomarker to enhance transcription may be identified by measuring the levels of biomarker-dependent transcription *in vivo* or *in vitro* in the presence and absence of the test compound.

[0136] Test compounds capable of modulating the activity of any of the biomarkers of Table I may be administered to patients who are suffering from or are at risk of developing Alzheimer's disease or other dementia's. For example, the administration of a test compound which increases the activity of a particular biomarker may decrease the risk of Alzheimer's in a patient if the activity of the particular biomarker *in vivo* prevents the accumulation of proteins for Alzheimer's disease. Conversely, the administration of a test compound which decreases the activity of a particular biomarker may decrease the risk of Alzheimer's disease in a patient if the increased activity of the biomarker is responsible, at least in part, for the onset of Alzheimer's disease.

[0137] In an additional aspect, the invention provides a method for identifying compounds useful for the treatment of disorders such as Alzheimer's disease which are associated with increased levels of modified forms of any of the biomarkers of Tables I, II, IV-A, IV-B or V, such as cystatin C. For example, in one embodiment, cell extracts or expression libraries may be screened for compounds which catalyze the cleavage of full-length biomarkers, *e.g.*, cystatin C, to form truncated forms of the biomarkers, *e.g.*, Cystatin C ΔN1-8. For example, in one embodiment of such a screening assay, cleavage of cystatin C may be detected by attaching a fluorophore to cystatin C which remains quenched when

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cystatin C is uncleaved but which fluoresces when the protein is cleaved between positions 8 and 9. Alternatively, a version of full-length cystatin C modified so as to render the amide bond between amino acids 8 and 9 uncleavable may be used to selectively bind or "trap" the cellular protease which cleaves full-length cystatin C at that site *in vivo*. Methods for screening and identifying proteases and their targets are well-documented in the scientific literature, *e.g.*, in Lopez-Ottin et al. (Nature Reviews, 3:509-519 (2002)).

[0138] In yet another embodiment, the invention provides a method for treating or reducing the progression or likelihood of a disease, *e.g.*, Alzheimer's disease, which is associated with the increased levels of one or more of the biomarkers described herein, for example, Cystatin C Δ1-8. For example, after one or more proteins have been identified which cleave full-length cystatin C between amino acids 8 and 9, combinatorial libraries may be screened for compounds which inhibit the cleavage activity of the identified proteins. Methods of screening chemical libraries for such compounds are well-known in art. *See, e.g.*, Lopez-Otin et al. (2002). Alternatively, inhibitory compounds may be intelligently designed based on the structure of cystatin C.

[0139] The compounds tested as modulators of the relative levels of full-length versus truncated biomarkers, *e.g.*, cystatin C versus truncated Cystatin C Δ1-8, can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Alternatively, modulators can be proteases or genetically engineered proteases. Typically, test compounds will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential modulator or binding compound in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (*e.g.*, in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs, Switzerland) and the like.

[0140] Where the modification of a particular biomarker is associated with Alzheimer's disease or a non-Alzheimer's dementia, and where the modification is non-proteolytic, *e.g.*, where the biomarker is glycosylated, acetylated, or phosphorylated, the modulating enzyme can be similarly targeted by compounds which inhibit the modulating enzyme's activity, specifically or generally. Likewise, where an increased concentration of

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an unmodified form of a particular biomarker is associated with Alzheimer's disease or a non-Alzheimer's dementia, the activity of the appropriate modulating enzyme may be increased by the addition of exogenous compounds which enhance the activity of the modulating enzyme, directly or indirectly, or by the recombinant addition of the appropriate modulating enzyme(s). Note that virtually any activity which affects the amount of a biomarker or the extent to which the biomarker found in modified form can be targeted. For example, chromogranin peptide fragments that are generated by disease regulated prohormone convertases (PC) can be modulated by targeting the activity of the prohormone convertases.

[0141] In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or binding compounds). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

[0142] A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[0143] Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.,* U.S. Patent No. 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication No. WO 93/20242), random bio-oligomers (*e.g.*, PCT Publication No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen

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et al., *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.,* U.S. Patent No. 5,539,083), antibody libraries (*see, e.g.,* Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.,* Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent No. 5,593,853), small organic molecule libraries (*see, e.g.,* benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; pyrrolidines, U.S. Patent Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent No. 5,506,337; benzodiazepines, U.S. Patent No. 5,288,514, and the like).

[0144] Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.,* ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, MO, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, *etc.*).

[0145] Full-length cystatin C is believed to bind to and inhibit the activity of human lysosomal proteases such as cathepsins B and L. N-terminal truncations of cystatin C are thought to diminish cystatin C's protease inhibitory activity. *See, e.g.*, Abrahamson et al. (Biochem. J. 273:621–626 (1991)). Compounds which impart truncated cystatin C with the functionality of full-length cystatin C are likely therefore to be useful in treating conditions, such as Alzheimer's disease, which are associated with the truncated form of cystatin C. Therefore, in a further embodiment, the invention provides methods for identifying compounds which increase the affinity of truncated cystatin C for its target proteases, *e.g.*, various cathepsins. For example, compounds may be screened for their ability to impart truncated cystatin C with the protease inhibitory activity of full-length cystatin C. Test compounds capable of modulating the inhibitory activity of cystatin C or the activity of molecules which interact with cystatin C may then be tested *in vivo* for their ability to slow or stop the progression of Alzheimer's disease in a subject.

[0146] At the clinical level, screening a test compound includes obtaining samples from test subjects before and after the subjects have been exposed to a test compound. The levels in the samples of one or more of the biomarkers listed in Table I may be measured and analyzed to determine whether the levels of the biomarkers change after exposure to a test compound. The samples may be analyzed by mass spectrometry, as

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described herein, or the samples may be analyzed by any appropriate means known to one of skill in the art. For example, the levels of one or more of the biomarkers listed in Table I may be measured directly by Western blot using radio- or fluorescently-labeled antibodies which specifically bind to the biomarkers. Alternatively, changes in the levels of mRNA encoding the one or more biomarkers may be measured and correlated with the administration of a given test compound to a subject. In a further embodiment, the changes in the level of expression of one or more of the biomarkers may be measured using *in vitro* methods and materials. For example, human tissue cultured cells which express, or are capable of expressing, one or more of the biomarkers of Tables I, II, IV-A, IV-B or V may be contacted with test compounds. Subjects who have been treated with test compounds will be routinely examined for any physiological effects which may result from the treatment. In particular, the test compounds will be evaluated for their ability to diminish the likelihood of disease in a subject. Alternatively, if the test compounds are administered to subjects who have previously been diagnosed with Alzheimer's disease, test compounds will be screened for their ability to slow or stop the progression of the disease.

[0147] The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of non-critical parameters that can be changed or modified to yield essentially the same results.

X. USE OF BIOMARKERS FOR IMAGING

[0148] Non-invasive medical imaging techniques such as Positron Emisson Tomography (PET) or single photon emission computerized tomography (SPECT) imaging are particularly useful for the detection of cancer, coronary artery disease and brain disease. PET and SPECT imaging shows the chemical functioning of organs and tissues, while other imaging techniques – such as X-ray, CT and MRI – show structure. The use of PET and SPECT imaging has become increasingly useful for qualifying and monitoring the development of brain diseases such as Alzheimer's disease. In some instances, the use of PET or SPECT imaging allows Alzheimer's disease to be detected several years earlier than the onset of symptoms.

[0149] Different strategies are being used to develop compounds suitable for in vivo imaging of amyloid deposits in human brains. Monoclonal antibodies against A-beta and peptide fragments have had limited uptake by the brain when tested in patients with AD.

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The small molecular approach for amyloid imaging has so far been most successful, as described by, *e.g.*, Nordberg A, Lancet Neurol., 3(9):519-27 (2004); Kung MP *et al*, Brain Res.,1025(1-2):98-105 (2004); Herholz K *et al.*, Mol Imaging Biol., 6(4):239-69 (2004); Neuropsychol Rev., Zakzanis KK *et al.*, 13(1):1-18 (2003); Herholz K, Ann Nucl Med., 17(2):79-89 (2003).

[0150] The peptide biomarkers disclosed herein, or fragments thereof, can be used in the context of PET and SPECT imaging applications. After modification with appropriate tracer residues for PET or SPECT applications, peptide biomarkers which interact with amyloid plaque proteins can be used to image the deposition of amyloid plaques in Alzheimer's patients. For example, the AD biomarker alpha(1)-antichymotrypsin (ACT) is associated with neurotoxic amyloid deposits. The M4357 ACT CT fragment described in Table IV-B, or a sub-fragment thereof, may therefore be used as a probe for PET or SPECT imaging applications.

XI. EXAMPLES

EXAMPLE 1. DISCOVERY OF BIOMARKERS FOR ALZHEIMER'S DISEASE

The protocols described in the Example below were used to generate mass spectra from 65 Swedish patient samples, 30 of whom were diagnosed with Alzheimer's disease and 35 of whom did not exhibit dementia. For this study, patients were diagnosed as AD patients according to NINCDS-ADRDA criteria, which includes cognitive testing, routine blood and urine tests, MRI or CT imaging when applicable, and measurements of CSF Tau and A-Beta(42). Severity of dementia assessed using Mini Mental State Examination (MMSE). The 30 Alzheimer's samples were taken from patients with an average MMSE of 21 (range from 5 to 30). The 35 control patients were age-matched, with a mean MMSE of 29 (range from 25-30). MMSE scores greater than 18 are considered evidence of mild dementia and patients with MMSE scores greater than 24 are considered to be extremely mild cases.

1. Anion Exchange Fractionation

Buffer List for anion exchange fractionation:

U1 (1M urea, 0.22% CHAPS, 50mM Tris-HCl pH9)

50mM Tris-HCl with 0.1% OGP pH9 (Wash buffer 1)

50mM Hepes with 0.1% OGP pH7 (Wash buffer 2)

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100mM NaAcetate with 0.1% OGP pH5 (Wash buffer 3)

100mM NaAcetate with 0.1% OGP pH4 (Wash buffer 4)

33.3% isopropanol/ 16.7% acetonitrile/ 0.1% trifluoracetic acid (Wash buffer 5)

Note: do not aliquot wash buffer 5 into the buffer tray until wash buffer 4 is being applied to the resin. This ensures that evaporation of the volatile organic solvents will not be an issue.

Material List:

Filter plate

5 v-well 96 well dishes, labeled F1-F5.

a. Wash resin

Prepare resin by washing Hyper Q DF resin (BioSepra, Cergy, France) 3 times with 5 bed volumes 50mM Tris-HCl pH9. Then store in 50mM Tris-HCl pH9 in a 50% suspension.

b. Equilibrate resin

Add 125µL Hyper Q DF to each well in filter plate

Filter buffer

Add 150µL U1 to each well

Filter buffer

Add 150µL U1 to each well

Filter buffer

Add 150µL U1 to each well

Filter buffer

c. Bind CSF with resin

Pipet 150µL of sample from each tube to appropriate well in filter plate

Vortex 30' at 4°

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d. Collect fractions

Place v-well 96 well plate F1 under filter plate

Collect flow-through in plate F1

Add 100µL of wash buffer 1 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Collect pH 9 eluant in plate F1

Fraction 1 contains the flow through and the pH 9 eluant.

Add 100µL of wash buffer 2 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Place v-well 96 well plate F2 under filter plate

Collect fraction 2 in plate F2

Add 100µL of wash buffer 2 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Collect remainder of fraction 2 in plate F2

Fraction 2 contains the pH 7 eluant.

Add 100µL of wash buffer 3 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Place v-well 96 well plate F3 under filter plate

Collect fraction 3 in plate F3

Add 100µL of wash buffer 3 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Collect remainder of fraction 3 in plate F3

Fraction 3 contains the pH 5 eluant

Add 100µL of wash buffer 4 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Place v-well 96 well plate F4 under filter plate

Collect fraction 4 in plate F4

Add 100µL of wash buffer 4 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Collect remainder of fraction 4 in plate F4

Fraction 4 contains the pH 4 eluant.

Add 100µL of wash buffer 5 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Place v-well 96 well plate F5 under filter plate

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Collect fraction 5 in plate F5

Add 100μL of wash buffer 5 to each well of filter plate

Vortex 10' at Room Temperature (RT)

Collect remainder of fraction 5 in plate F5

Fraction 5 contains the organic solvent eluant.

Freeze until proceeding with chip binding protocol

2. Chip Binding Protocol.

Bind CSF fractions to chips

Add 60μL of corresponding buffer into each well

Add 20μL of Q column fraction

Chip Washing Buffer list:

IMAC3 array (Ciphergen Biosystems, Inc.)

100mM $CuSO_4$

100mM Sodium Phosphate + 0.5M NaCl pH 7

WCX2 array (Ciphergen Biosystems, Inc.)

100mM Sodium Acetate pH 4

H50 array (Ciphergen Biosystems, Inc.)

10% Acetonitrile + 0.1% TFA

Array preparation

Place arrays into bioprocessor

Load IMAC arrays with copper

Load 50 μl of $CuSO_4$ onto each spot of the IMAC3 array

Vortex 5′ at Room Temperature (RT)

Remove $CuSO_4$ and repeat

Water rinse

Equilibrate arrays

Add 100 μl chip washing buffer appropriate to the array to each well

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Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex

Bind CSF fractions to arrays
Add 60 µl chip washing buffer appropriate to the array to each well
Add 20 µl CSF fraction
Vortex 30′ at RT
Remove sample and buffer

Wash arrays
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Water rinse 2 times

Add matrix
Remove Bioprocessor top and gasket
Allow the arrays to dry
SPA:
Add 1 µl 50% SPA (sinapinic acid) in 50% Acetonitrile and 0.5% TFA
Air dry
Add 1 µl 50% SPA
Air dry

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3. **Data acquisition settings:**

Energy absorbing molecule: 50% SPA

Set high mass to 100000 Daltons, optimized from 2000 Daltons to 100000 Daltons

Set starting laser intensity to 200

Set starting detector sensitivity to 8

Focus mass at 8000 Daltons

Set Mass Deflector to 1000 Daltons

Set data acquistion method to Seldi Quantitation

Set Seldi acquisition parameters 20. delta to 4. transients per to 10 ending position to 80

Set warming positions with 2 shots at intensity 225 and do not include warming shots

Process sample.

4. **Determination of Biomarker Identity.**

[0151] The spectra obtained were analyzed by Ciphergen Express^{tm} Data Manager Software with Biomarker Wizard and Biomarker Pattern Software from Ciphergen Biosystems, Inc. The mass spectra for each group were subjected to scatter plot analysis. A Mann-Whitney test analysis was employed to compare Alzheimer's disease and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p<0.0001$) between the two groups.

[0152] Examples of the biomarkers thus discovered are presented in Table I below. The "ProteinChip assay" column refers to chromatographic fraction in which the biomarker is found, the type of biochip to which the biomarker binds and the wash conditions.

TABLE I

Marker	P-Value	Up or down regulated in Alzheimer's Disease	ProteinChip® assay
M2579.3	<0.0001	Down	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M2986.5	<0.0001	Down	Fraction 4, WCX, wash with 100 mM Na acetate pH 4
M3147.2	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M3205.4	<0.0001	Down	Fraction 4, WCX, wash with

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Marker	P-Value	Up or down regulated in Alzheimer's Disease	ProteinChip® assay
			100 mM Na acetate pH 4
M3258.4	<0.0001	Down	Fraction 4, WCX, wash with 100 mM Na acetate pH 4
M3733.3	<0.0001	Down	Fraction 4, WCX, wash with 100 mM Na acetate pH 4
M4636.6	<0.0001	Up	Fraction 5, WCX, wash with 100 mM Na acetate pH 4
M4934.8	<0.0001	Up	Fraction 5, WCX, wash with 100 mM Na acetate pH 4
M5865.3	<0.0001	Up	Fraction 4, IMAC, wash with 100mM Na Phosphate 0.5M NaCl pH 7
M5974.5	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M6876.4	<0.0001	Up	Fraction 4, IMAC, wash with 100mM Na Phosphate 0.5M NaCl pH 7
M7466.2	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M7748.6	<0.0001	Up	Fraction 4, IMAC, wash with 100mM Na Phosphate 0.5M NaCl pH 7
M8295.7	<0.0001	Down	Fraction 5, WCX, wash with 100 mM Na acetate pH 4
M8623.2 (C4ades-Arg, SEQ ID NO:1)	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M9758.9	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M9786.8	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M11465.5	<0.0001	Up	Fraction 4, WCX, wash with 100 mM Na acetate pH 4
M11498.2	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M11621.2	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M11728.3 (β2 microglobulin)	< 0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M11938.4	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M12193	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M13059.2	<0.0001	Up	Fraction 3, WCX, wash with

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Marker	P-Value	Up or down regulated in Alzheimer's Disease	ProteinChip® assay
			100 mM Na acetate pH 4
M13175.6	<0.0001	Up	Fraction 4, WCX, wash with 100 mM Na acetate pH 4
M13212.7	= 0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M15827.5	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M15983.9	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M16037.5	<0.0001	Up	Fraction 3, WCX, wash with 100 mM Na acetate pH 4
M44698.7	<0.0001	Down	Fraction 3, IMAC, wash with 100mM Na Phosphate 0.5M NaCl pH 7
M59127.8	<0.0001	Down	Fraction 4, IMAC, wash with 100mM Na Phosphate 0.5M NaCl pH 7
M66550.5 (Albumin)	< 0.0001	Down	Fraction 3, WCX, wash with 100 mM Na acetate pH 4

[0153] The identity of biomarkers were determined as follows. Proteins were separated on an acrylamide gel and a band containing the biomarker was cut out of the gel. The protein in the band was destained. The gel was dried using acetonitrile and then subject to digestion in a solution of trypsin. The digest fragments were analyzed on a Ciphergen PBSII mass spectrometer. The determined masses were used to interrogate a protein database, which identified the protein having the same tryptic digest pattern. All of these identifications were further confirmed by tandem MS analysis. Finally, the identity of β-microglobulin was determined by antibody capture. Antibodies were bound to Ciphergen PS20 ProteinChip arrays, which have reactive epoxide surfaces. Sample containing the biomarker was applied to the antibody spot. Unbound proteins were removed and the arrays were read with a Ciphergen PBSII reader. The detection of a protein having the mass of the target confirmed identity.

[0154] The identity of M8623.24 was found to be the C4ades-Arg protein, which is a cleavage product of anaphylatoxin C4a. Anaphylatoxin C4a is a biologically active fragment of Complement C4 (Swiss-Prot accession number P01028 http://us.expasy.org/cgi-bin/niceprot.pl?P01028). In serum as well as CSF, the carboxypeptidase N rapidly cleaves off the C-terminal arginine, thereby generating the

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relatively stable protein C4ades-Arg. The amino acid sequence of C4ades-Arg was determined by direct sequencing. The amino acid sequence of C4ades-Arg is as follows:

NVNFQKAINEKLGQYASPTAKRCCQDGVTRLPMMRSCEQRAARVQQ
PDCREPFLSCCQFAESLRKKSRDKGQAGLQ (SEQ ID NO:1)

(theoretical MW is 8607.88 Da).

[0155] In immunoassay experiments, it was found that an antibody against human C4ades-Arg, *i.e.*, an affinity-purified anti-C4ades-Arg antibody, specifically pulls down the 8607 Da protein from CSF.

EXAMPLE 2. VALIDATION STUDY OF B2 MICROGLOBULIN AS A MARKER FOR ALZHEIMER'S DISEASE

[0156] To validate the use of β2 microglobulin as a marker for Alzheimer's disease, 158 cerebrosprinal fluid (CSF) samples were taken from pre-diagnosed subjects in three groups: (1) Alzheimer's disease (AD), (2) Control, and (3) non-Alzheimer's dementia (Non AD). The distribution of samples in these groups is shown in Table III, below.

Table III. Distribution of Subjects in β2 Microglobulin Validation Study

Subject Classification		# of Subjects
Alzheimer's Disease	AD Mild (MMSE > 24)	56
	AD severe	10
Control	Depression	6
	Control	45
Non-Alzheimer's Dementia	FTD	21
	LBD	20
	Total	158

[0157] Briefly, of the 158 samples, sixty six were taken from patients suffering from mild forms of Alzheimer's disease (characterized by a Mini-Mental State Examination (MMSE) score greater than twenty four) or suffering from more severe forms of Alzheimer's disease. Fifty one "control" samples were obtained from non-dementia patients, including six patients diagnosed as "depressed." Samples taken from forty one patients

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suffering from non-Alzheimer's dementia included twenty samples taken from patients suffering from Lewy body dementia (LBD) and twenty one samples taken from patients suffering from frontotemporal dementia (FTD).

[0158] SELDI-MS measurements of β2 microglobulin in each of the samples were obtained using a Ciphergen H50 ProteinChip according to the following binding protocol, using 50% SPA as an EAM for reading the chip:

Bulk Wash H50 arrays in 50% acetonitrile for 30 minutes and then air dry for 30 minutes.

Add 100 μL of Binding buffer (10% Acetonitrile + 0.1% Trifluoroacetic acid) into each well

Shake 5 min at Room Temperature (RT)

Remove buffer after shaking

Add 100 μL of Binding buffer into each well

Shake 5 min at Room Temperature (RT)

Remove buffer after shaking

Add 45 μl of Binding buffer into each well

Add 5 μl of neat CSF sample

Shake 30 min at Room Temperature (RT)

Remove sample buffer after shaking

Add 100 μL of Binding buffer into each well

Shake 5 min at Room Temperature (RT)

Remove buffer after shaking

Add 100 μL of Binding buffer into each well

Shake 5 min at Room Temperature (RT)

Remove buffer after shaking

Add 1 μl 50% SPA (sinapinic acid) in 50% Acetonitrile and 0.5% TFA

Air dry

Add 1 μl 50% SPA

Air dry

Analyze arrays.

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[0159] The results (FIG. 3) show that (1) the levels of β2 microglobulin in subjects suffering from Alzheimer's disease are significantly higher than the levels in subjects suffering from non-Alzheimer's dementia; (2) the levels of β2 microglobulin in subjects suffering from Alzheimer's disease are significantly higher than the levels in subjects who do not show symptoms of dementia; and (3) the levels of β2 microglobulin in subjects suffering from non-Alzheimer's dementia are significantly lower than the levels in subjects who do not show symptoms of dementia.

EXAMPLE 3. DISCOVERY OF ADDITIONAL BIOMARKERS FOR ALZHEIMER'S DISEASE

For this Example, 237 CSF samples from Swedish and Finnish patients were used. These samples included: 98 samples from patients with Alzheimer's Disease (including 83 very mild cases with MMSE >24), 31 samples from patients with Frontotemporal Dementia (FTD), 29 samples from patients with dementia with Lewy Body (DLB), and 79 age-matched normal individuals including 9 depression controls. Diagnoses were made according to the NINCDS-ADRDA criteria discussed in the preceding Examples. The 237 samples were randomly divided into sets for training (2/3) and blind testing (1/3).

The anion exchange prefractionation step utilized in Example 1, above, was omitted for these CSF samples (note that for serum samples, the use of a pre-fractionation step is preferred). Instead, 5 μL of neat CSF sample was used per well on each chip. The chips used were Ciphergen's IMAC30 (activated with copper or nickel), Q10, CM10 and H50 ProteinChips. The samples were run in triplicate using multiple data collection settings using two different types of Energy Absorbing Molecules (EAM). A pooled normal reference CSF sample was run in parallel with clinical samples to monitor inter- and intra-assay reproducibility. All samples were processed and analyzed using a ProteinChip AutoBiomarker System that includes a Biomek® 2000 liquid-handling robot and ProteinChip TOF-MS Reader (Model PBS IIC) with chip Autoloader. Samples were randomized across different bioprocessors to eliminate any systematic bias.

1. General Chip Binding Protocol.

Bind CSF fractions to chips

Add 45μL of corresponding buffer into each well

Add 5μL of neat CSF

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Chip Washing Buffer list:

IMAC30 array (Ciphergen Biosystems, Inc.):
100mM $CuSO_4$ or $NiSO_4$, as appropriate
100mM Sodium Phosphate + 0.5M NaCl pH 7

H50 array (Ciphergen Biosystems, Inc.):
10% Acetonitrile + 0.1% TFA

Q10 array (Ciphergen Biosystems, Inc.):
100 mM Tris pH 9.0

CM10 array (Ciphergen Biosystems, Inc.):
100mM Sodium Acetate pH 4

Array preparation
Place arrays into bioprocessor

Load IMAC30 arrays with copper or nickel, as appropriate
Load 50 µl of $CuSO_4$ (or $NiSO_4$) onto each spot of the IMAC30 array
Vortex 5′ at Room Temperature (RT)
Remove $CuSO_4$ (or $NiSO_4$) and repeat
Water rinse

Equilibrate arrays
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex

Bind CSF fractions to arrays

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Add 45 µl chip washing buffer appropriate to the array to each well
Add 5 µl CSF
Vortex 30′ at RT
Remove sample and buffer

Wash arrays
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Add 100 µl chip washing buffer appropriate to the array to each well
Vortex 5′ at RT
Remove buffer after vortex
Water rinse 2 times

Add matrix
Remove Bioprocessor top and gasket
Allow the arrays to dry
SPA:
Add 1µl 50% SPA (sinapinic acid) in 50% Acetonitrile and 0.5% TFA
Air dry
Add 1µl 50% SPA
Air dry
CHCA
Add 1µl 50% CHCA dissolved in 50% Acetonitrile + 0.25% TFA
Air dry
Add 1µl 50% CHCA
Air dry

2. Specific Chip Binding Protocols

Q10 Chip

Equilibrate arrays

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1. Add 100 µL 100 mM Tris pH9 to each well.
2. Mix 5 min at room temperature.
3. Remove buffer after mixing.
4. Add 100 µL 100 mM Tris pH9 to each well.
5. Mix 5 min at room temperature.
6. Remove buffer after mixing.

Add sample to arrays

1. Add 50 µL 100 mM Tris pH9 to each well.
2. Add 5 µL CSF.
3. Mix 30 min at room temperature.
4. Remove sample and buffer.

Wash arrays

1. Add 100 µL 100 mM Tris pH9 to each well.
2. Mix 5 min at room temperature.
3. Remove buffer after mixing.
4. Add 100 µL 100 mM Tris pH9 to each well.
5. Mix 5 min at room temperature.
6. Remove buffer after mixing.
7. Add 100 µL 100 mM Tris pH9 to each well.
8. Mix 5 min at room temperature.
9. Remove buffer after mixing.
10. Rinse twice with deionized water

Add EAM

1. Remove Bioprocessor reservoir and gasket.
2. Briefly allow the arrays to dry.
3. Apply EAM:

For SPA

a. Add 400 µL of 50% acetonitrile, 0.5% TFA to SPA tube.

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b. Mix 5 min at room temperature.

c. Add 1µL to each spot.

d. Air dry.

e. Add 1µL to each spot.

f. Air dry.

For CHCA

a. Add 200 µL of 50% ACN, 0.25% TFA to CHCA tube.

b. Mix 5 min at room temperature.

c. Centrifuge for 1 min at 10,000 rpm at room temperature.

d. Remove the supernatant and dilute with an equal volume of 50% acetonitrile,

0.25% TFA.

e. Apply 1µL to each spot.

f. Air dry.

g. Apply 1µL to each spot.

h. Air dry.

CM10

Equilibrate arrays

1. Add 100 µL 100 mM Sodium acetate pH 4 to each well.
2. Mix 5 min at room temperature.
3. Remove buffer after mixing.
4. Add 100 µL 100 mM Sodium acetate pH 4 to each well.
5. Mix 5 min at room temperature.
6. Remove buffer after mixing.

Add sample to arrays

1. Add 50 µL 100 mM Sodium acetate pH 4 to each well.
2. Add 5 µL CSF.
3. Mix 30 min at room temperature.
4. Remove sample and buffer.

Wash arrays

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1. Add 100 µL 100 mM Sodium acetate pH 4 to each well.
2. Mix 5 min at room temperature.
3. Remove buffer after mixing.
4. Add 100 µL 100 mM Sodium acetate pH 4 to each well.
5. Mix 5 min at room temperature.
6. Remove buffer after mixing.
7. Add 100 µL 100 mM Sodium acetate pH 4 to each well.
8. Mix 5 min at room temperature.
9. Remove buffer after mixing.
10. Rinse twice with deionized water

Add EAM

1. Remove Bioprocessor reservoir and gasket.
2. Briefly allow the arrays to dry.
3. Apply EAM:

For SPA

a. Add 400 µL of 50% acetonitrile, 0.5% TFA to SPA tube.
b. Mix 5 min at room temperature.
c. Add 1µL to each spot.
d. Air dry.
e. Add 1µL to each spot.
f. Air dry.

For CHCA

a. Add 200 µL of 50% ACN, 0.25% TFA to CHCA tube.
b. Mix 5 min at room temperature.
c. Centrifuge for 1 min at 10,000 rpm at room temperature.
d. Remove the supernatant and dilute with an equal volume of 50% acetonitrile, 0.25% TFA.
e. Apply 1µL to each spot.

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f. Air dry.

g. Apply 1μL to each spot.

h. Air dry.

IMAC30 and H50 ProteinChips

See the protocols in Examples 1 and 2, respectively, for IMAC 30 and H50 ProteinChip protocols. The protocol for the IMAC30 ProteinChips is essentially the same as that given for the IMAC3 ProteinChip except that, as with the all the chips in this Example, the anion exchange fractionation step as been eliminated. The IMAC30 ProteinChip is an alternative to the IMAC3 arrays with the added feature of a hydrophobic barrier for sample containment. Like the IMAC3 arrays, the IMAC30 arrays are activated with transition metals (*e.g.*, copper or nickel) prior to use.

3. **Data acquisition settings:**

The following conditions were used for data acquisition.

IMAC30 Cu: CHCA, SPA low, SPA high (50% SPA used as the energy absorbing molecule)

IMAC30 Ni: CHCA; SPA low, SPA high

Q10: CHCA, SPA low, SPA high

CM10: CHCA; SPA low, SPA high

H50: CHCA; SPA low, SPA high

CHCA

Set Detector Voltage 2850 Volts.

Set high mass to 200000 Daltons, optimized from 1000 Daltons to 200000 Daltons.

Set starting laser intensity to 170.

Set starting detector sensitivity to 7.

Focus mass at 4000 Daltons.

Set Mass Deflector to 1000 Daltons.

Set data acquistion method to Seldi Quantitation

Set Seldi acquisition parameters 22. delta to 5. transients per to 5 ending position to 82.

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Set warming positions with 2 shots at intensity 220 and Don't include warming shots.
Process sample.

SPA low

Set Detector Voltage 2850 Volts.
Set high mass to 200000 Daltons, optimized from 1000 Daltons to 200000 Daltons.
Set starting laser intensity to 194.
Set starting detector sensitivity to 8.
Focus mass at 4000 Daltons.
Set Mass Deflector to 1000 Daltons.
Set data acquistion method to Seldi Quantitation
Set Seldi acquisition parameters 20. delta to 5. transients per to 5 ending position to 80.
Set warming positions with 2 shots at intensity 220 and Don't include warming shots.
Process sample.

SPA high

Set Detector Voltage 2850 Volts.
Set high mass to 200000 Daltons, optimized from 10000 Daltons to 200000 Daltons.
Set starting laser intensity to 199.
Set starting detector sensitivity to 8.
Focus mass at 12000 Daltons.
Set Mass Deflector to 4000 Daltons.
Set data acquistion method to Seldi Quantitation
Set Seldi acquisition parameters 22. delta to 5. transients per to 5 ending position to 82.
Set warming positions with 2 shots at intensity 220 and Don't include warming shots.
Process sample.

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4. Data analysis:

[0160] Spectral data was collected using ProteinChip Software version 3.1. with large scale data handling and univariate analysis performed using CiphergenExpress™ Data Manager 2.1. Spectral pre-processing included baseline subtraction and internal molecular weight calibration using known masses from endogenous sample analytes. Normalization of peak intensity was performed by total ion current using an external coefficient of 0.2. Peak labelling and clustering across different spectra was done automatically by the software employing user-defined settings. Peak intensity P values for individual peaks across each group were calculated using a Mann-Whitney test for two group comparisons and Kruskal-Wallis Test for three or more groups. Multivariate data analysis was performed using the Biomarker PatternsTM Software 5.0 to best classify the samples based on pre-determined phenotype.

Results

[0161] Using the ProteinChips and conditions described above, a set of univariate biomarkers ($P<0.005$) was identified after analysis of the training set ("Study 2", i.e., 2/3 of the sample set described above) under all conditions. The results are summarized in Table II, below.

TABLE II

ROC area	p-value	P value comparison	Cluster mass	Assay	Up/Down in AD	ID
0.26	0.00000083	AD/N	4332.9 (4330.4)	IMAC 30 Cu SPA Low and Ni CHCA	Up	
0.39	0.00000300	AD/C+N	4342.1	IMAC 30 Ni SPA low	Up	
0.28	0.00000698	AD/N	3680.7	Q10 SPA low	Up	Secretogranin II peptide (secretoneurin)
0.28	0.00001000	AD/N	5078.4 (5078.3) (5082.6)	H50, IMAC 30 Cu, CM10 SPA high	Up	
0.32	0.00001278	AD/C+N	4757.9	H50 SPA low	Up	
0.34	0.00002000	AD/C+N	10362.1	H50 CHCA	Up	
0.38	0.00002055	AD/N	2431.2 (2433.1)	IMAC30 Cu CHCA, CM10 SPA low	Up	Truncated ABri/ADan amyloid peptide of Integral membrane protein 2B
0.37	0.00003000	AD/C	11753.4 (11763.7) (11747.0) (11749.4)	H50, IMAC 30 Ni, CM10 and Q10 SPA low	Up	Beta-2-microglobulin
0.37	0.00003000	AD/N	12583.4 (12544.7)	H50, IMAC 30 Cu, CM10 SPA	Up	8 aa truncated Cystatin C

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ROC area	p-value	P value comparison	Cluster mass	Assay	Up/Down in AD	ID
			(12556.3)	low		
0.35	0.00007220	AD/N	4352.4 (4353.7)	Q10 CHCA and SPA low	Up	alpha-1-antichymotrypsin C-terminal fragment
0.34	0.00007977	AD/C+N	4737.8	IMAC 30 Ni	Up	
0.36	0.00010089	AD/C+N	3234.3 (3235.5) (3236.8)	CM10 CHCA, IMAC 30 Cu and Ni CHCA	Up	
0.32	0.00010151	AD/N	6634.3	Q10 SPA low	Up	
0.63	0.00013746	AD/N	11127.8	CM10 CHCA	Down	
0.38	0.00016000	AD/C+N	5062.5 (5063.0) (5067.4)	CM10 CHCA and Q10 CHCA and SPA low	Up	
0.31	0.00016894	AD/N	3687.7	CM10 CHCA	Up	N-terminal fragment of Neurosecretory protein VGF (-3aa) (Fragment 2)
0.32	0.00021243	AD/N	3951.6 (3966.2)	CM10 CHCA and SPA low	Up	N-terminal fragment of Neurosecretory protein VGF (Fragment 1)
0.37	0.00021656	AD/C	4971.3	CM10 CHCA	Up	Thymosin beta-4 (N-acetylated)
0.33	0.00027133	AD/N	3912.8	Q10 SPA low	Up	
0.35	0.00030200	AD/C+N	9742.3 (9752.4)	Q10 CHCA and SPA low	Up	
0.43	0.00033000	AD/C+N	3982.6	IMAC 30 Ni CHCA	Up	
0.32	0.00039159	AD/C+N	4813.3	Q10 SPA low	Up	
0.34	0.00041439	AD/N	6501.9	IMAC 30 Ni CHCA	Up	
0.37	0.00050908	AD/C+N	8183.6	CM10 CHCA	Up	Ubiquitin truncated from C-terminus (-4aa)
0.37	0.00051300	AD/N	6527.1	IMAC 30 Ni SPA low	Up	
0.64	0.00056603	AD/C+N	3821.2	Q10 SPA low	Down	
0.33	0.00065202	AD/N	4184.3	IMAC 30 Cu CHCA	Up	
0.36	0.00067000	AD/C+N	4743.5 (4753.7)	CM10 CHCA and Q10 SPA low	Up	
0.36	0.00075331	AD/C	9789.1	H50 SPA low	Up	
0.36	0.00080000	AD/N	7269.3 (7276.9)	CM10 SPA low and IMAC30 Cu SPA high	Up	
0.61	0.00091828	AD/C	60976.2	IMAC 30 Cu SPA Low and Ni CHCA	Down	Hemopexin
0.38	0.00107103	AD/C	89874.1 (90605.3)	CM10 SPA high and Q10 SPA high	Up	
0.35	0.00115893	AD/N	2248.2	CM10 CHCA	Up	
0.34	0.00151443	AD/N	8933.2 (8936.9)	IMAC 30 Cu SPA low and Ni CHCA	Up	Complement 3a des-Arg
0.38	0.00165366	AD/C+N	21100.1	Q10 SPA high	Up	
0.35	0.00179305	AD/N	7281.7	Q10 SPA high	Up	
0.43	0.00193474	AD/C	5281.9	IMAC 30 Ni	Up	

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ROC area	p-value	P value comparison	Cluster mass	Assay	Up/Down in AD	ID
				SPA high		
0.39	0.00197628	AD/N	6519.2 (6515.2)	IMAC 30 Cu SPA high and Ni SPA low	Up	
0.41	0.00200779	AD/C	4019.8	IMAC 30 Ni	Up	
0.64	0.00205481	AD/N	6441.6	H50 SPA low	Down	
0.41	0.00237350	AD/C+N	4007.1	IMAC 30 Ni CHCA	Up	
0.39	0.00249026	AD/C+N	3514.5 (3511.3) (3517.7)	IMAC 30 Cu, CM10 CHCA and SPA low	Up	C-terminal fragment of Neuroendocrine protein 7B2
0.61	0.00256521	AD/C+N	14152.3	IMAC 30 Ni SPA low	Down	Transthyretin S-glutathionylated
0.35	0.00294281	AD/C	7676.9	IMAC 30 Cu CHCA	Down	
0.40	0.00299932	AD/C	8207.8	CM10 SPA high	Up	
0.35	0.00300866	AD/N	3253.5	IMAC 30 Ni CHCA	Up	
0.42	0.00342646	AD/C+N	3818.0	IMAC 30 Ni SPA low	Up	
0.37	0.00362052	AD/N	158656.8	CM10 SPA high	Up	
0.35	0.00385295	AD/N	2628.2	CM10 CHCA	Up	
0.38	0.00397209	AD/C+N	6642.3	IMAC 30 Cu SPA high	Up	
0.44	0.00418700	AD/C	4986.4	IMAC 30 Ni	Up	
0.39	0.00441947	AD/C+N	3705.0	CM10 SPA low	Up	
0.36	0.00448691	AD/N	8981.5	IMAC 30 Ni SPA low	Up	
0.38	0.00497339	AD/C	14619.6	IMAC 30 Ni SPA high	Up	

[0162] An additional analysis was performed utilizing a complete sample set (i.e., 236 samples comprising 98 AD (including 83 very mild cases with an MMSE > 24), 78 normals, 31 FTD and 29 Lewy Body Dementia cases). Using this complete sample set, a set of unique peaks were found which were able to separate the AD samples from one of the other groups with a P value of < 0.001. The biomarkers range in size from 2 – 90 kDa. These peaks are presented in Table IV-A, below. Note that the masses listed in Table IV-A differ slightly from those in Table II. The masses in Table IV-A reflect additional instrument calibration using the theoretical molecular weights of the biomarkers identified in earlier studies.

[0163] A further analysis was performed utilizing a subset of the samples derived solely from the Gothenburg site (i.e., 113 samples comprising 64 AD (including 49 very mild cases with an MMSE>24) and 49 clinically normal individuals). A set of unique peaks were found which were able to separate AD samples from clinically normal with a P value of <0.005. The biomarkers range in size from 3.5-92.1 kDa. These peaks are presented in Table IV-B, below. Reported in the table are masses using internal spectral calibration

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(using the masses of known proteins and peptides as calibrants) and theoretical mass predicted from sequenced where available.

Table IV-A

Cluster mass, Da	AD vs. Normal	AD vs. FTD	AD vs. DLB	Up/Down in AD	ProteinChip Assay	Identification
2429.8	0.95487612	**0.00017162**	0.26538695	Up	ICc	Integral Membrane 2B C-terminal fragment
3215.5	0.23273391	**0.00001642**	0.01118477	Down	Hc	
3235.8	**0.00006439**	0.00225532	0.14515129	Up	Cc + ICc + INc	
3315.9	0.69596856	**0.00002396**	0.03491240	Down	Hc	
3513.8	**0.00098304**	0.04572348	0.00386392	Up	Cs + ICc + ICs	Neuroendocrine protein 7B2 C-terminal fragment
3669.9	0.09454931	0.10767137	**0.00041166**	Up	Cc	
3681.3	0.11666086	0.00436488	**0.00001668**	Up	Qs	Secretogranin II fragment (secretoneurin)
3691.2	0.12619916	0.06923653	**0.00000279**	Up	Cc + Cs	VGF N-terminal fragment 2
3909.2	0.00648300	0.00955931	**0.00035033**	Up	Qs	Chromogranin A fragment
3932.9	0.01023885	**0.00002062**	0.00130144	Up	Qs	
3948.5	0.02027587	0.07735421	**0.00000212**	Up	Cc	VGF N-terminal fragment 1
3966.1	**0.00075979**	0.01030175	**0.00066025**	Up	Cs	
4146.5	0.02001680	**0.00051124**	**0.00032008**	Up	Qs	
4183.1	0.05179624	**0.00000000**	0.10283792	Up	Icc	
4335.4	**0.00032119**	**0.00000000**	**0.00051926**	Up	ICs + INc	
4353.2	0.02412351	0.00156337	**0.00005341**	Up	Qs	Alpha-1-antichymotrypsin C-terminal fragment
4746.0	0.036828	**0.000941**	**0.000370**	Up	Hs + Cs	
4809.5	0.39862870	0.03111856	**0.00001209**	Up	Qs	
4974.0	**0.00000187**	**0.00001239**	**0.00013077**	Up	Cc	Thymosin beta-4 (N-acetylated)
5003.5	**0.000081**	**0.000001**	**0.000219**	Up	Hs	
5059.7	**0.00089928**	0.00504850	**0.00067411**	Up	Cs + Qs + Ics+Hs	
6256.9	0.04206642	0.01734302	**0.00036644**	Up	Qs	
6273.8	0.00321745	**0.00013350**	0.00644068	Up	Cs + ICc + ICs + INc	
6446.3	0.565441	**0.000036**	0.017879	Down	Hs + Hc	Apolipoprotein C-I (2 aa deleted from N-terminus)
6502.9	**0.00090782**	**0.00050848**	0.00266677	Up	INc + INs	
6674.1	0.24876618	0.10787226	**0.00000515**	Up	Qs	
6681.5	0.77039619	**0.00022426**	0.31943288	Down	INc	
8291.9	**0.00000468**	0.03077283	0.01707923	Up	Cc	Ubiquitin (3 aa deleted from C-terminus)
8575.5	**0.00000969**	**0.00025594**	0.00876087	Up	Cc	Ubiquitin Full Length
8934.5	0.11176238	**0.00002279**	0.09368956	Up	ICs + INc + INs	C3a anaphylatoxin des-Arg
9759.9	0.00119119	0.01428452	**0.00089480**	Up	Qs	
9804.6	**0.000303**	0.004270	0.067531	Up	Hs	
10377.7	**0.000013**	**0.000125**	0.021733	Up	Hc + Hs	
11371.9	0.80246140	**0.00002687**	0.50685072	Up	INs	
11733.2	**0.00000240**	0.00532157	0.00496514	Up	Cs + Qs + ICc + INc +	Beta-2-Microglobulin

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12542.2	0.01464240	0.00000416	0.01333416	Up	Ins +Hc +Hs Cc + Cs + ICc + ICs + INc + Ins +Hc +Hs	Cystatin-C N-terminal truncation
13958.9	0.00096765	0.55798776	0.08726619	Down	INs	Transthyretin S-Cys and/or S-CysGly
14112.5	0.00081568	0.33747927	0.00238552	Down	INs	Transthyretin S-glutathionylated
14540.2	0.16249090	0.00005669	0.89261196	Up	INs	
21030.1	0.46655641	0.00015990	0.10034825	Up	Qs	Retinol Binding Protein
59280.8	0.00000119	0.67722736	0.17701643	Down	ICs + INc + INs	Hemopexin
66472.2	0.20360350	0.00014003	0.72727727	Up	Cc	Albumin
79098.4	0.00027794	0.00000018	0.00584688	Up	Cc	Transferrin
89388.0	0.01053139	0.00000151	0.83952605	Up	Cc + ICc	

Legend: IC - IMAC chip, activated with Cu; IN - IMAC chip, activated with Ni; H - H50 chip; C - CM10 chip; Q - Q10 chip; lower case "c" and "s" refer to the use of the energy absorbing molecules SPA and CHCA, respectively.

