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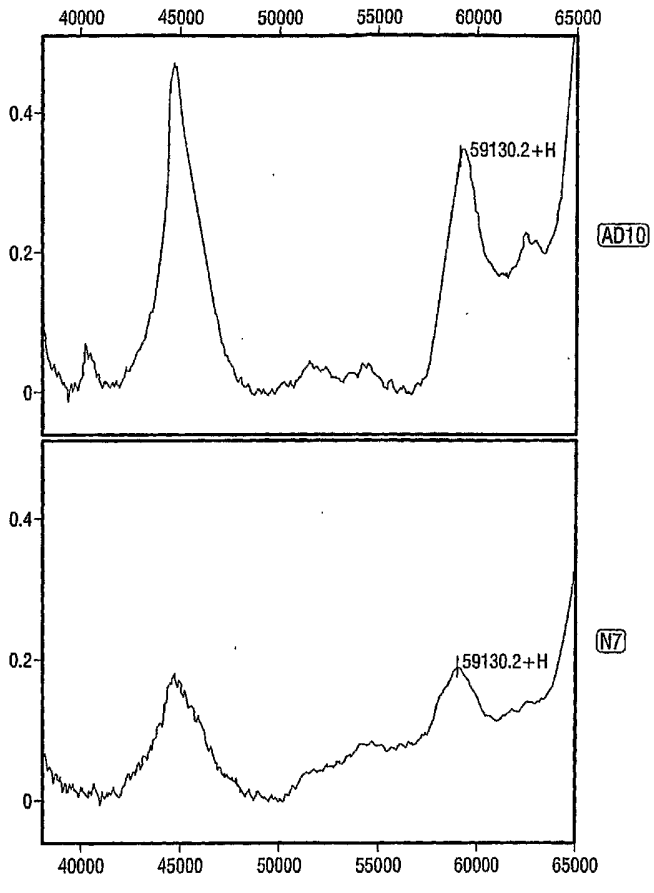

(57) Abstract: The present invention provides protein-based biomarkers and biomarker combinations that are useful in qualifying Alzheimer's disease status in a patient. In particular, the biomarkers of this invention are useful to classify a subject sample as Alzheimer's or non-Alzheimer's dementia or 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

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

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

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

(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).

[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

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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[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,

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.

[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.

[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

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.

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^{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. 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

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^{tm} 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

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.

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

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,

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

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.

[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.

[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

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.

[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

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

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

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.

[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.

[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

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

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

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

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

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.

[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.

[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

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

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

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

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.

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)

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°

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

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

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

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™ 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

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

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

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

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.

[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

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

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

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.

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

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.

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.

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.

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

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	

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

(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

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

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

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.

[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

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

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.

[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.

[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)

	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.

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.

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,

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).

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.

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.

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:

(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-

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)),

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).

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.

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.

68. A method for qualifying Alzheimer's diseases status in a subject, comprising

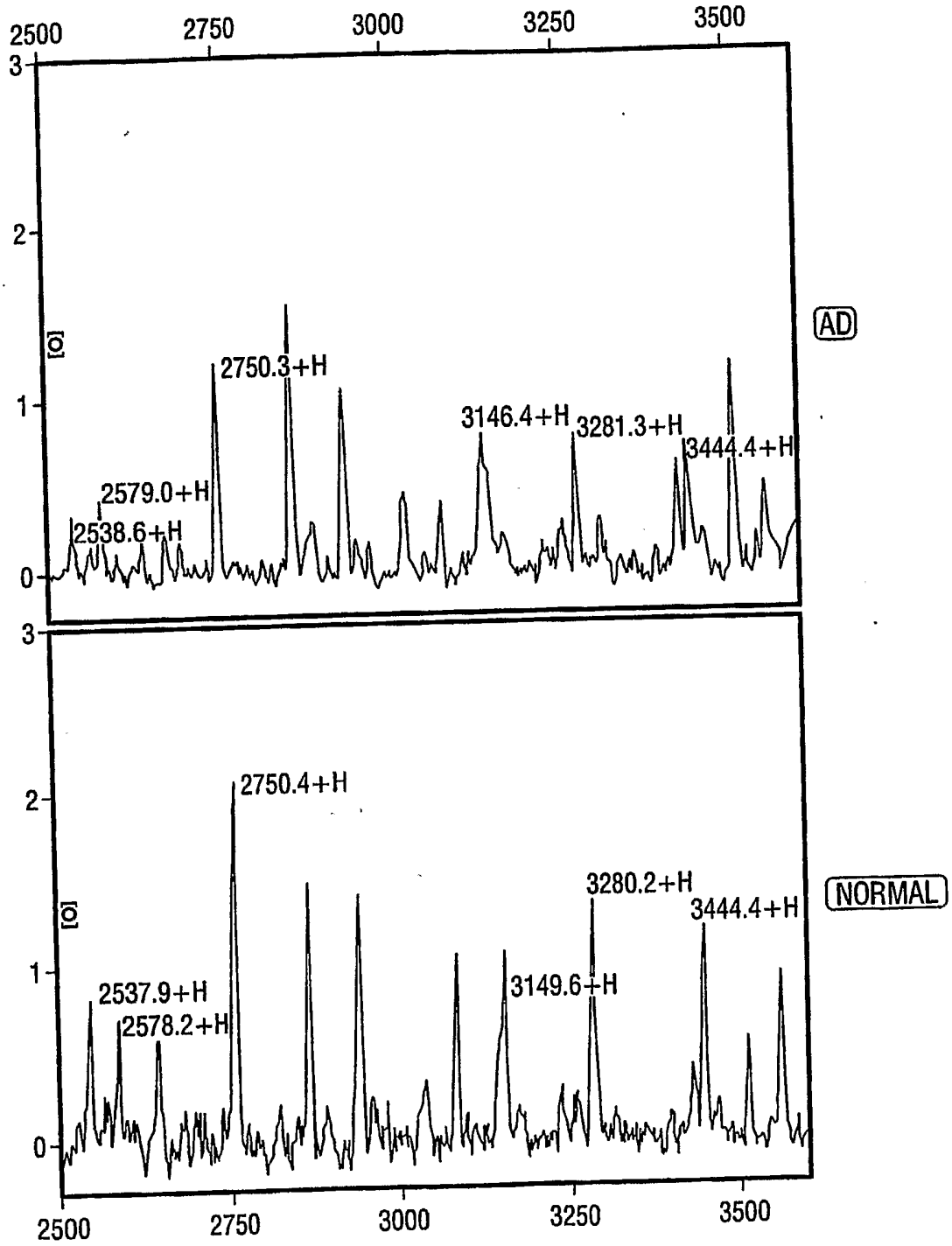
a. detectably labeling a biomarker selected from the group consisting of M60464.7 (Hemopexin), M3513.9 (7B2 CT fragment), M8291.0 (Ubiquitin -3aa from CT), M5044.2, M10379.8 (10.3 kDa), M9984.6 (related to 10.3 kDa), M10265.6 (related to 10.3 kDa), M9802.4 (EA-92 (ChrA peptide)), 9757.0 (related to 10.3 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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2538.6+H
AD
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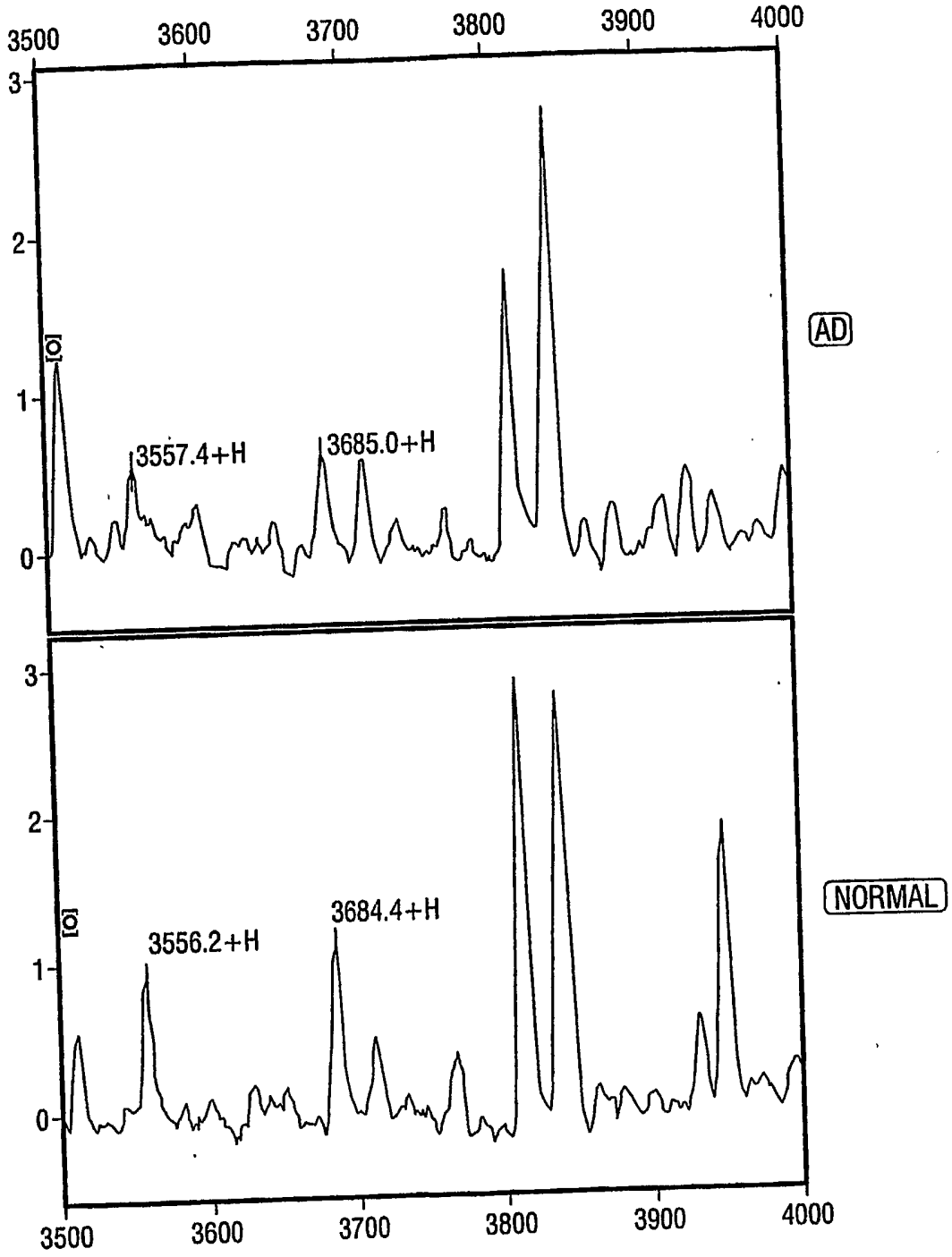
2537.9+H
2578.2+H
3149.6+H
NORMAL