Table IV-B

M/Z (int. cal)	P Total	ROC	AD v. N	Best Condition	# of Conditions	ID	Calculated MW, Da
60464.7	< 0.0001	0.8409	down	IM Ni SPA high	2	Hemopexin	glycosylated
3513.9	< 0.0001	0.8219	up	CM10 SPA low	6	7B2 CT fragment	3,512.84
8291.0	< 0.0001	0.819	up	CM10 SPA high	1	Ubiquitin -3aa from CT	8,294.55
11725.7	< 0.0001	0.8058	up	H50 SPA high	14	Beta-2-Microglobulin	11,731.17
5044.2	< 0.0001	0.7972	up	CM10 SPA high	12		
10379.8	< 0.0001	0.7943	up	H50 CHCA	1	10.3 kDa	
9984.6	< 0.0001	0.7832	up	H50 CHCA	5	related to 10.3 kDa	
10265.6	< 0.0001	0.7796	up	H50 CHCA	1	related to 10.3 kDa	
9802.4	< 0.0001	0.7757	up	Q10 SPA high	5	EA-92 (ChrA peptide)	9,730.18
9757.0	< 0.0001	0.7735	up	CM10 SPA high	2	related to 10.3 kDa	
16207.4	< 0.0001	0.7634	up	IM Cu SPA high	3	Pancreatic ribonuclease	glycosylated
14092.7	< 0.0001	0.7631	down	IM Ni SPA high	2	Transthyretin S-glutathionylated	14,067
13904.7	< 0.0001	0.758	down	IM Ni SPA high	6	Transthyretin S-Cys/S-CysGly	13,880/13,937
12545.9	< 0.0001	0.7503	up	CM10 SPA high	5	Cystatin-C -8aa from NT	12,540.22
8183.6	< 0.0001	0.7481	up	CM10 SPA high	2	Ubiquitin -4aa from CT	8,181.39
5227.4	< 0.0001	0.7477	up	H50 CHCA	1		
3687.0	< 0.0001	0.7363	up	Q10 SPA low	1	Secretoneurin (ChrC/SGII peptide)	3,679.01
3906.4	< 0.0001	0.7321	up	IM Cu CHCA	5	Vasostatin II (ChrA peptide)	3,908.13
78936.5	< 0.0001	0.7315	down	IM Ni SPA high	4	Transferrin	glycosylated
3806.2	< 0.0001	0.7312	up	IM Ni CHCA	3		
8955.1	< 0.0001	0.7312	up	Q10 SPA high	1		
5263.9	< 0.0001	0.7309	up	CM10 SPA low	1		
14565.1	< 0.0001	0.7286	up	IM Ni SPA high	1	Pancreatic ribonuclease	14,574.33
20839.2	< 0.0001	0.7254	up	IM Cu CHCA	4		
6509.6	< 0.0001	0.7235	up	IM Ni CHCA	1	Chromogranin B peptide	6,502.87
4320.6	< 0.0001	0.7213	up	IM Ni CHCA	3	A-beta 1-40	4329.86
7258.2	0.0002	0.7205	up	Q10 SPA high	3	Chromogranin B peptide	7,262.42
17349.3	0.0001	0.7199	down	CM10 SPA high	1	Apolipoprotein A-II dimer	17,379.82
58845.4	< 0.0001	0.7194	down	IM Cu CHCA	1		
8938.5	0.0001	0.713	up	IM Cu CHCA	1	C3a des-Arg	8,932.50
6608.9	0.0003	0.7115	up	Q10 SPA high	2		
13349.5	0.0001	0.7108	up	H50 SPA low	6	CysC	13,347.14
5838.3	0.0003	0.7083	up	Q10 SPA high	1		
23477.4	0.0004	0.7063	up	Q10 SPA high	1	Prostaglandin-D synthase	glycosylated
4357.0	0.0005	0.704	up	Q10 SPA low	2	Alpha-1-antichymotrypsin CT fragment	4,354.19
7653.2	0.0006	0.6901	up	IM Cu SPA high	11	Osteopontin CT fragment	7658.19

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16716.9	0.0007	0.6899	up	CM10 CHCA	5		
4812.5	0.0021	0.6791	up	Q10 SPA low	1	VGF(NCBI) peptide	4,808.80
4989.4	0.0012	0.6783	up	IM Cu SPA low	7	Thymosin beta-4 - acetylated	4,967.46
7878.7	0.0012	0.6779	up	IM Ni SPA low	1		
92082.4	0.0016	0.6738	up	IM Cu SPA high	4		
66479.2	0.0038	0.6685	down	Q10 SPA high	4	Albumin	
3967.6	0.0023	0.6677	up	IM Ni CHCA	1		
7718.8	0.0023	0.6677	up	IM Cu SPA high	4	Osteopontin CT fragment phosphorylated	7738.19
89707.1	0.0032	0.667	up	CM10 SPA high	1		
11579.2	0.0027	0.6652	up	H50 SPA high	1		
8585.9	0.0039	0.6635	up	CM10 SPA high	1	Ubiquitin	8564.84
4455.4	0.0034	0.6614	up	IM Cu CHCA	2		

[0164] Figure 4A-K shows the distribution of peak intensities observed for the various groups and the results of Mann-Whitney or Kruskal-Wallis tests used to determine the significance of any differences observed. Figures 5-8 also show results obtained from the analysis of (1) a set of case-control age-matched AD vs. Normal sample set (n=86) and (2) an Ab42/T-Tau pre-selected sample set (n=104) comprising 69 AD and 35 Normals (AD group with Tau >450 + Ab42 < 550 and normals the reverse). Figure 9 shows examples of mass spectra obtained for many of the biomarkers of Table IV-A and IV-B.

[0165] The identities of a number of the peaks detected in this study were established using methods similar to those described above. Biomarkers were purified using combinations of chromatography techniques employing a range of Biosepra sorbents typically followed by 1D-SDS-PAGE. The purification schemes were monitored using a ProteinChip system to track biomarkers of interest. For proteins smaller than 30 kDa, intact bands of interest were extracted from gels and reanalyzed using the ProteinChip Reader to confirm the mass matched with the original biomarker. The gel-extracted proteins were in-solution digested with trypsin and proteins larger than 30 kDa were in-gel digested. Tryptic digests were analyzed by peptide mapping using the ProteinChip Reader and by tandem MS using a Q-STAR (Applied Biosystems) instrument fitted with a PCI-1000 ProteinChip Interface. Biomarkers smaller than 4 kDa were enriched by combinations of chromatography techniques and identified directly by tandem MS without SDS-PAGE purification and/or trypsin digestion.

[0166] For instance, the following peptides/proteins were identified:

[0167] M11727: This protein was identified as β2 microglobulin (Swiss-Prot accession number P01884, http://us.expasy.org/cgi-bin/niceprot.pl?P01884), which finding is consistent with the findings of Example 1 and 2. β2 microglobulin (B2M) is a potential initiator of inflammatory responses in that (1) it directs intracellular transport of major histocompatibility complex class I molecules; and (2) it is modulated by interferons and

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certain cytokines that also play an important role in inflammation. Its role as a CSF biomarker for AD has previously been discussed. However, it has now been identified as a biomarker for AD in blood.

[0168] M3680.7: This peptide was identified as a Secretogranin II peptide (also known as secretoneurin), which has the italicized sequence of SEQ ID NO:2. Secretogranin II is a large dense-core synaptic vesicle protein. The levels of secretogranin II were observed to decrease in the temporal cortex of AD patients vs. age-matched controls. One of the main features of AD is a degeneration of synapses. The levels of secretoneurin peptide in CSF may therefore reflect synaptic loss. This loss of synapses, reflected by early cognitive impairments, precedes the appearance of extra cellular focal deposits of beta-amyloid peptide in the brain of patients.

[0169] M78677.3: This protein was identified as Transferrin (Swiss-Prot accession number P02787), which is consistent with the findings of Example 1. Transferrin is a major transport protein for iron, which is a major factor in free radical generation and oxidative stress in neurodegenerative diseases. Transferrin levels increase in AD frontal cortex, compared with elderly controls. C2 allele associated with AD in ApoE4 negative subjects. ApoE and Transferrin may be part of a complex mechanism in the pathogenesis of Alzheimer's disease.

[0170] M2431.2: This peptide was identified as a truncated ABri/ADan amyloid peptide of Integral Membrane Protein 2B (MMP2B or BRI), which has the underlined sequence of SEQ ID NO:3. The exact physiological role in the brain is yet to be fully understood. Mutations in BRI gene cause rare neurodegenerative conditions -- familial British and Danish dementia -- which involve deposition of the extended amyloidogenic peptides (ADan/ABri) and bear striking neuropathological similarities to AD. This is the first time the shorter WT form of the C-terminal peptide has been associated with a disease.

[0171] M13391 peptide was identified to be full-length Cystatin C, which has the italicized sequence of SEQ ID NO:4. Cystatin C is found in most bodily fluids and tissues and is a marker of renal function in urine. Cystatin C inhibits activity of lysosomal cysteine proteases (Cathepsins). Cystatin-C/Cathepsin balance is important for many disease processes including inflammation, cancer and AD. Cystatin C is associated with AD. For instance, CST3 B/B homozygosity is associated with an increased risk of developing AD; Cystatin C increases in AD brain at neuronal sites most susceptible to cell death in AD independent of cystatin C genotype; and Cystatin C co-localizes with A-beta in AD brain deposits.

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[0172] M12583.4: This peptide was identified as a truncated Cystatin C peptide, which has the underlined sequence of SEQ ID NO:4. This truncated Cys-C, which lacks the first eight N-terminal residues, has a 20-fold lower affinity for Cathepsin B, but not other cathepsins. Cathepsin B plays key role in AD. It is identified in most early endosomes in Alzheimer brains, but detectable in only a minor proportion of endosomes in normal brain. Specific cathepsin B inhibitors abolish neurotoxic effects caused by Abeta42-activated microglial cells. It has been found that this truncated Cys-C is a biomarker for AD in both CSF and blood. This is the first time this 8aa N-terminal truncated version has been described in CSF or blood.

[0173] M3951.6: This peptide was identified to be a N-terminal fragment of the Neurosecretory protein VGF, which has the italicized sequence of SEQ ID NO:5. Similarly, the M3687.7 peptide was identified to be a N-terminal fragment of Neurosecretory protein VGF (-3amino acids), which has the bolded sequence of SEQ ID NO:5. The Neurosecretory protein VGF is a nerve growth factor selectively synthesized in neuroendocrine and neuronal cells. VGF mRNA levels are regulated by neuronal activity, including long-term potentiation, seizure, and injury. The sequence is rich in paired basic amino acid residues that are potential sites for proteolytic processing. Such fragments appear to be novel. A different fragment of the same protein was discovered by another group.

[0174] M60976.2: This protein was identified as Hemopexin (Swiss-Prot accession number P02790). Hemopexin is an acute phase reactant protein induced after inflammation by IL-6; a scavenger/transporter of heme to prevent heme-mediated oxidative stress; and also believed to play a role in nerve repair.

[0175] M8933.2: This peptide was identified to be C3a anaphylatoxin desArg, which has the underlined sequence of SEQ ID NO: 6. The full-length sequence for the C3a anaphylatoxin peptide is the italicized sequence of SEQ ID NO: 6. Complement activation known to occur in the AD brain: (1) contributes to the development of a local inflammatory state; and (2) correlated with cognitive dysfunction. Localization and potential mechanism for C3 in AD brain: (1) protein expression increases (5-10 fold) in cultured mice microglial cells in response to A-Beta synthetic peptides; and (2) inhibition of C3 in hAPP mice increases plaque deposition and neuronal degeneration – potential role in clearance. This peptide is a novel complement protein fragment marker of AD.

[0176] M3514.5: This peptide was identified as a C-terminal fragment of Neuroendocrine protein 7B2, which has the underlined sequence of SEQ ID NO: 7. The C-terminal fragment corresponds to amino acids 182-212; the full-length protein has the

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italicized sequence of SEQ ID NO: 7. Neuroendocrine protein 7B2 complexes with Prohormone Convertase 2 (PC2) in the endoplasmic reticulum (ER). PC2 processes somatostatin precursors. Once the proPC2/7B2 complex arrives at the trans-Golgi network, 7B2 is internally cleaved into two domains, the 21-kDa fragment and a carboxy-terminal 31 residue peptide. If proPC2 has not encountered 7B2 intracellularly, it cannot generate a catalytically active mature species. A marked decrease in the ratio of the PC2 precursor to the total enzymatic pool is observed in the frontal cortex of Alzheimer patients. This decrease coincides with an increase in the binding protein 7B2. A somatostatin deficit occurs in the cerebral cortex of Alzheimer's disease patients without a major loss in somatostatin-containing neurons. This deficit could be related to a reduction in the rate of proteolytic processing of peptide precursors. There is a body of evidence to suggest that certain forms of somatostatin in CSF correlate with dementia severity.

[0177] M3912.8: This peptide was identified as a fragment of chromogranin A (CMGA_HUMAN (SwissProt # P10645)) corresponding to the italicized sequence in SEQ ID NO: 8. The underlined sequence in SEQ ID NO: 8 is vasostatin I. The protein consisting of the underlined sequence and the italicized sequence is vasostatin II, a fragment of chromogranin A with vasoinhibitory properties. Chromogranin A is the major protein of large dense-core synaptic vesicles. The ratio of chromogranin A to secretogranin II in the temporal cortex is significantly correlated to the clinical severity of dementia and to the extent of neuropathological changes. The levels of the vasostatin II peptide in CSF may reflect synaptic loss.

[0178] M4352.4: This peptide was identified as a C-terminal fragment of alpha-1-antichymotrypsin (SwissProt # P01011; theoretical MW 4354.19 Da; SEQ ID NO: 9). This is an acute phase/inflammatory protein overproduced in the AD brain that can promote the formation of, and is associated with, neurotoxic amyloid deposits. Increase in brain levels is reflected by higher levels in the CSF. The peptide was directly sequenced and the identify was confirmed using an anti-alpha-1-antichymotrypsin antibody.

[0179] M21100.1: This protein was identified as full-length Retinol-binding protein (SwissProt # P02753; theoretical. MW 21,071.60 Da). The identity of this marker was established by direct sequencing of 8 tryptic-digest fragments and a pull-down assay using an anti-Retinol-binding protein antibody.

[0180] M8575, M8292, M8184: These proteins were identified as ubiquitin and C-terminus fragments thereof. Neurofibrillary tangles (NFT) are composed of a hyperphosphorylated and ubiquitinated form of tau protein. With maturation, tau-based

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neurofibrillary tangles are increasingly ubiquitinated. Levels of tau and conjugated ubiquitin are elevated both in AD brain and CSF. CSF-ubiquitin levels are also associated with increasing degree of cortical and central brain atrophy as measured by computerized tomography.

[0181] M4971: This protein was identified as an N-acetylated form of Thymosin beta-4 (the N-terminal serine is acetylated). It is thought that Thymosin beta-4 may act as a marker for activated microglia, a central part of the chronic inflammatory processes in AD. The amino acid sequence of this protein is shown as SEQ ID: 10 (SwissProt Accession Number P62328).

[0182] M13960 and M14110: These biomarkers were identified as full-length Transthyretin S-Cys (and/or S-CysGly) and Transthyretin S-gluathionylated proteins, respectively. Transthyretin has been previously characterized as an A-beta sequestering protein which is present at lower concentrations in the CSF of AD patients versus healthy controls. This decrease is negatively correlated with senile plaque abundance.

[0183] M6502 and 7262: These biomarkers were identified as fragments of Chromogranin B (ChB) peptide found in neuronal large dense-core vesicles. ChB is highly processed by prohormone convertase (PC) enzymes to form smaller peptides from the precursor protein. A disease associated imbalance in PC enzymes could lead to changes in the processing of chromogranin proteins. Chromogranin proteins (ChA, ChB and SecretograninII) show distinct changes in their distribution in the brains of AD patients and are often associated with amyloid plaques. Chromogranin peptides including ChB have a potential as neuronal markers for synaptic degeneration in Alzheimer's disease.

[0184] M7658 and M7738: These biomarkers are Osteopontin C-terminal fragments, unphosphorylated and singly phosphorylated species respectively. Osteopontin is a cytokine regulating tissue repair that may play a key role in the pathogenesis of neuroinflammation.

[0185] M17380: This biomarker is a dimer of Apolipoprotein A-II. ApoA-II forms a complex with the protein ApoE which binds strongly to a-beta possibly playing a role in clearance. Levels of ApoA-II have previously been shown to be decreased in the serum of dementia patients.

[0186] M23477: This biomarker is prostaglandin D-synthase, a glycoprotein also known as beta-trace protein that catalyzes the formation of prostaglandin D2 (PGD2) from PGH2.

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[0187] 7653 Da/7718 Da (IMAC-Cu SPA): This protein was identified as a C-terminal fragment of Osteopontin (SwissProt# P10451, MW 7658.19 Da; SEQ ID NO: 11). Osteopontin is known to be extensively phosphorylated at serine residues. The 7653 Da is an unphosphorylated form, while the 7718 Da is a phosphorylated peptide. The sequence highlighted in bold in SEQ ID NO: 11 corresponds to the 7653 Da biomarker.

[0188] 7258 Da (Q10 SPA): This protein is a processed fragment of Chromogranin B (Secretogranin I; SG1_HUMAN (SwissProt# P05060)). The sequence highlighted in bold in SEQ ID NO: 12 corresponds to the 7258 Da biomarker. Three underlined fragments were identified by MSMS with high scores. Y341 is sulfotyrosine, therefore the predicted MW is 7262.42 Da. The biochemistry to generate this peptide is exactly the same as for the 6502 Da peptide. The 7262 Da peptide sequence is flanked by cleavage sequences for prohormone convertase 1/3 and prohormone convertase 2: -KK-, -RR-, and -KR- (and carboxypeptidase H trims C-terminal Lys and Arg).

[0189] 23 kDa biomarker: This protein is Prostaglandin-H2 D-isomerase (SwissProt# P41222). The predicted MW is 18.7 kDa, but the protein is very heavily glycosylated.

[0190] 17.3 kDa biomarker: This protein is a Cys-Cys dimer of Apolipoprotein A-II (SwissProt# P02652). The MW of the plain monomer is 8707.91 Da, however the N-terminal Q is known to be modified to pyrrolidone carboxylic acid (-17 Da). Therefore the dimer of two full-length monomers is 17379.82 Da.

[0191] 9.8 kDa (Q10 SPA): The protein was identified as the EA-92 peptide of Chromogranin A (SwissProt# P10645, MW 9730.18 Da; SEQ ID NO: 13). The sequence highlighted in bold in SEQ ID NO: 13 corresponds to the 9730 Da biomarker. Three fragments underlined were identified by MSMS with high scores. The 9730 Da peptide sequence is flanked by cleavage sequences for prohormone convertase 1/3 and prohormone convertase 2. Note that the 9.8 kDa biomarker (Q10) is not the same as the 9750 Da biomarker which appears under other (CM10, H50) assay conditions.

[0192] 4812 Da (Q10): This biomarker was identified as processed fragment of VGF nerve growth factor inducible precursor (NCBI# gi17136078, MW 4808.80 Da; SEQ ID NO: 14). The sequence highlighted in bold in SEQ ID NO: 14 corresponds to the 4808 Da biomarker. Two fragments underlined were identified by MSMS and cover all the sequence of the peptide. The 4808 Da peptide sequence is flanked by cleavage sequences for prohormone convertase 1/3 and prohormone convertase 2.

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[0193] 4320 (IMAC-Ni): This biomarker was identified as A-beta 1-40 peptide (MW 4329.86 Da). This peak was found in Q fraction 3 using IMAC-Ni array, purified by RPC, YM30, and SDS-PAGE, digested with trypsin, and major ions in the digest were identified as fragments of Amyloid beta A4 precursor (SwissProt# P05067; SEQ ID NO: 15). The sequence in SEQ ID NO: 15 highlighted in bold corresponds to the 4330 Da biomarker. Three fragments underlined were identified by MSMS.

[0194] 16.2 kDa (IMAC-Cu): This biomarker is glycosylated Pancreatic Ribonuclease (SwissProt# P07998). All peaks after the 16.2 kDa peak co-purify as an entity and appear to be various glycosylation forms. Ribonuclease is known to be only partially glycosylated. The 14.6 kDa peak, which co-purifieds with the 16.2 kDa biomarker, corresponds to the plain non-glycosylated form of Pancreatic Ribonuclease (MW 14,574.33 Da).

[0195] 4146 Da (Q10): This polypeptide was identified as C-terminal fragment (SEQ ID NO: 16) of Protease C1 inhibitor (SwissProt # P05155, MW 4152.87 Da).

[0196] The foregoing biomarkers have a number of interesting mechanistic links to AD. For instance, the following are host response proteins and fragments: neurosectretory protein VGF (fragment); and Beta-2-microglobulin (full-length). The following are plaque "related" proteins/peptides: BRI membrane protein (fragment); A-Beta (fragments/forms); Cystatin C and truncated Cystatin-C (fragment); secreteoneurin; vasostatin II; ubiquitin and ubiquitin fragments; neuroendocrine protein 7B2; and Complement 3a protein (fragment). Finally, the following proteins play a role in iron transport and recycling: Transferrin (full-length); and Hemopexin (full-length). The mechanistic relationships between these biomarkers are summarized in Figure 10 and in Table V, below.

Table V

List of characterized markers with Neuropathological associations

Plaque Associated	Alpha1-antichymotrypsin (fragment)
	Retinol-binding protein
	Transthyretin (modified forms)
	Cystatin C (N-terminal truncation)
Neurofibrillary Tangle Associated	Ubiquitin full length (+ 2 fragments)
Synaptic Loss	Secretogranin II (fragment)
	Chromogranin A (fragment)
Neurotransmitter dysfunction	Neuroendocrine protein 7B2 fragment (fragment)
Lipid metabolism	Apolipoprotein C-I (truncated)
Inflammatory proteins	Alpha1-antichymotrypsin (fragment)
	Cystatin C (N-terminal truncation)

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	Beta-2-microglobulin
	Thymosin beta-4 (modified)
	Complement 3a (fragment)
Iron metabolism and Oxidative Stress	Transferrin
	Hemopexin
Unknown	Neurosecretory protein VGF (3 fragments)
	BRI protein C-terminal fragment

EXAMPLE 4. CYSTATIN C AND MODIFIED FORMS AS MARKERS FOR ALZHEIMER'S DISEASE

A. Sample Protocol

1. Manually apply 2 μL of a solution containing 0.25 mg/mL of Cystatin C antibody to each spot of an PS-20 array (Ciphergen Biosystems, Inc., Fremont, CA). For a negative control, use the same concentration of IgG on another spot.
2. Incubate for 2 h in a humidity chamber at room temperature to allow the antibodies to covalently bind to the spots.
3. Transfer the bioprocessor to Biomek 2000 liquid handling robot.
4. Block residual active sites by adding 25 μL 1 M ethanolamine. Incubate for 30 min at room temperature in a humidity chamber.
5. Remove unbound proteins by washing the array with 3 times 100 μL PBS + 0.5% Triton X-100 5 min. each.
6. Wash with 100 μL PBS for 5 min.
7. Apply 20 μL neat CSF to each spot and incubate at 4 °C overnight.
8. Wash the spots with 3 x 100 μL PBS + 0.5% Triton X-100 10 min each.
9. Wash with 3 x 100 μL PBS for 1 min each.
10. Rinse the array with 100 μL 1mM HEPES.
11. Remove the bioprocessor from the Biomek 2000 robot.
12. Remove the bioprocessor gasket and allow the spots to air dry.
13. Manually apply 2 x 0.8 μL saturated SPA solution to each spot of the array.
14. Analyze the array using the ProteinChip Reader.

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B. Validation of CysC Δ1-8 Marker

To validate the use of CysC Δ1-8 as a marker for Alzheimer's disease, 158 cerebrospinal fluid (CSF) samples were taken from pre-diagnosed subjects in three groups: (1) Alzheimer's disease (AD), Control, and Non-Alzheimer's dementia (Non AD). The distribution of samples in these groups is shown in Table III, above.

[0197] SELDI-MS measurements of CysC Δ1-8 in each of the samples were obtained according to standard protocols, using a Ciphergen H50 ProteinChip and 50% SPA as an EAM. The results are shown in the graph at the bottom left in Figure 11. Similar measurements of full-length CysC were also made using the same set of samples (Figure 11, bottom right). The results show that the levels of CysC Δ1-8 in subjects suffering from Alzheimer's disease are significantly higher than the levels in subjects suffering from non-Alzheimer's dementia.

EXAMPLE 5. MARKER COMBINATIONS FOR DETECTING ALZHEIMERS

[0198] As discussed above, a combination of biomarkers can provide greater predictive value of a particular status than single biomarkers alone. A plurality of biomarkers in a sample can increase the sensitivity and/or specificity of the test. A preferred set of biomarkers for qualifying Alzheimer's status in a patient is one in which the biomarkers of the set are regulated *in vivo* independently of each other. A preferred test has greater than 80% sensitivity and specificity. Even more preferred are tests where both the sensitivity and specificity are greater than 90%. One example of a set of biomarkers which is preferred for a combination test includes M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), and M3513.9 (7B2 CT fragment). Another example of a preferred set of biomarkers is the set which includes M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), M10379.8 (10.3 kDa) and M11725.7 (Beta-2-Microglobulin).

[0199] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

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WHAT IS CLAIMED IS:

1. A method for qualifying Alzheimer's disease status in a subject comprising:

a. measuring at least one biomarker in a biological sample from the subject, wherein the at least one biomarker is selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M9802.4 (EA-92 (ChrA peptide)), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M14565.1 (Pancreatic ribonuclease), M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M8938.5 (C3a des-Arg), M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), and M7718.8 (Osteopontin CT fragment phosphorylated); and

b. correlating the measurement with Alzheimer's disease status.

2. The method of claim 1, further comprising measuring at least one additional biomarker in a biological sample from said subject, wherein said at least one additional biomarker is selected from the group consisting of M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin).

3. A method for qualifying Alzheimer's disease status in a subject comprising:

a. measuring at least one biomarker in a biological sample from the subject, wherein the at least one biomarker is selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2,

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M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4; and

b. correlating the measurement with Alzheimer's disease status.

4. The method of claim 3, further comprising measuring at least one additional biomarker in a biological sample from said subject, wherein said at least one additional biomarker is selected from the group consisting of M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin).

5. The method of any of claims 1 or 3, wherein said at least one biomarker comprises M60464.7 (Hemopexin).

6. The method of claim 3, wherein said at least one biomarker comprises M10379.8 (10.3 kDa).

7. The method of any of claims 1 or 3, wherein said at least one biomarker comprises M17349.3 (Apolipoprotein A-II dimer).

8. The method of claim 3, further comprising measuring each of the following biomarkers: M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), M10379.8 (10.3 kDa) and M11725.7 (Beta-2-Microglobulin).

9. The method of claim 1, further comprising measuring each of the following biomarkers: M17349.3 (Apolipoprotein A-II dimer) and M60464.7 (Hemopexin).

10. The method of claim 1, further comprising measuring each of the following biomarkers: M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin) and M3513.9 (7B2 CT fragment).

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11. The method of any of claims 1, 2, 3 or 4, wherein the at least one biomarker is measured by capturing the biomarker on an adsorbent surface of a SELDI probe and detecting the captured biomarkers by laser desorption-ionization mass spectrometry.

12. The method of any of claims 1, 2, 3 or 4, wherein the at least one biomarker is measured by immunoassay.

13. The method of any of claims 1, 2, 3 or 4, wherein the sample is CSF.

14. The method of any of claims 1, 2, 3 or 4, wherein the sample is serum.

15. The method of any of claims 1, 2, 3 or 4, wherein the correlating is performed by a software classification algorithm.

16. The method of any of claims 1, 2, 3 or 4, wherein Alzheimer's disease status is selected from Alzheimer's disease and non-dementia.

17. The method of any of claims 1, 2, 3 or 4, further comprising (c) managing subject treatment based on the status.

18. The method of any of claims 1, 2, 3 or 4, wherein Alzheimer's disease status is selected from Alzheimer's disease, non-dementia, and non-Alzheimer's dementia.

19. The method of any of claims 1, 2, 3 or 4, wherein Alzheimer's disease status is selected from Alzheimer's disease and non-Alzheimer's dementia.

20. The method of claim 19 wherein non-Alzheimer's dementia is selected from dementia with Lewy bodies and frontotemporal dementia.

21. The method of claim 11, wherein the adsorbent is a cation exchange adsorbent.

22. The method of claim 11, wherein the adsorbent is a biospecific adsorbent.

23. The method of claim 11, wherein the adsorbent is a hydrophobic adsorbent.

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24. The method of claim 1, wherein the at least one biomarker is measured by a means of detection other than by mass.

25. The method of claim 24, wherein the at least one biomarker is measured by immunoassay.

26. The method of claim 16, wherein, if the measurement correlates with Alzheimer's disease, then managing subject treatment comprises administering a choline esterase inhibitor to the subject.

27. The method of claim 17, further comprising:

(d) measuring the at least one biomarker after subject management.

28. A kit comprising:

(a) a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds at least one biomarker selected from a first group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4; and

(b) instructions for using the solid support to detect a biomarker of Table II, Table IV-A or Table IV-B.

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29. The kit of claim 28, wherein the capture reagent binds at least one biomarker selected from a first group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M9802.4 (EA-92 (ChrA peptide)), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M14565.1 (Pancreatic ribonuclease), M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M8938.5 (C3a des-Arg), M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), and M7718.8 (Osteopontin CT fragment phosphorylated).

30. The kit of claim 28, further comprising a second solid support comprising a capture reagent attached thereto, wherein the capture reagent binds at least one additional biomarker selected from the group consisting of M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin)the biomarker Cystatin C (M13391).

31. The kit of claim 28, further comprising instructions for qualifying Alzheimer's disease status.

32. The kit of any of claims 28, 29 or 30, wherein the solid support comprising a capture reagent is a SELDI probe.

33. The kit of any of claims 28, 29 or 30, additionally comprising (c) a container containing at least one of the biomarkers of Table I, Table II, Table IV-A and Table IV-B.

34. The kit of claim 26, wherein the capture reagent is a cation exchange adsorbent.

35. The kit of any of claims 28, 29 or 30, additionally comprising (c) an anion exchange chromatography sorbent.

36. A kit comprising:

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(a) a solid support comprising at least one capture reagent attached thereto, wherein the capture reagents bind at least one biomarker selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4; and

(b) a container containing at least one of the biomarkers.

37. The kit of claim 36, wherein the capture reagent binds at least one biomarker selected from a first group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M9802.4 (EA-92 (ChrA peptide)) , M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M14565.1 (Pancreatic ribonuclease), M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M8938.5 (C3a des-Arg), M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), and M7718.8 (Osteopontin CT fragment phosphorylated).

38. The kit of claim36, further comprising a second solid support comprising a capture reagent attached thereto, wherein the capture reagent binds at least one additional biomarker selected from the group consisting of M11725.7 (Beta-2-

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Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin).

39. The kit of any of claims 36, 37 or 38, wherein the solid support comprising a capture reagent is a SELDI probe.

40. The kit of any of claims 36, 37 or 38, additionally comprising (c) an anion exchange chromatography sorbent.

41. The kit of claim 34, wherein the capture reagent is a cation exchange adsorbent.

42. A software product comprising:

a. code that accesses data attributed to a sample, the data comprising measurement of at least one biomarker in the sample, the biomarker selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin - 3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4; and

b. code that executes a classification algorithm that classifies the Alzheimer's disease status of the sample as a function of the measurement.

43. The software product of claim 42, wherein the classification algorithm classifies the Alzheimer's disease status of the sample as a function of the measurement of a biomarker selected from the group consisting of: M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M9802.4 (EA-92 (ChrA peptide)),

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M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M14565.1 (Pancreatic ribonuclease), M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M8938.5 (C3a des-Arg), M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), and M7718.8 (Osteopontin CT fragment phosphorylated).

44. The software product of claim 42, wherein the classification algorithm classifies the Alzheimer's disease status of the sample as a function of the measurement of at least one additional biomarker selected from the group consisting of M11725.7 (Beta-2-Microglobulin), M78936.5 (transferrin), M13349.5 (Cystatin C), M66479.2 (Albumin) and M8585.9 (Ubiquitin).

45. The software product of claim 42, wherein the classification algorithm classifies the Alzheimer's disease status of the sample as a function of the measurement of each of the following biomarkers: M17349.3 (Apolipoprotein A-II dimer), M60464.7 (Hemopexin), M10379.8 (10.3 kDa) and M11725.7 (Beta-2-Microglobulin).

46. An isolated modified Cystatin C polypeptide, wherein said modified Cystatin C polypeptide has the amino acid sequence of SEQ ID NO:2.

47. A method for qualifying Alzheimer's disease status in a subject comprising:

a. measuring at least one modified Cystatin C biomarker in a biological sample from the subject, wherein the at least one modified Cystatin C biomarker has the amino acid sequence of SEQ ID NO:2; and

b. correlating the measurement with Alzheimer's disease status.

48. The method of claim 47, further comprising measuring Cystatin (M13416).

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49. The method of claim 47, wherein the at least one modified Cystatin C biomarker is measured by capturing the biomarker on an adsorbent surface of a SELDI probe and detecting the captured biomarkers by laser desorption-ionization mass spectrometry.

50. The method of claim 47, wherein the at least one modified Cystatin C biomarker is measured by immunoassay.

51. The method of claim 47, wherein the sample is CSF.

52. The method of claim 47, wherein the sample is serum.

53. The method of claim 47, wherein the correlating is performed by a software classification algorithm.

54. The method of claim 47, wherein Alzheimer's disease status is selected from Alzheimer's disease and non-Alzheimer's dementia. .

55. The method of claim 54, wherein the non-Alzheimer's dementia is a member selected from the group consisting of Lewy body dementia (LBD) and frontotemporal dementia (FTD).

56. The method of claim 47, further comprising (c) managing subject treatment based on the status.

57. The method of claim 47, wherein the adsorbent is a hydrophobic adsorbent.

58. The method of claim 47, wherein the adsorbent is a biospecific adsorbent.

59. The method of claim 54 wherein, if the measurement correlates with Alzheimer's disease, then managing subject treatment comprises administering a choline esterase inhibitor to the subject.

60. The method of claim 56, further comprising:

(d) measuring the at least one biomarker after subject management.

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61. The method of claim 47, wherein the at least one modified Cystatin biomarker is captured with an antibody.

62. A method for identifying a compound that interacts with Cystatin C ΔN1-8, wherein said method comprises:

a) contacting Cystatin C ΔN1-8 with a test compound; and

b) determining whether the test compound interacts with Cystatin C ΔN1-8.

63. A method for identifying a compound that interacts with Cystatin C, wherein said method comprises:

a) contacting Cystatin C with a test compound; and

b) determining whether the test compound interacts with Cystatin C.

64. A method for modulating the concentration of Cystatin C in a cell, wherein said method comprises: contacting said cell with a protease inhibitor, wherein said protease inhibitor prevents cleavage of cystatin C between Arg8 and Leu9.

65. A method of treating Alzheimer's disease in a subject, wherein said method comprises: administering to a subject a therapeutically effective amount of a compound which modulates the expression or activity of a protease which cleaves Cystatin C between Arg8 and Leu9.

66. A method for qualifying dementia status in a subject, wherein dementia status is selected from non-dementia and non-Alzheimer's dementia, comprising:

a. measuring β2 microglobulin in a biological sample from the subject; and

b. correlating the measurement with non-Alzheimer's dementia status.

67. A method for qualifying Alzheimer's disease status in a subject comprising:

a. measuring at least one biomarker in a biological sample from the subject, wherein the at least one biomarker is selected from the group consisting of the biomarkers of Table I, Table II, Table IV-A and Table IV-B; and

b. correlating the measurement with Alzheimer's disease status.

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68. A method for qualifying Alzheimer's diseases status in a subject, comprising

a. detectably labeling a biomarker selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 kDa), M16207.4 (Pancreatic ribonuclease), M14092.7 (Transthyretin S-glutathionylated), M13904.7 (Transthyretin S-Cys/S-CysGly), M12545.9 (Cystatin-C -8aa from NT), M8183.6 (Ubiquitin -4aa from CT), M5227.4, M3687.0 (Secretoneurin (ChrC/SGII peptide)), M3906.4 Vasostatin II (ChrA peptide), M3806.2, M8955.1, M5263.9, M14565.1 (Pancreatic ribonuclease), M20839.2, M6509.6 (Chromogranin B peptide), M4320.6 (A-beta 1-40), M7258.2 (Chromogranin B peptide), M17349.3 (Apolipoprotein A-II dimer), M58845.4, M8938.5 (C3a des-Arg), M6608.9, M5838.3, M23477.4 (Prostaglandin-D synthase), M4357.0 (Alpha-1-antichymotrypsin CT fragment), M7653.2 (Osteopontin CT fragment), M16716.9, M4812.5 (VGF(NCBI) peptide), M4989.4 (Thymosin beta-4 - acetylated), M7878.7, M92082.4, M66479.2 (Albumin), M3967.6, M7718.8 (Osteopontin CT fragment phosphor), M89707.1, M11579.2, and M4455.4; and

b. treating a subject with said labeled biomarker;

c. utilizing Positron Emission Tomography (PET) to visualize a pattern of localization of said biomarker in said subject;

d. correlating said pattern of localization with Alzheimer's disease status.

69. The method of claim 68, wherein said biomarker co-localizes with beta-amyloid deposits in the brains of Alzheimer's patients.