FIG. 1A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3700
3800
3900
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3
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1
0
[o]
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AD
3
2
1
0
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3556.2+H
3684.4+H
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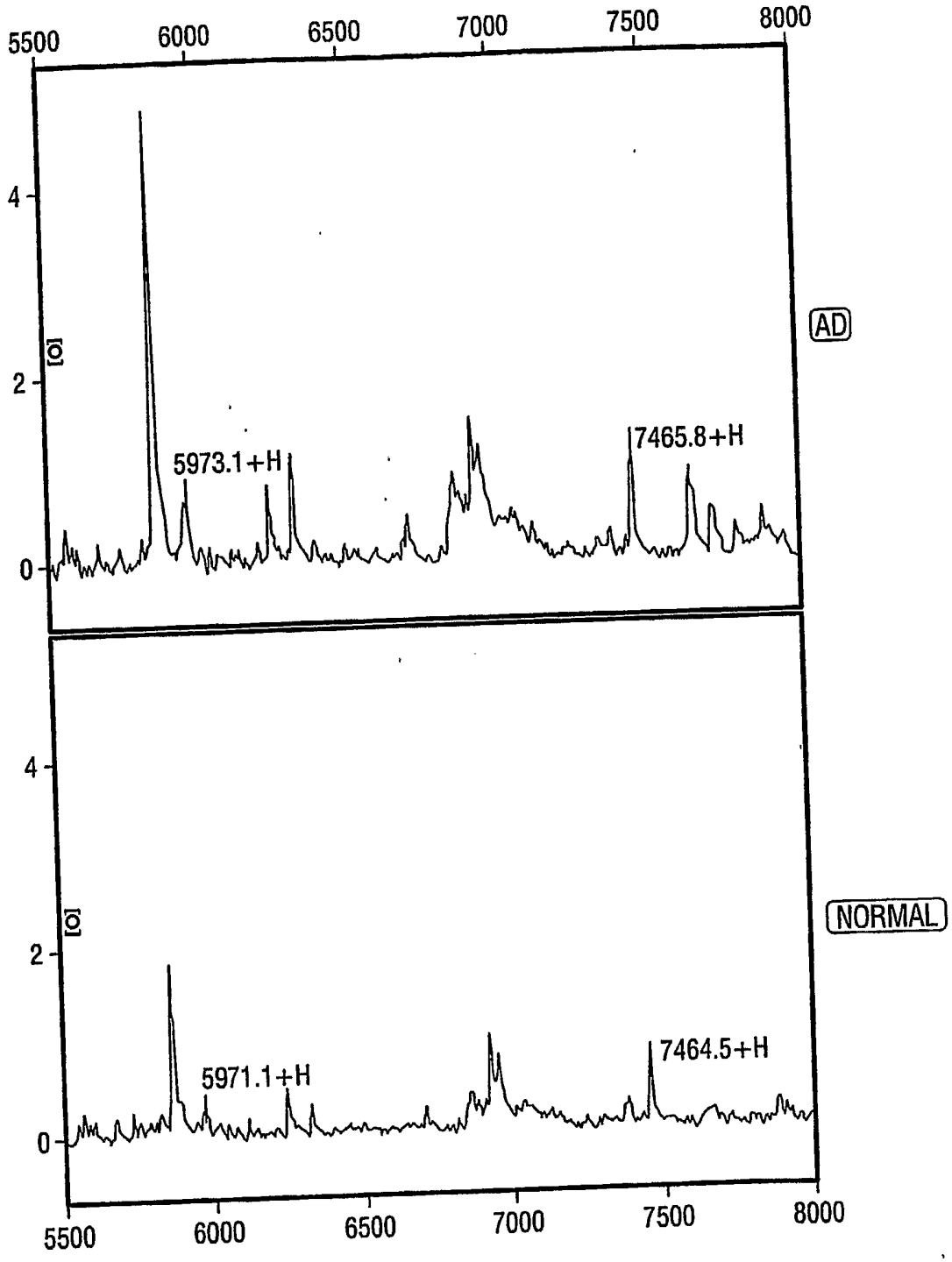
3600
3700
3800
3900
4000

FIG. 1B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8000
4
2
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7465.8+H
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2
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5971.1+H
7464.5+H
NORMAL
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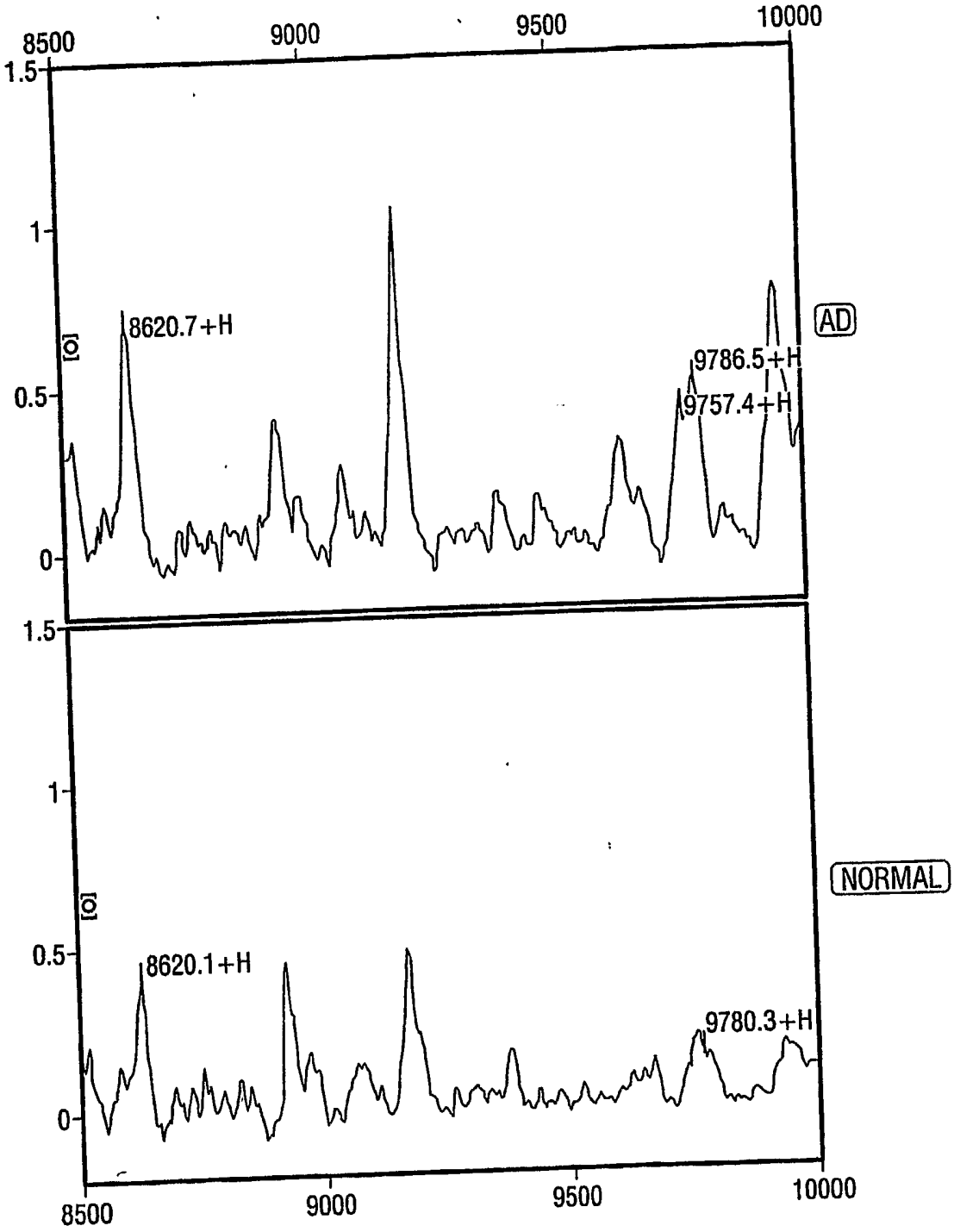
6000
6500
7000
7500
8000