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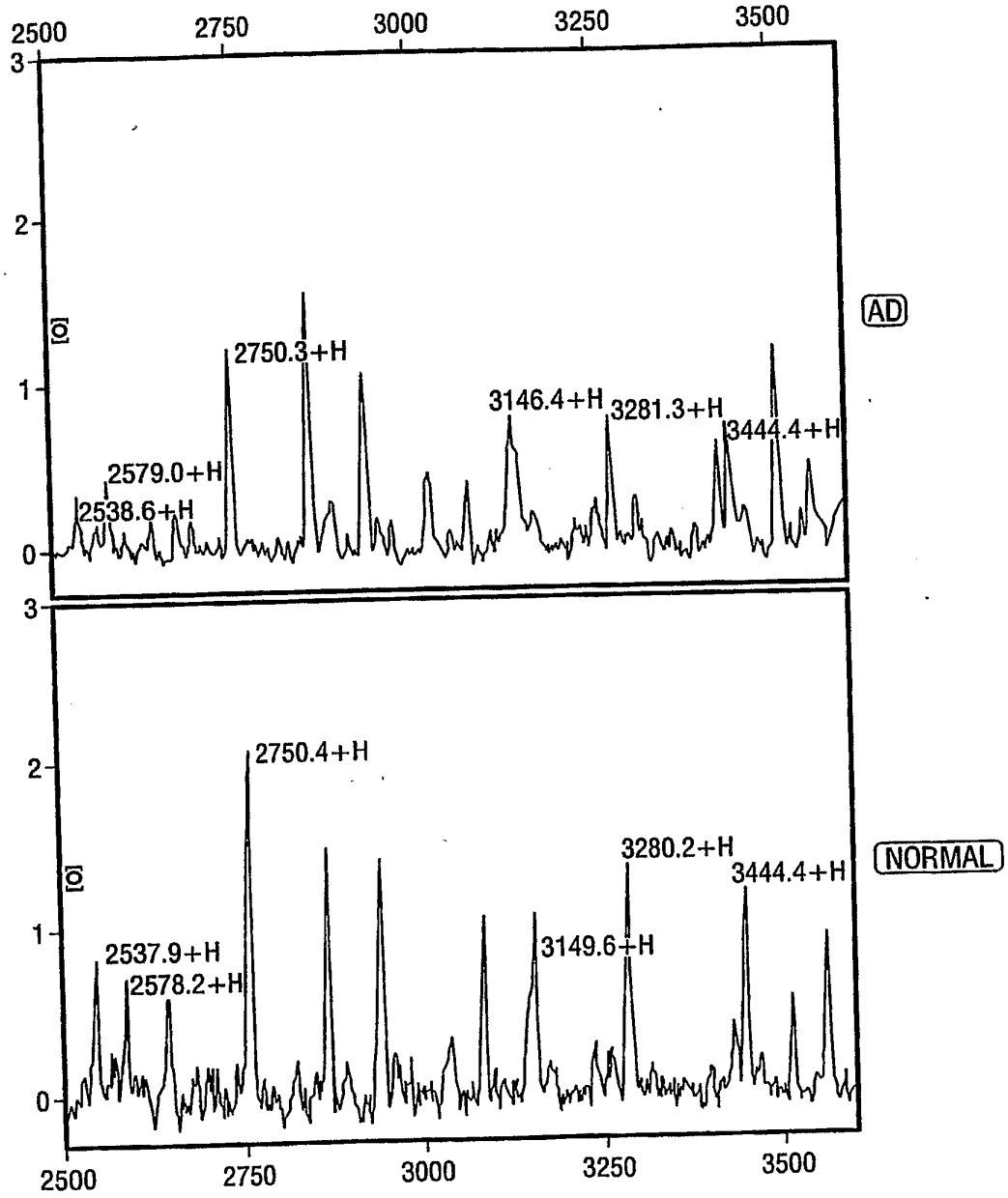


FIG. 1A

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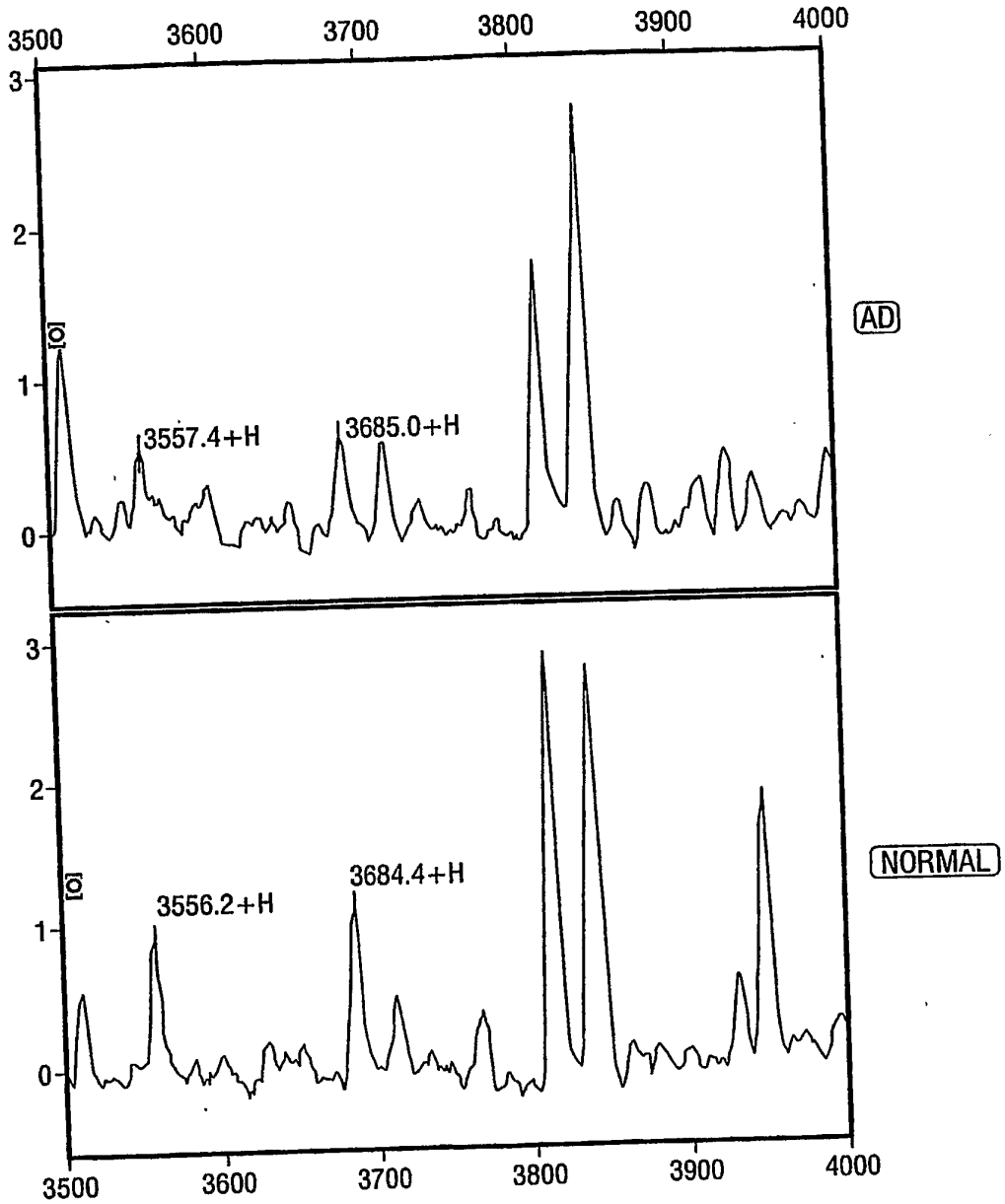


FIG. 1B

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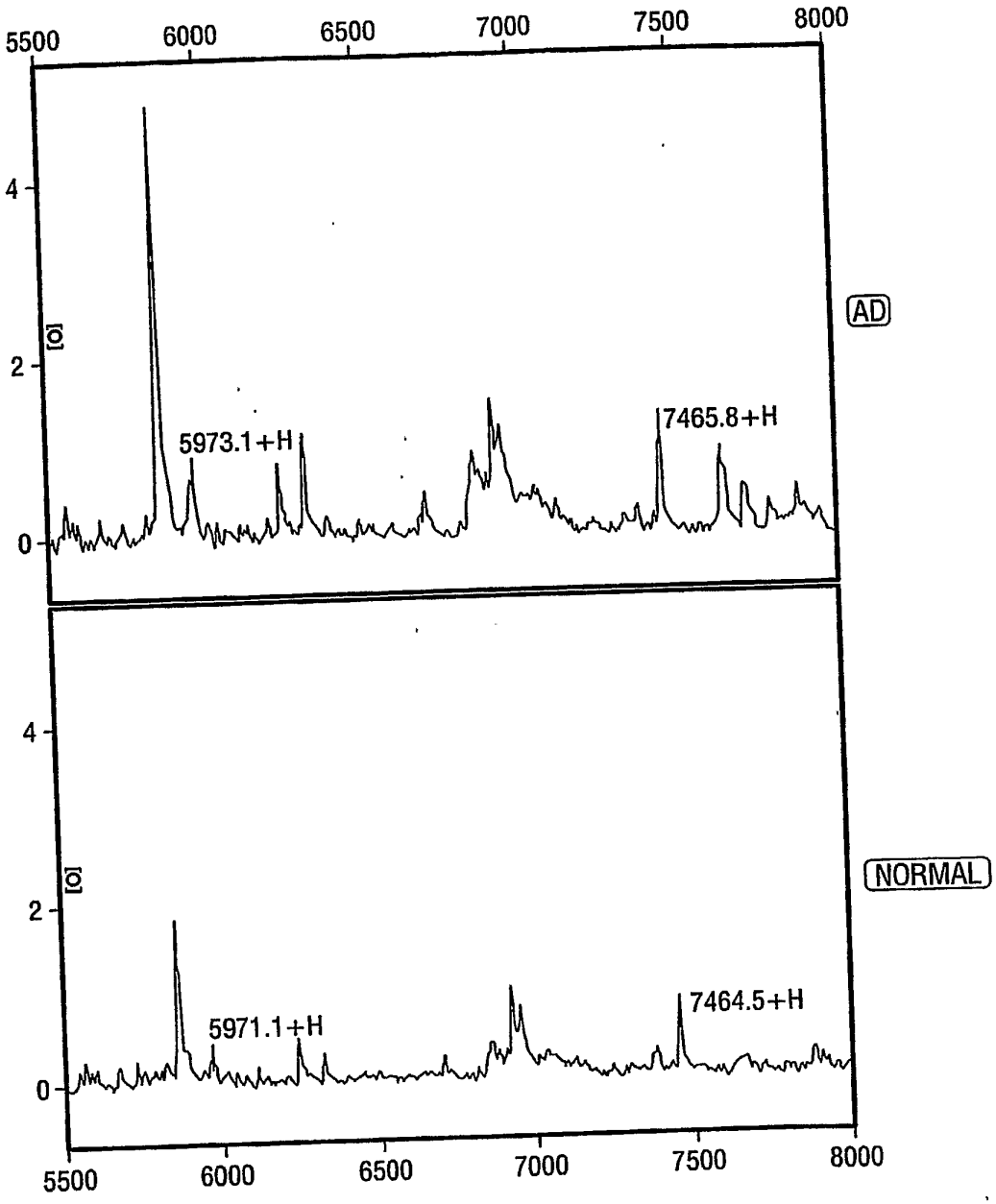


FIG. 1C

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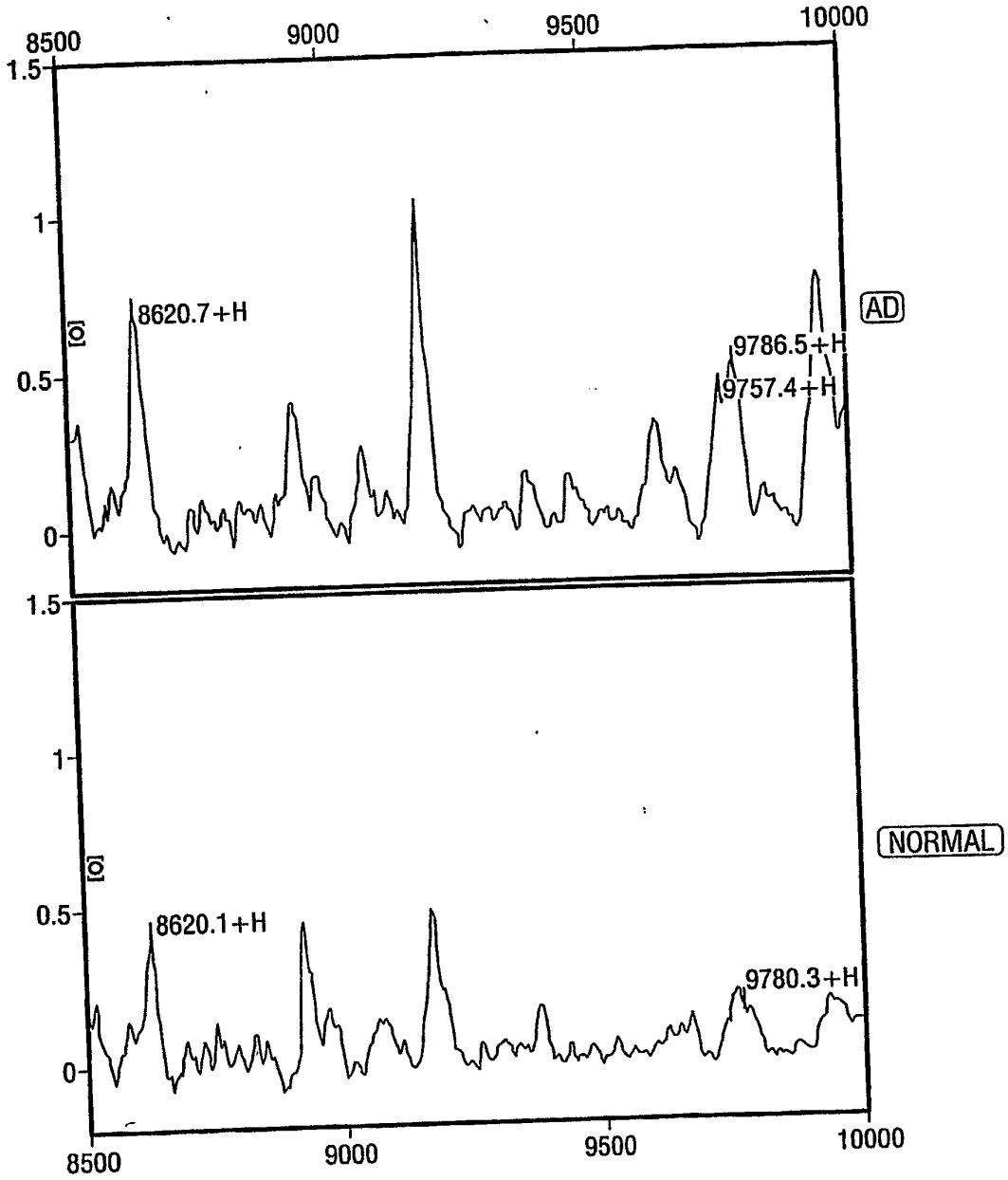


FIG. 1D

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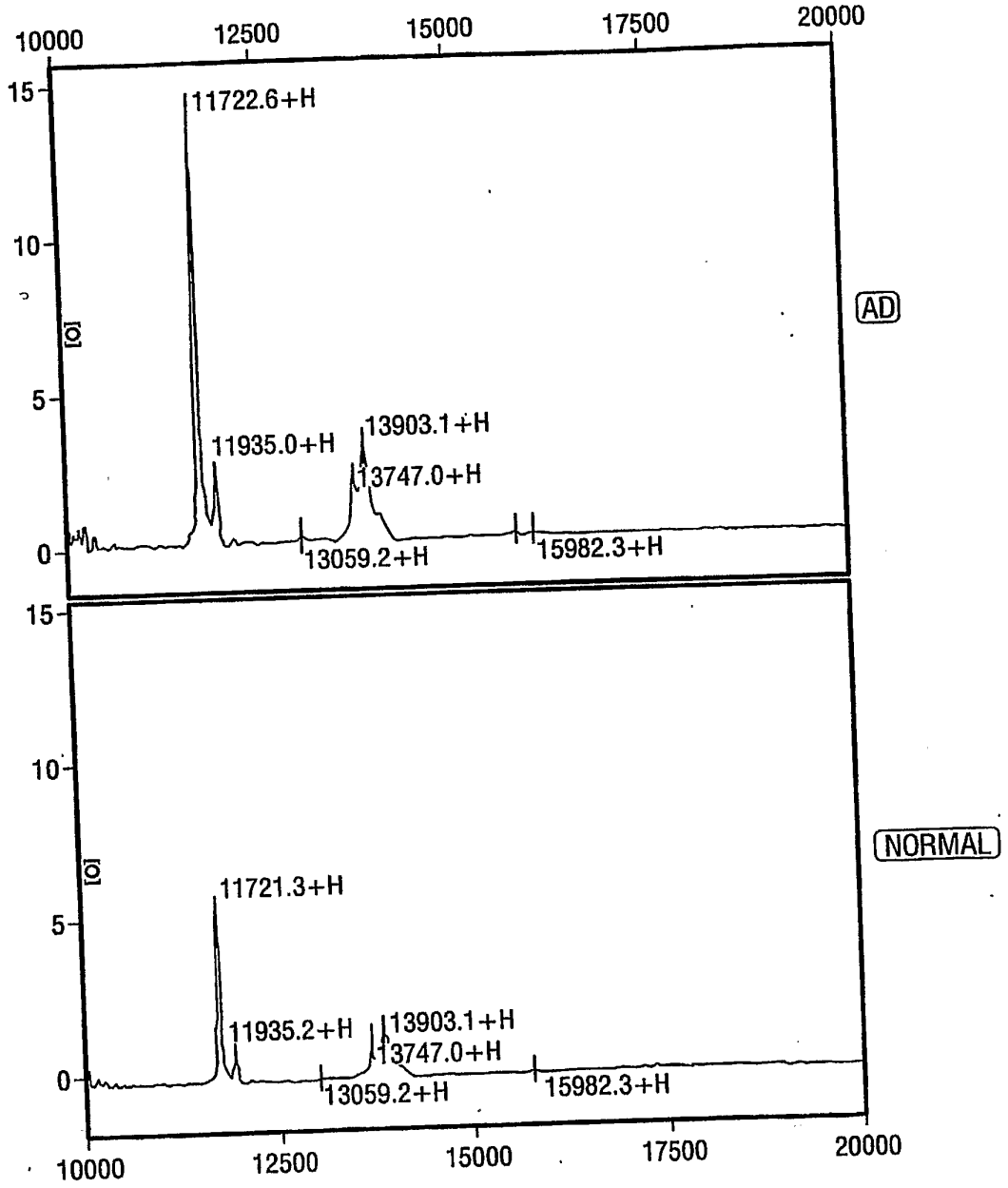


FIG. 1E

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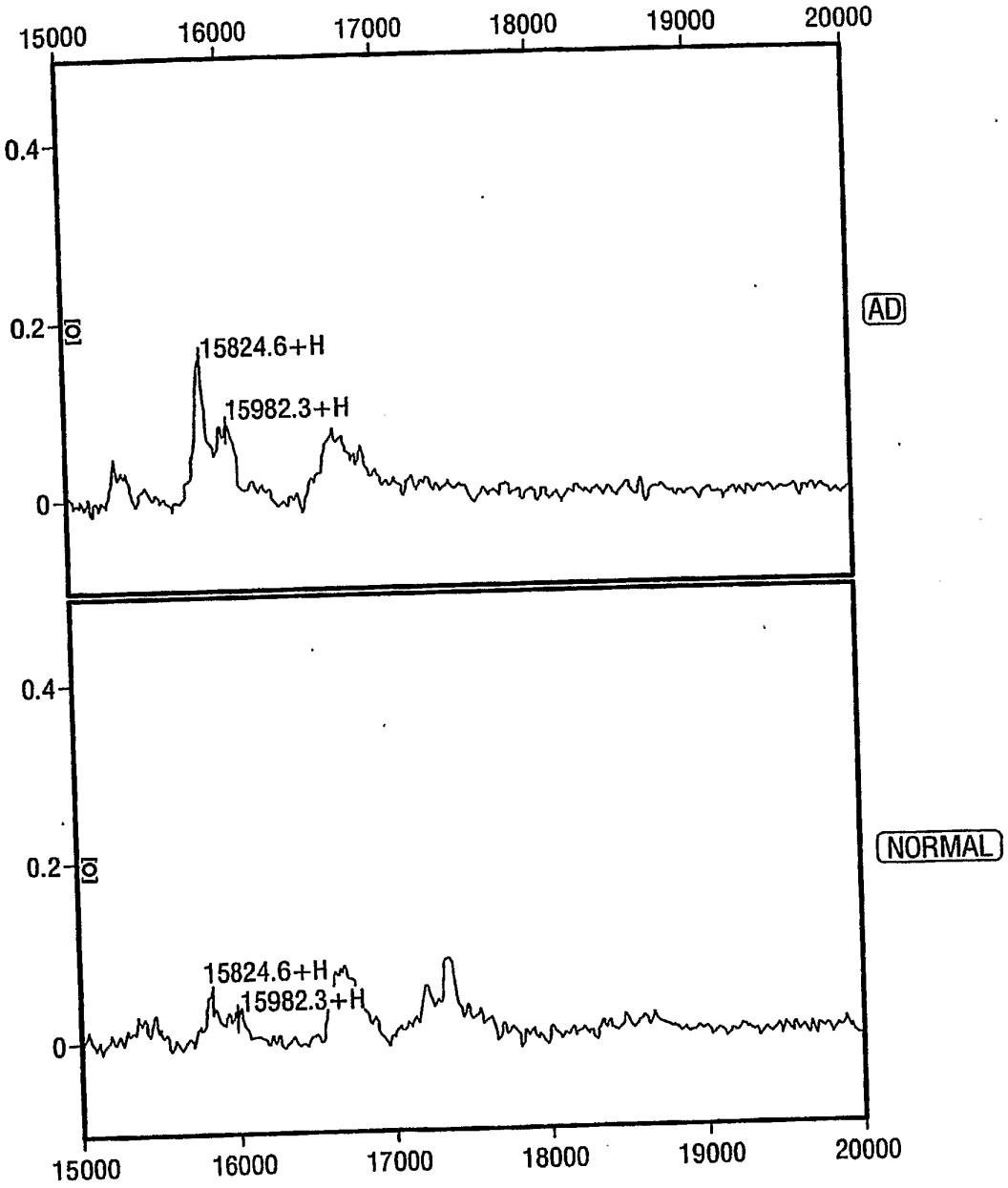


FIG. 1F

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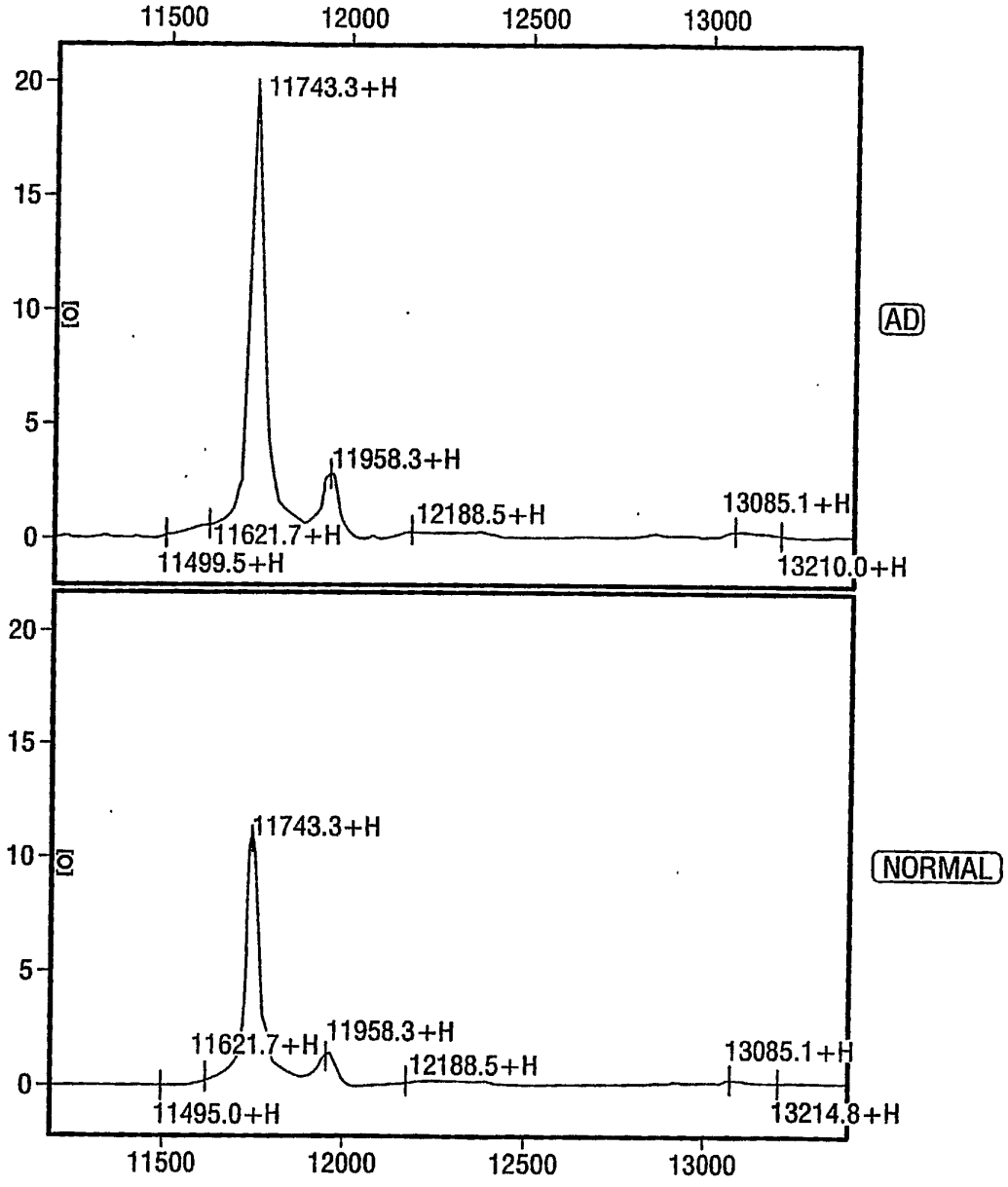


FIG. 1G

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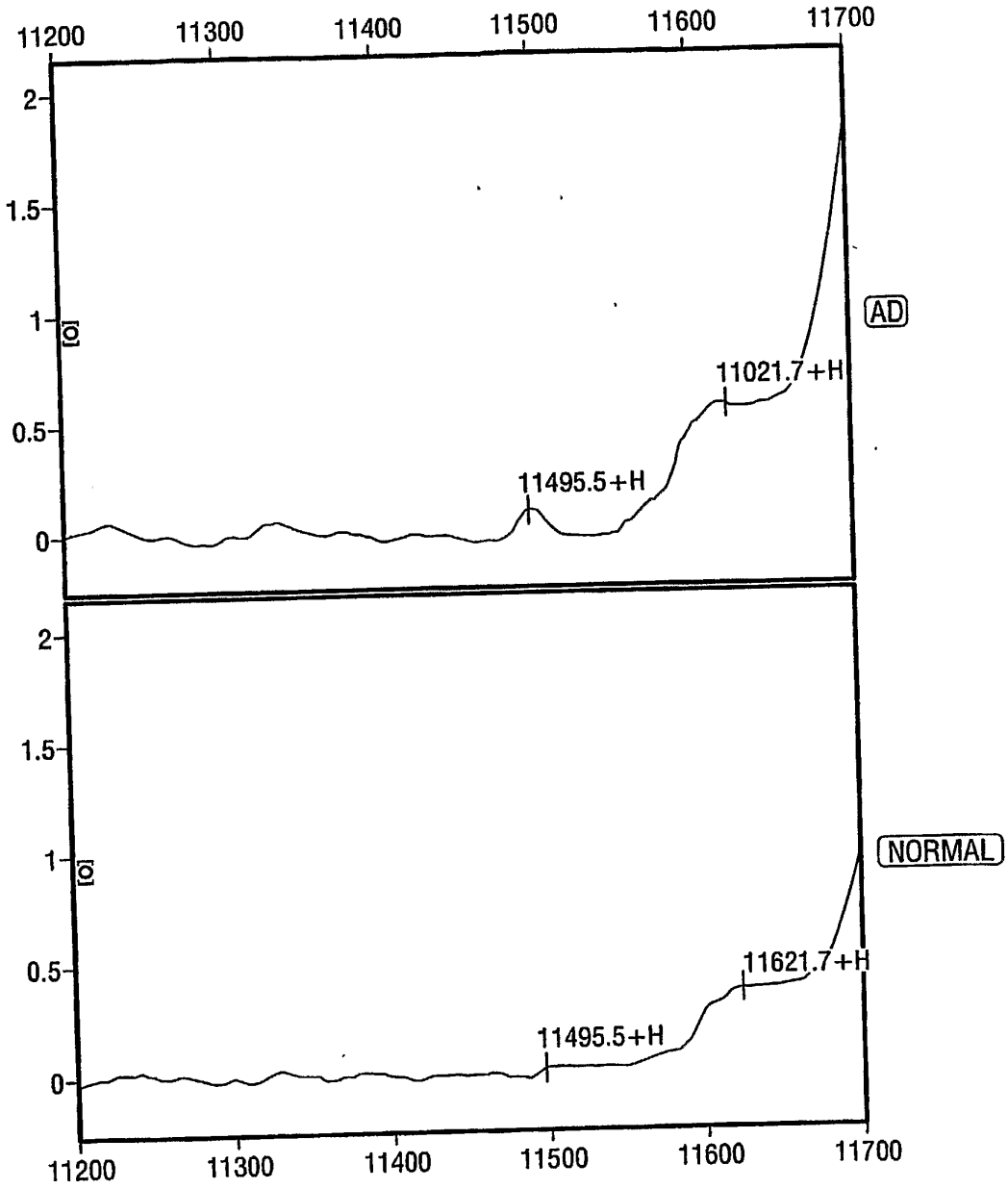


FIG. 1H

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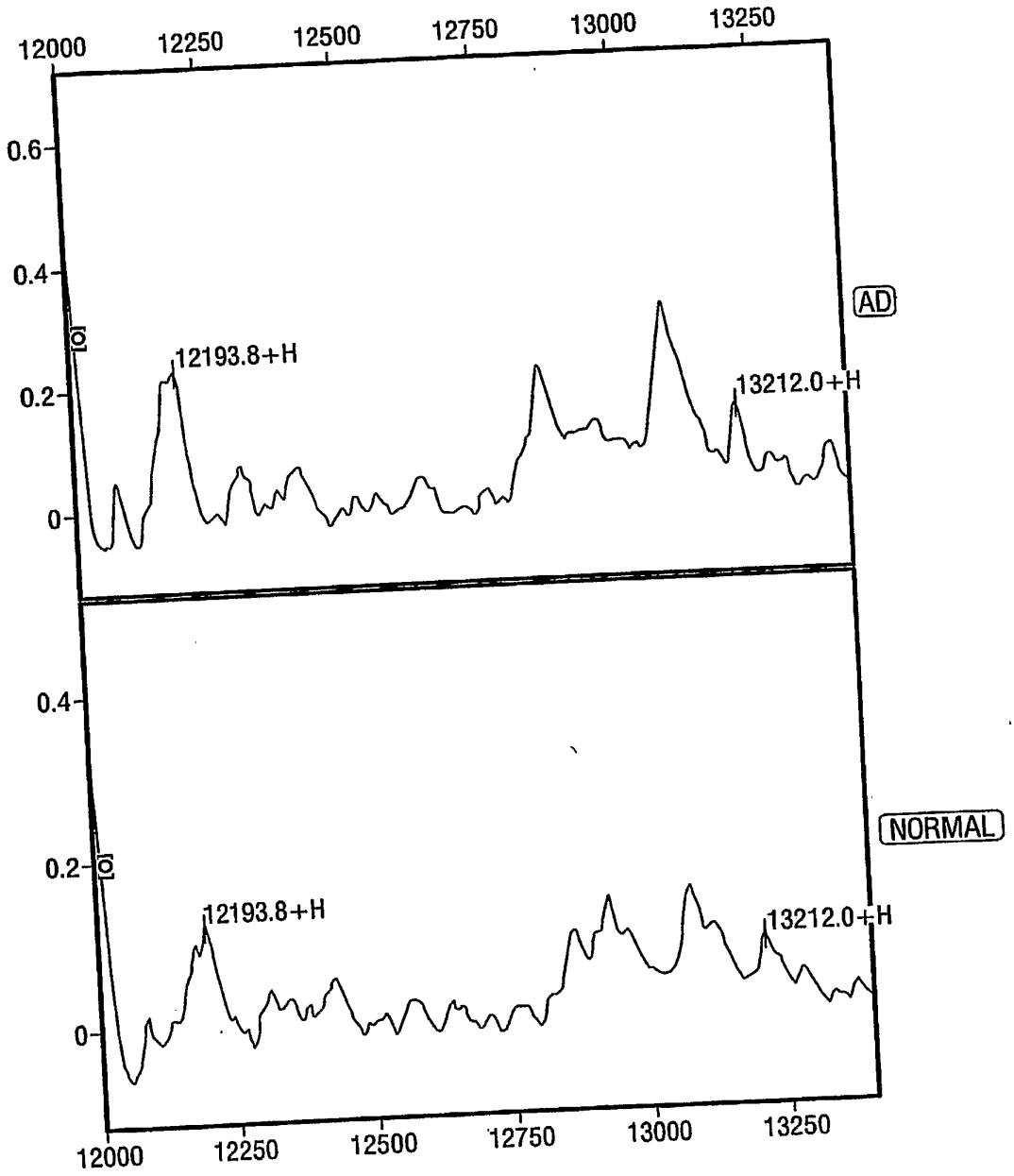


FIG. 1I

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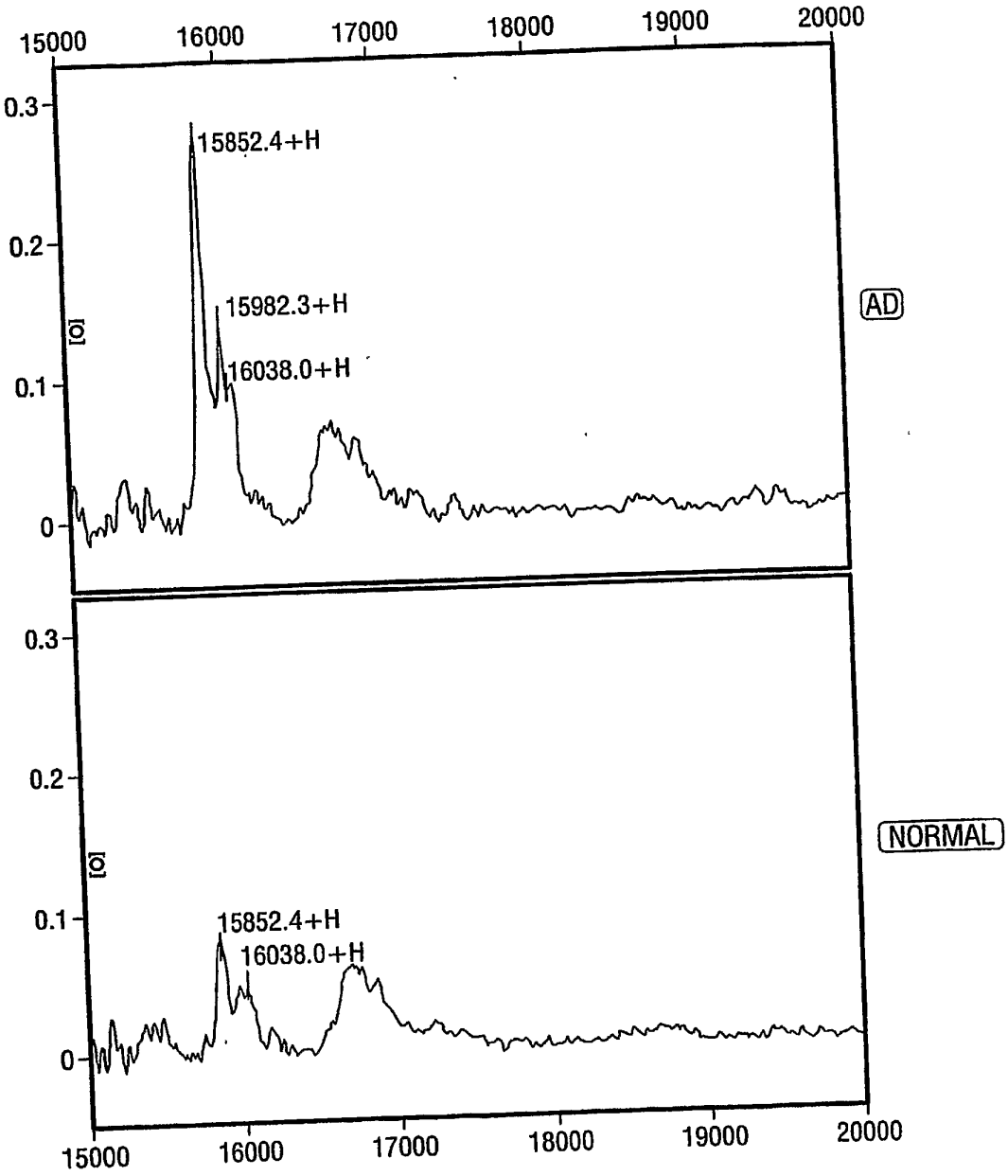


FIG. 1J

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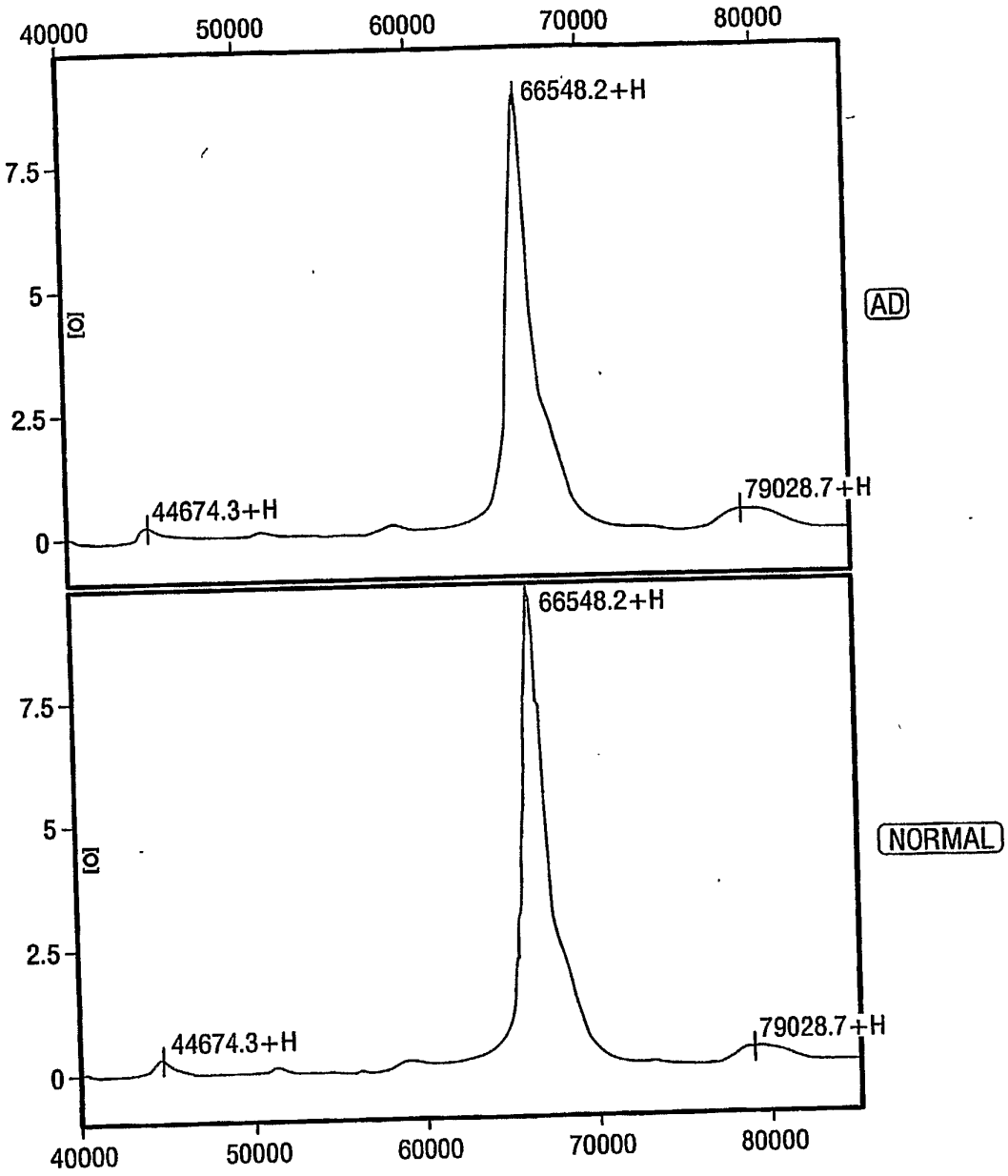


FIG. 1K

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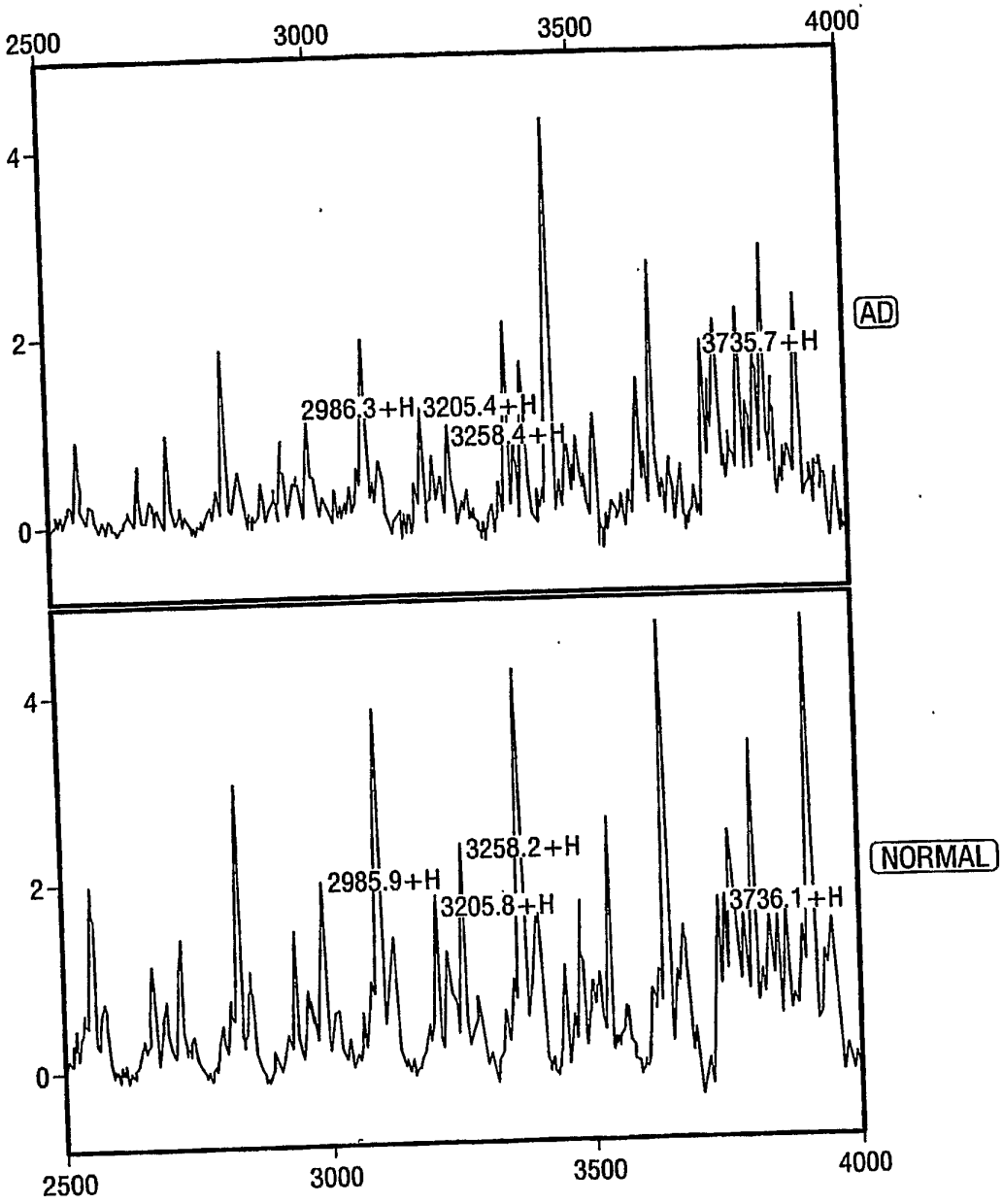


FIG. 1L

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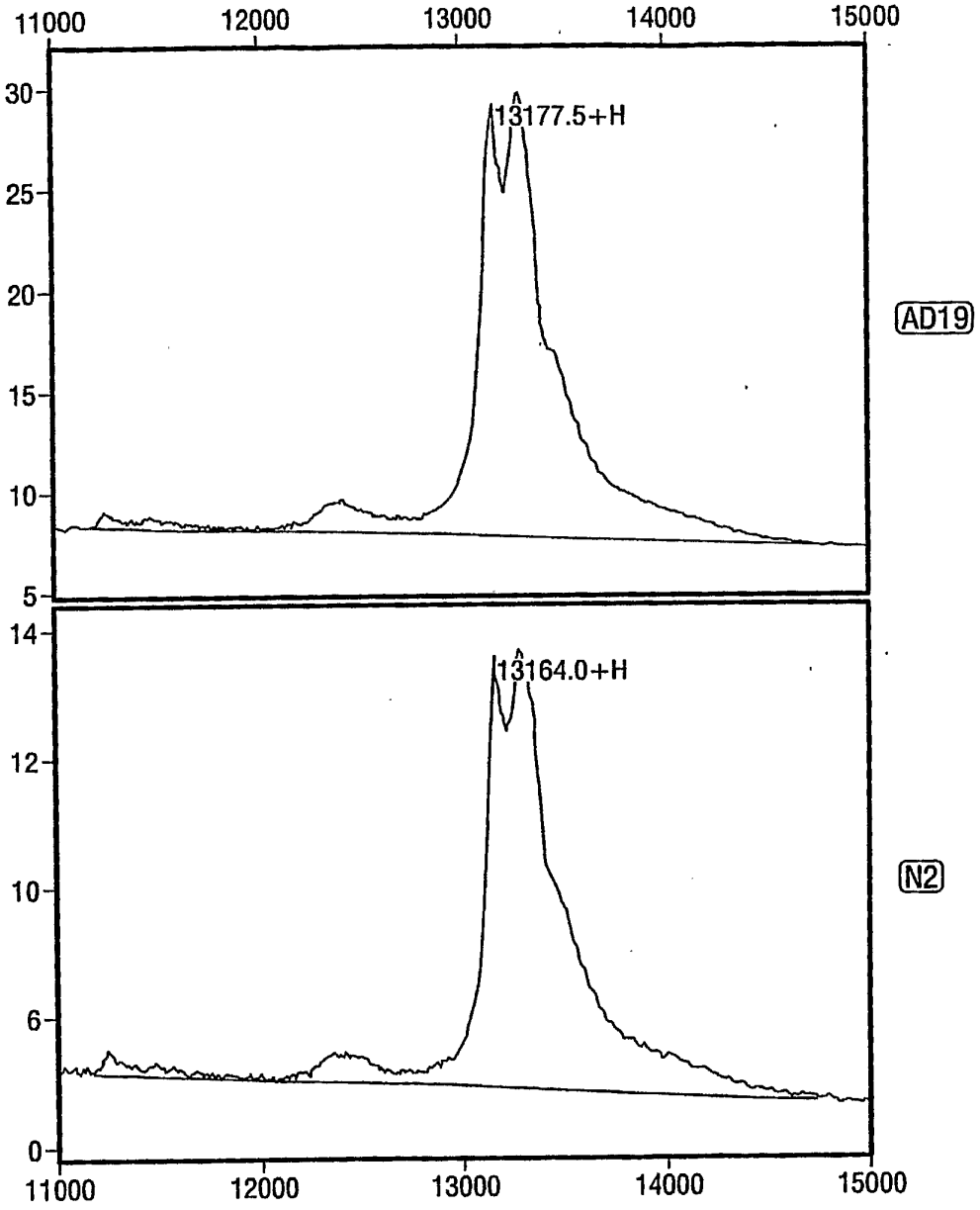