FIG. 1C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1
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9757.4+H
AD
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1
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NORMAL
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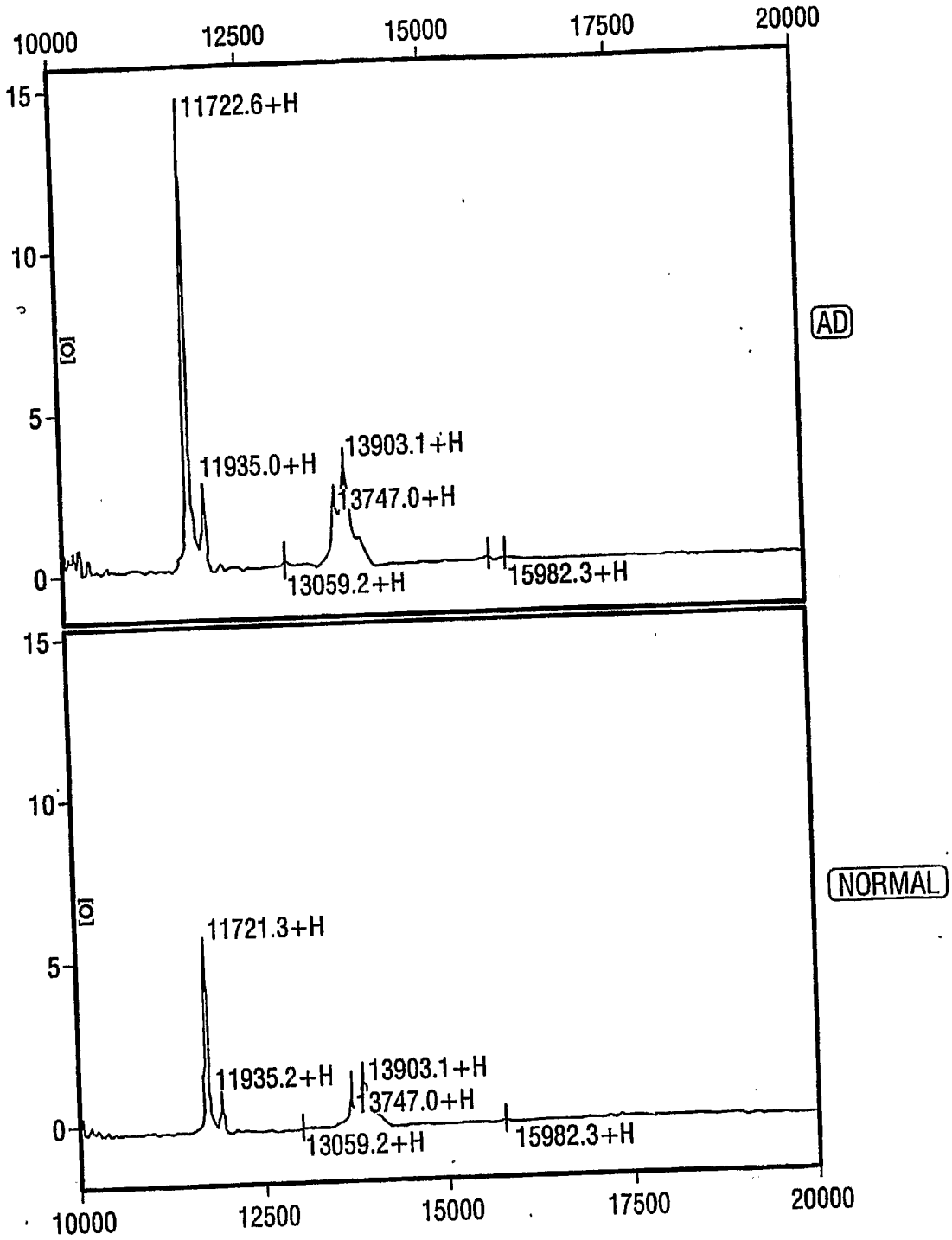
9000
9500
10000

FIG. 1D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15000
17500
20000
15
10
5
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13903.1+H
13747.0+H
13059.2+H
15982.3+H
AD
15
10
5
0
[o]
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11935.2+H
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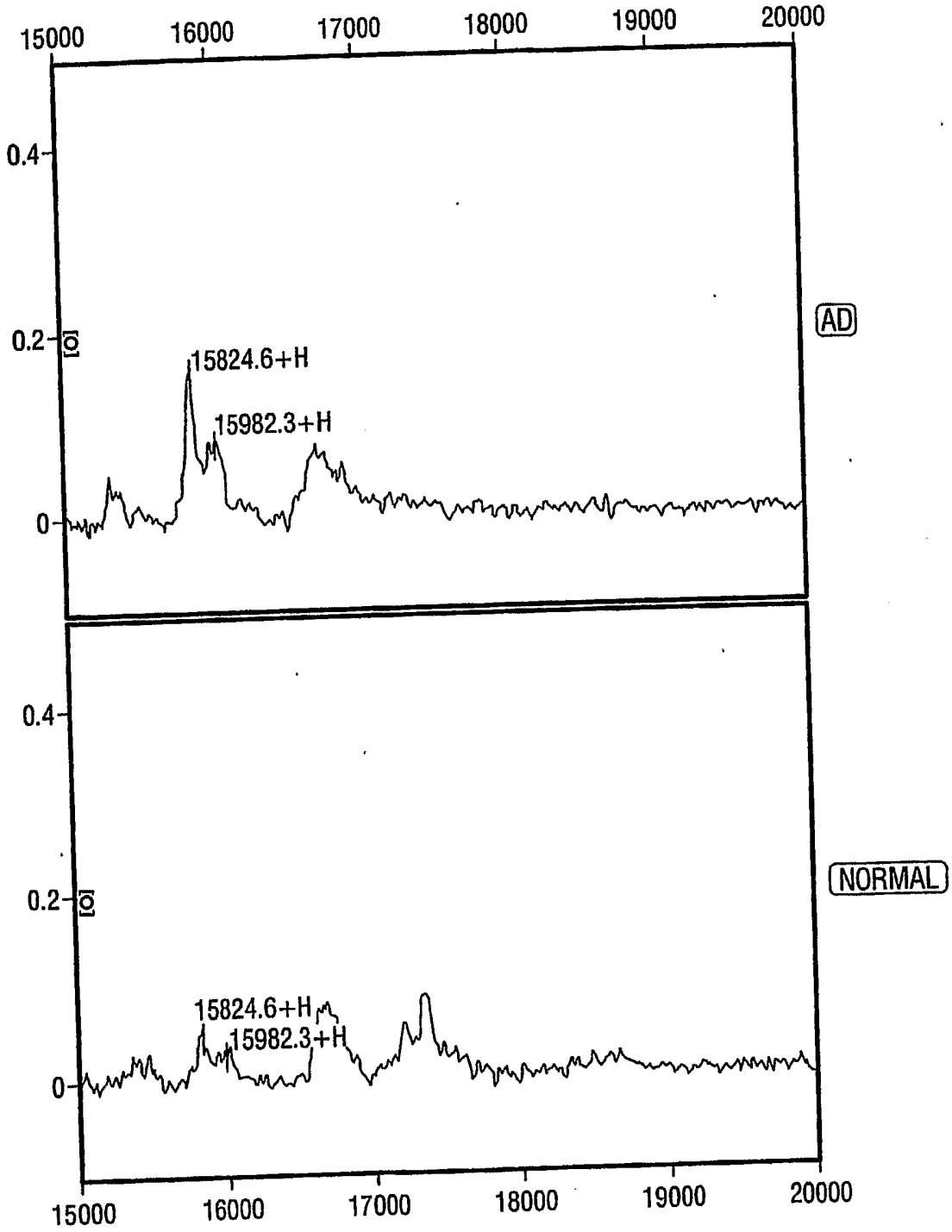
17500
20000

FIG. 1E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19000
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15982.3+H
AD
0.4
0.2
[o]
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15982.3+H
NORMAL
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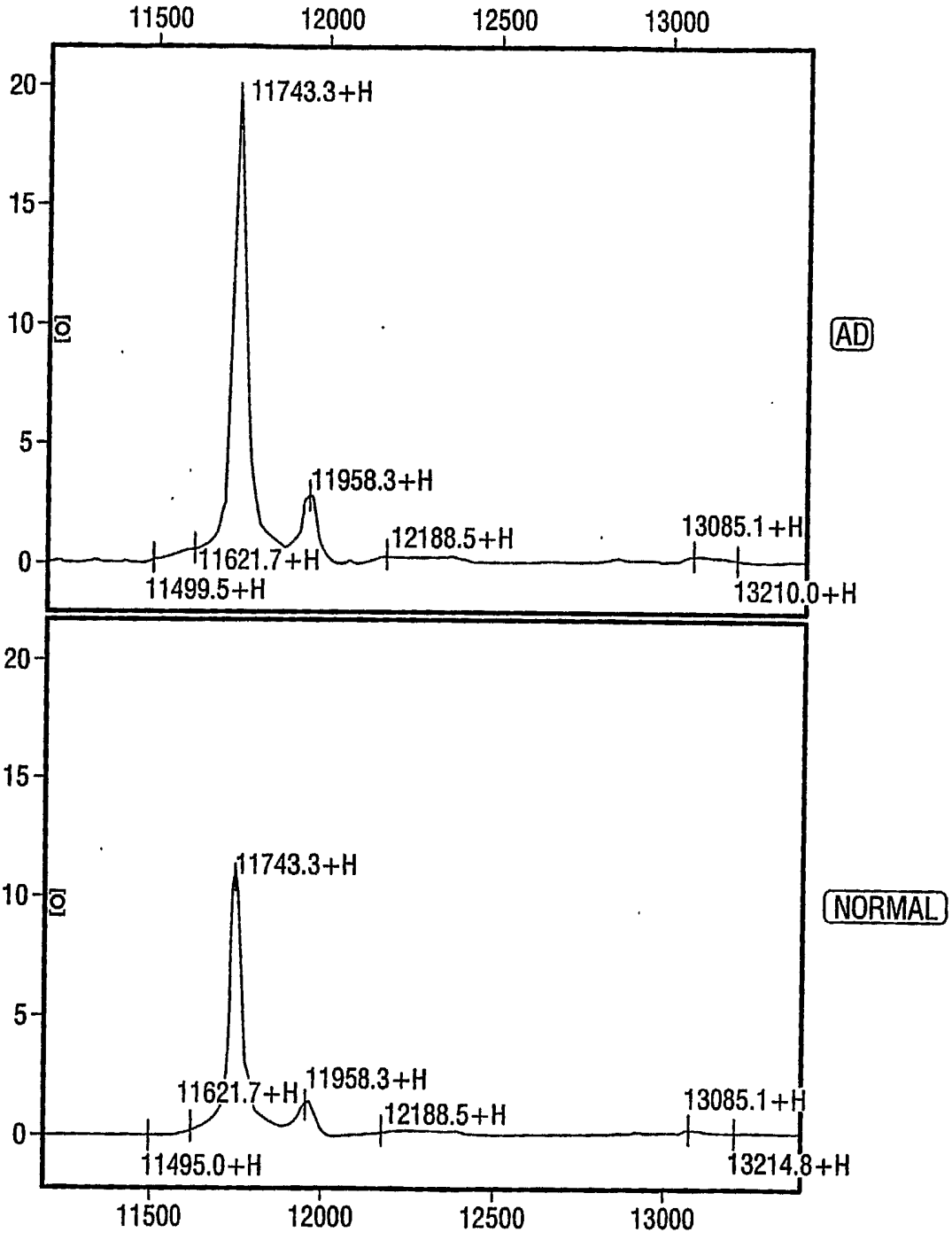
17000
18000
19000
20000