FIG. 1M

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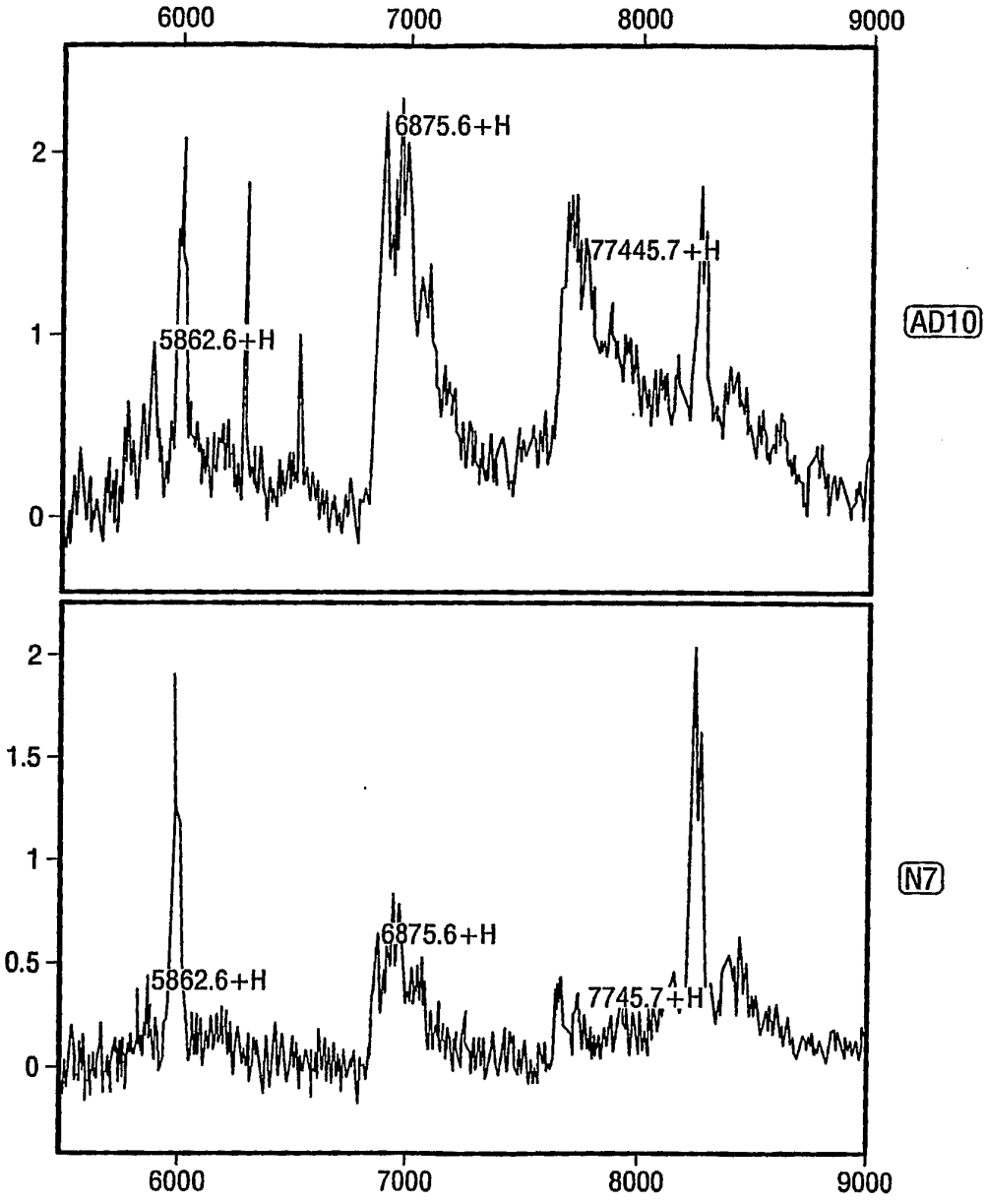


FIG. 1N

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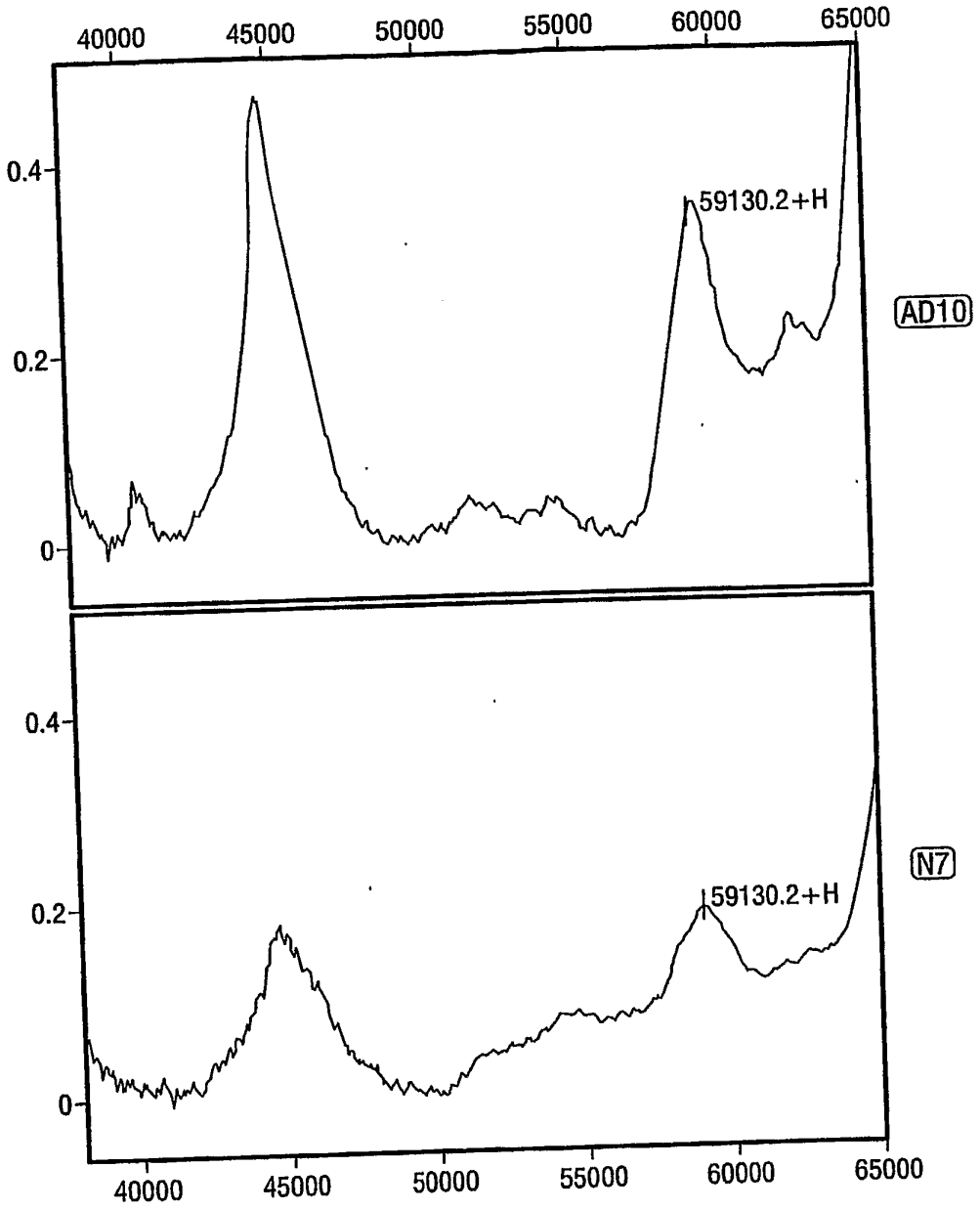


FIG. 10

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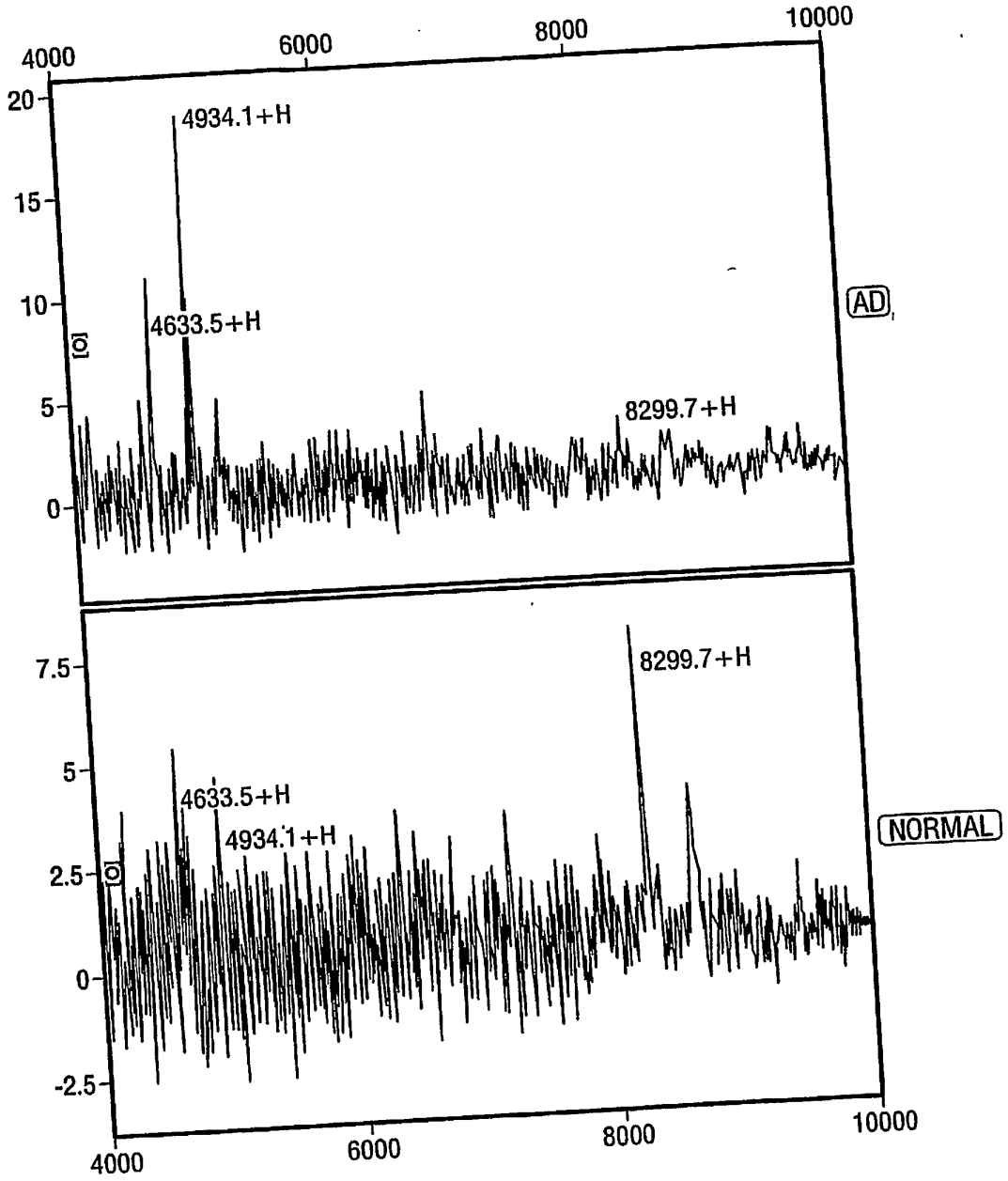


FIG. 1P

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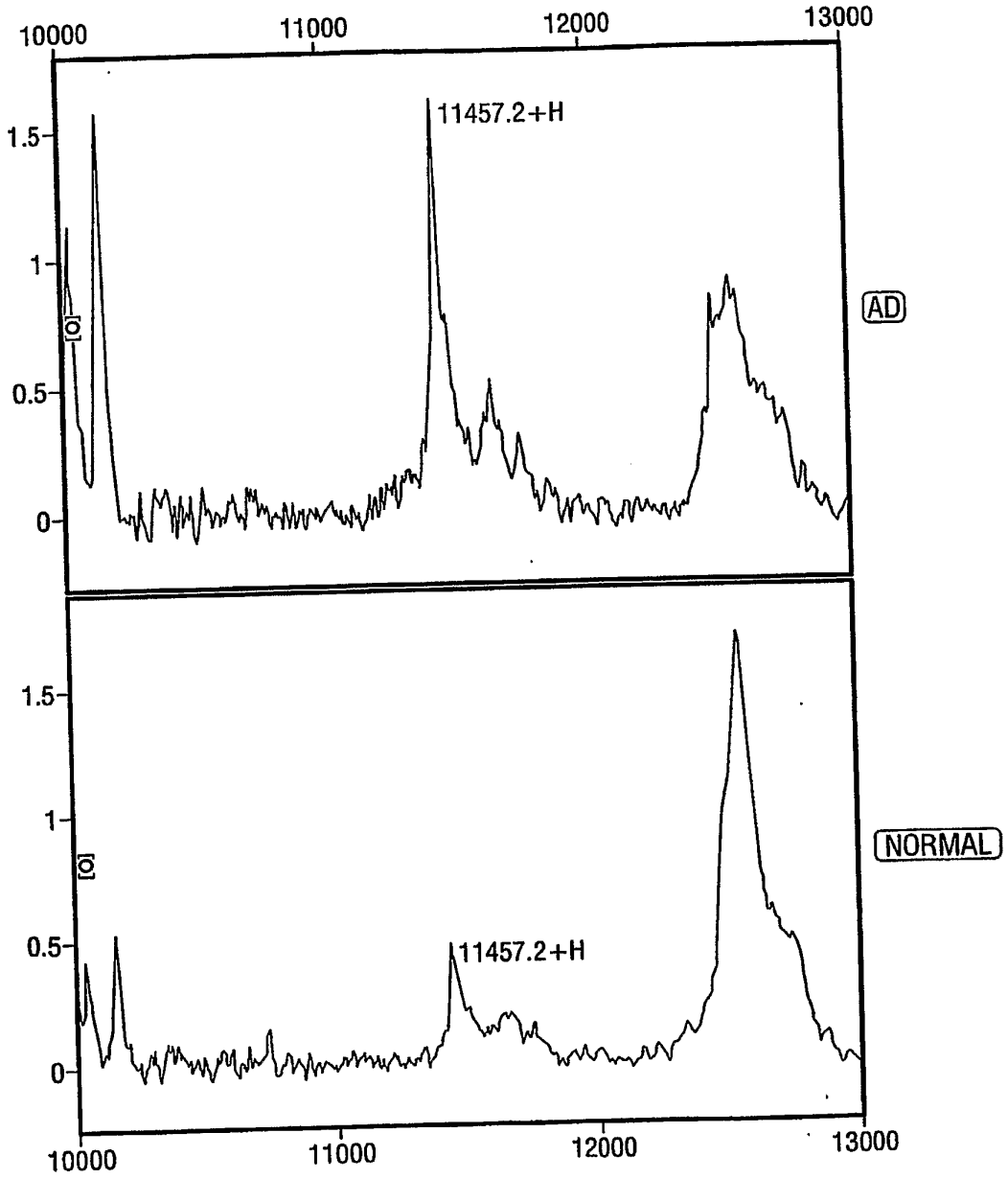


FIG. 1Q

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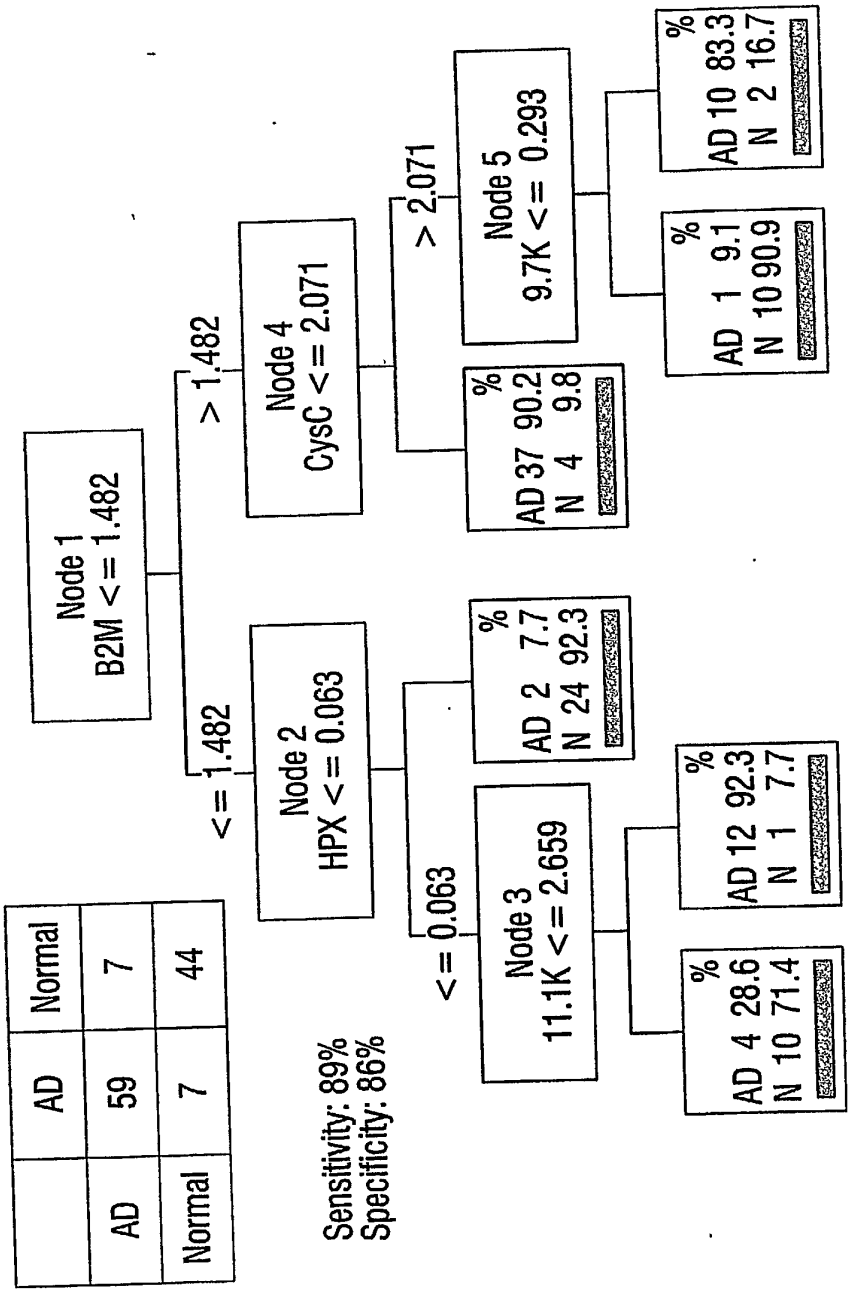
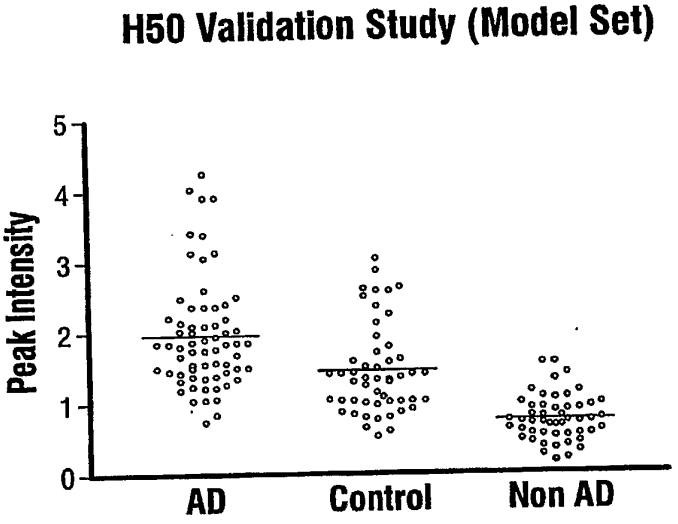


FIG. 2

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7519

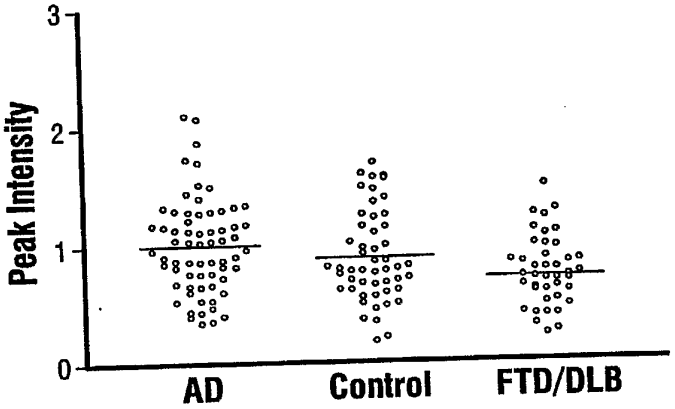


Kruskal Wallis p value	Mann Whitney p value		
A/C/N	A-C	A-N	C-N
<0.0001	<0.0001	<0.0001	P<0.0001

FIG. 3

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7520

C-terminal Fragment of Integral Membrane Protein 2B: *2431 Da on IMAC30-Cu- also found as a Marker on CM10*



AD vs. Control:	P=0.1586
AD vs. FTD/DLB:	P=0.0026
FTD/DLB vs. Control:	P=0.1669
AD vs. Cont. vs. FTD/DLB:	P=0.0126