FIG. 1F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13210.0+H
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15
10
5
0
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11743.3+H
11621.7+H
11958.3+H
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11495.0+H
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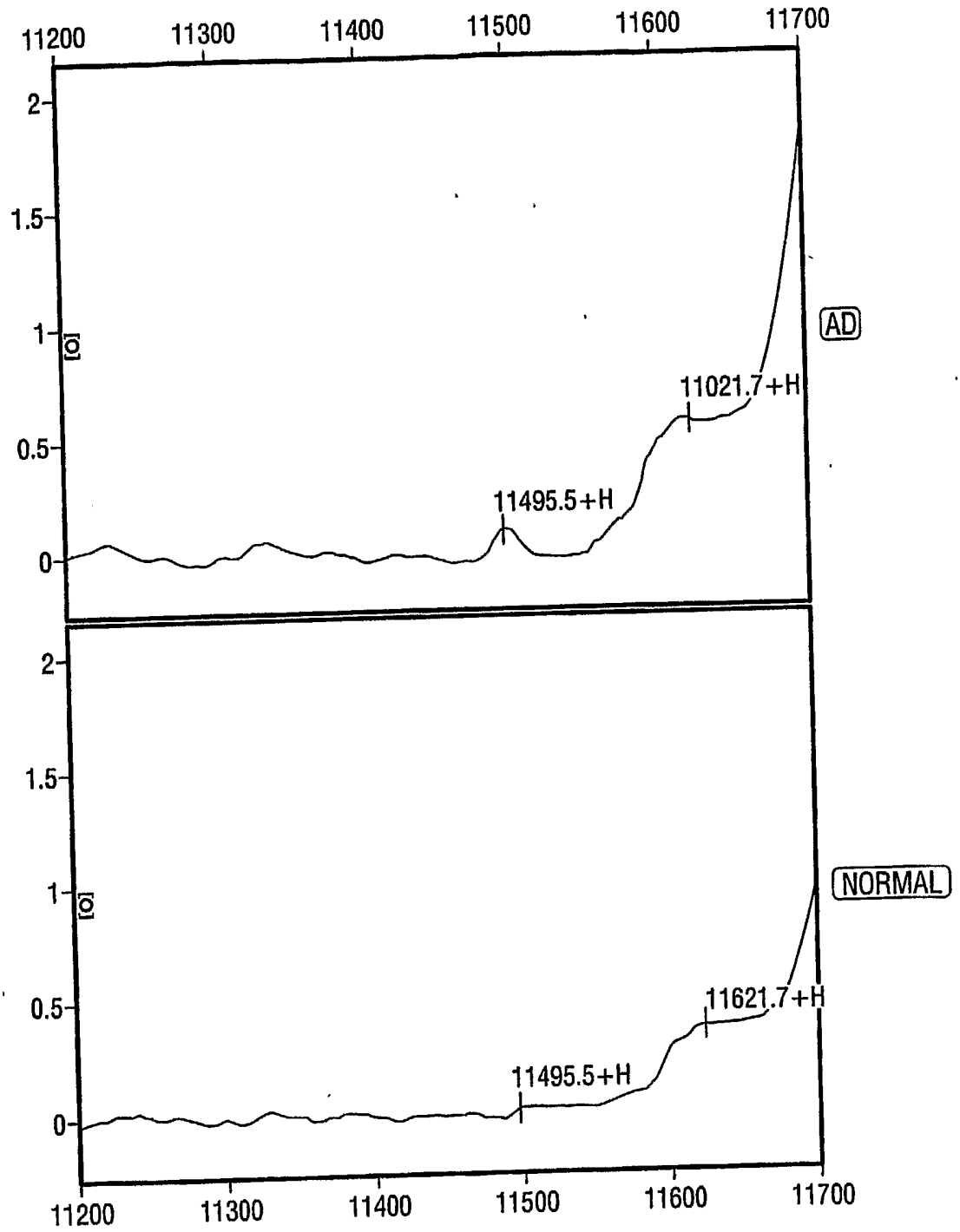
13000

FIG. 1G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0
[o]
AD
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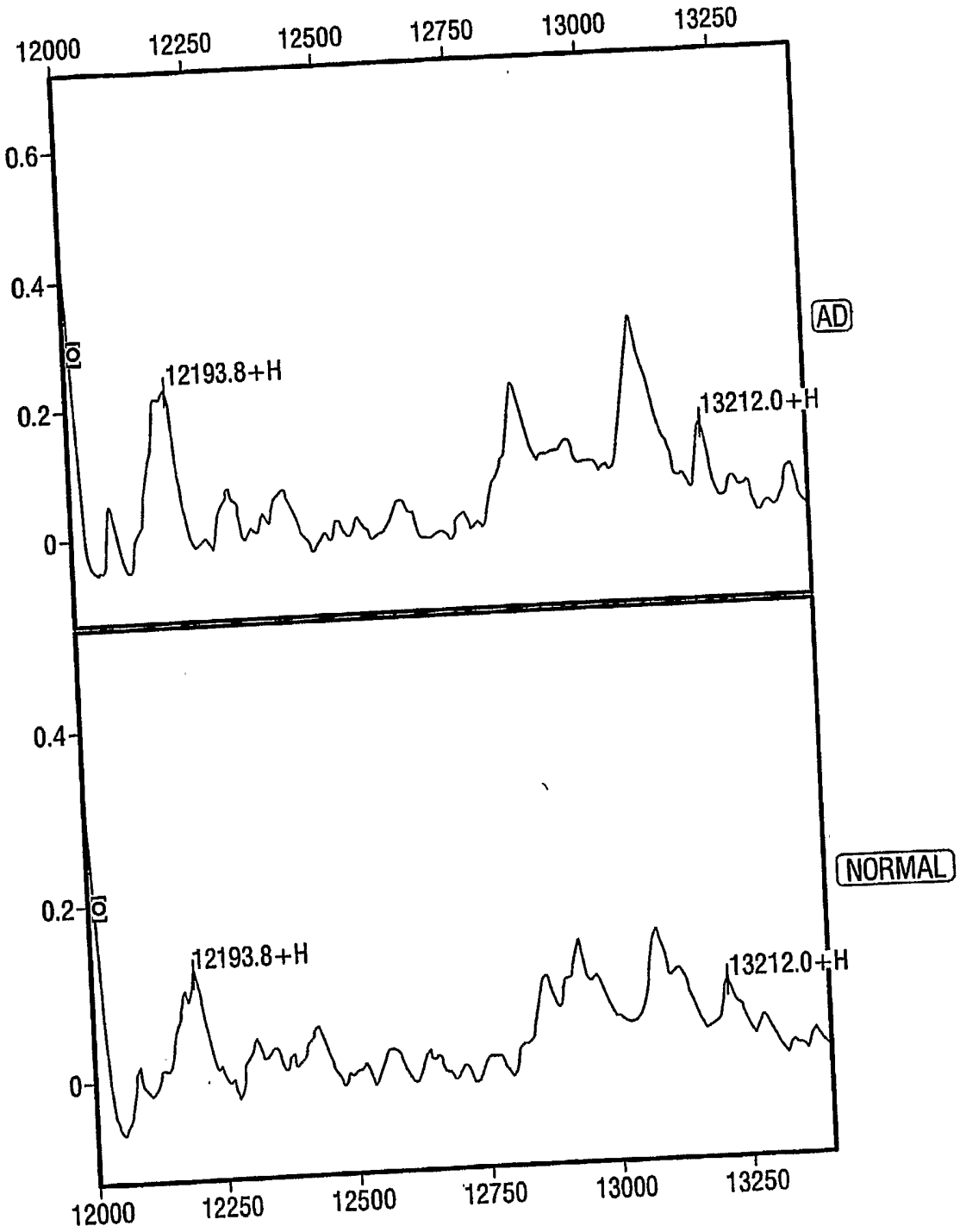
11495.5+H
NORMAL
11621.7+H