FIG. 4A

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7521

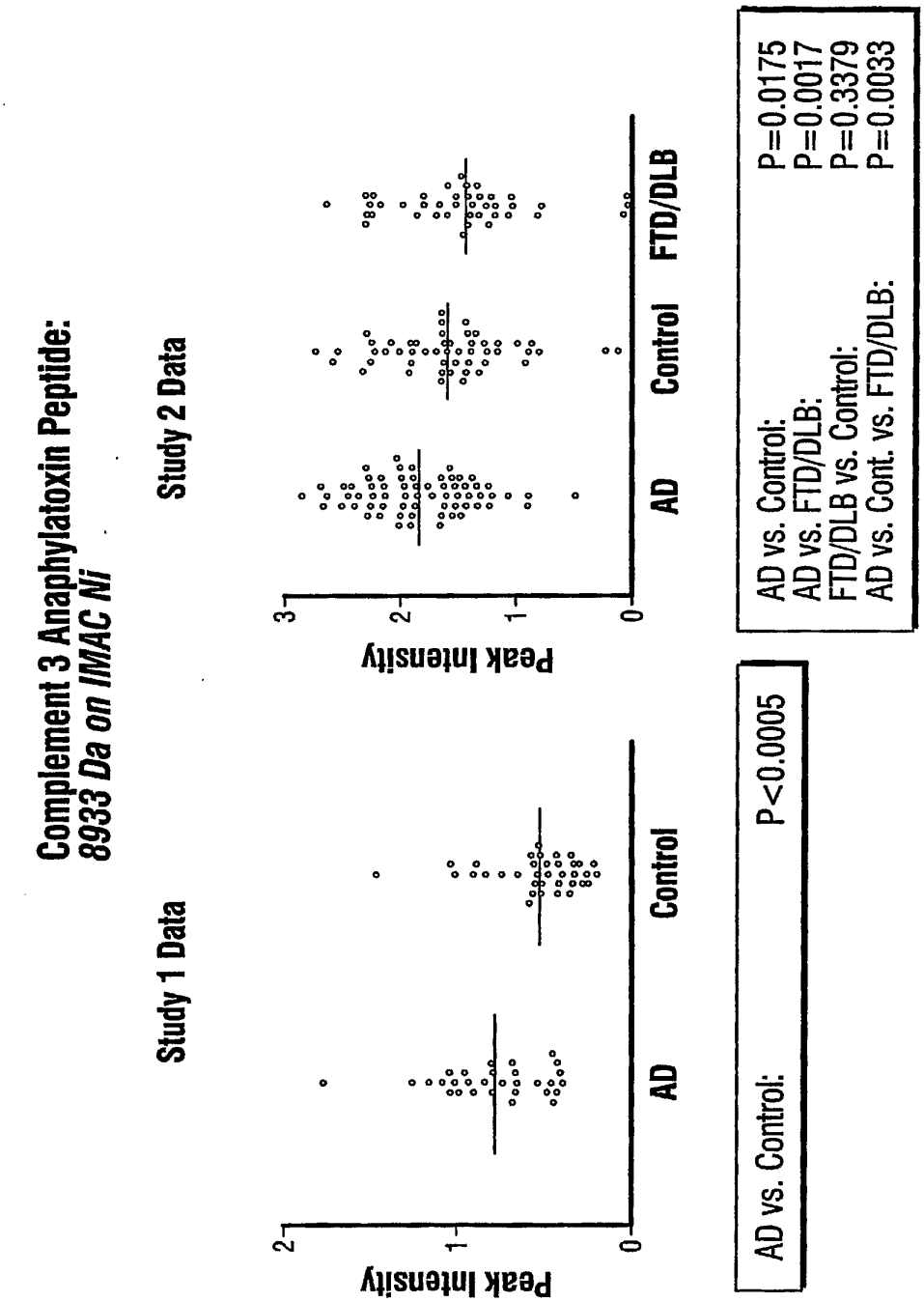


FIG. 4B

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7522

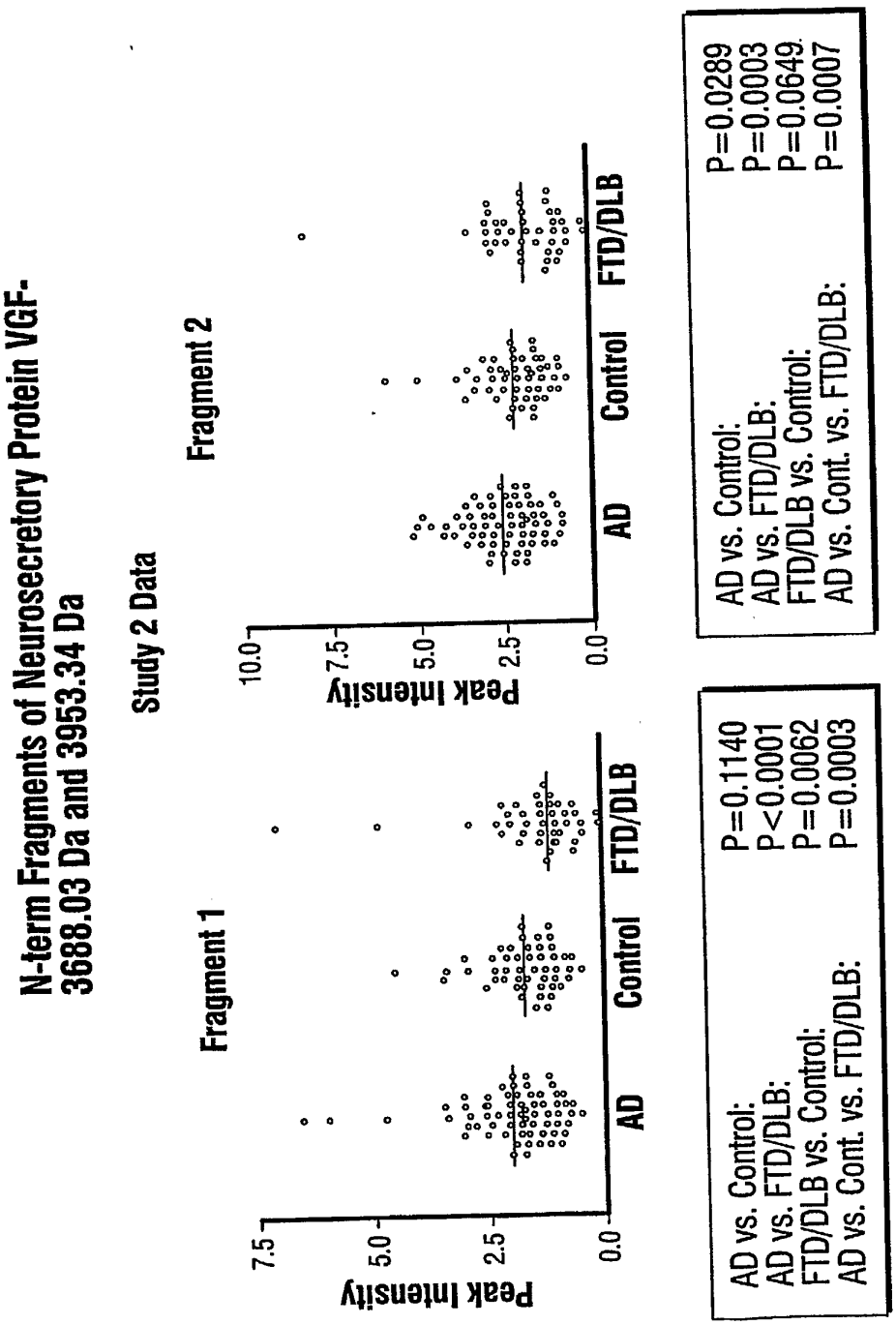


FIG. 4C

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7523

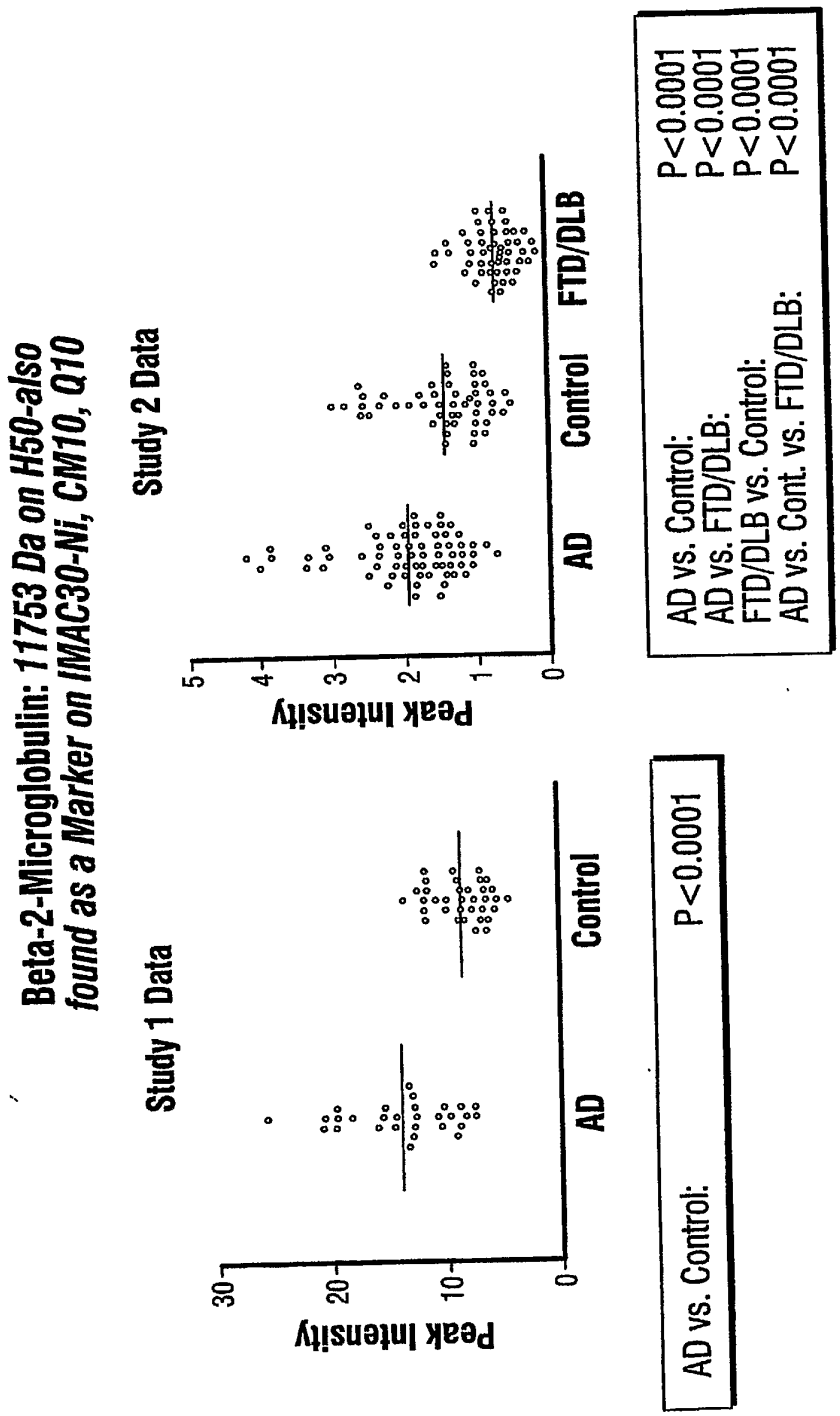


FIG. 4D

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7524

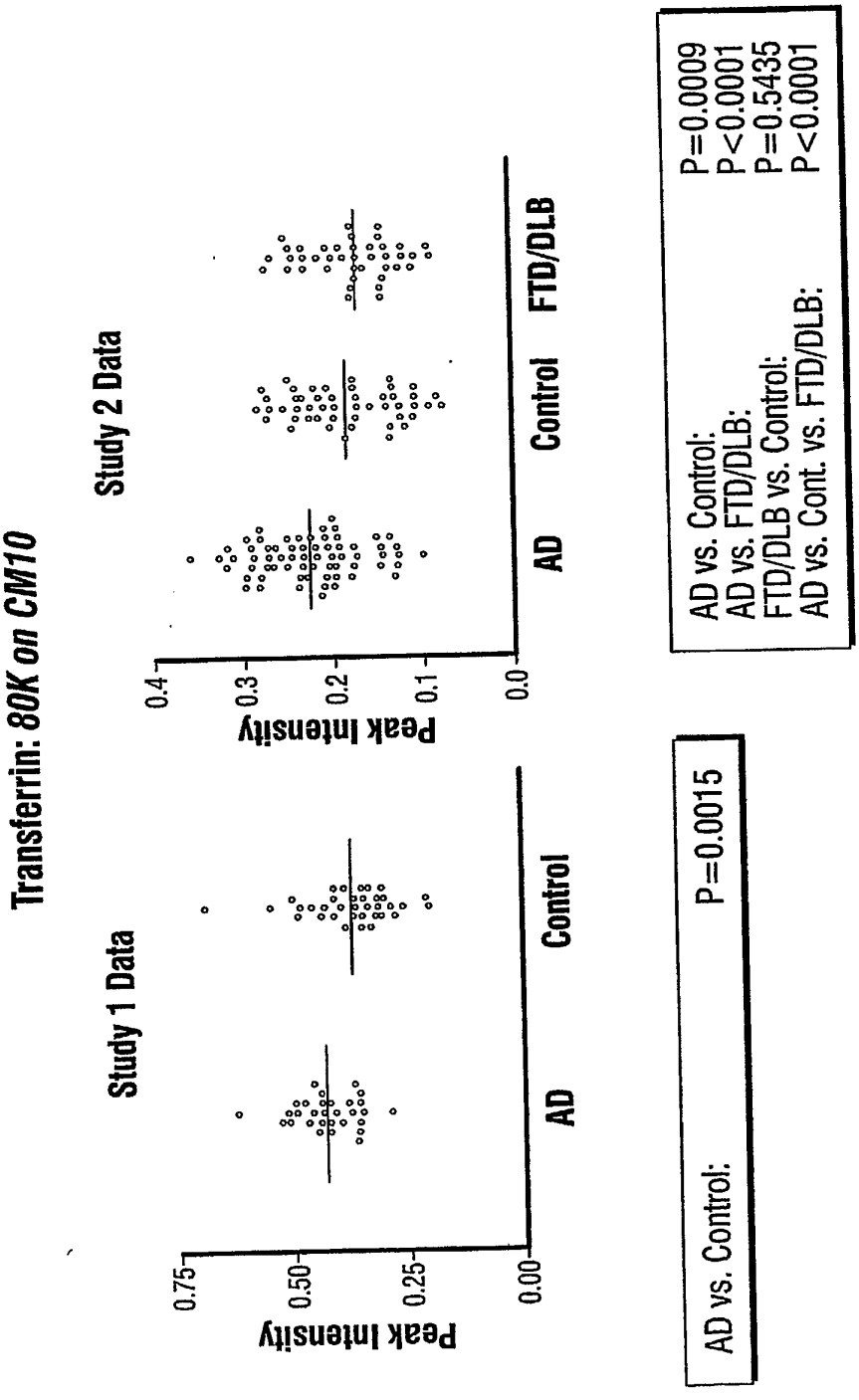


FIG. 4E

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7525

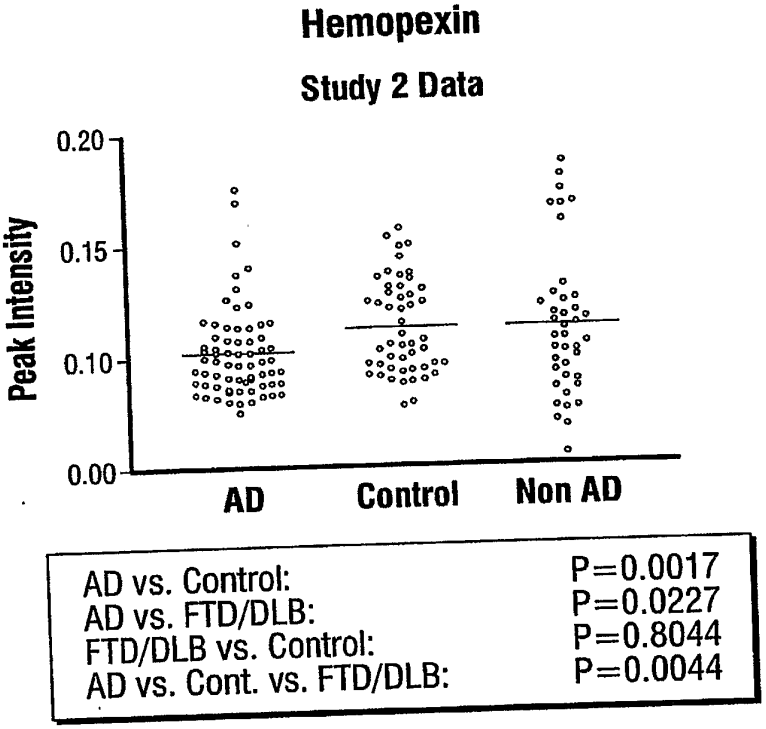


FIG. 4F

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7526

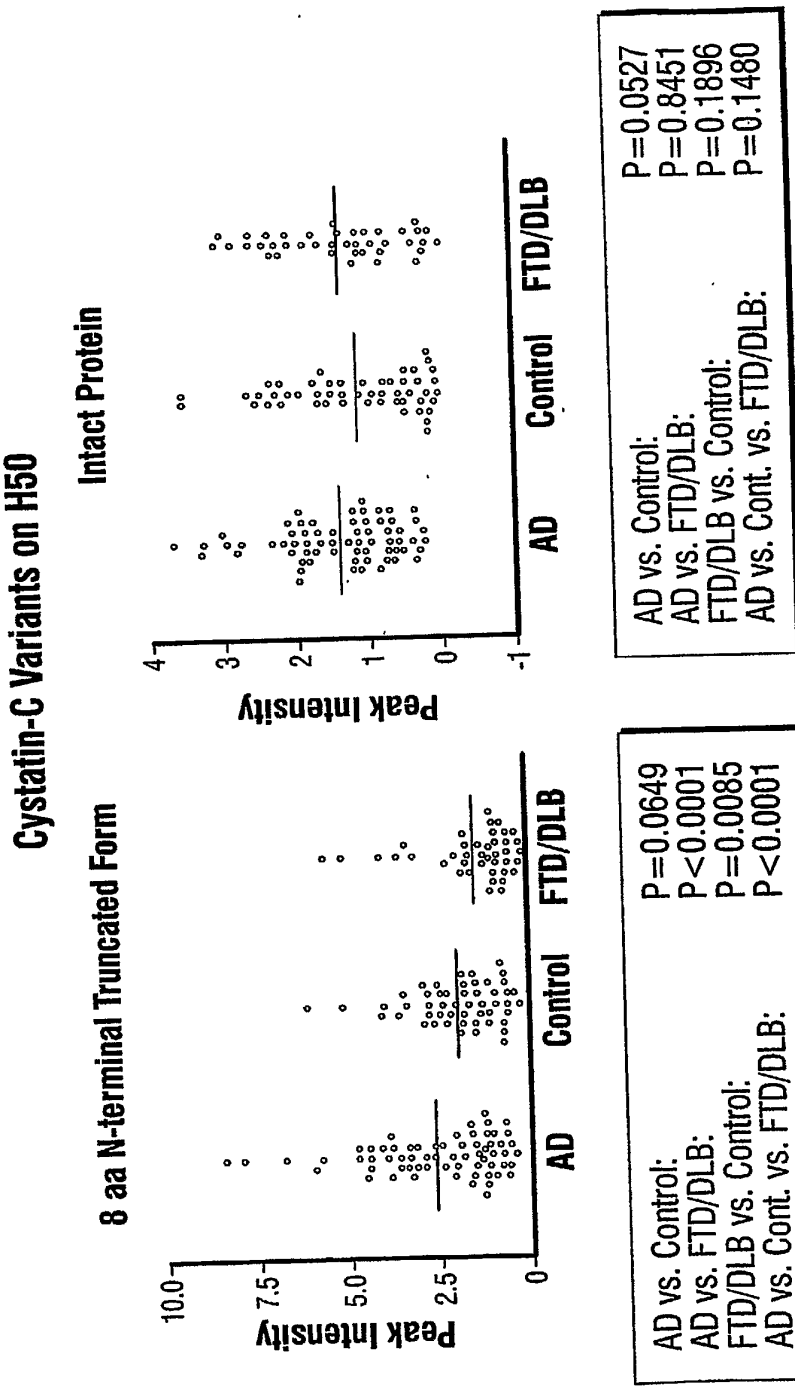


FIG. 4G

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7527

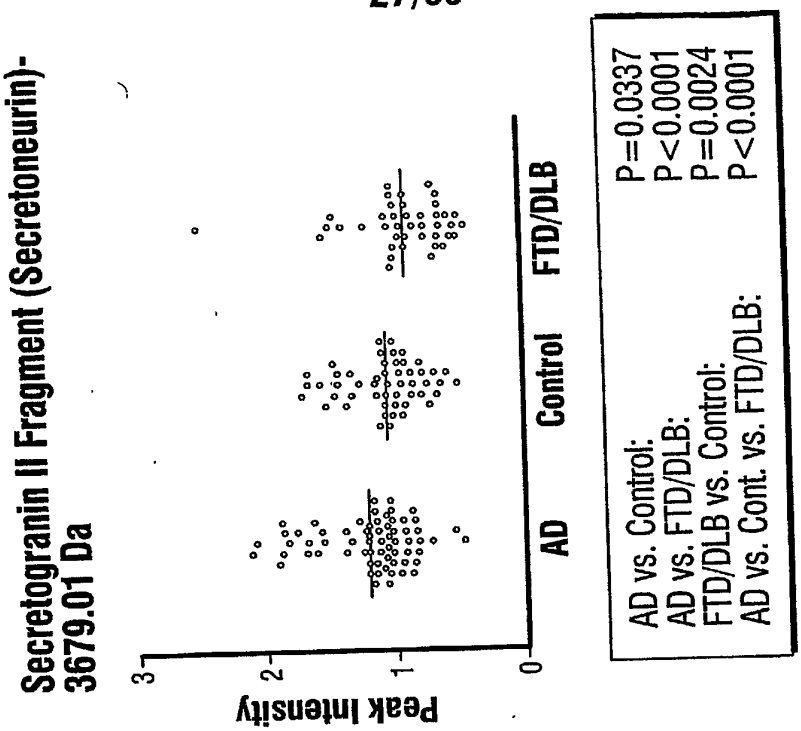


FIG. 4I

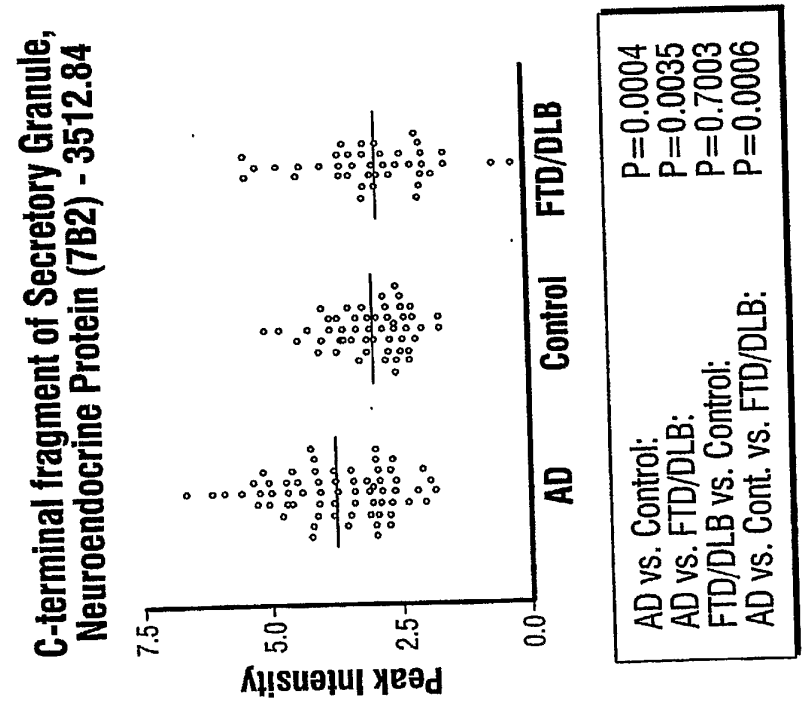


FIG. 4H

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7528

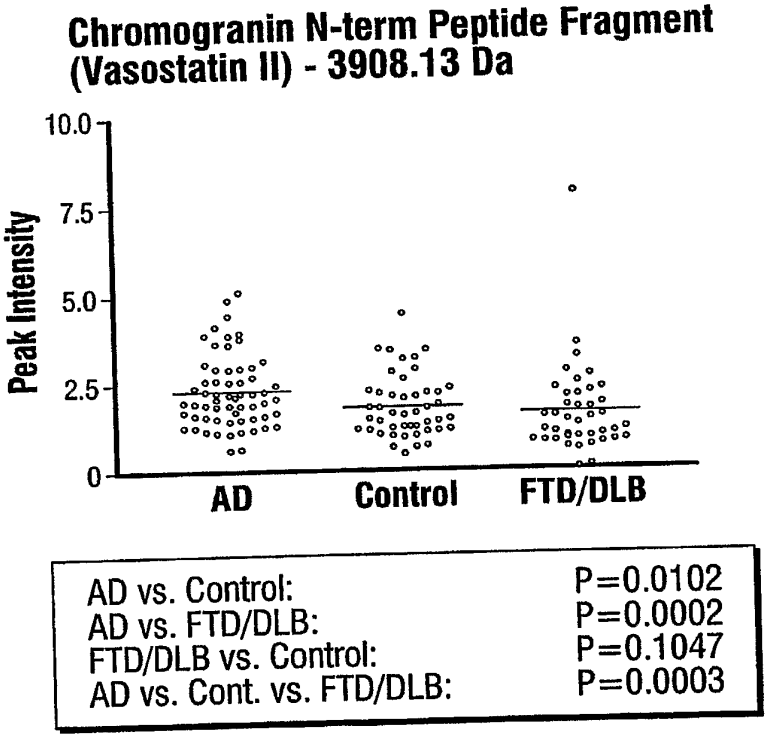


FIG. 4J

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7529

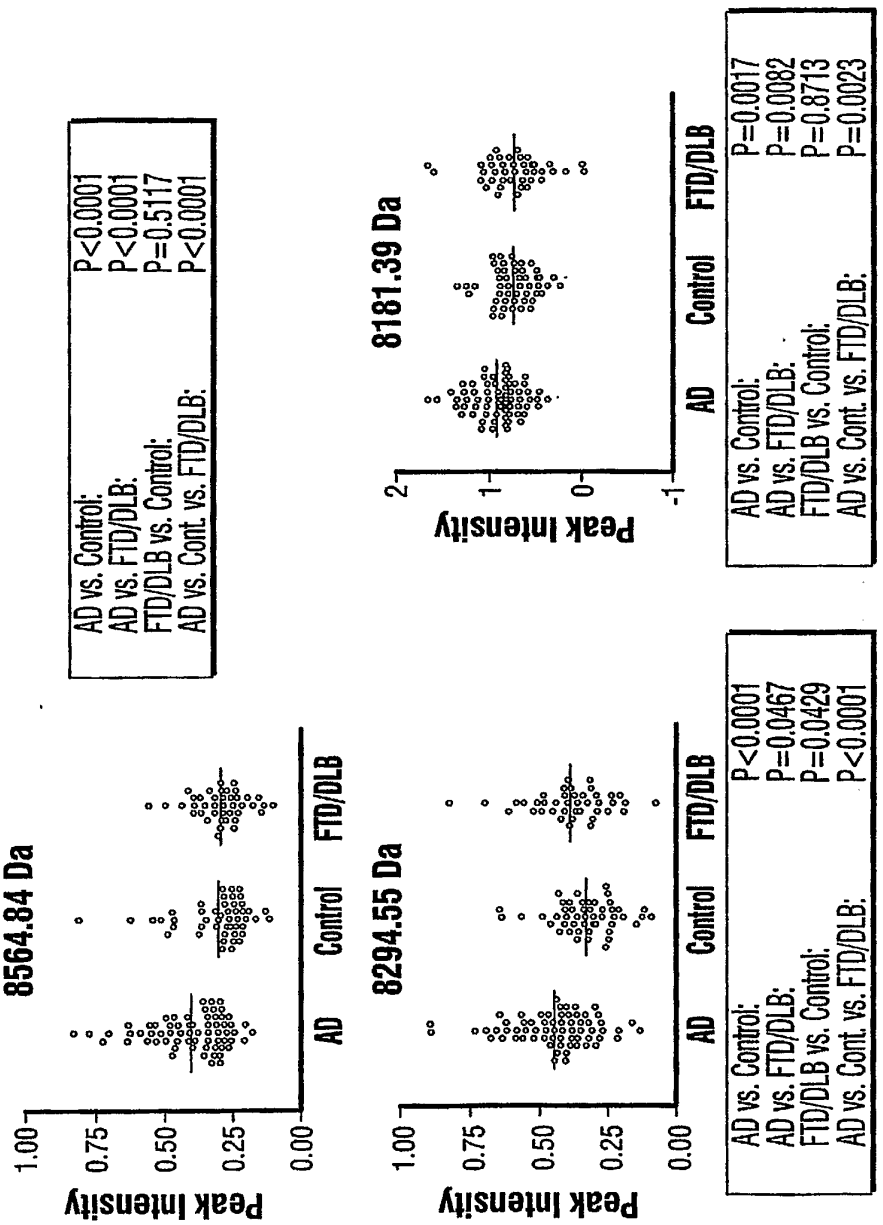


FIG. 4K

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7530

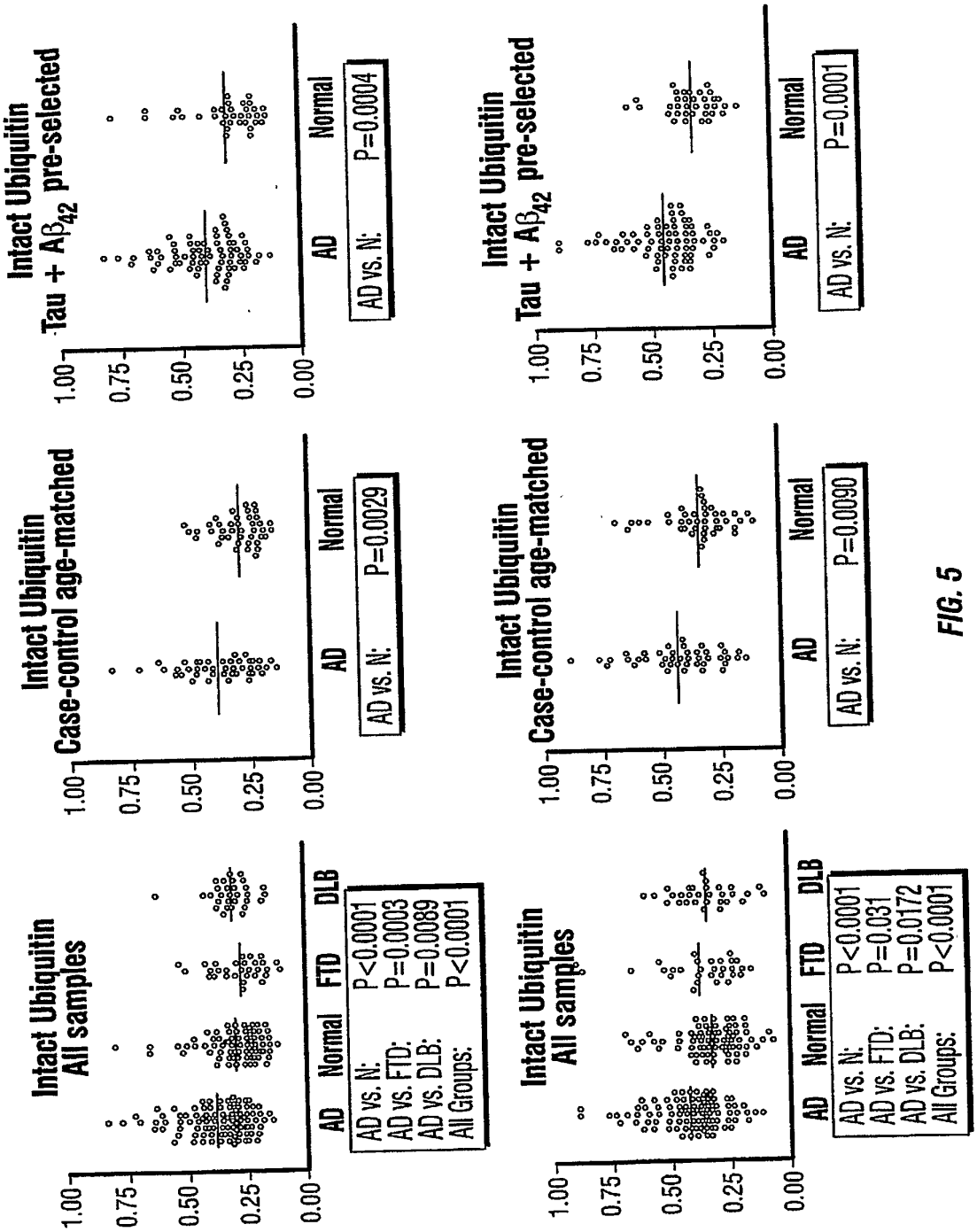


FIG. 5

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7531

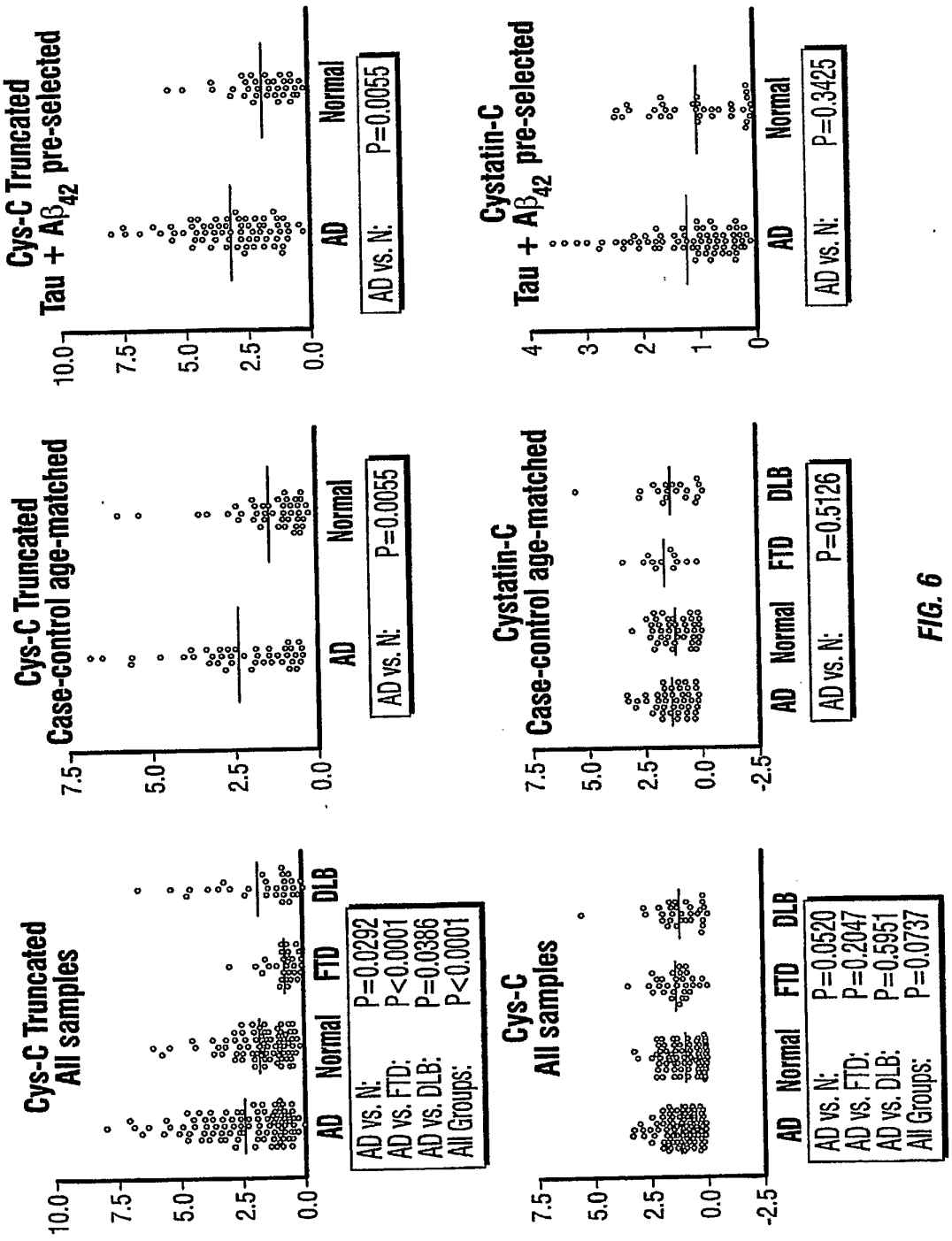


FIG. 6

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7532

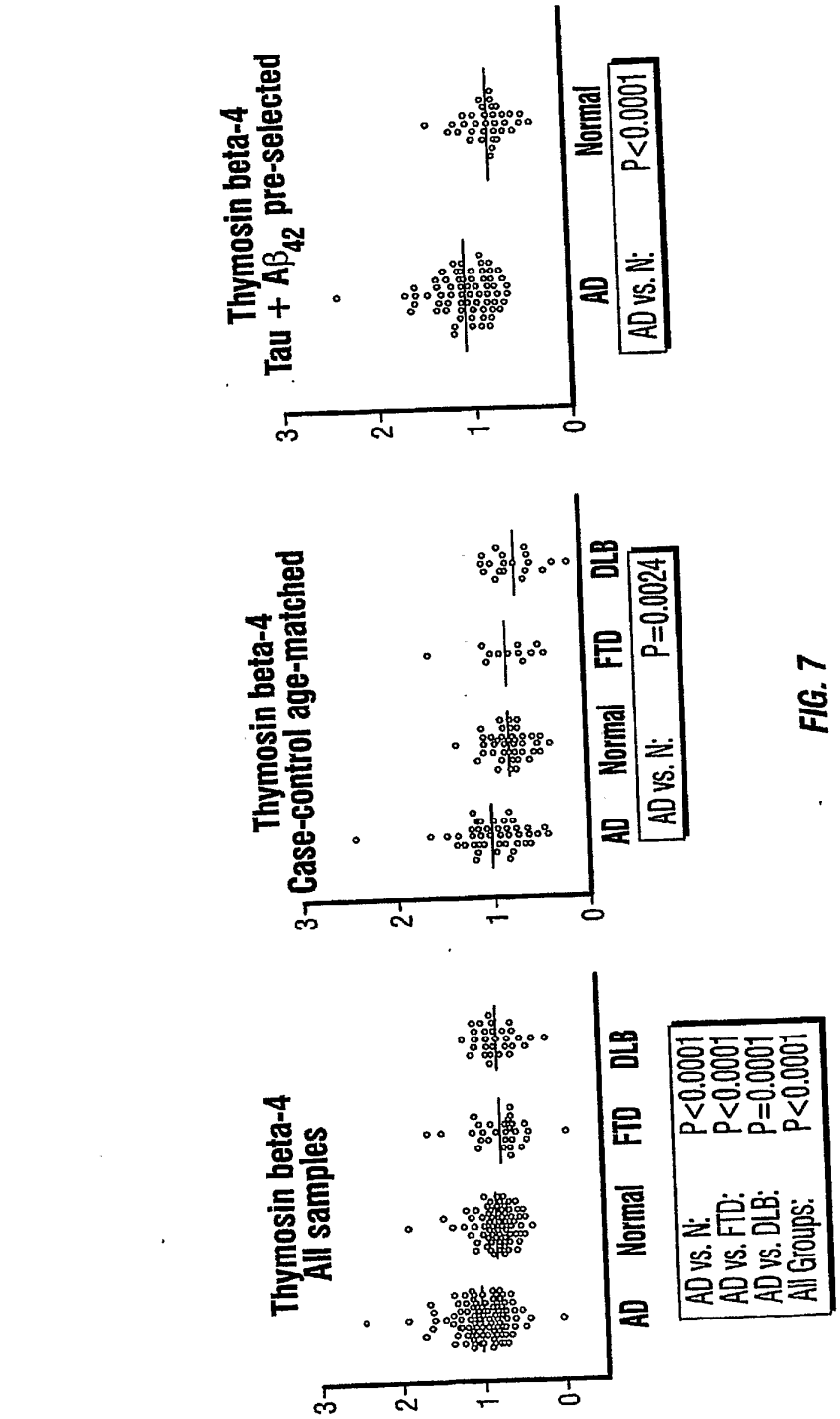


FIG. 7

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7533

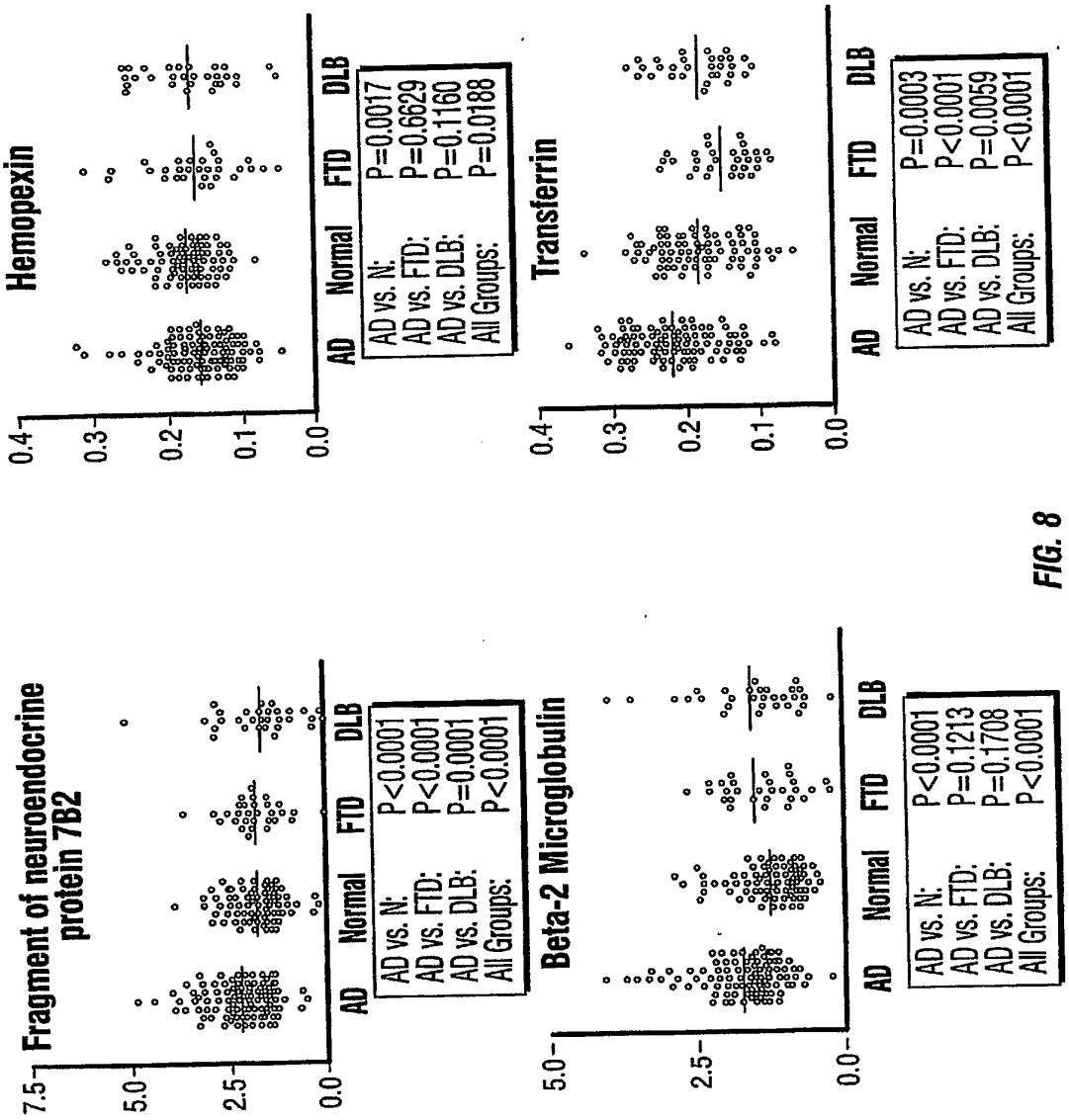


FIG. 8

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7534

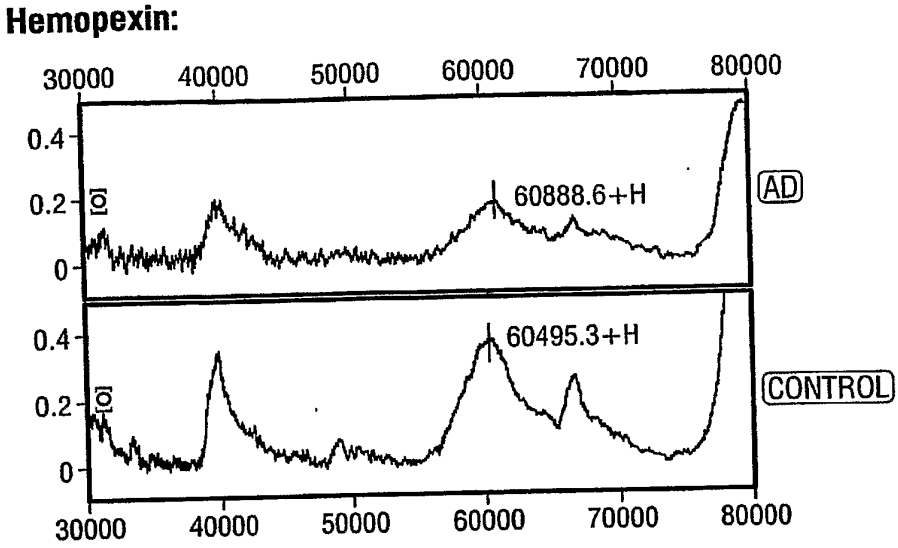


FIG. 9A

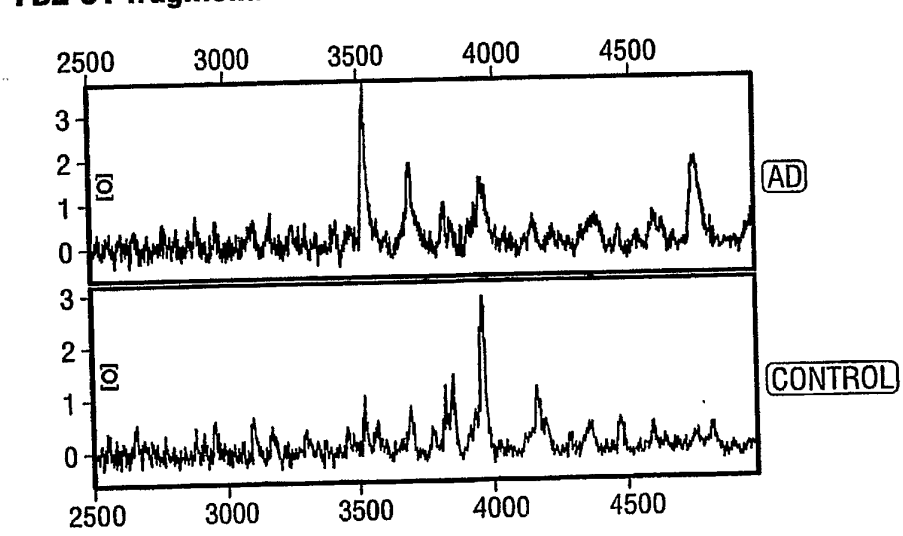


FIG. 9B

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7535

Ubiquitin -3aa from CT:

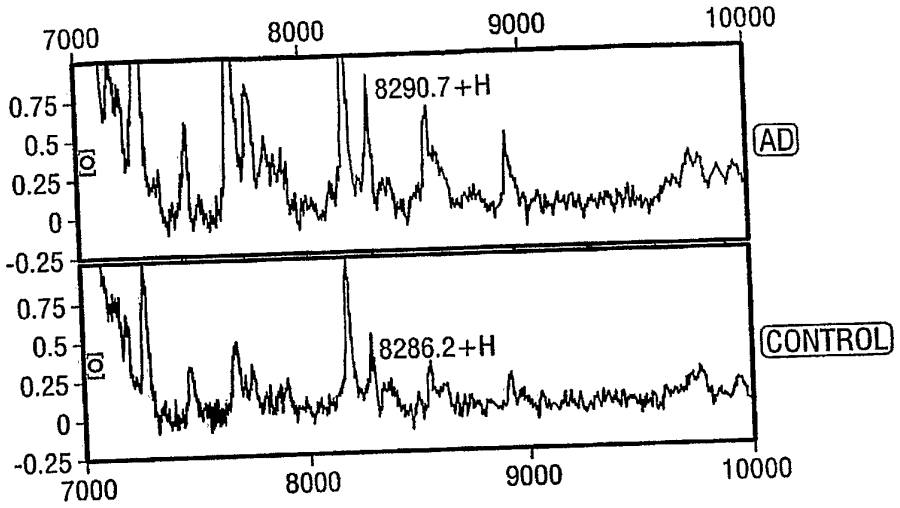


FIG. 9C

Beta-2-microglobulin:

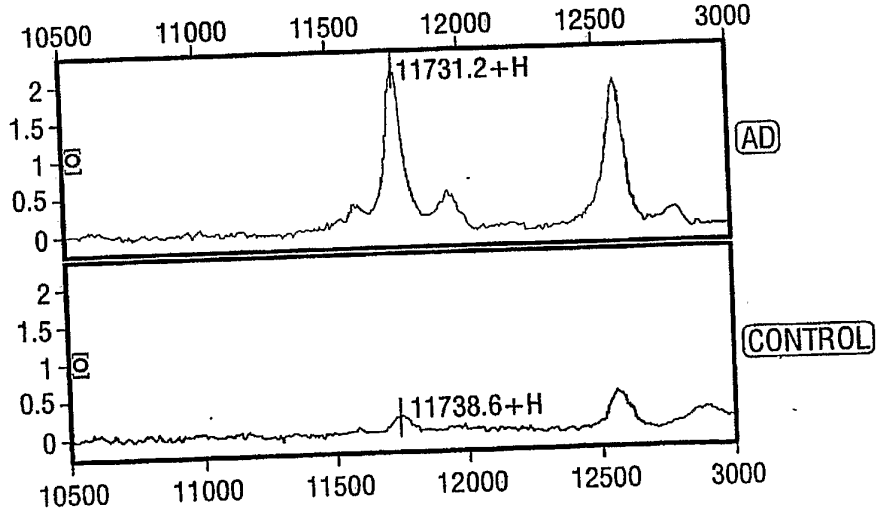


FIG. 9D

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7536

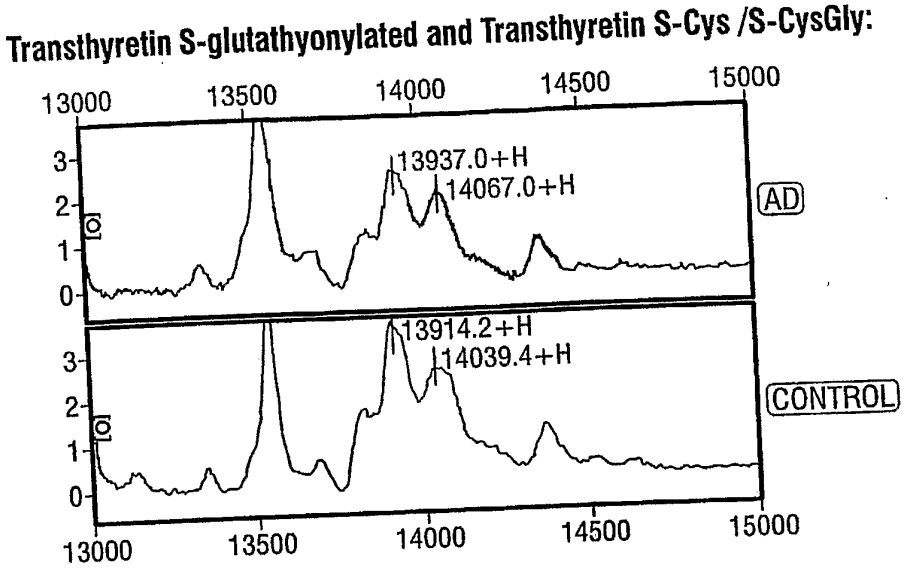


FIG. 9E

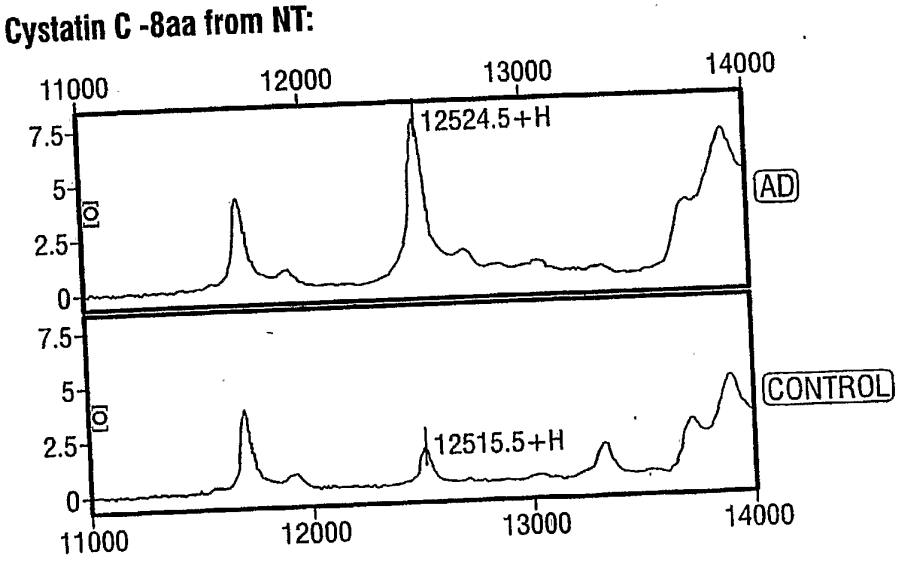


FIG. 9F

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7537

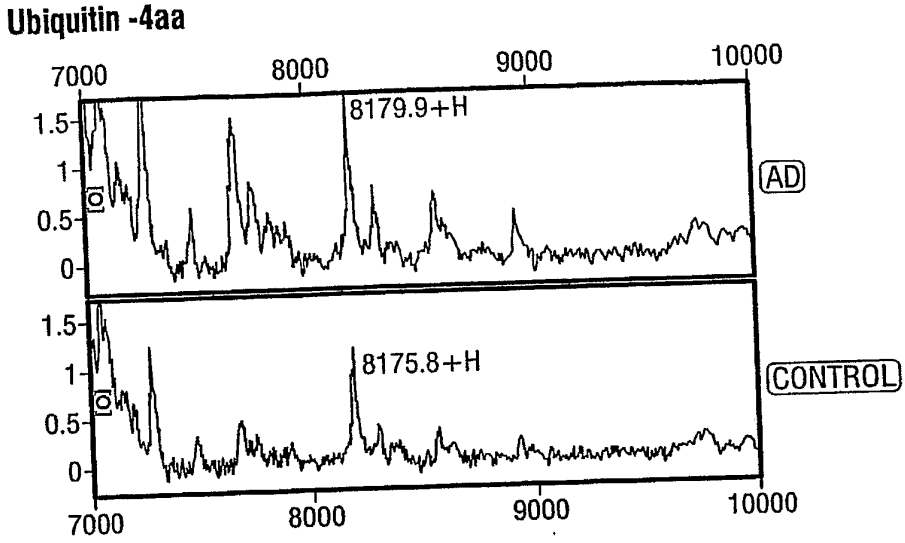


FIG. 9G

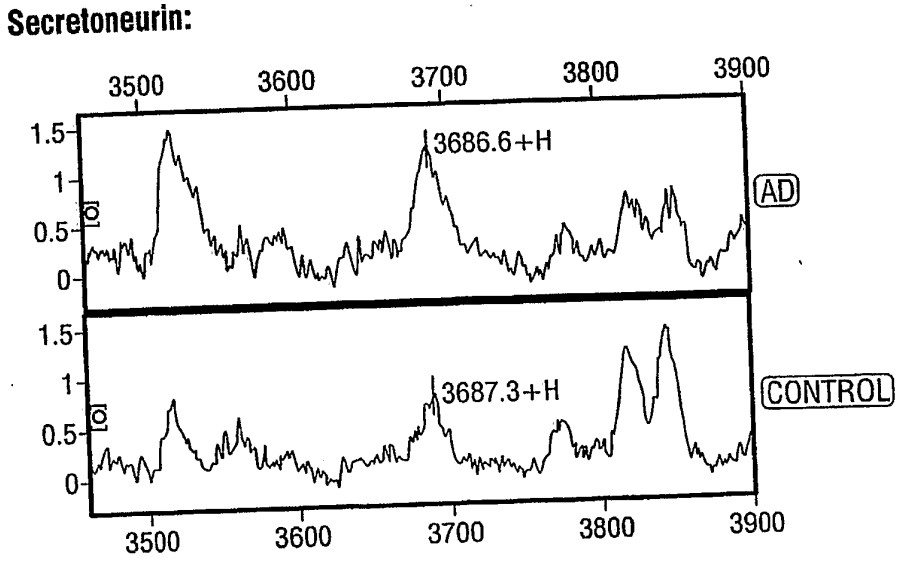


FIG. 9H

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7538

Secretoneurin:

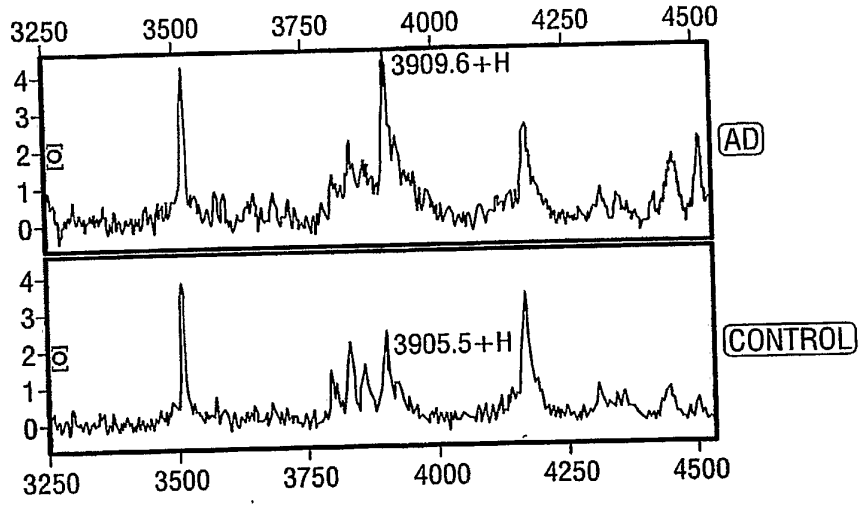


FIG. 9I

Transferrin:

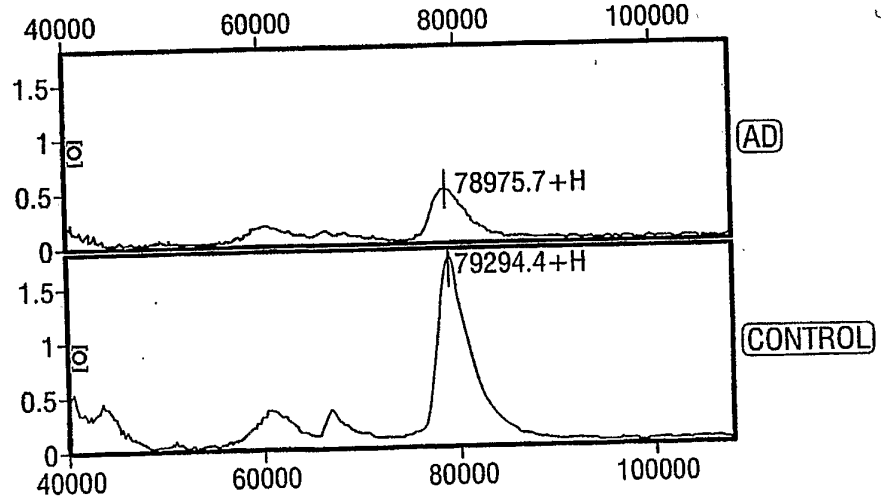


FIG. 9J

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7539

Chromogranin B peptide 6.5 kDa:

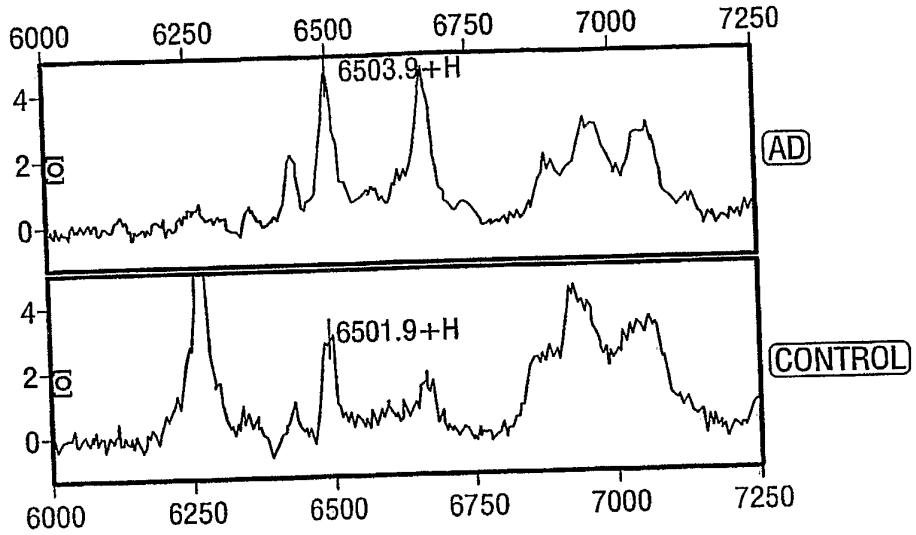


FIG. 9K

A-beta 1-40:

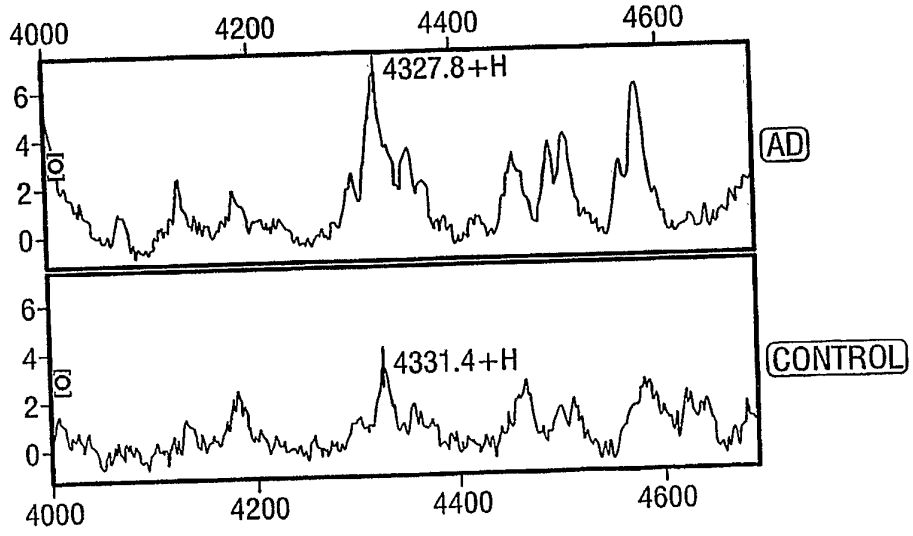


FIG. 9L

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7540

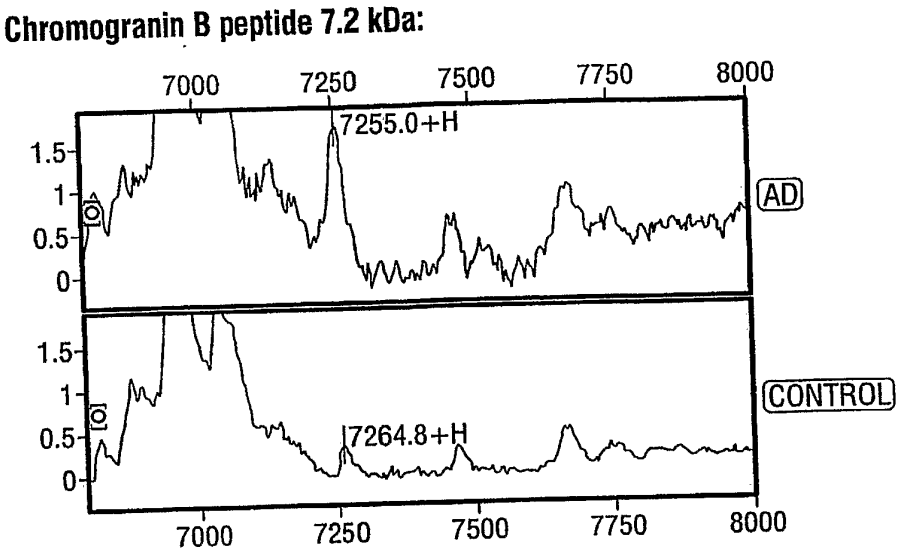


FIG. 9M

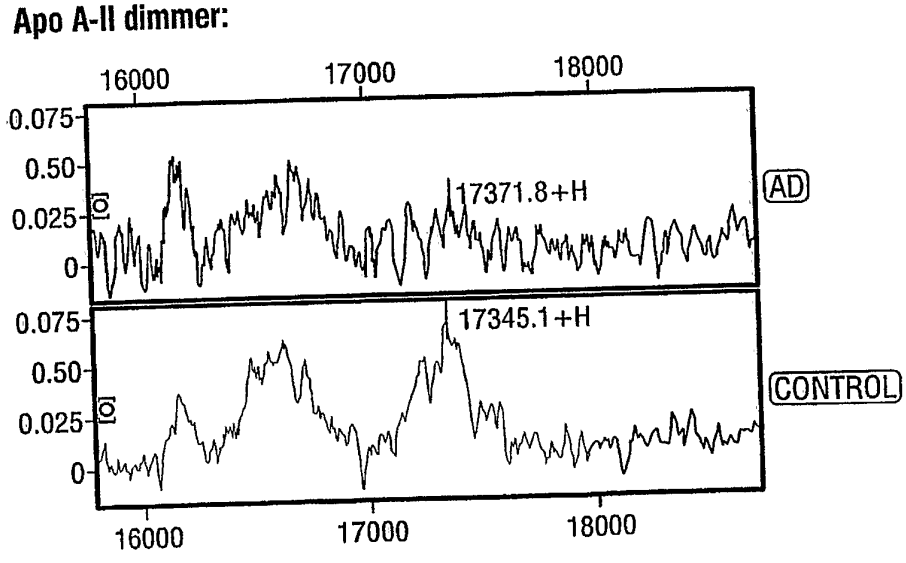


FIG. 9N

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7541

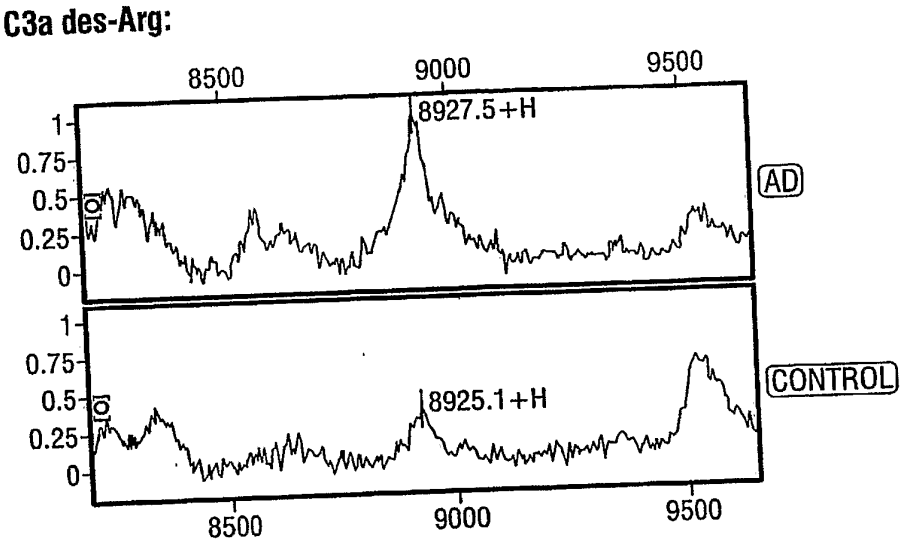


FIG. 9O

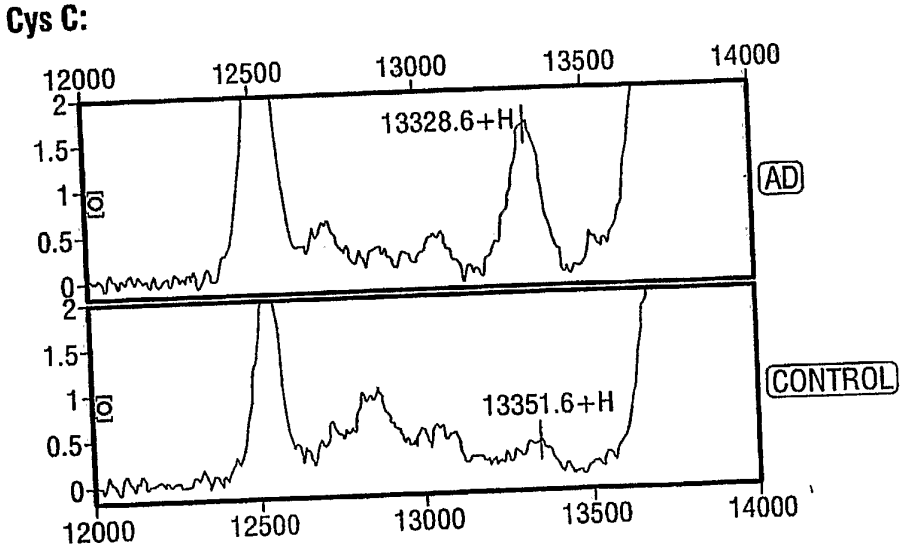


FIG. 9P

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7542

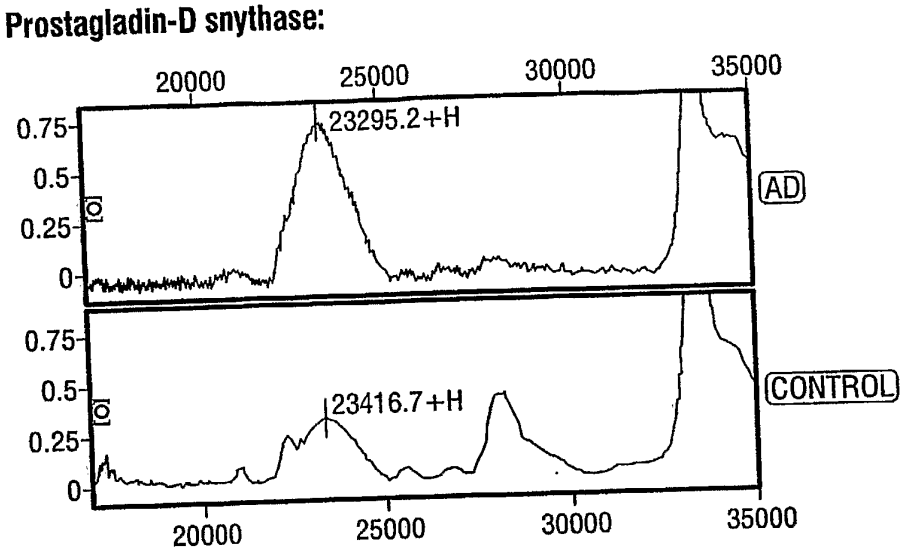


FIG. 9Q

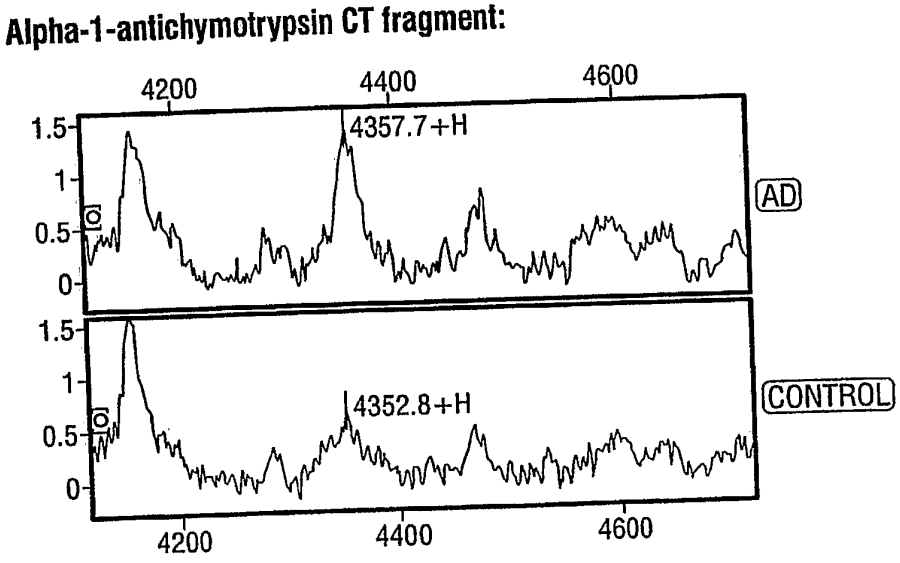


FIG. 9R

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7543

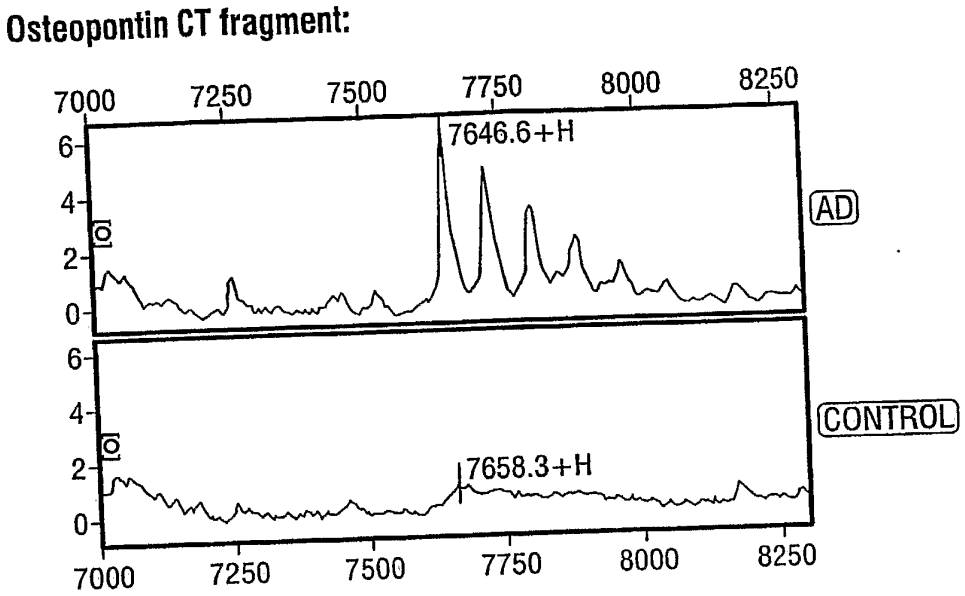


FIG. 9S

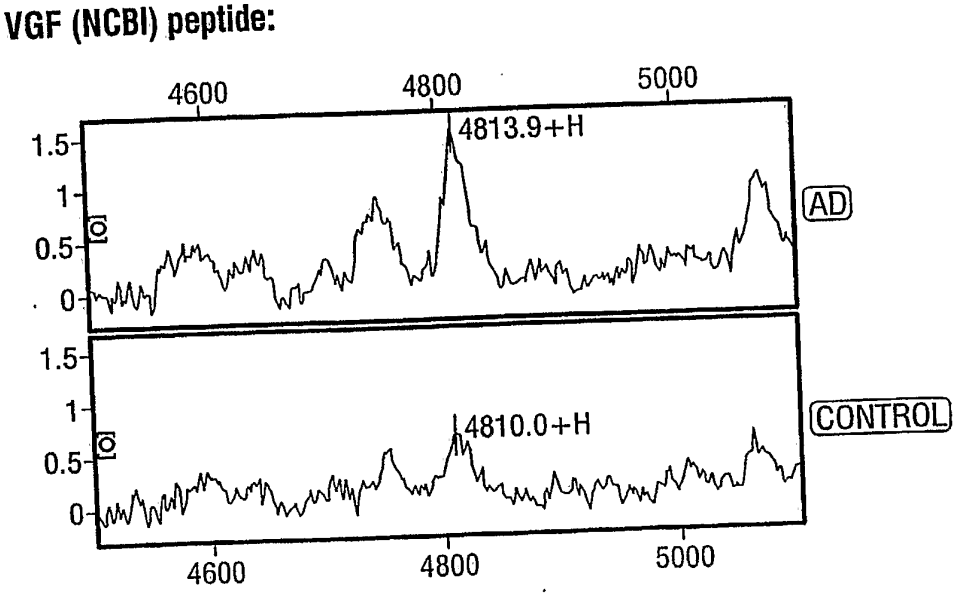


FIG. 9T

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7544

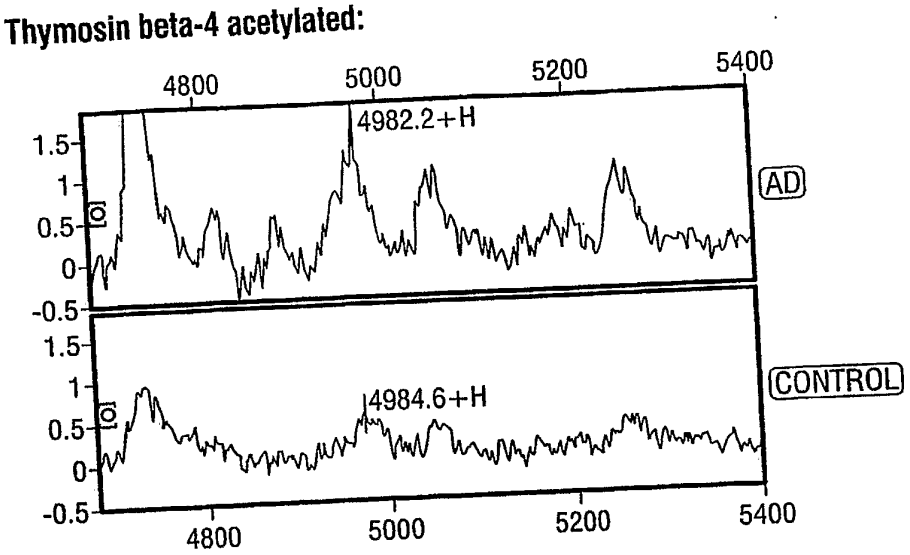


FIG. 9U

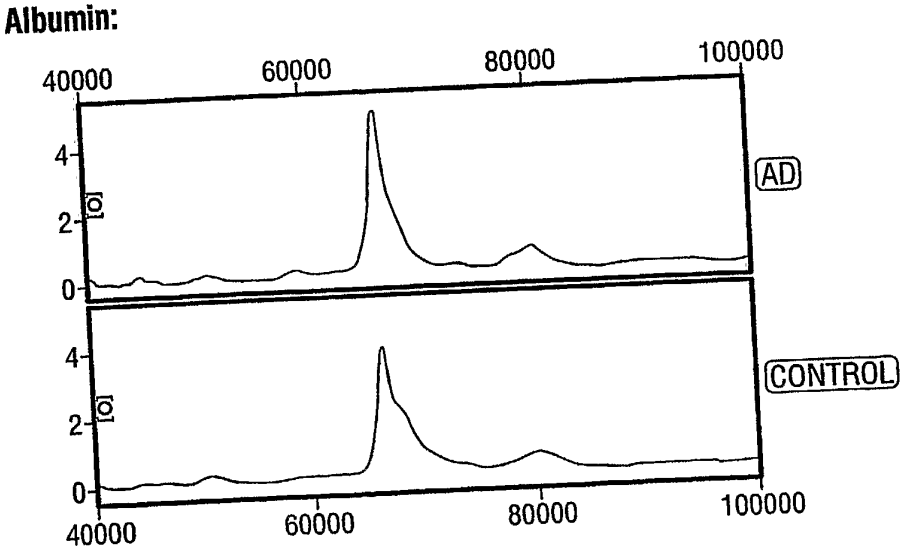


FIG. 9V

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7545

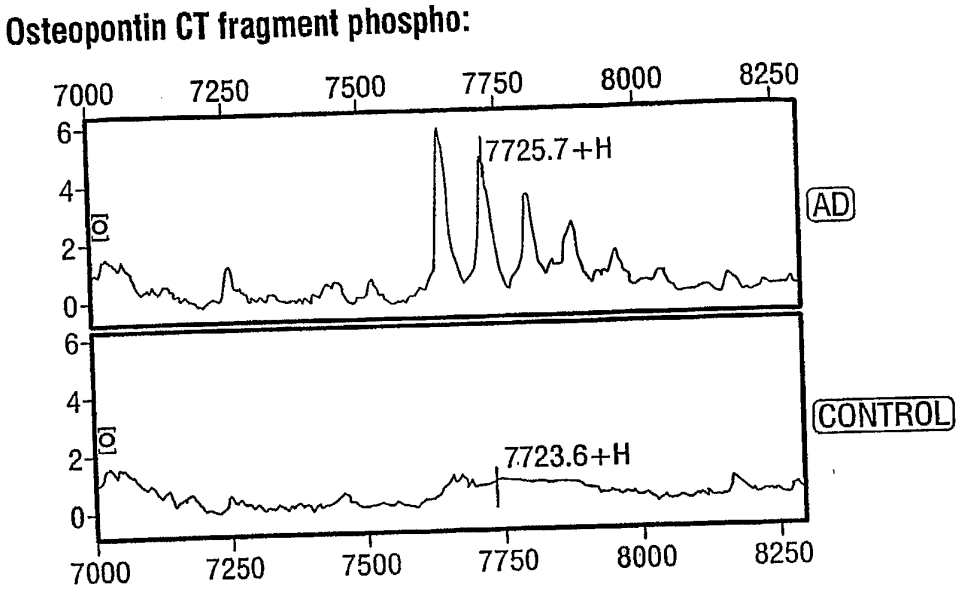


FIG. 9W

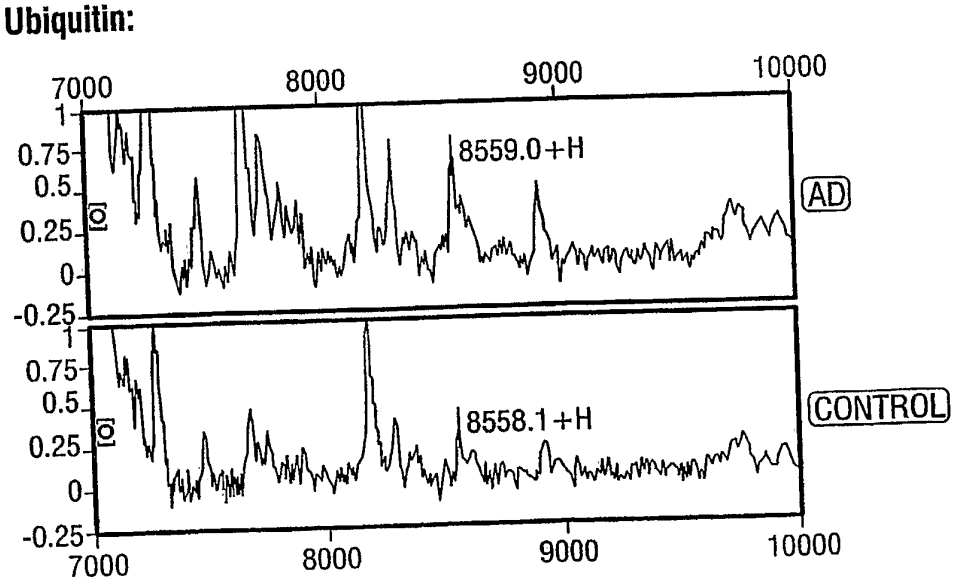


FIG. 9X

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7546

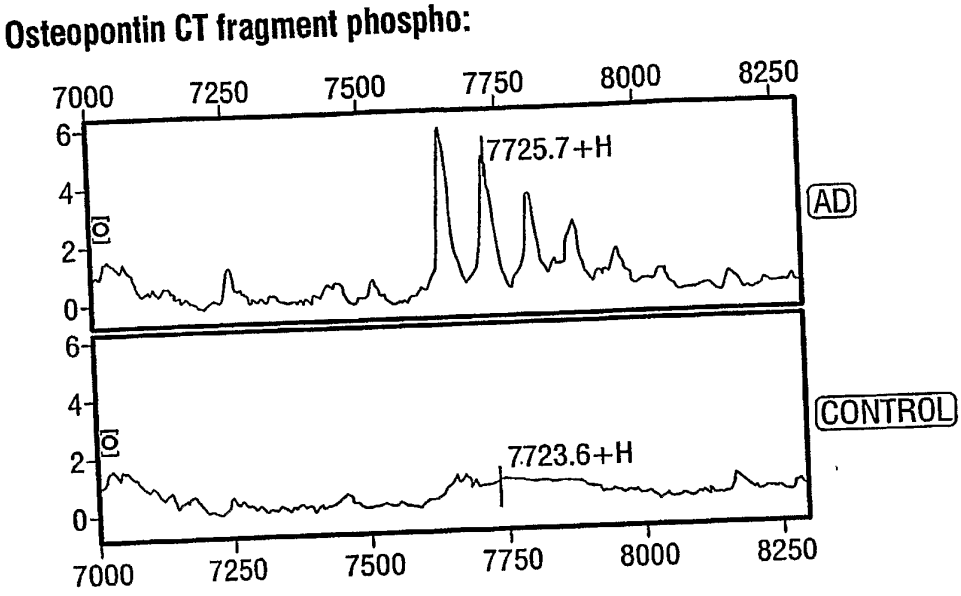


FIG. 9W

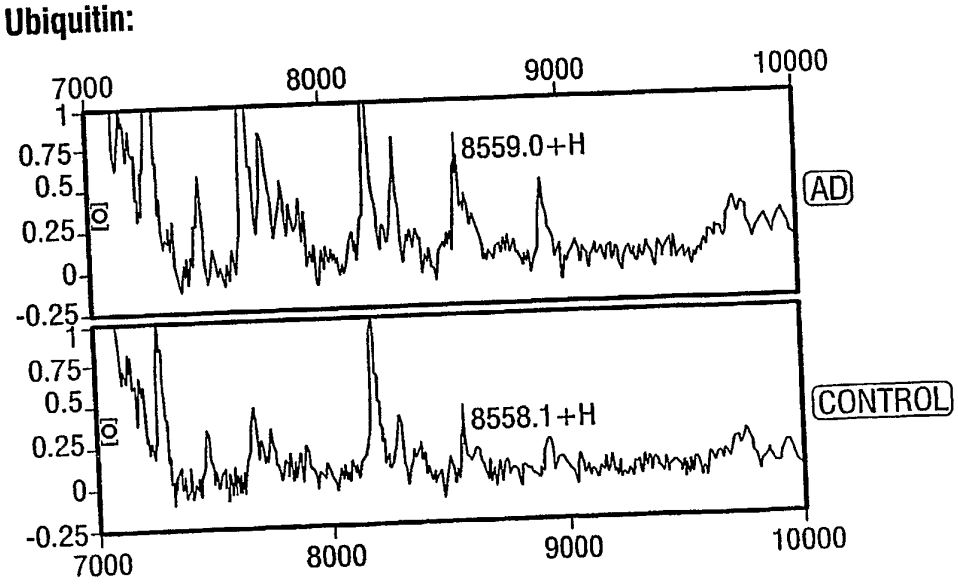


FIG. 9X

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7547

5.0 kDa:

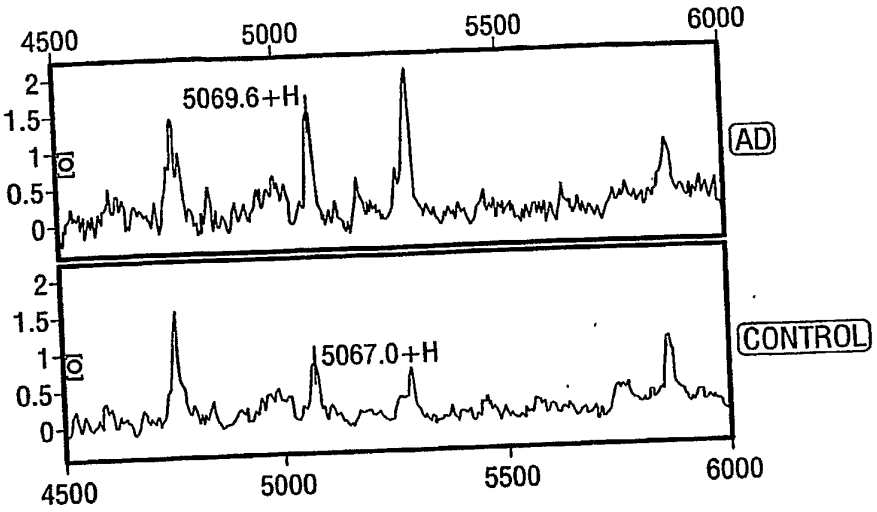


FIG. 9Y

9.7, 9.9, 10.2 and 10.3 kDa:

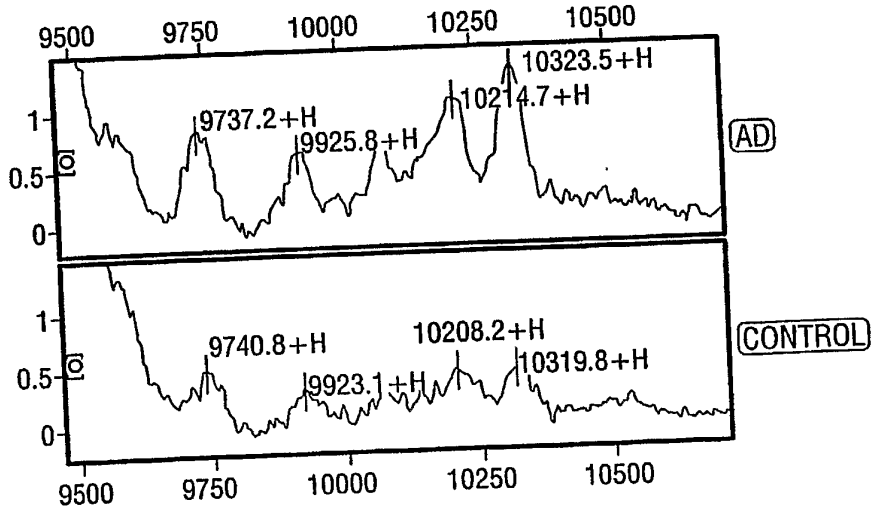


FIG. 9Z

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7548

EA-92 Chromogranin A peptide:

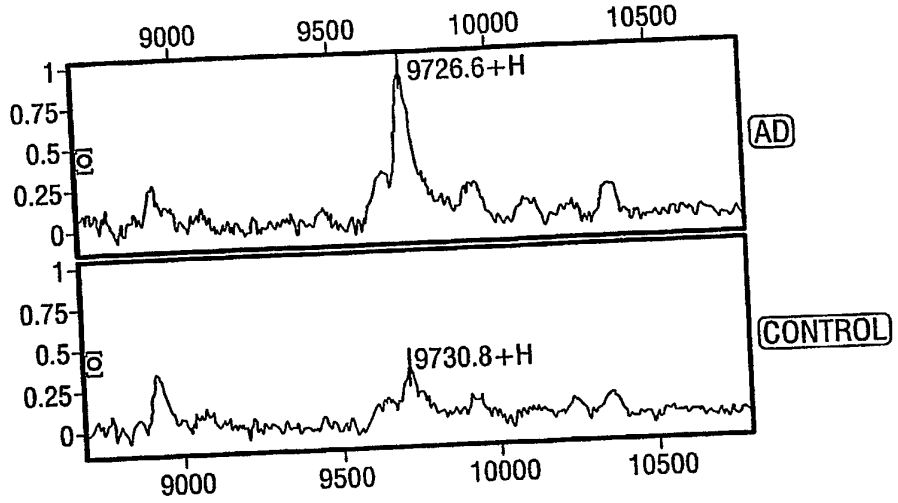


FIG. 9AA

Pancreatic ribonuclease:

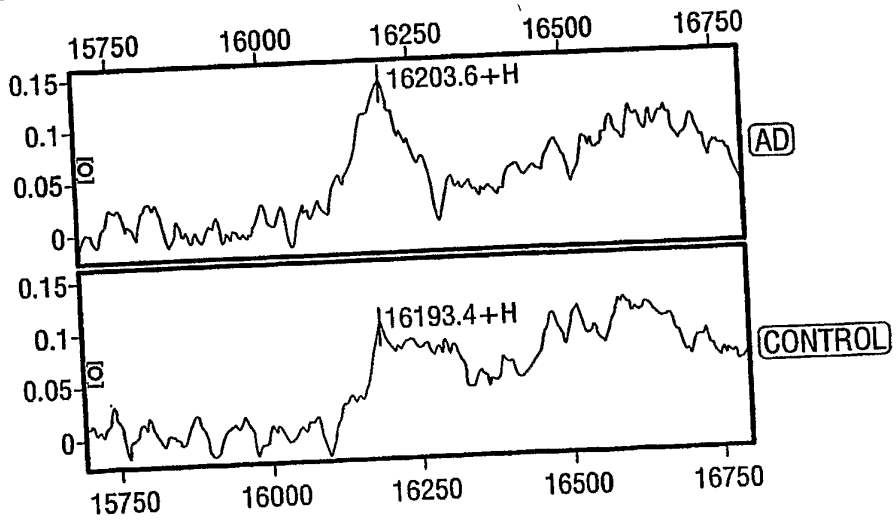


FIG. 9BB

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7549

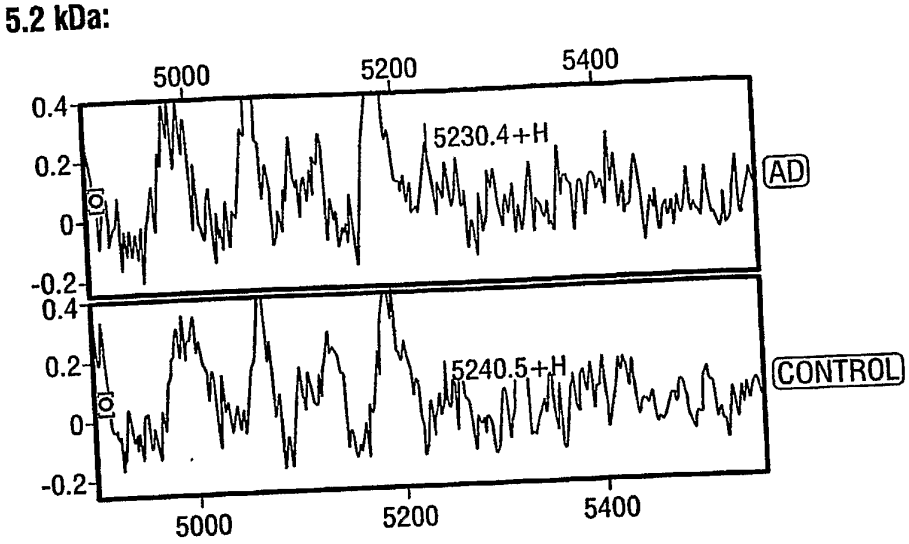


FIG. 9CC

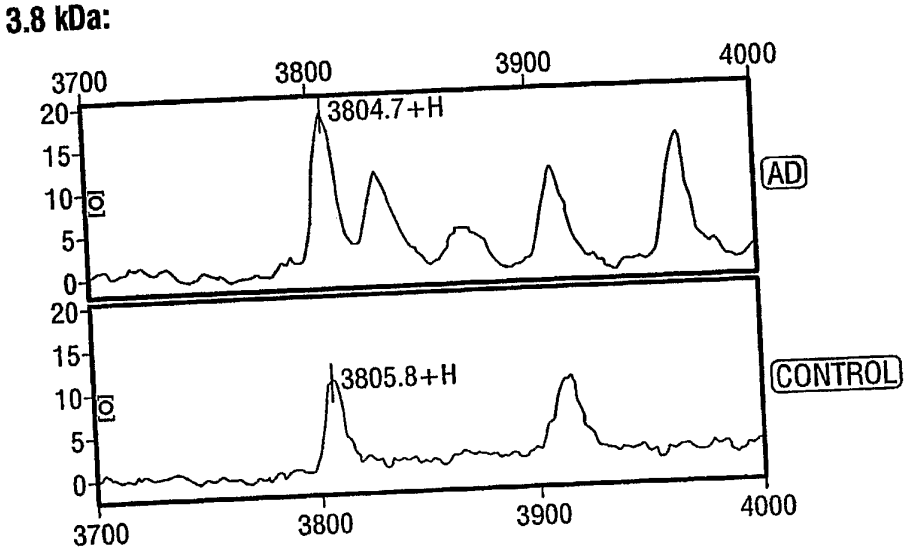


FIG. 9DD

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7550

8.9 kDa:

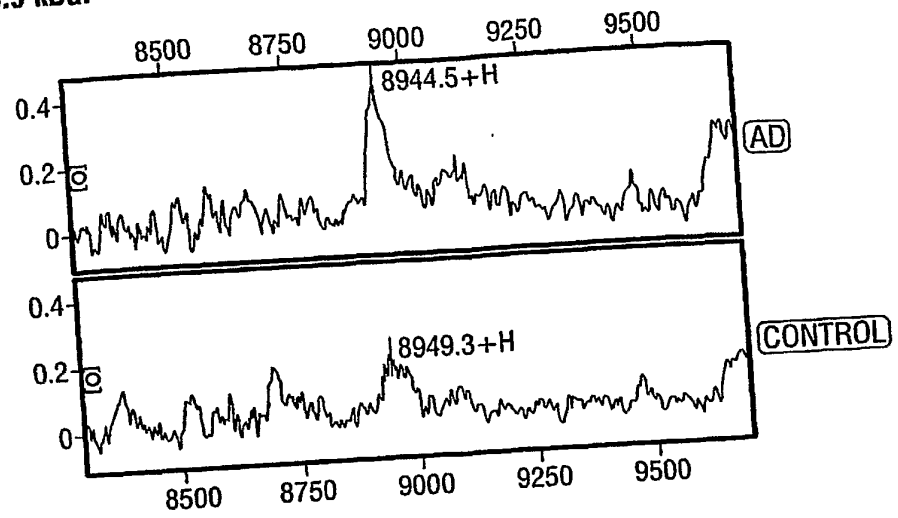


FIG. 9EE

5.2 kDa:

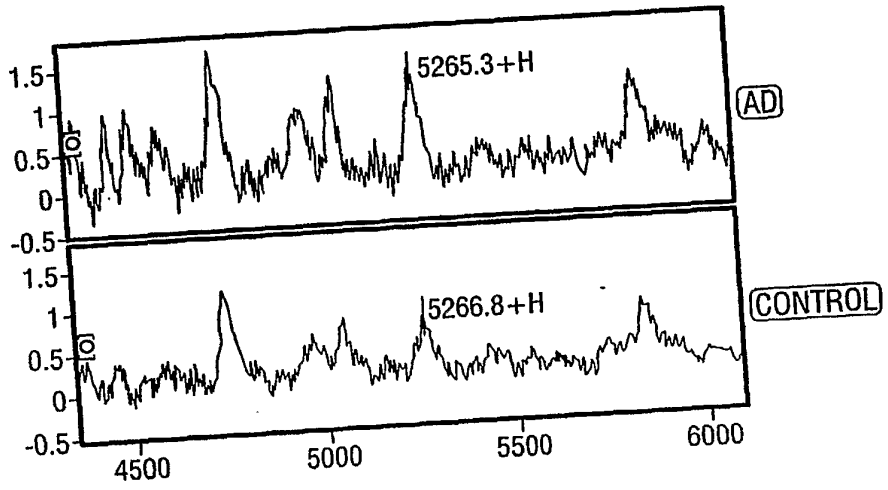


FIG. 9FF

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7551

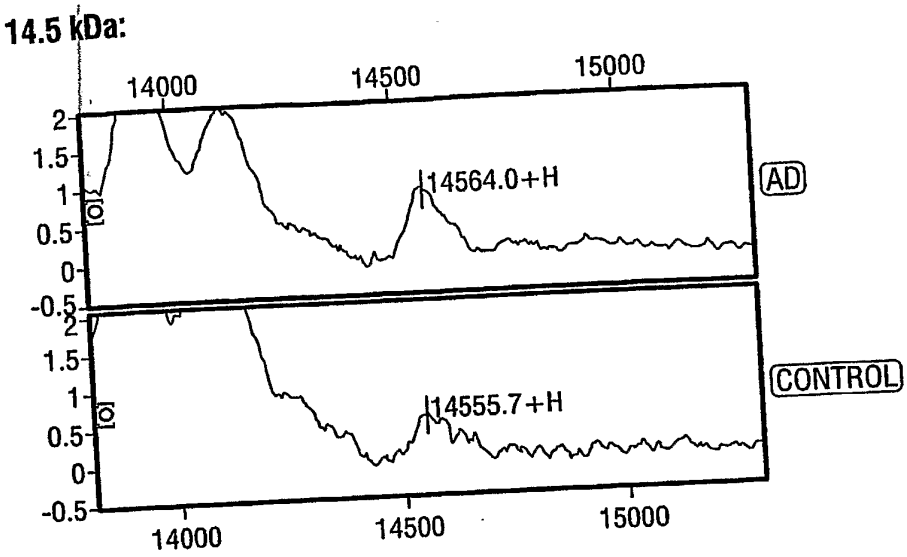


FIG. 9GG

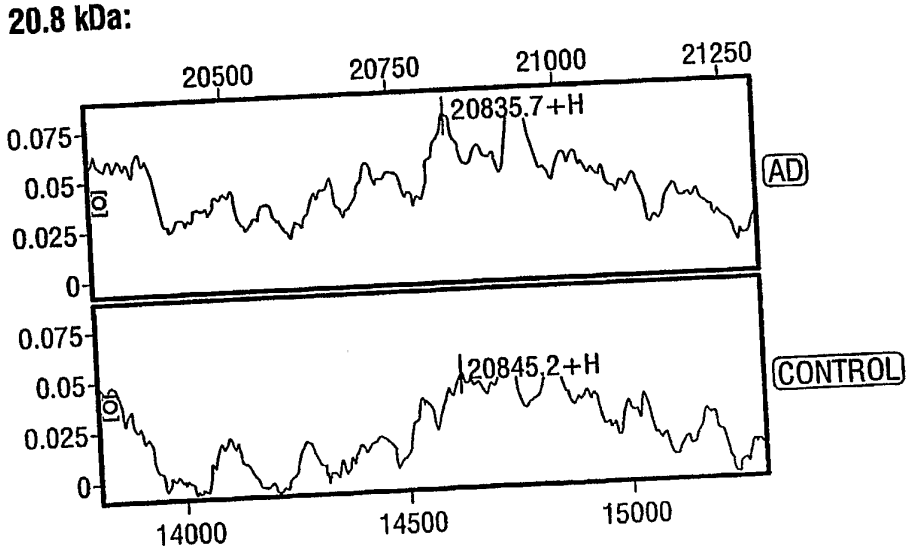


FIG. 9HH

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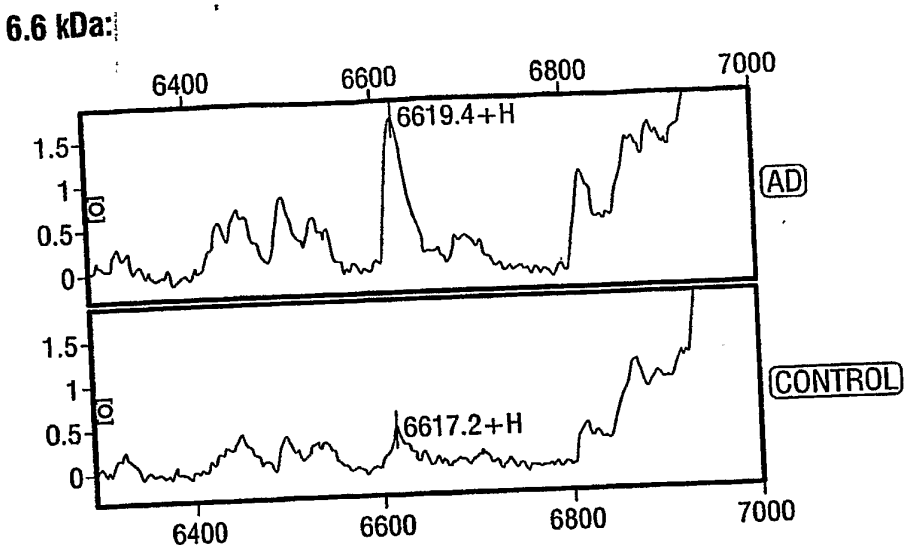


FIG. 9II

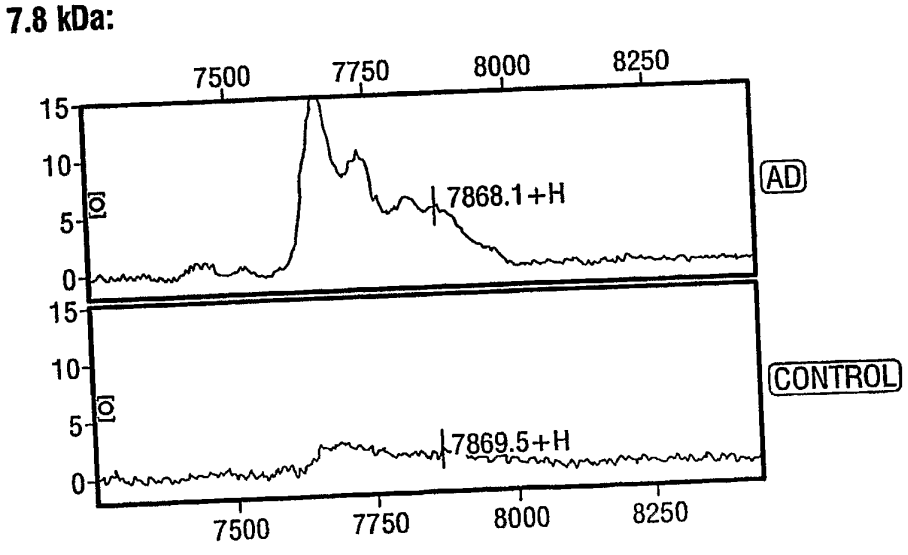


FIG. 9JJ

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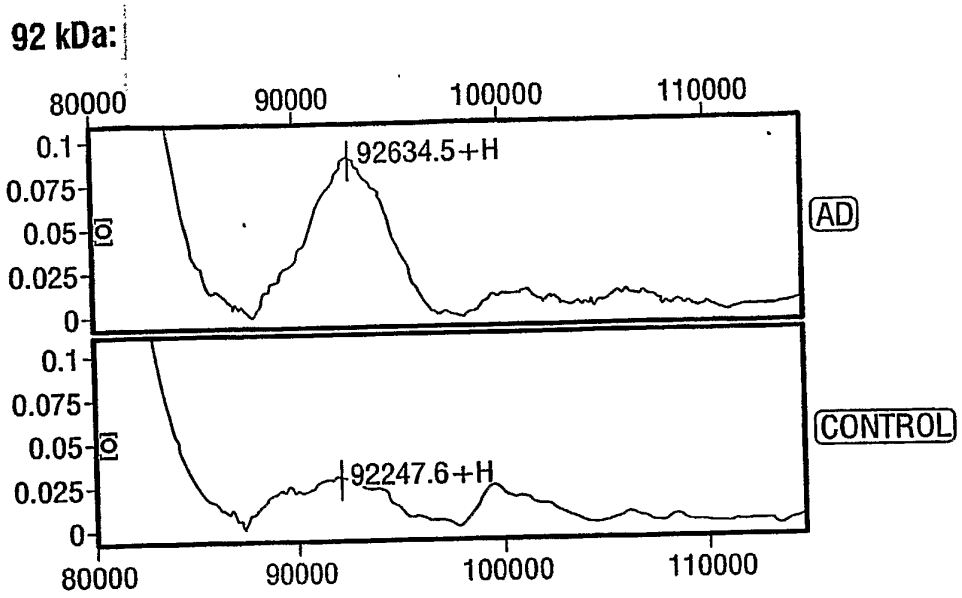