FIG. 1H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0.2
0
101
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13212.0+H
AD
0.4
0.2
0
101
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13212.0+H
NORMAL
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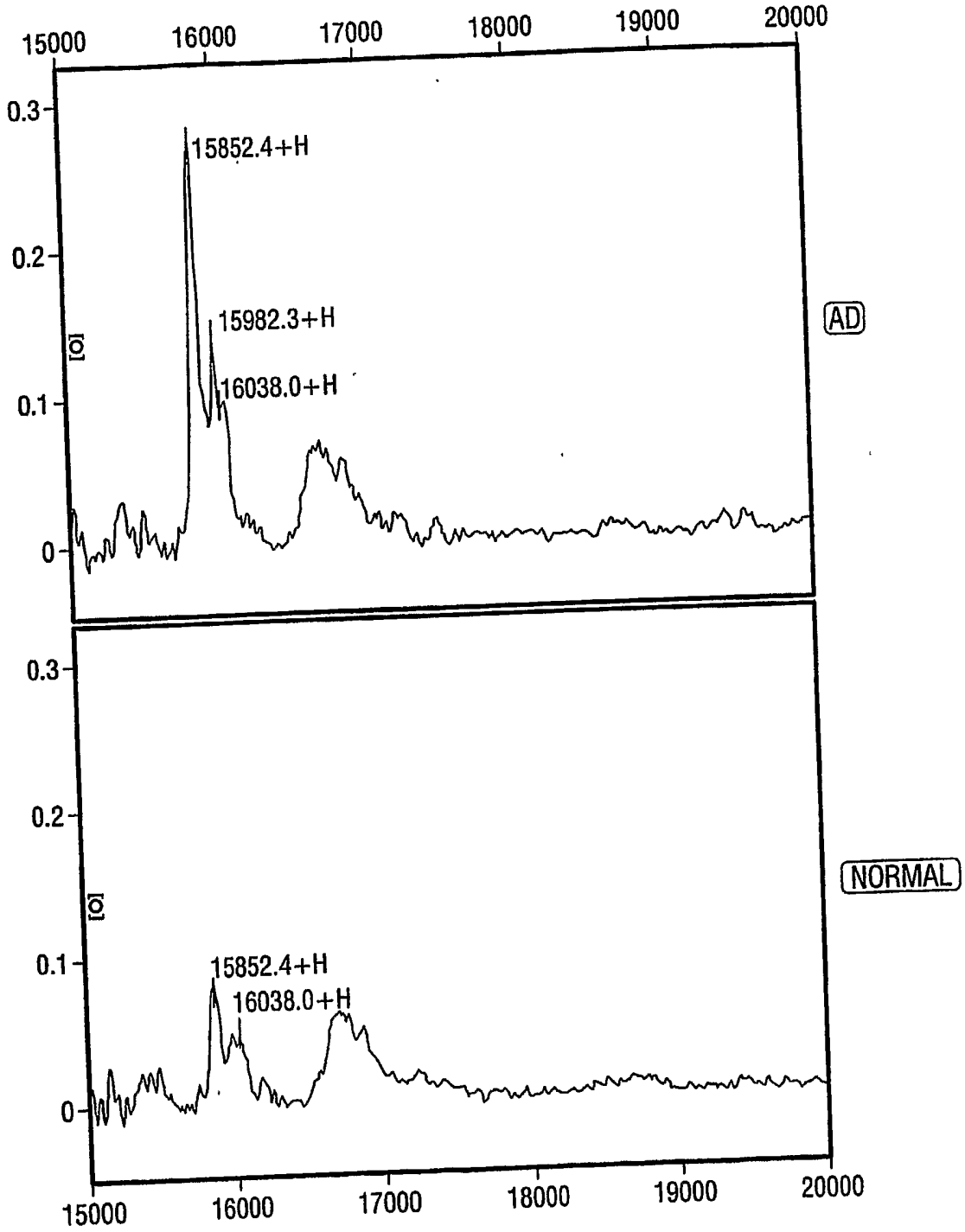
12250
12500
12750
13000
13250

FIG. 1I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15982.3+H
16038.0+H
[o]
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0.2
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16038.0+H
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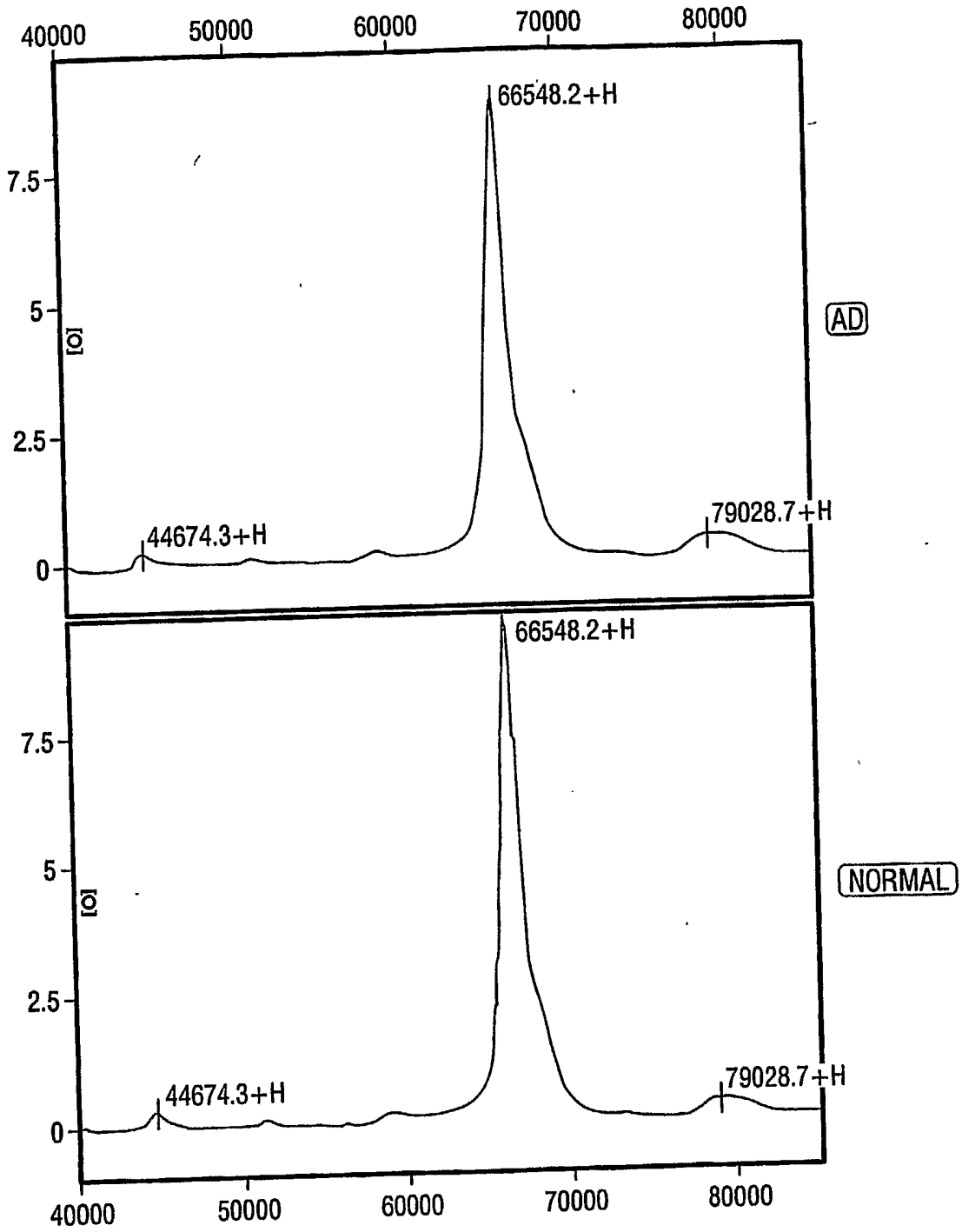
20000

FIG. 1J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50000
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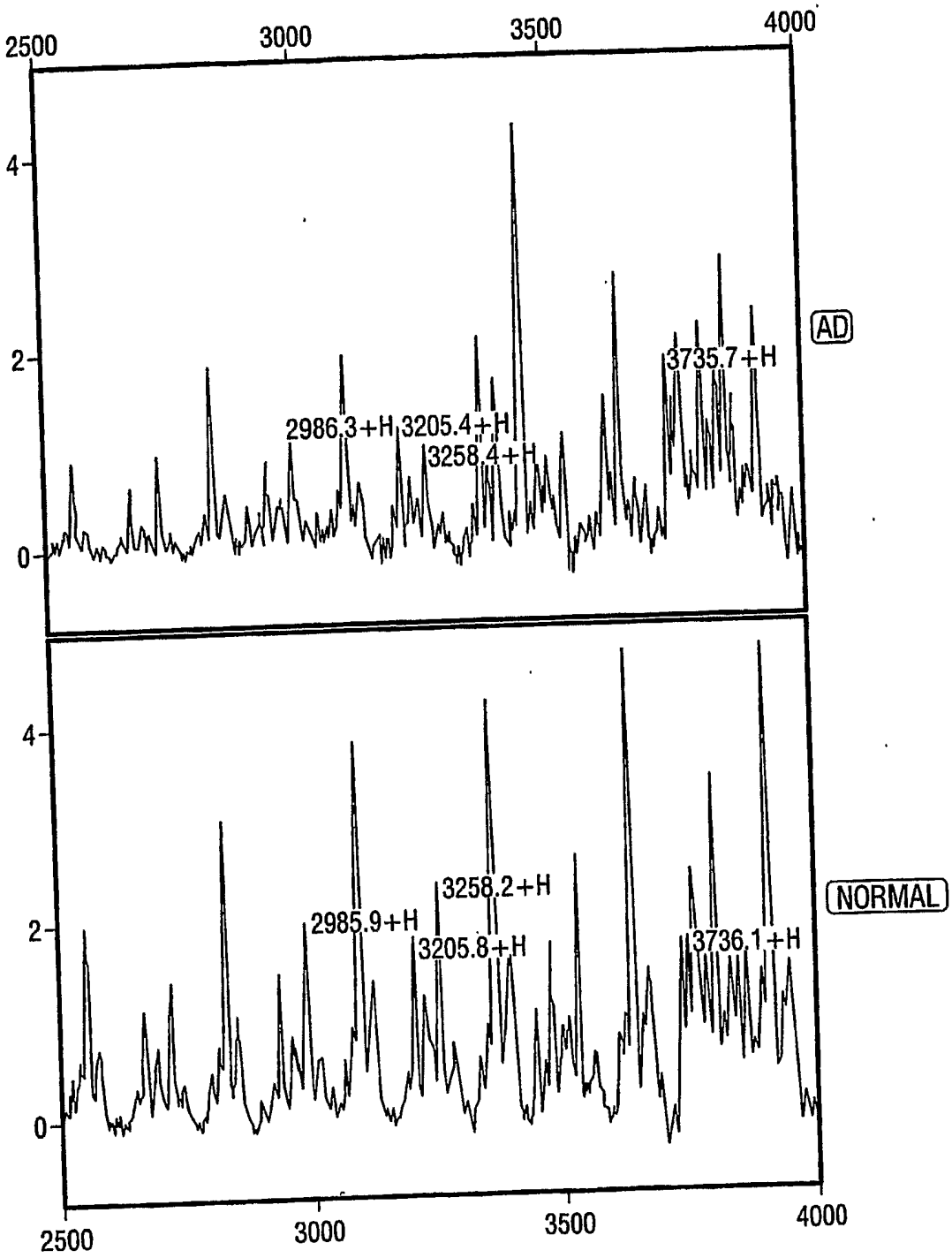
80000