FIG. 9KK

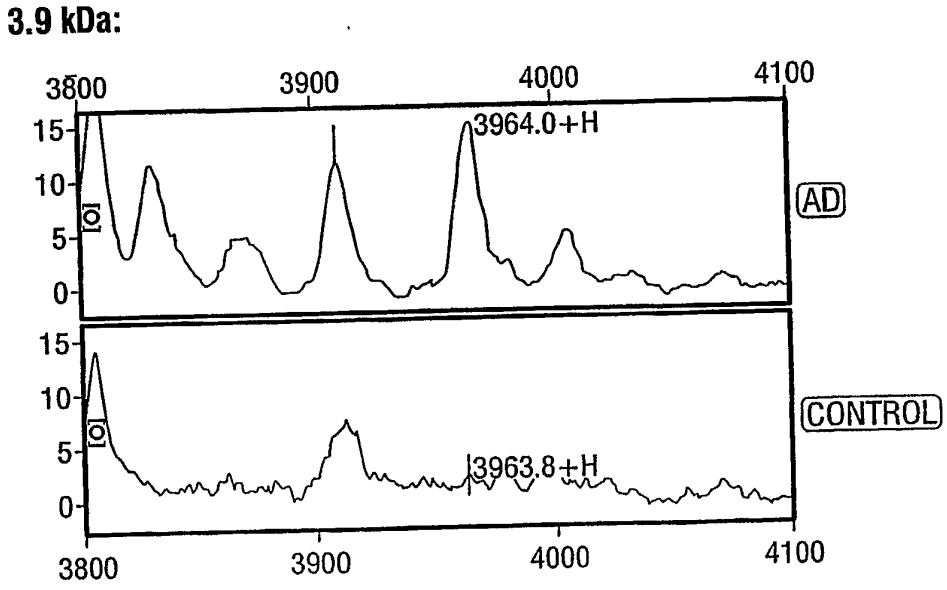


FIG. 9LL

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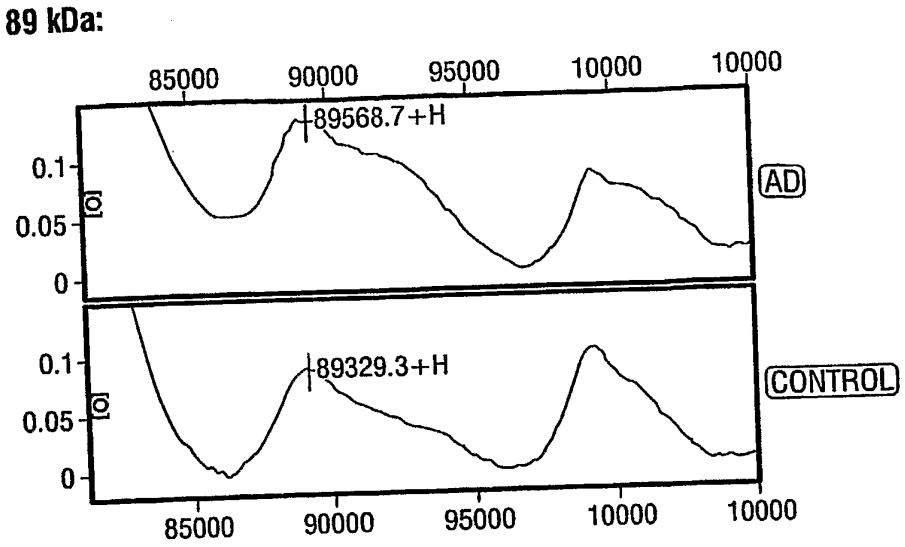


FIG. 9MM

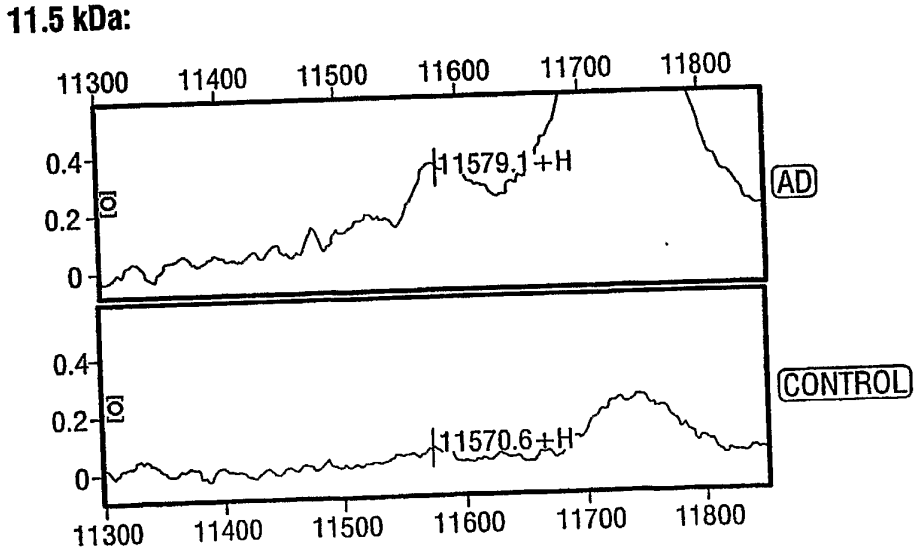


FIG. 9NN

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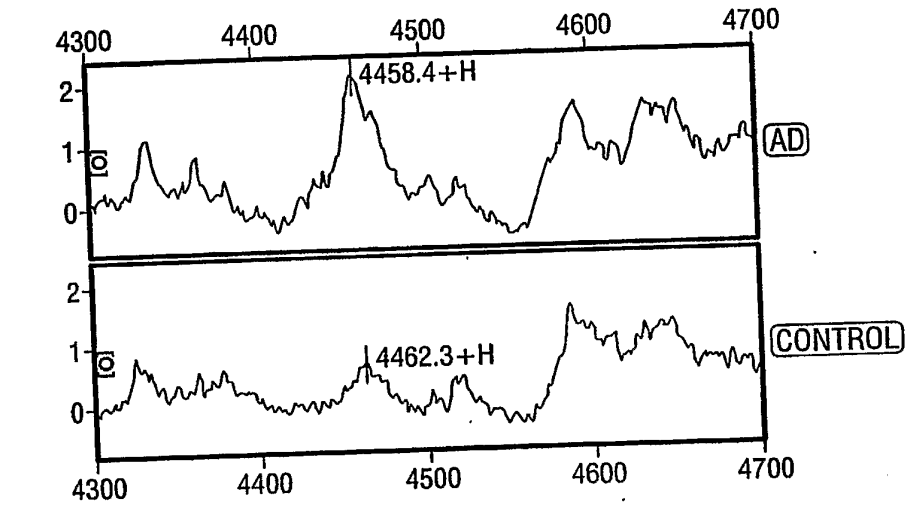


FIG. 900

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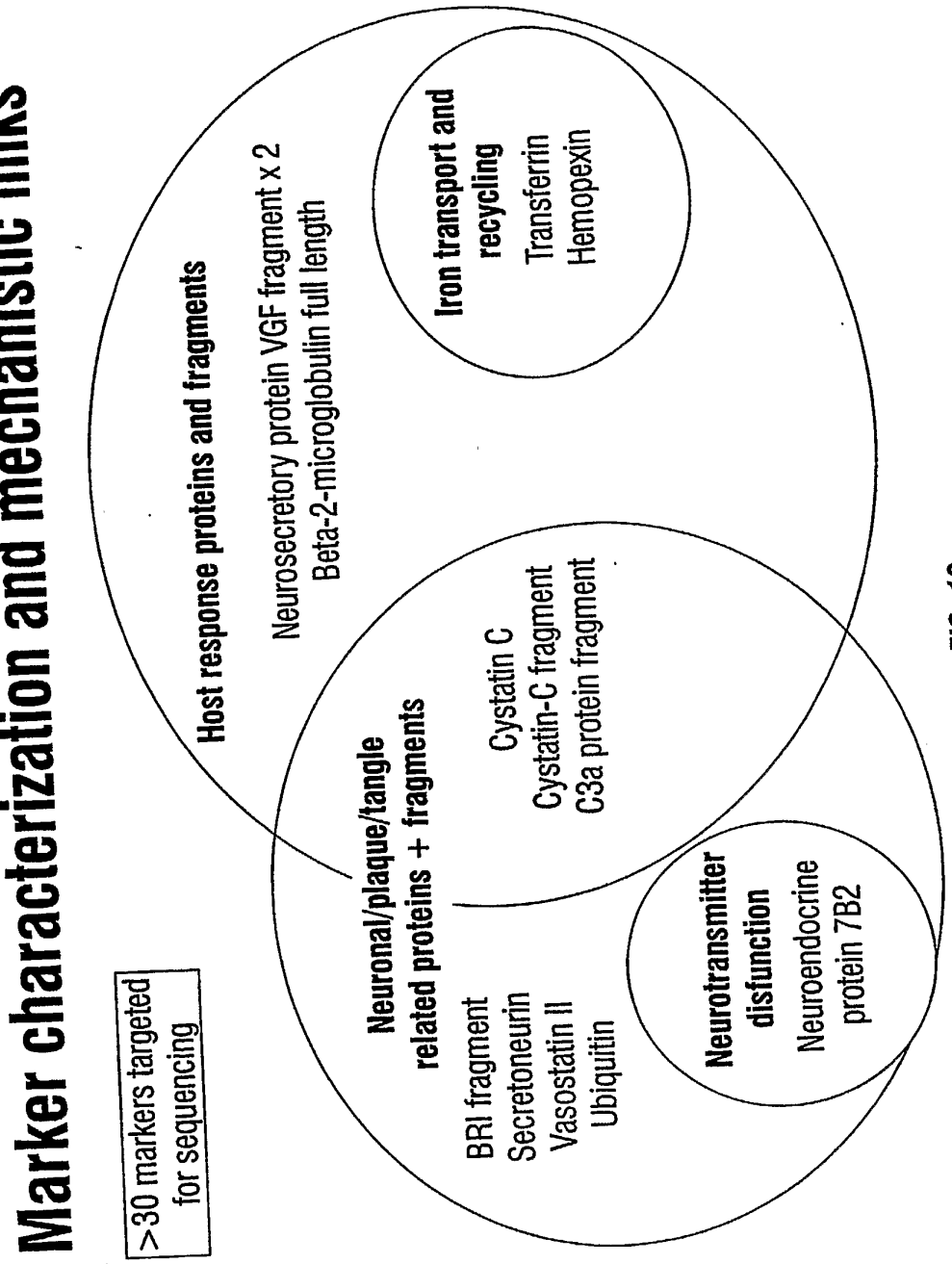


FIG. 10

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Marker validation

12583.8 Da marker

Kruskal Wallis p value	Mann Whitney p value		
A/C/N	A/C	A-N	C-N
0.0001	0.0649	<0.0001	0.0085

13391.9 Da marker

Kruskal Wallis p value	Mann Whitney p value		
A/C/N	A/C	A-N	C-N
0.148	0.0527	0.08451	0.1896

FIG. 11

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SEQUENCE LISTING

SEQ ID NO:1

NVNFQKAINEKLGQYASPTAKRCCQDGVTRLPMMRSCEQRAARVQQPDCREPFLSCCQFAESL
RKKSRDKGQAGLQ

SEQ ID NO:2

```
   1 MAEAKTHWLG AALSLIPLIF LISGAEAASF QRNQLLQKEP DLRLENVQKF PSPEMIRALE  60
  61 YIENLRQQAH KEESSPDYNP YQGVSVPLQQ KENGDESHLP ERDSLSEEDW MRIILEALRQ 120
 121 AENEPQSAPK ENKPYALNSE KNFPMDMSDD YETQQWPERK LKHMQFPPMY EENSRDNPFK 180
 181 RTNEIVEEQY TPQSLATLES VFQELGKLTG PNNQKRERMD EEQKLYTDDE DDIYKANNIA 240
 241 YEDVVGGEDW NPVEEKIESQ TQEEVRDSKE NIGKNEQIND EMKRSGQLGI QEEDLRKESK 300
 301 DQLSDDVSKV IAYLKRLVNA AGSGRLQNGQ NGERATRLFE KPLDSQSIYQ LIEISRNLQI 360
 361 PPEDLIEMLK TGEKPNGSVE PERELDLPVD LDDISEADLD HPDLFQNRML SKSGYPKTPG 420
 421 RAGTEALPDG LSVEDILNLL GMESAANQKT SYFPNPYNQE KVLPRLPYGA GRSRSNQLPK 480
 481 AAWIPHVENR QMAYENLNDK DQELGEYLAR MLVKYPEIIN SNQVKRVPGQ GSSEDDLQEE 540
 541 EQIEQAIKEH LNQGSSQETD KLAPVSKRFP VGPPKNDDTP NRQYWDEDLL MKVLEYLNQE 600
 601 KAEKGREHIA KRAMENM
```

SEQ ID NO:3

```
   1 MVKVTFNSAL AQKEAKKDEP KSGEEALIIP PDAVAVDCKD PDDVVPVGQR RAWCWCMCFG  60
  61 LAFMLAGVIL GGAYLYKYFA LQPDDVYYCG IKYIKDDVIL NEPSADAPAA LYQTIEENIK 120
 121 IFEEEEVEFI SVPVPEFADS DPANIVHDFN KKLTAYLDLN LDKCYVIPLN TSIVMPPRNL 180
 181 LELLINIKAG TYLPQSYLIH EHMVITDRIE NIDHLGFFIY RLCHDKETYK LQRRETIKGI 240
 241 QKREASNCFA IRHFENKFAV ETLICS
```

SEQ ID NO:4

```
   1 MAGPLRAPLL LLAILAVALA VSPAAGSSPG KPPRLVGGPM DASVEEEGVR RALDFAVGEY  60
  61 NKASNDMYHS RALQVVRARK QIVAGVNYFL DVELGRTTCT KTQPNLDNCP FHDQPHLKRK 120
 121 AFCSFQIYAV PWQGTMTLSK STCQDA
```

SEQ ID NO:5

```
   1 MKALRLSASA LFCLLLINGL GAAPPGRPEA QPPPLSSEHK EPVAGDAVPG PKDGSAPEVR  60
  61 GARNSEPQDE GELFQGVDPR ALAAVLLQAL DRPASPPAPS GSQQGPEEEA AEALLTETVR 120
 121 SQTHSLPAAG EPEPAAPPRP QTPENGPEAS DPSEELEALA SLLQELRDFS PSSAKRQQET 180
 181 AAAETETRTH TLTRVNLESP GPERVWRASW GEFQARVPER APLPPPAPSQ FQARMPDSGP 240
 241 LPETHKFGEG VSSPKTHLGE ALAPLSKAYQ GVAAPFPKAR RAESALLGGS EAGERLLQQG 300
 301 LAQVEAGRRQ AEATRQAAAQ EERLADLASD LLLQYLLQGG ARQRGLGGRG LQEAAEERES 360
 361 AREEEEAEQE RRGGEERVGE EDEEAAEAAE AEADEAERAR QNALLFAEEE DGEAGAEDKR 420
 421 SQEETPGHRR KEAEGTEEGG EEEDDEEMDP QTIDSLIELS TKLHLPADDV VSIIEEVEEK 480
 481 RNRKKKAPPE PVPPPRAAPA PTHVRSPQPP PPPPSARDEL PDWNEVLPPW DREEDEVYPP 540
 541 GPYHPFPNYI RPRTLQPPSA LRRRHYHHAL PPSRHYPGRE AQARHAQQEE AEAEERRLQE 600
 601 QEELENYIEH VLLRRP
```

SEQ ID NO:6

```
   1 MGPTSGPSLL LLLLTHLPLA LGSPMYSIIT PNILRLESEE TMVLEAHDAQ GDVPVTVTVH  60
  61 DFPGKKLVLS SEKTVLTPAT NHMGNVTFTI PANREFKSEK GRNKFVTVQA TFGTQVVEKV 120
 121 VLVSLQSGYL FIQTDKTIYT PGSTVLYRIF TVNHKLLPVG RTVMVNIENP EGIPVKQDSL 180
 181 SSQNQLGVLP LSWDIPELVN MGQWKIRAYY ENSPQQVFST EFEVKEYVLP SFEVIVEPTE 240
```


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```
 241 KFYYIYNEKG LEVTITARFL YGKKVEGTAF VIFGIQDGEQ RISLPESLKR IPIEDGSGEV  300
 301 VLSRKVLLDG VQNLRAEDLV GKSLYVSATV ILHSGSDMVQ AERSGIPIVT SPYQIHFTKT  360
 361 PKYFKPGMPF DLMVFVTNPD GSPAYRVPVA VQGEDTVQSL TQGDGVAKLS INTHPSQKPL  420
 421 SITVRTKKQE LSEAEQATRT MQALPYSTVG NSNNYLHLSV LRTELRPGET LNVNFLLRMD  480
 481 RAHEAKIRYY TYLIMNKGRL LKAGRQVREP GQDLVVLPLS ITTDFIPSFR LVAYYTLIGA  540
 541 SGQREVVADS VWVDVKDSCV GSLVVKSGQS EDRQPVPGQQ MTLKIEGDHG ARVVLVAVDK  600
 601 GVFVLNKKNK LTQSKIWDVV EKADIGCTPG SGKDYAGVFS DAGLTFTSSS GQQTAQRAEL  660
 661 QCPQPAARRR RSVQLTEKRM DKVGKYPKEL RKCCEDGMRE NPMRFSCQRR TRFISLGEAC  720
 721 KKVFLDCCNY ITELRRQHAR ASHLGLARSN LDEDIIAEEN IVSRSEFPES WLWNVEDLKE  780
 781 PPKNGISTKL MNIFLKDSIT TWEILAVSMS DKKGICVADP FEVTVMQDFF IDLRLPYSVV  840
 841 RNEQVEIRAV LYNYRQNQEL KVRVELLHNP AFCSLATTKR RHQQTVTIPP KSSLSVPYVI  900
 901 VPLKTGLQEV EVKAAVYHHF ISDGVRKSLK VVPEGIRMNK TVAVRTLDPE RLGREGVQKE  960
 961 DIPPADLSDQ VPDTESETRI LLQGTPVAQM TEDAVDAERL KHLIVTPSGC GEQNMIGMTP 1020
1021 TVIAVHYLDE TEQWEKFGLE KRQGALELIK KGYTQQLAFR QPSSAFAAFV KRAPSTWLTA 1080
1081 YVVKVFSLAV NLIAIDSQVL CGAVKWLILE KQKPDGVFQE DAPVIHQEMI GGLRNNNEKD 1140
1141 MALTAFVLIS LQEAKDICEE QVNSLPGSIT KAGDFLEANY MNLQRSYTVA IAGYALAQMG 1200
1201 RLKGPLLNKF LTTAKDKNRW EDPGKQLYNV EATSYALLAL LQLKDFDFVP PVVRWLNEQR 1260
1261 YYGGGYGSTQ ATFMVFQALA QYQKDAPDHQ ELNLDVSLQL PSRSSKITHR IHWESASLLR 1320
1321 SEETKENEGF TVTAEGKGQG TLSVVTMYHA KAKDQLTCNK FDLKVTIKPA PETEKRPQDA 1380
1381 KNTMILEICT RYRGDQDATM SILDISMMTG FAPDTDDLKQ LANGVDRYIS KYELDKAFSD 1440
1441 RNTLIIYLDK VSHSEDDCLA FKVHQYFNVE LIQPGAVKVY AYYNLEESCT RFYHPEKEDG 1500
1501 KLNKLCRDEL CRCAEENCFI QKSDDKVTLE ERLDKACEPG VDYVYKTRLV KVQLSNDFDE 1560
1561 YIMAIEQTIK SGSDEVQVGQ QRTFISPIKC REALKLEEKK HYLMWGLSSD FWGEKPNLSY 1620
1621 IIGKDTWVEH WPEEDECQDE ENQKQCQDLG AFTESMVVFG CPN
```

SEQ ID NO:7

```
   1 MVSRMVSTML SGLLFWLASG WTPAFAYSPR TPDRVSEADI QRLLHGVMEQ LGIARPRVEY  60
  61 PAHQAMNLVG PQSIEGGAHE GLQHLGPFGN IPNIVAELTG DNIPKDFSED QGYPDPPNPC 120
 121 PVGKTADDGC LENTPDTAEF SREFQLHQHL FDPEHDYPGL GKWNKKLLYE KMKGGERRKR 180
 181 RSVNPYLQGQ RLDNVVAKKS VPHFSDEDKD PE
```

SEQ ID NO:8

```
   1 MRSAAVLALL LCAGQVTALP VNSPMNKGDT EVMKCIVEVI SDTLSKPSPM PVSQECFETL  60
  61 RGDERILSIL RHQNLLKELQ DLALQGAKER AHQQKKHSGF EDELSEVLEN QSSQAELKEA 120
 121 VEEPSSKDVM EKREDSKEAE KSGEATDGAR PQALPEPMQE SKAEGNNQAP GEEEEEEEEA 180
 181 TNTHPPASLP SQKYPGPQAE GDSEGLSQGL VDREKGLSAE PGWQAKREEE EEEEEEAEAG 240
 241 EEAVPEEEGP TVVLNPHPSL GYKEIRKGES RSEALAVDGA GKPGAEEAQD PEGKGEQEHS 300
 301 QQKEEEEEMA VVPQGLFRGG KSGELEQEEE RLSKEWEDSK RWSKMDQLAK ELTAEKRLEG 360
 361 QEEEEDNRDS SMKLSFRARA YGFRGPGPQL RRGWRPSREE DSLEAGLPLQ VRGYPEEKKE 420
 421 EEGSANRRPE DQELESLSAI EAELEKVAHQ LQALRRG
```


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SEQ ID NO:9

VETRTIVRF NRPFLMIIVP TDTQNIFFMS KVTNPKQA

SEQ ID NO:10

SDKPDMAEIE KFDKSKLKKT ETQEKNPLPS KETIEQEKQA GES

SEQ ID NO:11

```
      1          11         21         31         41         51
    1 MRIAVICFCL LGITCAIPVK QADSGSSEEK QLYNKYPDAV ATWLNPDPSQ KQNLLAPQNA 60
   61 VSSEETNDFK QETLPSKSNE SHDHMDDMDD EDDDDHVDSQ DSIDSNDSDD VDDTDDSHQS 120
  121 DESHHSDESD ELVTDFPTDL PATEVFTPVV PTVDTYDGRG DSVVYGLRSK SKKFRRPDIQ 180
  181 YPDATDEDIT SHMESEELNG AYKAIPVAQD LNAPSDWDSR GKDSYETSQL DDQSAETHSH 240
  241 KQSRLYKRKA NDESNEHSDV IDSQELSKVS REFHSHEFHS HEDMLVVDPK SKEEDKHLKF 300
  301 RISHELDSAS SEVN
```

SEQ ID NO:12

```
      1          11         21         31         41         51
    1 MQPTLLLLSLL GAVGLAAVNS MPVDNRNHNE GMVTRCIIEV LSNALSKSSA PPITPECRQV  60
   61 LKTSRKDVKD KETTENENTK FEVRLLRDPA DASEAHESSS RGEAGAPGEE DIQGPTKADT 120
  121 EKWAEGGGHS RERADEPQWS LYPSDSQVSE EVKTRHSEKS QREDEEEEEG ENYQKGERGE 180
  181 DSSEEKHLEE PGETQNAFLN ERKQASAIKK EELVARSETH AAGHSQEKTH SREKSSQESG 240
  241 EEAGSQENHP QESKGQPRSQ EESEEGEEDA TSEVDKRRTR PRHHHGRSRP DRSSQGGSLP 300
  301 SEEKGHPQEE SEESNVSMAS LGEKRDHHST HYRASEEEPE YGEEIKGYPG VQAPEDLEWE 360
  361 RYRGRGSEEY RAPRPQSEES WDEEDKRNYP SLELDKMAHG YGEESEEERG LEPGKGRHHR 420
  421 GRGGEPRAYF MSDTREEKRF LGEGHHRVQE NQMDKARRHP QGAWKELDRN YLNYGEEGAP 480
  481 GKWQQQGDLQ DTKENREEAR FQDKQYSSHH TAEKRKRLGE LFNPYYDPLQ WKSSHFERRD 540
  541 NMNDNFLEGE EENELTLNEK NFFPEYNYDW WEKKPFSEDV NWGYEKRNLA RVPKLDLKRQ 600
  601 YDRVAQLDQL LHYRKKSAEF PDFYDSEEPV STHQEAENEK DRADQTVLTE DEKKELENLA 660
  661 AMDLELQKIA EKFSQRG
```

SEQ ID NO:13

```
      1          11         21         31         41         51
    1 MRSAAVLALL LCAGQVTALP VNSPMNKGDT EVMKCIVEVI SDTLSKPSPM PVSQECFETL  60
   61 RGDERILSIL RHQNLLKELQ DLALQGAKER AHQQKKHSGF EDELSEVLEN QSSQAELKEA 120
  121 VEEPSSKDVM EKREDSKEAE KSGEATDGAR PQALPEPMQE SKAEGNNQAP GEEEEEEEEA 180
  181 TNTHPPASLP SQKYPGPQAE GDSEGLSQGL VDREKGLSAE PGWQAKREEE EEEEEEAEAG 240
  241 EEAVPEEEGP TVVLNPHPSL GYKEIRKGES RSEALAVDGA GKPGAEEAQD PEGKGEQEHS 300
  301 QQKEEEEEMA VVPQGLFRGG KSGELEQEEE RLSKEWEDSK RWSKMDQLAK ELTAEKRLEG 360
  361 QEEEEDNRDS SMKLSFRARA YGFRGPGPQL RRGWRPSREE DSLEAGLPLQ VRGYPEEKKE 420
  421 EEGSANRRPE DQELESLSAI EAELEKVAHQ LQALRRG
```

SEQ ID NO:14

```
      1 MKALRLSASA LFCLLLINGL GAAPPGRPEA QPPPLSSEHK EPVAGDAVPG PKDGSAPEVR
     61 GARNSEPQDE GELFQGVDPR ALAAVLLQAL DRPASPPAPS GSQQGPEEEA AEALLTETVR
    121 SQTHSLPAPE SPEPAAPPRP QTPENGPEAS DPSEELEALA SLLQELRDFS PSSAKRQQET
    181 AAAETETRTH TLTRVNLESP GPERVWRASW GEFQARVPER APLPPPAPSQ FQARMPDSGP
    241 LPETHKFGEG VSSPKTHLGE ALAPLSKAYQ GVAAPFPKAR RPESALLGGS EAGERLLQQG
    301 LAQVEAGRRQ AEATRQAAAQ EERLADLASD LLLQYLLQGG ARQRGLGGRG LQEAAEERES
    361 AREEEEAEQE RRGGEERVGE EDEEAAEAEA EAEEAERARQ NALLFAEEED GEAGAEDKRS
    421 QEETPGHRRK EAEGTEEGGE EEDDEEMDPQ TIDSLIELST KLHLPADDVV SIIEEVEEKR
    481 KRKKNAPPEP VPPPRAAPAP THVRSPQPPP PAPAPARDEL PDWNEVLPPW DREEDEVYPP
    541 GPYHPFPNYI RPRTLQPPSA LRRRHYHHAL PPSRHYPGRE AQARRAQEEA EAEERRLQEQ
    601 EELENYIEHV LLRRP
```


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SEQ ID NO:15