FIG. 1K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3258.4+H
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NORMAL
3258.2+H
2985.9+H
3205.8+H
3736.1+H
2500
3000
3500
4000

FIG. 1L

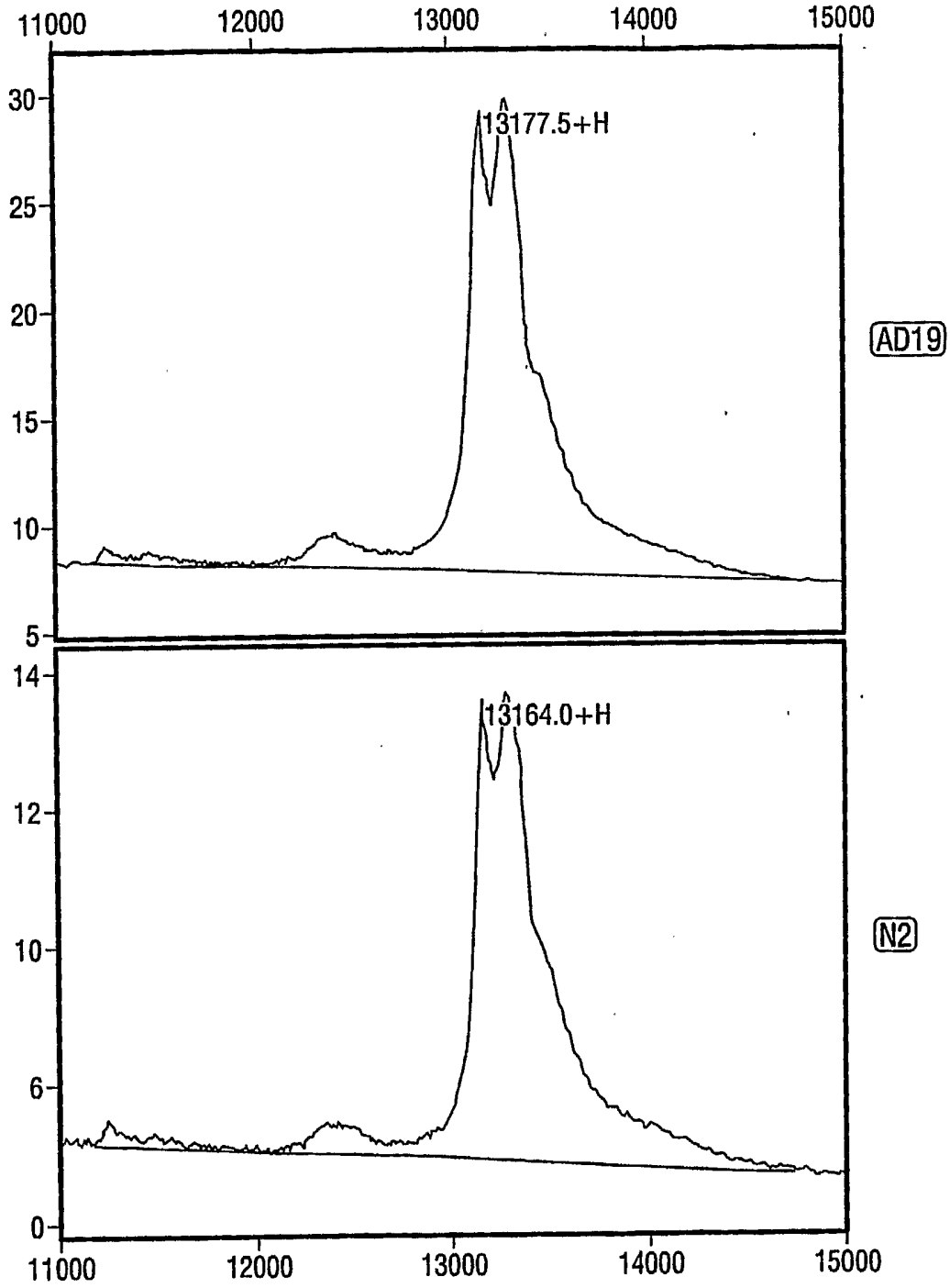

11000
12000
13000
14000
15000
30
25
20
15
10
5
13177.5+H
AD19
14
12
10
6
0
13164.0+H
N2
11000
12000
13000
14000
15000

FIG. 1M

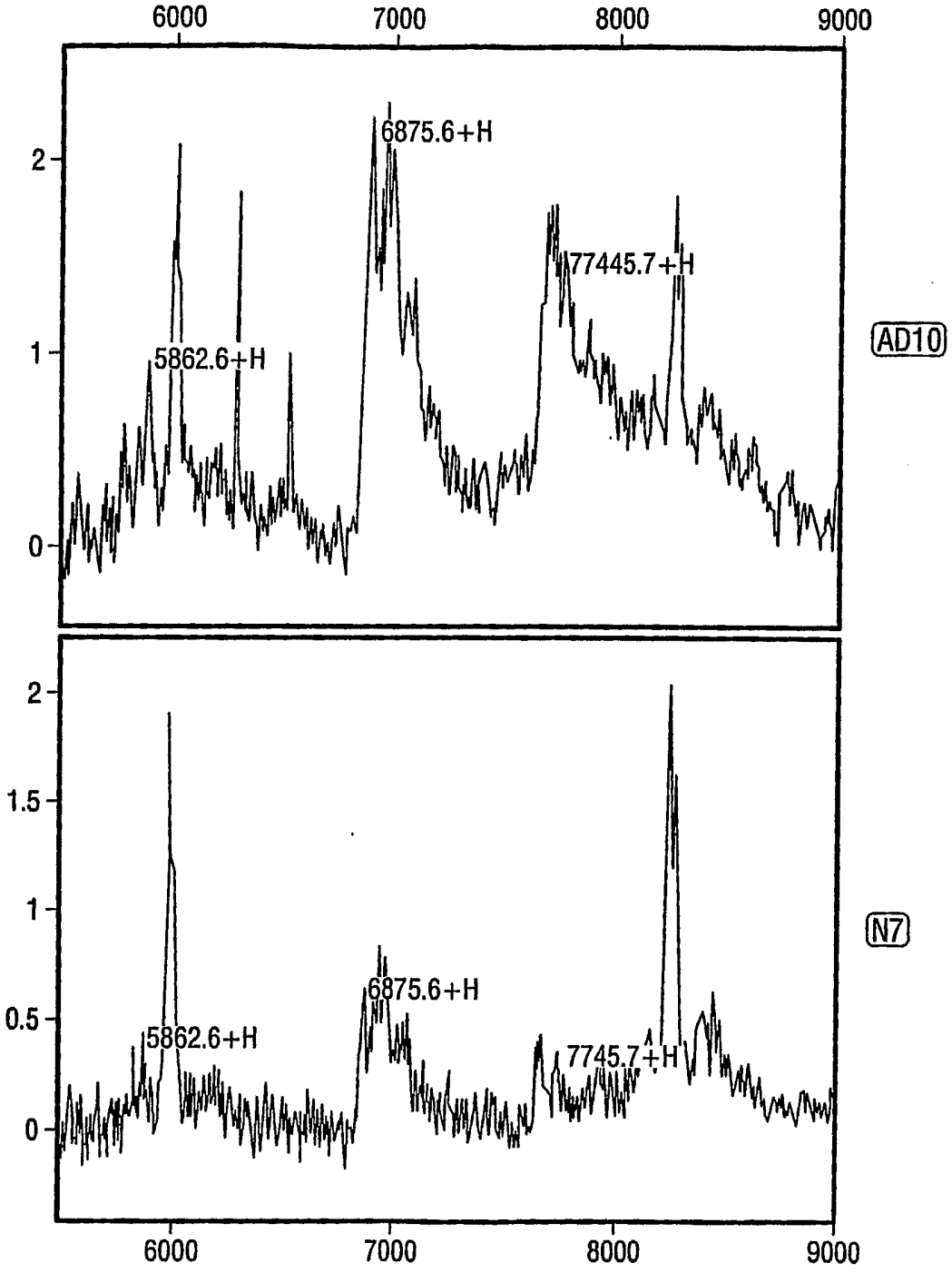

6000
7000
8000
9000
6875.6+H
77445.7+H
5862.6+H
AD10
2
1
0
2
1.5
1
0.5
0
6875.6+H
5862.6+H
7745.7+H
N7
6000
7000
8000
9000

FIG. 1N

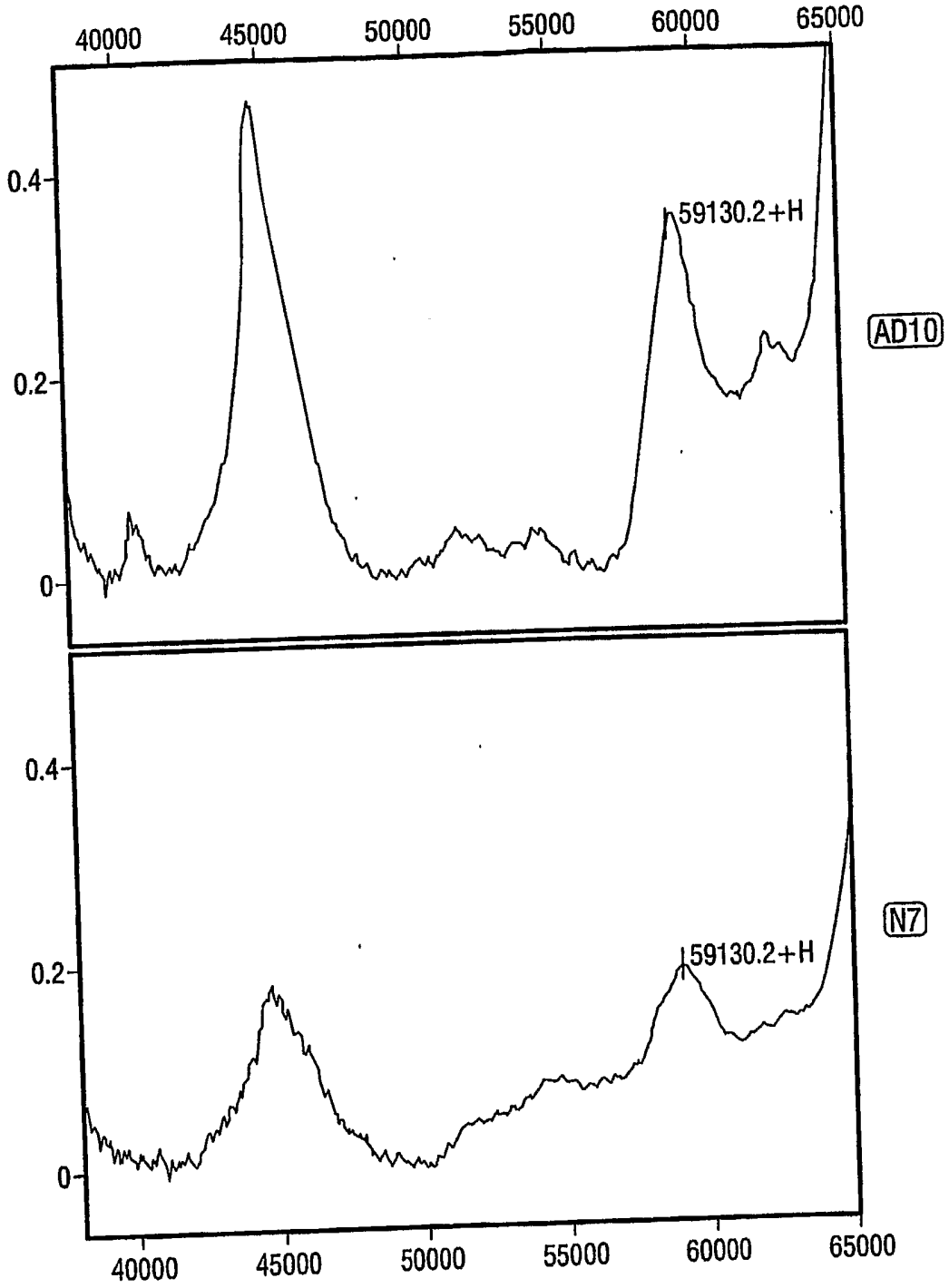

40000
45000
50000
55000
60000
65000
0.4
0.2
0
59130.2+H
AD10
0.4
0.2
0
59130.2+H
N7
40000
45000
50000
55000
60000
65000

FIG. 10

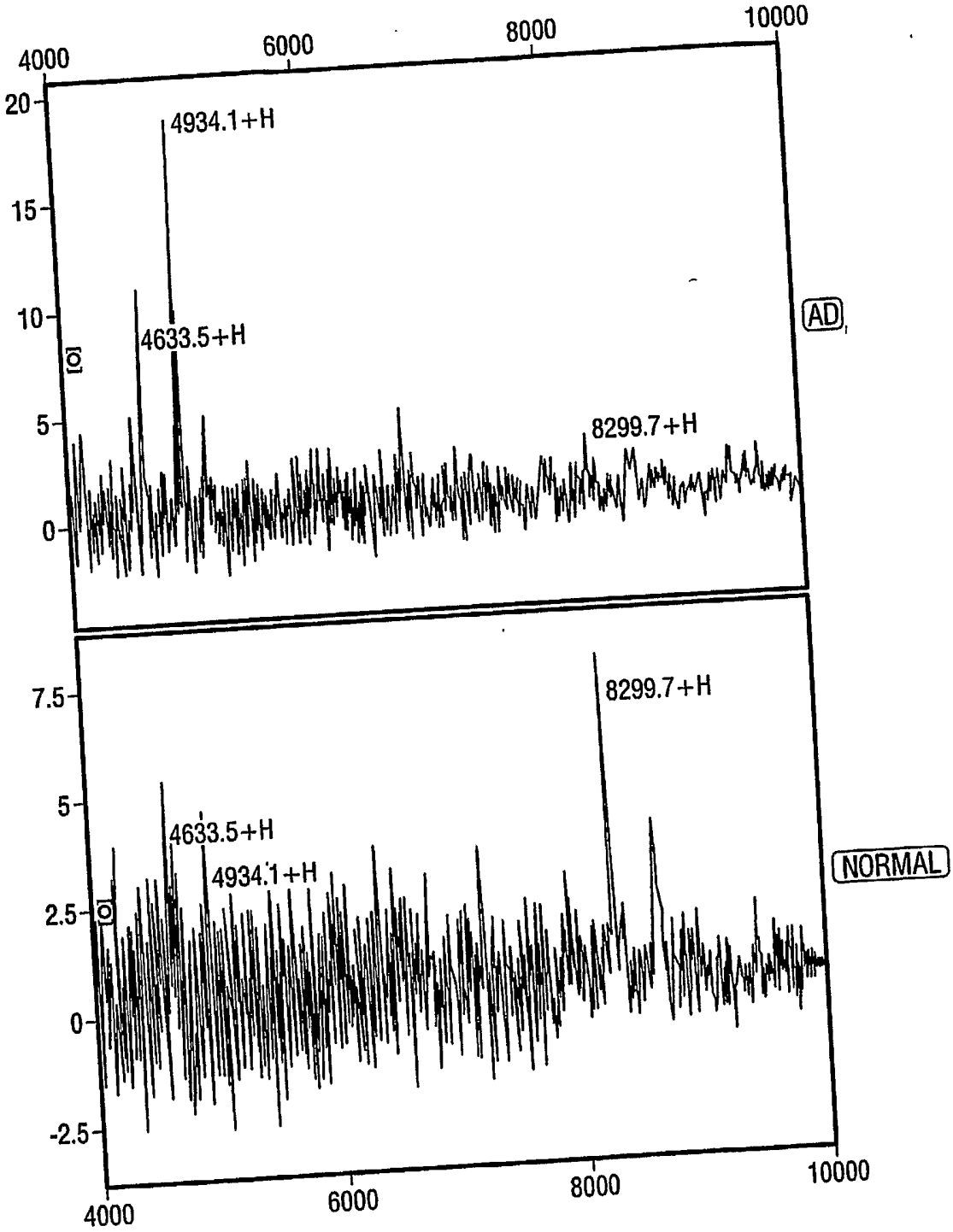

4000
6000
8000
10000
20
15
10
5
0
4934.1+H
4633.5+H
8299.7+H
AD
7.5
5
2.5
0
-2.5
4633.5+H
4934.1+H
8299.7+H
NORMAL

FIG. 1P

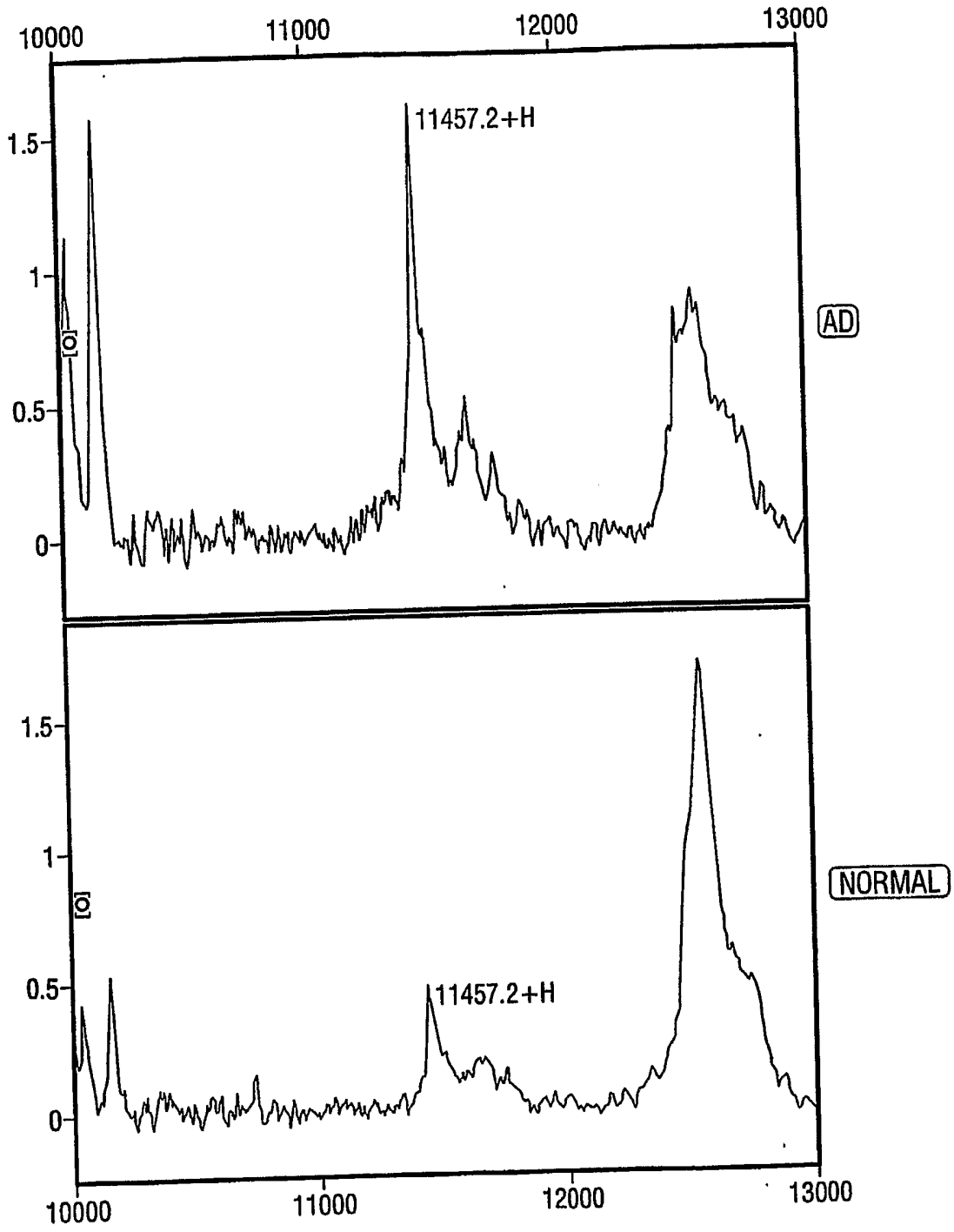


FIG. 1Q

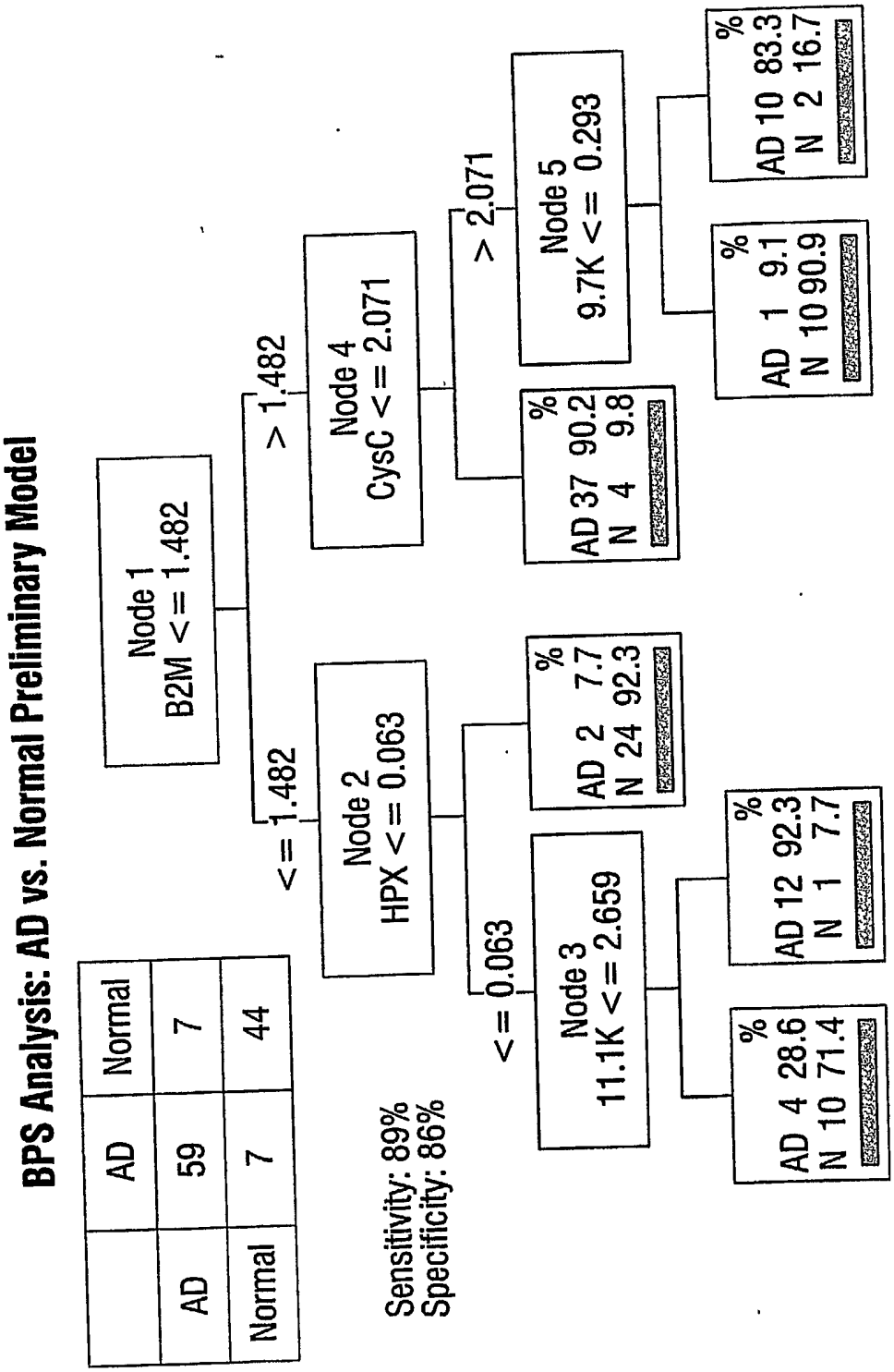
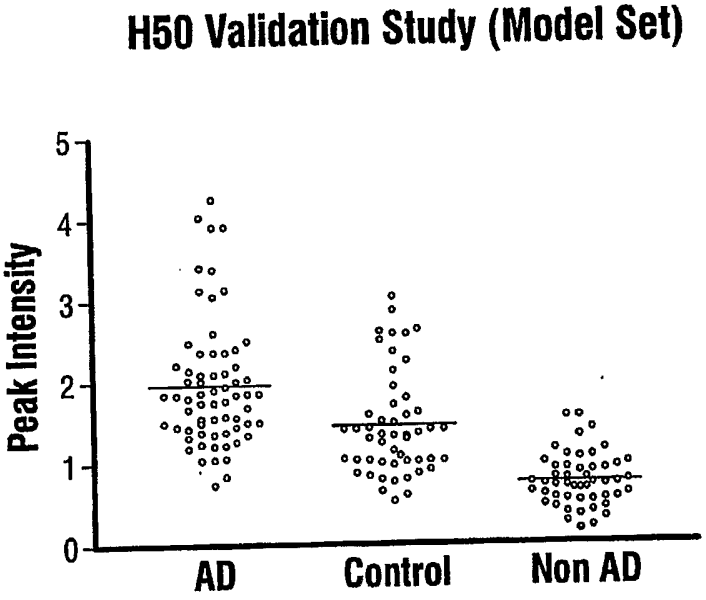

BPS Analysis: AD vs. Normal Preliminary Model