```
      1          11         21         31         41         51
    1 MLPGLALLLL AAWTARALEV PTDGNAGLLA EPQIAMFCGR LNMHMNVQNG KWDSDPSGTK  60
   61 TCIDTKEGIL QYCQEVYPEL QITNVVEANQ PVTIQNWCKR GRKQCKTHPH FVIPYRCLVG 120
  121 EFVSDALLVP DKCKFLHQER MDVCETHLHW HTVAKETCSE KSTNLHDYGM LLPCGIDKFR 180
  181 GVEFVCCPLA EESDNVDSAD AEEDDSDVWW GGADTDYADG SEDKVVEVAE EEEVAEVEEE 240
  241 EADDDEDDED GDEVEEEAEE PYEEATERTT SIATTTTTTT ESVEEVVREV CSEQAETGPC 300
  301 RAMISRWYFD VTEGKCAPFF YGGCGGNRNN FDTEEYCMAV CGSAMSQSLL KTTQEPLARD 360
  361 PVKLPTTAAS TPDAVDKYLE TPGDENEHAH FQKAKERLEA KHRERMSQVM REWEEAERQA 420
  421 KNLPKADKKA VIQHFQEKVE SLEQEAANER QQLVETHMAR VEAMLNDRRR LALENYITAL 480
  481 QAVPPRPRHV FNMLKKYVRA EQKDRQHTLK HFEHVRMVDP KKAAQIRSQV MTHLRVIYER 540
  541 MNQSLSLLYN VPAVAEEIQD EVDELLQKEQ NYSDDVLANM ISEPRISYGN DALMPSLTET 600
  601 KTTVELLPVN GEFSLDDLQP WHSFGADSVP ANTENEVEPV DARPAADRGL TTRPGSGLTN 660
  661 IKTEEISEVK MDAEFRHDSG YEVHHQKLVF FAEDVGSNKG AIIGLMVGGV VIATVIVITL 720
  721 VMLKKKQYTS IHHGVVEVDA AVTPEERHLS KMQQNGYENP TYKFFEQMQN
```

SEQ ID NO: 16

TLLVFEVQQPFLFVLWDQQHKFPVFMGRVYDPRA

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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** AMYLOID BETA PEPTIDE BINDING POLYPEPTIDE

(57) **Abstract:** An amyloid beta peptide binding polypeptide is provided, which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having 1 to about 20 substitution mutations. Nucleic acid encoding the polypeptide, expression vector comprising the nucleic acid, and host cell comprising the expression vector are also provided. Also provided are methods comprising a step of affinity separation or detection, in which step a polypeptide according to the invention is used. Such methods may be used for reducing the content of amyloid beta peptide in a body fluid. The use of an amyloid beta peptide binding polypeptide as a medicament is also provided.

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AMYLOID BETA PEPTIDE BINDING POLYPEPTIDE

Field of the invention

The present invention is related to a new polypeptide, which binds to an amyloid beta peptide, and to the use of such polypeptide in a method of affinity separation, for example in a method for reducing the content of amyloid beta peptide in a body fluid, in diagnostics of Alzheimer's disease, in histochemical analyses and in other areas of application. The invention also relates to the use of such an amyloid beta peptide binding polypeptide as a medicament. The polypeptide is related to a domain of staphylococcal protein A (SPA), in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having at least one substitution mutation.

Background

Affibody® molecules

Molecules related to protein Z, derived from domain B of staphylococcal protein A (SPA) (Nilsson B *et al* (1987) Protein Engineering 1, 107-133), have been selected from a library of randomized such molecules using different interaction targets (see e g WO95/19374; WO00/63243; Nord K *et al* (1995) Prot Eng 8:601-608; Nord K *et al* (1997) Nature Biotechnology 15, 772-777). Different target molecules have been used to select such protein Z derivatives, e g as described in Nord K *et al* (1997, *supra*). The experiments described in this reference outline principles of the general technology of selecting protein Z derivatives against given targets, rather than being a study directed towards the express objective of obtaining a molecule with high enough affinity for use in a specific therapeutic or biotechnological application.

Amyloid beta peptide and its role in Alzheimer's disease

Many different diseases, such as Alzheimer's disease, type II diabetes, primary and secondary systemic amyloidosis, and familial amyloid polyneuropathy 1, have been recognized as belonging to the ever-growing family of amyloid diseases. All amyloid diseases have in common the presence of extracellular protein aggregates that may or may not be fibrillar. An attractive strategy for developing therapies for amyloid diseases is thus to inhibit or reverse protein/peptide aggregation (Mason J *et al*, Curr Opin Struct Biol 13:526-532 (2003)). A recent review of therapeutic strategies for combating amyloid diseases in general is Sacchettini and Kelly, Nat Rev Drug Discov 1:267-275 (2002).

There is great need for a treatment that targets the amyloidogenic intermediates of these diseases and not merely through their stabilization, but rather through their increased clearance. Alzheimer's disease is one of the most studied amyloid diseases, primarily due to its widespread occurrence and the fact that there is currently neither a cure nor a treatment available to reverse its effects. Alzheimer's disease is among the most common diseases of advanced age, affecting almost one out of ten individuals who survive beyond the age of 65 years, and another 10 % for each additional decade of the life span. Alzheimer's Disease International estimates that there are 18 million cases of dementia worldwide, of which 12 million cases are Alzheimer's disease. Current treatments for Alzheimer's disease provide only modest symptomatic relief, for periods between six and eighteen months. Since current compounds only provide symptomatic relief, there is great need for therapies that slow the course of the disease and prevent or delay the disease in susceptible individuals.

The exact cause of Alzheimer's disease is unknown, but it has been suggested that a key event in Alzheimer's disease pathogenesis is the conversion of the amyloid

beta peptide (Aβ) from soluble to aggregated form, as well as protein deposition in tissues. This process is believed to give rise to amyloid plaques, which are characteristic for Alzheimer's disease. Aβ is predominantly found to be 39-43 amino acids in length, appearing most frequently in a 40 amino acid form and/or a 42 amino acid form. It is a normal, soluble product of proteolysis of the amyloid precursor protein (APP), and is generated in different body tissues and fluids (Neve RL *et al*, Brain Research 886:54-66 (2000)).

Among treatments for Alzheimer's disease are antibody-mediated chronic therapies, i e passive immunization schemes, wherein antibodies to e g the amyloid beta peptide are administered to the patient. Currently however, few antibodies have been approved for long-term treatment. Bard *et al*, Proc Natl Acad Sci USA 100:2023-2028 (2003), is a publication of a recent study of antibodies directed against Aβ. This article provides an overview of the current state of this technology, and the references cited therein can be consulted for further information on passive immunization. As an example of antibodies for use as therapeutic agents against Alzheimer's disease, F Hoffmann - La Roche and MorphoSys AG have developed phage display-derived human antibodies against Aβ, that bind to human amyloid plaques *in vitro* and *in vivo* in a mouse model of Alzheimer's disease. The antibodies were selected from a synthetic human combinatorial antibody library based on phage display (poster by Bohrmann B *et al*, IBC 14th Annual International Conference on Antibody Engineering (30 Nov - 3 Dec 2003) San Diego, CA, USA). Drawbacks of the approach of passive immunization with antibodies include the facts that only a transient effect is achieved, that the antibodies used are large proteins meaning they are problematic to administrate, and that the cost of such transient treatments with protein pharmaceuticals is exceedingly high.

Another method for treatment of Alzheimer's disease is through active immunization, wherein Aβ itself is administered to a subject, leading to the formation of endogenous antibodies towards Aβ. Recent clinical studies conducted by Elan Corporation and Wyeth, however, have given rise to signs of central neuroinflammation and were therefore discontinued in the exploratory phase IIA study. At the moment, it is unclear whether it is possible to develop immunization treatments in Alzheimer's disease without similar harmful side effects. A representative, recent publication on the approach of active immunization is Hock *et al*, Neuron 38:547-554 (2003).

There is evidence for a high significant correlation between plasma levels and brain levels of Aβ. Hence, an active lowering of the levels of Aβ in plasma may initiate a net efflux of Aβ from the brain into the periphery, so that this lowering would act as a peripheral sink (DeMattos RB *et al*, Science 295:2264 (2002)). Furthermore, it has been suggested that this lowering of the Aβ levels in the brain of Alzheimer's disease patients could be achieved via extracorporeal removal of plasma Aβ through immunoabsorption using Aβ-specific antibodies (see e g a project proposal by Germino K, Northwestern University, Chicago, IL, USA, available at least from 9 Dec 2003 to 5 Feb 2004, on http://mstp.northwestern.edu/M2JC_2003/Germino_Proposal.pdf). The significance of extracorporeal removal of Aβ peptides is supported by the studies on active immunization performed in humans (e g Hock *et al*, *supra*). During these trials, it was observed that Aβ immunization generated antibodies that were able to bind Aβ peptides, locally as well as in the periphery, thereby reducing the aggregation of the Aβ peptides and as such neutralizing their pathogenicity. Extracorporeal removal of Aβ in a body fluid of a subject have the advantage to other treatments of Alzheimer's disease of being non-invasive, hence also non-toxic to the subject.

Whether the object is to find a novel therapeutic strategy for treating amyloid diseases, to establish novel methods for the separation of Aβ from other constituents in a sample, or some other application relying on Aβ binding, the provision of molecules having a binding affinity for the amyloid beta peptide is critical. Previously known antibodies and antibody related derivatives against Aβ are not always the optimal choice, due to the complexity of the antibody molecule. Thus, there is a continued need for novel and alternative molecules with a high affinity for Aβ, which can be used as reagents in various assays and processes where such an affinity is needed.

Disclosure of the invention

It is an object of the present invention to meet this need through the provision of a polypeptide that exhibits specific binding to an amyloid beta peptide.

A related object of the invention is an amyloid beta peptide binding polypeptide which exhibits little or no non-specific binding.

It is another object of the invention to provide an amyloid beta peptide binding polypeptide that can readily be used as a moiety in a fusion polypeptide.

Another object is the provision of an amyloid beta peptide binding polypeptide, which does not exhibit the known problems of stability experienced with antibody reagents, but provides a stable and robust structure with the ability to withstand harsh environmental conditions.

Furthermore, it is an object to provide an amyloid beta peptide binding polypeptide, the properties of which enables easy coupling thereof to a matrix.

A related object is to provide an amyloid beta peptide binding polypeptide, which enables efficient separation of Aβ from other constituents of a sample. Preferably, such a polypeptide could also be used for reducing the content of Aβ in a body fluid of a human.

It is also an object to provide a molecule which can be used as a reagent for the detection of Aβ at a low detection limit.

A further object is to provide a novel medicament for the treatment of Alzheimer's disease.

These and other objects are met by the invention as claimed in the appended claims. Thus, in a first aspect, the invention provides an amyloid beta peptide binding polypeptide, which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having 1 to about 20 substitution mutations.

In accordance herewith, the present inventors have found that it is possible to obtain an amyloid beta peptide binding polypeptide through substitution mutagenesis of a domain from SPA. An embodiment of the polypeptide of the invention may have the ability to interact with Aβ with a K_D value of at most 5×10^{-6} M. Preferably, the polypeptide of the invention has the ability to interact with Aβ with a K_D value of at most 1×10^{-6} M. More preferably, the polypeptide of the invention has the ability to interact with Aβ with a K_D value of at most 9×10^{-7} M.

"An amyloid beta peptide" (abbreviated Aβ) refers to a peptide which may be from 1 to about 43 amino acids in length, but which is predominantly either 40 or 42 amino acids in length. As used herein, an amyloid beta peptide is a normal, soluble proteolytic product of the amyloid precursor protein (APP). In the experiments presented below for illustration, $A\beta_{40}$ and $A\beta_{42}$ refers to an amyloid beta peptide being 40 and 42 amino acids in length, respectively. These peptides are also sometimes referred to in the literature as $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively. For an overview of the processing of APP to Aβ peptides, see e g Golde TE *et al*, Science 255:728-730 (1992); Shoji M *et al*, Science 258:126-129 (1992); or Neve RL *et al*, *supra*.

"Binding affinity for an amyloid beta peptide" refers to a property of a polypeptide which may be tested e

g by the use of surface plasmon resonance technology, such as in a Biacore® instrument. Aβ binding affinity may be tested in an experiment wherein Aβ is immobilized on a sensor chip of the instrument, and a sample containing the polypeptide to be tested is passed over the chip. Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing Aβ is passed over the chip. The skilled person may then interpret the sensorgrams obtained to establish at least a qualitative measure of the polypeptide's binding affinity for the amyloid beta peptide. If a quantitative measure is sought, e g with the purpose to establish a certain K_D value for the interaction, it is again possible to use surface plasmon resonance methods. Binding values may e g be defined in a Biacore® 2000 instrument (Biacore AB). Aβ peptide is immobilized on a sensor chip of the instrument, and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected in random order. K_D values may then be calculated from the results, using e g the 1:1 Langmuir binding model of the BIAevaluation 3.2 software provided by the instrument manufacturer.

The polypeptide according to the invention may prove useful in any method relying on affinity for Aβ of a reagent. Thus, the polypeptide may be used as a detection reagent, a capture reagent or a separation reagent in such methods, but also as a therapeutic agent in its own right or as a means for targeting other therapeutic agents to the amyloid beta peptide. Methods that employ the polypeptide according to the invention *in vitro* may be performed in different formats, such as in microtiter plates, in protein arrays, on biosensor surfaces, on tissue sections, and so on. Different modifications of, and/or additions to, the polypeptide according to the invention may be performed in order to tailor the polypeptide to the specific use intended, without departing from the scope of the present invention. Such modifications

and additions are described in more detail below, and may comprise additional amino acids comprised in the same polypeptide chain, or labels and/or therapeutic agents that are chemically conjugated or otherwise bound to the polypeptide according to the invention. Furthermore, the invention also encompasses fragments of the polypeptide that retain the capability of binding to Aβ.

As stated above, the sequence of the polypeptide according to the present invention is related to the SPA domain sequence in that 1 to about 20 amino acid residues of said SPA domain have been substituted for other amino acid residues. However, the substitution mutations introduced should not affect the basic structure of the polypeptide. That is, the overall folding of the C_α backbone of the polypeptide of the invention will be substantially the same as that of the SPA domain to which it is related, e g having the same elements of secondary structure in the same order etc. Thus, polypeptides fall under the definition of having the same fold as the SPA domain if basic structural properties are shared, those properties e g resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant. This requirement of essentially conserving the basic structure of the SPA domain, upon mutation thereof, places restrictions on what positions of the domain may be subject to substitution. When starting from the known structure of the Z protein, for example, it is preferred that amino acid residues located on the surface of the Z protein may be substituted, whereas amino acid residues buried within the core of the Z protein "three-helix bundle" should be kept constant in order to preserve the structural properties of the molecule. The same reasoning applies to other SPA domains, and to the case when a fragment of an SPA domain is used.

The invention also encompasses polypeptides in which an Aβ binding polypeptide described above is present as an Aβ binding domain, to which additional amino acid

residues have been added at either terminal. These additional amino acid residues may play a role in the binding of Aβ by the polypeptide, but may equally well serve other purposes, related for example to one or more of the production, purification, stabilization, coupling or detection of the polypeptide. Such additional amino acid residues may comprise one or more amino acid residues added for purposes of chemical coupling, e g to a chromatographic resin to obtain an affinity matrix. An example of this is the addition of a cysteine residue at the very first or very last position in the polypeptide chain, i e at the N or C terminus. Such additional amino acid residues may also comprise a "tag" for purification or detection of the polypeptide, such as a hexahistidyl (His_6) tag, or a "myc" tag or a "flag" tag for interaction with antibodies specific to the tag. The skilled person is aware of other alternatives.

The "additional amino acid residues" discussed above may also constitute one or more polypeptide domain(s) with any desired function, such as the same binding function as the first, Aβ-binding domain, or another binding function, or an enzymatic function, or a fluorescent function, or mixtures thereof.

Thus, the invention encompasses multimers of the polypeptide with affinity for Aβ. It may be of interest, e g in a method of purification of Aβ, to obtain even stronger binding of Aβ than is possible with one polypeptide according to the invention. In this case, the provision of a multimer, such as a dimer, trimer or tetramer, of the polypeptide may provide the necessary avidity effects. The multimer may consist of a suitable number of polypeptides according to the invention. These polypeptide domains according to the invention, forming monomers in such a multimer, may all have the same amino acid sequence, but it is equally possible that they have different amino acid sequences. The linked polypeptide "units" in a multimer according to the invention may be connected

by covalent coupling using known organic chemistry methods, or expressed as one or more fusion polypeptides in a system for recombinant expression of polypeptides, or joined in any other fashion, directly or mediated by a linker comprising a number of amino acids.

Additionally, "heterogenic" fusion polypeptides, in which an Aβ binding polypeptide constitutes a first domain, or first moiety, and the second and further moieties have other functions than binding Aβ, are also contemplated and fall within the ambit of the present invention. The second and further moiety/moieties of the fusion polypeptide may comprise a binding domain with affinity for another target molecule than Aβ. Such a binding domain may well also be related to an SPA domain through substitution mutation at 1 to about 20 positions thereof. The result is then a fusion polypeptide having at least one Aβ-binding domain and at least one domain with affinity for said other target molecule, in which both domains are related to an SPA domain. This makes it possible to create multispecific reagents that may be used in several biotechnological applications. The preparation of such multispecific multimers of SPA domain related polypeptides, in which at least one polypeptide domain has affinity for Aβ, may be effected as described above for the multimer of several Aβ binding "units". In other alternatives, the second or further moiety or moieties may comprise an unrelated, naturally occurring or recombinant, protein (or a fragment thereof retaining the binding capability of the naturally occurring or recombinant protein) having a binding affinity for a target. An example of such a binding protein, which has an affinity for human serum albumin and may be used as fusion partner with an Aβ binding SPA domain derivative of the invention, is the albumin binding domain of streptococcal protein G (SPG) (Nygren P-Å *et al* (1988) Mol Recogn 1:69-74). A fusion polypeptide between an amyloid beta peptide binding, SPA domain-related polypeptide and the albumin

binding domain of SPG thus falls within the scope of the present invention. When the polypeptide according to the invention is administered to a human subject as a therapeutic agent or as a targeting agent, the fusion thereof to a moiety which binds serum albumin may prove beneficial, in that the half-life *in vivo* of such a fusion protein may likely prove to be prolonged as compared to the half-life of the SPA domain related, Aβ binding moiety in isolation (this principle has been described e g in WO91/01743).

Other possibilities for the creation of fusion polypeptides are also contemplated. Thus, an Aβ binding SPA domain-related polypeptide according to the first aspect of the invention may be covalently coupled to a second or further moiety or moieties, which in addition to or instead of target binding exhibit other functions. One example is a fusion between one or more Aβ binding polypeptide(s) and an enzymatically active polypeptide serving as a reporter or effector moiety. Examples of reporter enzymes, which may be coupled to the Aβ binding polypeptide to form a fusion protein, are known to the skilled person and include enzymes such as β-galactosidase, alkaline phosphatase, horseradish peroxidase, carboxypeptidase. Other options for the second and further moiety or moieties of a fusion polypeptide according to the invention include fluorescent polypeptides, such as green fluorescent protein, red fluorescent protein, luciferase and variants thereof.

In regard to the description above of fusion proteins incorporating Aβ binding polypeptide according to the invention, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between Aβ binding moiety or moieties on the one hand, and moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein.

Thus, for example, said first moiety may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein.

The invention also encompasses polypeptides in which an Aβ binding polypeptide as described above has been provided with a label group, such as at least one fluorophore, biotin or a radioactive isotope, for example for purposes of detection of the polypeptide.

As examples of SPA related domains that are useful as starting points for the creation of a polypeptide according to the invention may be mentioned the five domains of naturally occurring staphylococcal protein A, i e a domain selected from the E domain, the D domain, the A domain, the B domain and the C domain (see for example Uhlén *et al*, J Biol Chem 259:1695-1702 (1984), reporting the original cloning of SPA).

Another example of an SPA related domain for use as a starting point for the creation of a polypeptide according to the invention is protein Z, derived from domain B of staphylococcal protein A. As pointed out in the Background section, this protein has previously been used as a scaffold structure for the creation of molecules, denoted Affibody® molecules, capable of binding to a variety of targets. The 58 amino acid sequence of unmodified protein Z, denoted Z_{wt}, is set out in SEQ ID NO:1 and illustrated in Figure 1.

In an embodiment of the polypeptide according to the invention, it is related to a domain of SPA in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having 4 to about 20 substitution mutations. Other embodiments may have 1 to about 13 substitution mutations, or 4 to about 13 substitution mutations.

In a more specific embodiment of the polypeptide according to the invention, its sequence corresponds to the sequence set forth in SEQ ID NO:1 having 1 to about 20 substitution mutations, such as 4 to about 20, 1 to about 13 or 4 to about 13 substitution mutations.

The polypeptide according to the invention may in some embodiments correspond to the sequence set forth in SEQ ID NO:1, which sequence comprises substitution mutations at one or more of the positions 17, 18, 24, 27, 28 and 35. Additionally, the sequence of the polypeptide according to the invention may comprise substitution mutations at one or more of the positions 10, 14, 25 and 32 of the sequence of SPA protein Z in SEQ ID NO:1. The sequence may furthermore comprise substitution mutations at one or more of the positions 9, 11 and 13 of the sequence of SPA protein Z in SEQ ID NO:1.

For example in an amyloid beta binding polypeptide in accordance with the invention which is related to protein Z, the amino acid at position 3 corresponds to the amino acid at position 3 in the original (or "wild-type") sequence of protein Z shown in SEQ ID NO:1 when the amyloid binding polypeptide has 58 amino acids, but, when the polypeptide has an additional 10 amino acid N terminal extension, the amino acid at position 13 of that polypeptide corresponds to the amino acid at position 3 of the protein Z sequence in SEQ ID NO:1.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 27 in SEQ ID NO:1 from arginine to leucine.

The sequence of a polypeptide according to yet another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 17 in SEQ ID NO:1 from leucine to valine.

The sequence of a polypeptide according to a further embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 24 in SEQ ID NO:1 from glutamic acid to alanine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 18 in SEQ ID NO:1 from histidine to an amino acid residue selected from tyrosine and phenylalanine, more preferably to tyrosine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 28 in SEQ ID NO:1 from asparagine to an amino acid residue selected from cysteine and serine.

In another embodiment of the invention, the sequence of the polypeptide corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 35 in SEQ ID NO:1 from lysine to an amino acid selected from glutamic acid and glutamine, more preferably from lysine to glutamic acid.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 10 in SEQ ID NO:1 from glutamine to glycine.

Moreover, the sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position corresponding to position 14 in SEQ ID NO:1 from tyrosine to an amino acid residue selected from glycine and proline.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising from 1 to about 20 substitution mutations, and having an acidic amino acid at a position corresponding to position 25 in SEQ ID NO:1.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at a position

corresponding to position 32 in SEQ ID NO:1 from glutamine to an amino acid residue selected from lysine, arginine and histidine, preferably selected from lysine and arginine.

Furthermore, the sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least one substitution mutation at a position corresponding to one of positions 13-14 and 24-25 in SEQ ID NO:1 from the amino acid residue in the sequence according to SEQ ID NO:1 to proline.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least the following mutations: L17V, H18Y, E24A and R27L.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least the following mutations: P20K, H18V, L17F and I16F.

A polypeptide according to another embodiment of the invention is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having from 1 to about 20 substitution mutations so that it contains the amino acid motif KLVFF.

Examples of specific sequences of polypeptides according to the invention, each comprising one or more of the specific mutations described above, are set out in SEQ ID NO:2-46 and illustrated in Figure 1. Aβ binding characteristics of these polypeptides, and of polypeptides in which these polypeptides are present as Aβ binding domains, are disclosed in the examples that follow.

Thus, as non limiting examples of the amyloid beta peptide binding polypeptides of the invention, the invention encompasses any amyloid beta peptide binding polypeptide, or any amyloid beta peptide binding domain, whose amino acid sequence fulfils one definition selected from the following:

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a) it is selected from SEQ ID NO:2-46;

b) it is an amino acid sequence having 85 % or greater identity to a sequence selected from SEQ ID NO:2-46;

As evident from this definition, in addition to a polypeptide whose amino acid sequence is selected from SEQ ID NO:2-46, the present invention also encompasses variants thereof. The amino acid sequences of such encompassed variants exhibit small differences only in comparison with SEQ ID NO:2-46. One definition of such variants is given in b) above, i e amyloid beta peptide binding polypeptide with an amino acid sequence having at least 85 % identity to a sequence selected from SEQ ID NO:2-46. In some embodiments of the invention, the amino acid sequence has at least 90 % identity, at least 95 % identity, or at least 98 % identity to a sequence selected from SEQ ID NO:2-46.

As discussed above, the polypeptide according to the invention may be present as a moiety or domain in a fusion protein, or be provided with a tag of additional amino acid residues. In the experimental section of the present disclosure, the amyloid beta peptide binding properties of several such constructs are tested. The polypeptides described are all included in the scope of the present invention.

Thus, the invention encompasses an amyloid beta peptide binding moiety fused to an albumin binding domain, wherein the amino acid sequence of the expressed product corresponds to a sequence selected from SEQ ID NO:47-65 (see Example 2 and Figure 3). The sequences SEQ ID NO:47-65 may also be represented as:

Ala-Gln-His-Asp-Glu-Ala-Leu-Glu-[$Z_{A\beta}$]-Val-Asp-Tyr-[ABD]

wherein [$Z_{A\beta}$] is a sequence selected from SEQ ID NO:6, 7, 12, 13, 15, 19, 24-28, 31 and 38-44 and [ABD] is the al-

bumin binding domain from streptococcal protein G (Nygren P-Å *et al* (1988) Mol Recogn 1:69-74).

Furthermore, the invention encompasses an amyloid beta peptide binding moiety fused to a His_6 tag, wherein the amino acid sequence of the expressed product corresponds to a sequence selected from SEQ ID NO:66-75 (see Example 3 and Figure 5). The sequences SEQ ID NO:66-75 may also be represented as:

Met-Gly-Ser-Ser-His-His-His-His-His-His-Tyr-Tyr-Leu-Glu-[$Z_{A\beta}$]-Val-Asp

wherein [$Z_{A\beta}$] is a sequence selected from SEQ ID NO:6, 12, 13, 15, 19, 38 and 41-44.

Furthermore, the invention encompasses a dimer of two amyloid beta peptide binding moieties fused to a His_6 tag and a "myc" tag, wherein the amino acid sequence of the expressed product corresponds to a sequence selected from SEQ ID NO:76-81 (see Example 4 and Figure 7). The sequences SEQ ID NO:76-81 may also be represented as:

Met-Gly-Ser-Ser-His-His-His-His-His-His-Tyr-Tyr-Leu-Glu-[$Z_{A\beta}$]-Val-Asp-[$Z_{A\beta}$]-Val-Asp-Glu-Gln-Lys-Leu-Ile-Ser-Gln-Gln-Asp-Leu

wherein [$Z_{A\beta}$] is a sequence selected from SEQ ID NO:6, 12, 13, 15, 42 and 43.

As an alternative to using an unmodified SPA domain as a starting point, the SPA domain may also be subjected to mutagenesis in order to increase the stability thereof in alkaline conditions. Such stabilization involves the site-directed substitution of any asparagine residues appearing in the unmodified sequence with amino acid residues that are less sensitive to alkaline conditions. When using the polypeptide according to the invention as an affinity ligand in affinity chromatography, this property

of having a reduced sensitivity to alkali provides benefits; affinity chromatography columns are frequently subjected to harsh alkali treatment for cleaning in place (CIP) between separation runs, and the ability to withstand such treatment prolongs the useful lifetime of the affinity chromatography matrix. As an example, making use of protein Z as starting point, the polypeptide according to the invention may, in addition to the substitution mutations conferring Aβ binding, have modifications in that at least one asparagine residue selected from N3, N6, N11, N21, N23, N28, N43 and N52 has been substituted with an amino acid residue that is less sensitive to alkaline treatment. Non-limiting examples of such polypeptides are those having the following sets of mutations (with respect to the sequence of Z_{wt}): N3A; N6D; N3A, N6D and N23T; N3A, N6D, N23T and N28A; N23T; N23T and N43E; N28A; N6A; N11S; N11S and N23T; N6A and N23T. Thus, these SPA domains, as well as other SPA domains that have been subjected to asparagine mutation for stability reasons, may all be subjected to further substitution mutation of amino acid residues in order to obtain an Aβ binding polypeptide of the invention. Alternatively, an Aβ binding polypeptide of the invention which comprises asparagine residues may be subjected to further mutation to replace such residues. Evidently, this latter alternative is only possible to the extent that Aβ binding capability of such a molecule is retained.

The invention also encompasses polypeptides that have been derived from any of the polypeptides described above through generation of a fragment of the above polypeptides, which fragment retains amyloid beta peptide affinity. The fragment polypeptide is such that it remains stable, and retains the specificity to bind Aβ. The possibility to create fragments of a wild-type SPA domain with retained binding specificity to immunoglobulin G is shown by Braisted AC and Wells JA in Proc Natl Acad Sci USA 93:5688-5692 (1996). By using a structure-based de-

sign and phage display methods, the binding domain of a three-helix bundle of 59 residues was reduced to a resulting two-helix derivative of 33 residues. This was achieved by stepwise selection of random mutations from different regions, which caused the stability and binding affinity to be iteratively improved. Following the same reasoning with the polypeptides according to the first aspect of the invention, the skilled man would be able to obtain a "minimized" amyloid beta peptide binding polypeptide with the same binding properties as that of the "parent" amyloid beta peptide binding polypeptide. Hence, a polypeptide constituting a fragment of a polypeptide according to the above aspect of the invention, which fragment retains binding affinity for an amyloid beta peptide, is a further aspect of the invention.

Another aspect of the present invention relates to a nucleic acid molecule comprising a sequence which encodes a polypeptide according to the invention.

A further aspect of the present invention relates to an expression vector comprising the nucleic acid molecule of the previous aspect, and other nucleic acid elements that enable production of the polypeptide according to the invention through expression of the nucleic acid molecule.

Yet another aspect of the present invention relates to a host cell comprising the expression vector of the previous aspect.

The latter three aspects of the invention are tools for the production of a polypeptide according to the invention, and the skilled person will be able to obtain them and put them into practical use without undue burden, given the information herein concerning the polypeptide that is to be expressed and given the current level of skill in the art of recombinant expression of proteins. As an example, a plasmid for the expression of unmodified protein Z (see e g Nilsson B *et al* (1987), *supra*) may be used as starting material. The desired sub-

stitution mutations may be introduced into this plasmid, using known techniques, to obtain an expression vector in accordance with the invention.

However, the polypeptide according to the invention may also be produced by other known means, including chemical synthesis or expression in different prokaryotic or eukaryotic hosts, including plants and transgenic animals. When using chemical polypeptide synthesis, any of the naturally occurring amino acid residues in the polypeptide as described above may be replaced with any corresponding, non-naturally occurring amino acid residue or derivative thereof, to the extent that the Aβ binding capacity of the polypeptide is not substantially affected. Such non-classical amino acids, or synthetic amino acid analogs, include, but are not limited to, the D-isomers of the common amino acids, α-amino isobutyric acid, 4-amino butyric acid, 2-amino butyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoroamino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid residues can be present in D or L form.

The present invention also concerns different aspects of using the above-described Aβ binding polypeptide, as well as various methods for treatment, diagnosis and detection in which the polypeptide is useful due to its binding characteristics. When referring to the "Aβ binding polypeptide" in the following description of these uses and methods, this term is intended to encompass the Aβ binding polypeptide alone, but also all those molecules based on this polypeptide described above that e g constitute fragments thereof and/or incorporate the

Aβ binding polypeptide as a moiety in a fusion protein and/or are conjugated to a label or therapeutic agent and/or are provided with additional amino acid residues as a tag or for other purposes. As explained above, such fusion proteins, derivatives, fragments etc form a part of the present invention.

According to another aspect of the present invention, a method of separation, removal and/or purification of Aβ is provided. The method comprises a step of affinity separation, in which step a polypeptide according to the first aspect of the invention is used. Thus, the invention provides the use of the polypeptide as described above in a method of affinity separation. Suitably, the method involves a separation device, such as chosen among chromatographic media, membranes, cellulose, silica, agarose, polyacrylamide, magnetic beads, two-phase systems and other such materials commonly used in separation. In an embodiment, the polypeptide according to the invention is coupled to the separation device. The thus obtained separation device, having polypeptide according to the invention coupled thereto, is referred to as an affinity matrix.

For the purposes of purification of Aβ from a sample, the sample containing Aβ to be purified is suitably applied to such an affinity matrix under conditions that are conducive to binding of Aβ to the matrix. Thereafter, the affinity matrix is washed under conditions such that the binding of Aβ to the matrix is maintained, but most, ideally all, other proteins and contaminants bound to the matrix are washed away. In an elution step, the matrix is treated such that Aβ is released from the matrix in an Aβ enriched fraction denoted "Aβ fraction", which may be recovered.

If, conversely, the purpose of the separation is the removal of Aβ, essentially the same steps as above are suitably followed, with some exceptions. The sample containing Aβ to be removed is suitably applied to an affin-

ity matrix under conditions that are conducive to binding of Aβ to the matrix. Thereafter, the affinity matrix is washed under conditions such that the binding of Aβ to the matrix is maintained, but most, ideally all, other proteins are recovered in the flow-through, thus obtaining a "depleted fraction" with a substantial reduction in Aβ content, which is recovered. Thus, the non-Aβ constituents of the sample, that were discarded in the purification method above, may instead be retained and used and/or processed further.

Another method of the invention, also performed with the purpose of removing Aβ from a sample but with the added requirement that the "depleted fraction" should not contain any substances or solvents not present in the original sample, comprises a similar sequence of steps. The sample containing Aβ to be removed is brought to interact with the affinity matrix under conditions that are conducive to binding of Aβ to the matrix, and subsequently recovered. This yields a sample ("depleted fraction") with a substantial reduction in Aβ content, which furthermore is essentially free from anything that was not present in the sample before application thereof to the affinity matrix.

As a further alternative of the inventive method, both the "depleted fraction" and the "Aβ fraction" may be recovered from the same separation run. Then, once again, the sample containing Aβ is suitably applied to an affinity matrix under conditions that are conducive to binding of Aβ to the matrix. Thereafter, the affinity matrix is washed, under conditions such that the binding of Aβ to the matrix is maintained, but most, ideally all, other proteins are recovered in the flow-through. The thus obtained "depleted fraction" with a substantial reduction in Aβ content is recovered. In an elution step, the matrix is treated such that Aβ is released from the matrix in an Aβ enriched fraction denoted "Aβ fraction", which is recovered.

Another related aspect of the invention is a method for reducing the content of Aβ in a portion of a body fluid of a human, comprising the steps to: a) provide a portion of a body fluid from a human; b) apply the portion to an affinity matrix comprising an amyloid beta peptide binding polypeptide as described herein, under conditions enabling binding of the Aβ to the affinity matrix, thereby causing a reduction of the content of Aβ in the portion of body fluid; and c) return at least a part of said portion of body fluid to said human. The method according to this aspect of the invention may be directed to reducing the content of Aβ in a body fluid of a subject afflicted by Alzheimer's disease, whereby the symptoms of Alzheimer's disease are alleviated by performing the method. The body fluid may for example be whole blood, plasma or serum. Hence, by using the method according to the invention, subjects afflicted by Alzheimer's disease could be treated by extracorporeal removal of Aβ. The skilled person with experience in the art of extracorporeal devices, e g for immunoadsorption, could use this method with the inventive affinity matrix for treatment of a subject afflicted with Alzheimer's disease, by extracorporeal removal of Aβ from for example a sample of blood from said subject. Affinity adsorption treatment of humans is described in many previous publications, *inter alia* in US patents US5753227, US6264623 and US6676622, all to Strahilevitz M.

In a further aspect, the invention is directed to an affinity matrix comprising an amyloid beta binding polypeptide according to the invention as described above.

Yet another aspect of the present invention is constituted by the use of an Aβ binding polypeptide as described herein in a method for detecting Aβ in a biological fluid sample. This method comprises the steps of (i) providing a biological fluid sample from a patient to be tested, for example a blood plasma sample for the measurement of plasma Aβ levels, (ii) applying an Aβ binding

polypeptide as described herein to the sample under conditions such that binding of the polypeptide to any Aβ present in the sample is enabled, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide. The amount of the detected bound polypeptide is correlated to the amount of Aβ present in the sample. In step (ii), the application of Aβ binding polypeptide to the sample may be performed in any suitable format, and includes for example the situation when Aβ binding polypeptide is immobilized on a solid support with which the sample is brought into contact, as well as set-ups in which Aβ binding polypeptide is present in solution. The method according to this aspect of the invention may suitably be performed in a standard 96-well format, in analogy to existing ELISA tests. As a preferred alternative, the polypeptide according to the invention is used as one or more reagent(s) in a sandwich assay, whereas a monoclonal or polyclonal antibody directed against Aβ may be used as other reagents. A sandwich assay using the SPA domain derived Aβ binding molecule as either capture or detection agent shows several advantages compared to using conventional antibody reagents for both capture and detection. One specific such advantage is the elimination of false positive results in the absence of Aβ, which false positives are due to crosslinking between capture and detection antibodies by for example heterophilic anti-animal Ig antibodies (HAIA).

As an additional aspect, the invention provides the use of an Aβ binding polypeptide as described herein in a method of detection of Aβ in tissue samples. This method comprises the steps of (i) providing a tissue sample suspected of containing Aβ, (ii) applying an Aβ binding polypeptide according to the invention to said sample under conditions conducive for binding of the polypeptide to any Aβ present in the sample, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide. The

amount of the detected bound polypeptide is correlated to the amount of Aβ present in the sample.

Another aspect of the present invention is the use of an amyloid beta peptide binding polypeptide as described herein as a medicament.

In addition, the invention provides the use of an amyloid beta peptide binding polypeptide as described herein in the preparation of a medicament for the treatment of a disease characterized by an over-representation of Aβ. A method for treatment of a disease characterized by an over-representation of Aβ, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a composition comprising an amyloid beta peptide binding polypeptide as described herein is also provided.

A particular such disease, characterized by an over-representation of Aβ, is Alzheimer's disease. Thus, the use of the polypeptide in the preparation of a medicament for the treatment of Alzheimer's disease is an embodiment of the invention. Another embodiment is a method for treatment of Alzheimer's disease, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a composition comprising an amyloid beta peptide binding polypeptide as described herein.

In analogy to the approach of passive immunization with Aβ antibodies, the administration of a polypeptide according to the invention to a subject afflicted with a disease characterized by an over-representation of Aβ would be likely to bring about a lowering of the concentration of free and circulating Aβ peptide in the subject. This, in turn, would also reduce the amount of insoluble Aβ in the body, because of the shift in equilibrium between soluble and aggregated forms of the amyloid beta peptide. Lowering the amounts of Aβ in this fashion serves to reduce, and perhaps entirely eliminate, the very source of the disease itself (DeMattos RB *et al*,

Proc Natl Acad Sci USA 98:8850 (2001); DeMattos RB *et al*, Science (2002), *supra*). One advantage of the present invention in this regard, which is shown in Example 5, is that the Aβ binding polypeptides according to the present invention do not appear bind to the Amyloid Precursor Protein (APP) on cell surfaces. This is an important feature to consider in using the Aβ binding polypeptides as a medicament.

Brief description of the drawings

Figure 1 shows an alignment of the sequences of the sequence listing. The amino acid positions that have been subjected to modification in the polypeptides $Z_{A\beta}$ according to the invention (represented by SEQ ID NO:2-46) are indicated in bold.

Figure 2A and 2B is a diagram of the A_{405} signals for Aβ binding by the obtained $Z_{A\beta}$ polypeptides, in an ELISA experiment. Molecules positive for binding to $A\beta_{40}$ are colored green (pale gray) and the background binding is colored brown (dark gray).

Figure 3 is a schematic illustration of the amino acid sequence of a fusion polypeptide according to the invention. $Z_{A\beta}$ represents an Aβ binding domain with a sequence selected from the sequences of $Z_{A\beta 1-20}$ and ABD represents the albumin binding domain of streptococcal protein G.

Figure 4 shows Biacore sensorgrams obtained after injection of the indicated $Z_{A\beta}$-ABD fusion proteins over sensor chip surfaces having $A\beta_{40}$ or $A\beta_{42}$ immobilized thereto. A: Binding of $Z_{A\beta\ 1-10}$-ABD to $A\beta_{42}$; B: Binding of $Z_{A\beta\ 11-20}$-ABD to $A\beta_{42}$; C: Binding of $Z_{A\beta\ 1-10}$-ABD to $A\beta_{40}$; D: Binding of $Z_{A\beta\ 11-20}$-ABD to $A\beta_{40}$.

Figure 5 is a schematic illustration of the amino acid sequences of a tagged polypeptide according to the invention. His_6 represents a hexahistidyl tag and $Z_{A\beta}$

represents an Aβ binding domain with a sequence selected from the sequence of $Z_{Aβ\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$.

Figure 6 shows Biacore sensorgrams obtained after duplicated injections of different concentrations of the indicated His_6-$Z_{Aβ}$ fusion proteins over sensor chip surfaces having $Aβ_{42}$ immobilized thereto. A: His_6-$Z_{Aβ\ 1}$; B: His_6-$Z_{Aβ\ 3}$; C: His_6-$Z_{Aβ\ 4}$; D: His_6-$Z_{Aβ\ 5}$; E: His_6-$Z_{Aβ\ 12}$; F: His_6-$Z_{Aβ\ 16}$; G: His_6-$Z_{Aβ\ 17}$; H: His_6-$Z_{Aβ\ 18}$; I: His_6-$Z_{Aβ\ 19}$; J: His_6-$Z_{Aβ\ 20}$.

Figure 7 is a schematic illustration of the amino acid sequences of the polypeptide according to the invention in a tagged dimeric construct. His_6 represents a hexahistidyl tag and $Z_{Aβ}$ represents an Aβ binding domain with a sequence selected from the sequence of $Z_{Aβ\ 1,\ 3,\ 4,\ 12,\ 18,\ 19}$. Myc represents a myc-tag.

Figure 8 shows Biacore sensorgrams obtained after injection of the indicated His_6-$(Z_{Aβ})_2$-myc fusion proteins over sensor chip surfaces having $Aβ_{42}$ immobilized thereto.

Figure 9 shows SDS-PAGE analysis of fractions from affinity chromatography of $Aβ_{42}$-peptide using His_6-$(Z_{Aβ1,\ 3,\ 4,\ or\ 12})_2$-myc columns on silver-stained NuPAGE® gel (12 %). 1, 3, 4, and 12 in the figure indicate gels with samples originating from the different His_6-$(Z_{Aβ1,\ 3,\ 4,\ or\ 12})_2$-myc columns respectively. Gels with samples from the experiments with PBS supplemented with $Aβ_{42}$ are marked with A and gels with samples from the $Aβ_{42}$-spiked plasma experiment are marked with B. Lane 1: Molecular weight marker; Lane 2: $Aβ_{42}$ and HSA reference (1 µg); Lane 3 Flow-through fraction; Lane 4-6: Fractions after washing with 0.3 M HAc pH 3.5; Lane 7-10: Fractions after elution with 0.3 M HAc pH 2.8; Lane 11: Fraction after regeneration of the columns with 0.3 M HAc pH 2.8; Lane 12: Fraction after column re-equilibration using PBS.

Figure 10 shows immunofluorescence staining on APP^+ human neuroblastoma SH-SY5Y cells. A and B show SH-SY5Y cells stained with goat-α-APP antibodies. Cells were fixed after the first step incubation and then stained in

the absence (A) or presence (B) of saponin. C and D show SH-SY5Y cells stained with the Aβ-specific polypeptides in the absence (C) or presence (D) of saponin.

Figure 11 shows SDS-PAGE analysis of fractions from affinity chromatography using His_6-$(Z_{A\beta3\ C28S})_2$-Cys columns of (A) 1 ml serum spiked with 100 µg $A\beta_{42}$ peptide and (B) 1 ml unspiked serum.

Figure 12 shows SDS-PAGE analysis of fractions from affinity chromatography using His_6-$(Z_{A\beta3\ C28S})_2$-Cys columns of 1 ml serum spiked with 100 ng $A\beta_{42}$ peptide.

Figure 13 shows the experimental setup of the *in vitro* assay described in Example 7.

The invention will now be illustrated further through the non-limiting recital of experiments conducted in accordance therewith. In these experiments, several Aβ binding polypeptides according to the invention were selected from a library of a multitude of different SPA domain related polypeptides, and subsequently characterized, and used in chromatography and cellular studies.

Example 1
Selection and ELISA study of Aβ binding polypeptides

Library panning and clone selection

A combinatorial phage display library was prepared essentially as described in Nord K *et al* (1995, *supra*). The pool of this library which was used in the present study comprised 3.3 x 10^9 variants of protein Z (Affibody® molecules), with random amino acid residues at positions 9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32 and 35. Aβ-binding Affibody® molecules were selected in four panning cycles using human biotin-conjugated $A\beta_{40}$ as the target (Usbio (Biosite) A2275). After the four selection cycles, in total 384 clones were picked for phage Enzyme Linked ImmunoSorbent Assay (ELISA) in order to perform an analysis of their Aβ binding activity.

Phage ELISA for analysis of Aβ binding

Phages from the clones obtained after four rounds of selection were produced in 96 well plates, and an ELISA was used for screening for phages expressing $A\beta_{40}$-binding Z mutants. Single colonies were used to inoculate 500 µl TSB+YE medium (30.0 g Tryptic Soy Broth (Merck), 5.0 g yeast extract, water to a final volume of 1 l, autoclaved) supplemented with 2 % glucose and 100 µg/ml ampicillin in deep well 96 well plates and grown on a shaker overnight at 37 °C. 30 µl overnight culture was added to 720 µl TSB+YE medium supplemented with 100 µg/ml ampicillin in a new plate. After growing at 37 °C for 2 h, 100 µl TSB+YE medium containing 5 x 10^9 pfu helper phage M13K07 (New England Biolabs, #NO315S) were added to each well, and the plates were incubated without shaking at 37 °C for 30 minutes. 50 µl TSB+YE supplemented with 1.8 µM IPTG (isopropyl-β-D-thiogalactopyranosid), 450 µg/ml kanamycin and 100 µg/ml ampicillin were added to each well, and the plates were incubated on a shaker overnight at 30 °C. Cells were pelleted by centrifugation at 3000 rpm for 10 minutes and 700 µl of the supernatant was transferred to a new plate with 175 µl precipitation buffer (20 % PEG, 2,5 M NaCl). After 4 h of incubation on ice, the phages were centrifuged for 30 minutes at 4300 rpm. The pellet was dissolved in 300 µl PBS (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and stored at 4 °C until it was used in ELISA. 100 µl of 2 µg/ml of biotin-conjugated $A\beta_{40}$ in ELISA coating buffer (0.1 M $NaCO_3$, pH 9.5) was added to strepavidin coated microtiter plates (Nunc #236001), and incubated overnight at 4 °C. Blocking buffer (1 % skim milk powder in PBS) was added and incubated for 2 h at room temperature. 100 µl phage-containing supernatant and 50 µl blocking buffer were added. The plates were incubated for 1.5 h at room temperature. A polyclonal antibody (rabbit anti-M13, Abcam #ab6188) was diluted 1:1000 or 1:200 times in block-

ing buffer, and 100 µl were added to each well. The plate was incubated at room temperature for 1 h. A goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma #A-3687) was diluted 1:10000 in blocking buffer, after which 100 µl were added to each well and incubated for 1 h at room temperature. Developing solution was prepared by dissolving Sigma-104 substrate in a 1:1 mixture of water and 1 M diethanolamine, 5 mM $MgCl_2$, pH 9.8 (1 tablet/5 ml). Thereafter, 100 µl of the developing solution was added to each well. Wells were washed three times with PBS-T (PBS + 0.1 % Tween-20) before addition of each new reagent. 30 minutes after addition of substrate, the plates were read at A_{405} in an ELISA spectrophotometer (Basic Sunrise, Tecan). The results obtained by the ELISA analysis are illustrated in Figure 2.

Aβ binders were identified using a threshold criterion of an ELISA value of A_{405} above 0.2. The binders giving an ELISA signal above this value were selected for further DNA sequence analysis.

DNA sequence analysis

Sequencing of DNA encoding these $Z_{A\beta}$ variants was performed with ABI PRISM® dGTP, BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations, using the biotinylated oligonucleotides AFFI-71 (5'-biotin-TGCTTCCGGCTCGTATGTTGTGTG) and AFFI-72 (5'-biotin-CGGAACCAGAGCCACCACCGG). The sequences were analyzed on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems. A number of clones did not give readable sequences and identical phagemid inserts occurred between 1 to 38 times. Thus, the sequence analysis resulted in 43 unique sequences. The sequences of the $Z_{A\beta}$ variants expressed by the clones selected in the ELISA binding assay are given in Figure 1, and identified in the sequence listing as SEQ ID NO:2-44.

Example 2
Expression and characterization of Aβ binding ABD-fusion polypeptides

From the 44 phage clones identified in Example 1 as expressing Aβ binding Z variants, 20 were selected for further study, and denoted $Z_{Aβ1}$-$Z_{Aβ20}$. In the experiments of this Example, these polypeptides are collectively denoted $Z_{Aβ}$. All experiments were individually conducted with all 20.

Expression and purification of fusion polypeptides

Fusion polypeptides were expressed in *E. coli* RV308 cells (Maurer R *et al*, J Mol Biol 139 (1980), 147-161, ATCC #31608), by adapting the methods of Nilsson B *et al*, Eur J Biochem 224 (1994), 103-108 and using conventional molecular biology methods for cloning. The expression vector used encodes a fusion polypeptide as schematically illustrated in Figure 3, in which $Z_{Aβ}$ represents the different Aβ binding domains with the sequence of $Z_{Aβ1}$-$Z_{Aβ20}$ (see Figure 1), and ABD represents the albumin binding domain of streptococcal protein G. Colonies of transformed cells were used to inoculate 100 ml TSB+YE medium supplemented with 100 µg/ml ampicillin. The cultures were grown at 37 °C to an OD_{600} ≈ 0.7-1, followed by induction with a final concentration of 0.5 mM IPTG and incubation at room temperature overnight. The cells were harvested by centrifugation at 6000 *g* for 8 minutes and periplasmic proteins were released by sonication. Cell pellets were resuspended in 10 ml TST buffer (25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.05 % Tween-20, pH 8.0) and the cells were lysed by freezing and thawing the samples followed by sonication. Cell debris was removed by centrifugation at 6000 x *g* for 10 min and the supernatants were allowed to pass through a 0.45 µm filter.

The ABD-fusion polypeptides were purified using affinity chromatography on HSA-Sepharose (CNBr-activated

Sepharose 4FF, Amersham Biosciences #17-0981-03, with HSA, Pharmacia & Upjohn #818476-01/5). A HR 5/5 column (Amersham Biosciences) was packed with 1 ml HSA-Sepharose and connected on an ÄKTA™explorer 100 chromatography system (Amersham Biosciences). The column was equilibrated with TST buffer. Sterile filtered cell lysates were separately applied to the column at a flow rate of 0.5 ml/min. After washing with 12 ml TST buffer, proteins were eluted with 0.5 M HAc, pH 2.8. The flow rate was 1 ml/min during the wash and elution steps. Protein content in eluted fractions was determined spectrophotometrically using absorption at 280 nm, and relevant fractions were pooled. Protein concentration of pooled samples was calculated from the measured absorption value at 280 nm and the theoretical extinction coefficient of the respective protein (calculated by using VectorNTI). Protein preparations were analyzed on 10-15 % Phast gels® using the PhastSystem (Amersham Biosciences) under reducing conditions.

Biosensor analysis of fusion polypeptides

Binding of the purified fusion polypeptides to Aβ was analyzed using surface plasmon resonance in a Biacore® 2000 instrument (Biacore AB). $A\beta_{42}$ and biotinylated $A\beta_{40}$ were immobilized in different flow cells by amine coupling onto the carboxylated dextran layer on surfaces of CM-5 chips (research grade, Biacore AB), according to the manufacturer's recommendations. One cell surface on each chip was activated and deactivated for use as reference cell during injections. Immobilization of $A\beta_{42}$ and biotinylated $A\beta_{40}$ to CM-5 chip surfaces resulted in approximately 3200 and 1000 resonance units (RU), respectively. Samples of fusion polypeptides were diluted in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005 % surfactant P-20, pH 7.4) to a final concentration of 1 µM, and injected in random order as duplicates at a constant flow-rate of 10 µl/min for 5 minutes.

After each injection, the surfaces were regenerated with 50 mM NaOH. When injected sequentially over the surfaces in this manner, all 20 fusion polypeptides ($Z_{A\beta}$-ABD) exhibited binding to $A\beta_{42}$ and biotinylated $A\beta_{40}$ (Figures 4A-4D). Based on this experiment, the 10 different fusion polypeptides $Z_{A\beta\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$-ABD were selected for further, more detailed analysis of the Aβ binding kinetics. The main selection criteria were a fast "on rate", and a medium to slow "off rate" for the binding to Aβ.

Example 3
Expression and characterization of Aβ binding hexahistidyl fusion polypeptides

In this Example, the Aβ binding polypeptides $Z_{A\beta\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$ were studied further, and are sometimes collectively referred to as $Z_{A\beta}$. All experiments were performed with each of these Aβ binding Z variants.

Expression and purification

$Z_{A\beta}$ polypeptides were expressed in *E. coli* BL-21(DE3) cells (Novagen #69450-4), using expression vectors encoding constructs that are schematically illustrated in Figure 5. In the figure, His_6 represents a hexahistidyl tag, and $Z_{A\beta}$ represents any one of the Aβ binding domains corresponding to the sequences of $Z_{A\beta\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$. Expression was followed by purification by Immobilized Metal ion Affinity Chromatography (IMAC).

E. coli BL-21(DE3) cells harboring the expression plasmids were grown in 10 ml TSB medium supplemented with 50 µg/ml kanamycin in baffled shaker flasks at 200 rpm at 37 °C overnight. The following day the cultures were diluted 1000 times in 200 ml TSB+YE medium supplemented with 50 µg/ml kanamycin in 2 liter baffled shaker flasks. The cultures were grown at 37 °C to an $OD_{600} \approx 0.7$-1, and

production was induced by adding IPTG to a final concentration of 0.5 mM followed by incubation at room temperature overnight. Cultures were harvested by centrifugation at 6000 x *g* for 8 minutes and pellets were stored in the freezer until protein preparation. Cell pellets were thawed and resuspended in 10 ml IMAC binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 6 M guanidinium HCl, pH 8.0). The solutions were transferred to 50 ml Falcon tubes and cells were thereafter lysed by sonication. Cell debris was removed by centrifugation at 12000 x *g* for 8 min and supernatants were filtrated using a 0.45 µm filter. The produced proteins were isolated using IMAC as follows: 3 ml Talon Metal Affinity Resin (Clontech, #8901) for each protein was washed twice with IMAC binding buffer (the resin was recovered in each step by centrifugation at 700 x *g* for 2 minutes after addition of buffer). The supernatants were added to the washed resin and incubated with head-over-tail rotation for 1 h at room temperature. Unbound proteins were removed by washing twice with 30 ml IMAC binding buffer, and the resin was resuspended in 10 ml IMAC binding buffer and transferred to an empty PD-10 column (Amersham Biosciences). After washing with an additional volume of 20 ml IMAC binding buffer, proteins were eluted with 5 ml IMAC elution buffer (250 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 6 M guanidinium HCl, pH 8.0) in 0.5 ml fractions. Protein content in eluted fractions was determined spectrophotometrically at A_{280} using a Smartspec 3000 spectrophotometer, Bio-Rad, and relevant fractions were pooled. To perform buffer exchange, PD-10 columns (Amersham Biosciences) were equilibrated with PBS, and samples were passed over these according to the manufacturer's recommendations. Protein concentration was determined using absorption at A_{280} and the extinction coefficient of the respective $Z_{A\beta}$ polypeptide. The purity of the proteins was analyzed by SDS-PAGE on 20 % polyacrylamide gels under reducing (DTT) and non reducing conditions and stained

with Coomassie brilliant blue R-250, using the Phast™ system (Amersham Biosciences) according to the manufacturer's recommendations.

Biosensor analysis

The interactions between the produced Z variants and $A\beta_{42}$ were analyzed using surface plasmon resonance on a Biacore® 2000 system. $A\beta_{42}$ was immobilized in different flow cells by amine coupling onto the carboxylated dextran layer on surfaces of a CM-5 chip, according to the manufacturer's recommendations. Immobilization of $A\beta_{42}$ resulted in 470 resonance units (RU). One flow cell surface on each chip was activated and deactivated for use as reference cell during injections. Six or seven different concentrations were measured as duplicates using two-fold dilutions in HBS-EP, and injected in random order in duplicates at a constant flow-rate of 30 µl/minute. The ability of the purified His_6-$Z_{A\beta\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$ to interact with Aβ was confirmed, as illustrated by the sensorgrams of Figures 6A-6J.

Furthermore, affinity constant K_D values were determined for His_6-$Z_{A\beta\ 1,\ 3,\ 4,\ 5,\ 12,\ 16,\ 17,\ 18,\ 19,\ and\ 20}$. Six or seven different Aβ concentrations (10 µM - 0.156 µM) were prepared in HBS-EP and injected as duplicates at a flow-rate of 30 µl/minute. The total injection time was 5 minutes (association) followed by a wash during 10 minutes (dissociation). The surfaces were regenerated with 2 injections of 50 mM NaOH. The responses measured in reference cells (activated/deactivated surface) were subtracted from the response measured in the cells with immobilized $Z_{A\beta}$ polypeptides. K_D values were calculated using the 1:1 Langmuir binding model of the BIAevaluation 3.2 software (Biacore AB), and are presented in Table 1.

TABLE 1

Protein	K_D (nM)
His_6-$Z_{A\beta\ 1}$	100-500
His_6-$Z_{A\beta\ 3}$	700
His_6-$Z_{A\beta\ 4}$	900
His_6-$Z_{A\beta\ 5}$	500
His_6-$Z_{A\beta\ 12}$	300
His_6-$Z_{A\beta\ 16}$	2000
His_6-$Z_{A\beta\ 17}$	900
His_6-$Z_{A\beta\ 18}$	600
His_6-$Z_{A\beta\ 19}$	900
His_6-$Z_{A\beta\ 20}$	2900

Example 4

Expression and characterization of dimers of tagged Aβ binding polypeptides, and use thereof as capture ligands in affinity chromatography

In this Example, the Aβ binding polypeptides $Z_{A\beta1\ ,3,\ 4,\ 12,\ 18,\ 19}$ were further studied, except for the example regarding affinity chromatography, where $Z_{A\beta1\ ,3,\ 4,\ 12}$ were studied. In this experiment, the polypeptides are sometimes collectively referred to as $Z_{A\beta}$.

Expression and purification

$Z_{A\beta}$ polypeptides were expressed in *E. coli* BL21(DE3) (Novagen #69450-4), using expression vectors encoding dimeric His_6-$(Z_{A\beta1,\ 3,\ 4,\ 12,\ 18\ and\ 19})_2$-myc constructs schematically illustrated in Figure 7. In the figure, His_6 represents a hexahistidyl tag, and $Z_{A\beta}$ represents any of the Aβ binding domains corresponding to the sequences of $Z_{A\beta1,\ 3,\ 4,\ 12,\ 18,\ 19}$. A "myc" tag was added to the end (amino acid sequence EQKLISEEDL). The cells harboring the expression

plasmid were separately inoculated in 10 ml TSB medium supplemented with 50 µg/ml kanamycin. The cultures were grown overnight at 200 rpm and 37 °C. The following day 500 µl overnight culture were inoculated with 500 ml TSB+YE medium supplemented with 50 µg/ml kanamycin in 5 liter baffled shaker flasks. The cultures were grown at 37 °C to an $OD_{600} \approx 0.7$-1, followed by addition of IPTG to a final concentration of 0.5 mM and incubated at room temperature overnight. Each culture was divided into two samples and harvested by centrifugation at 6000 x *g* for 8 min. The pellets were stored in the freezer until protein preparation. A pellet of each protein construct was thawed and resuspended in 35 ml IMAC binding buffer containing 6 M guanidinium HCl. The solutions were transferred to 50 ml Falcon tubes and sonicated on ice. Cell debris was removed by centrifugation at 12000 x *g* for 8 min and the supernatants were filtrated using a 0.45 µm filter.

The ÄKTA™ 3D Kit together with the ÄKTA™explorer 100 chromatography system (Amersham Biosciences) was used for purification of His_6-$(Z_{Aβ1, 3, 4, 12, 18 and 19})_2$-myc fusion proteins. Six samples could be purified on IMAC-columns in a single run followed by subsequent desalting on a HiPrep 26/10 desalting column (Amersham Biosciences). The IMAC columns consisted of HiTrap® Chelating HP columns prepacked with 5 ml Chelating Sepharose™ High Performance charged with Ni^{2+}. The desalting column was equilibrated with PBS manually before starting the purification run. Protocols supported by the ÄKTA™ 3D Kit were used for the automatic two-step purification as well as for metal ion charging and stripping of the HiTrap® Chelating HP columns. The crude samples of His_6-$(Z_{Aβ1 , 3, 4, 12, 18, 19})_2$-myc were added to appropriate vials and subsequently loaded on the ÄKTA™explorer. Automatic purification of the samples included pre-equilibration of HiTrap® columns with IMAC binding buffer, sample loading, sample wash with IMAC binding buffer, elution by IMAC elution buffer onto