AD
Normal
AD
59
7
Normal
7
44
Sensitivity: 89%
Specificity: 86%
Node 1
B2M <= 1.482
<= 1.482
> 1.482
Node 2
HPX <= 0.063
Node 4
CysC <= 2.071
<= 0.063
> 2.071
Node 3
11.1K <= 2.659
Node 5
9.7K <= 0.293
% AD 2 7.7 N 24 92.3
% AD 37 90.2 N 4 9.8
% AD 4 28.6 N 10 71.4
% AD 12 92.3 N 1 7.7
% AD 1 9.1 N 10 90.9
% AD 10 83.3 N 2 16.7

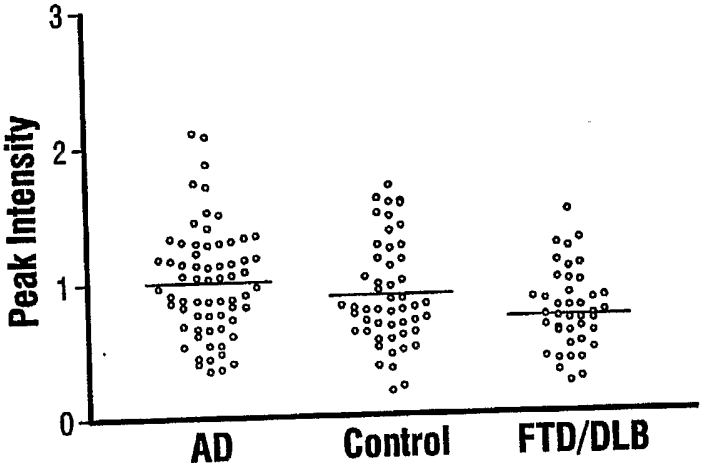
FIG. 2



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

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

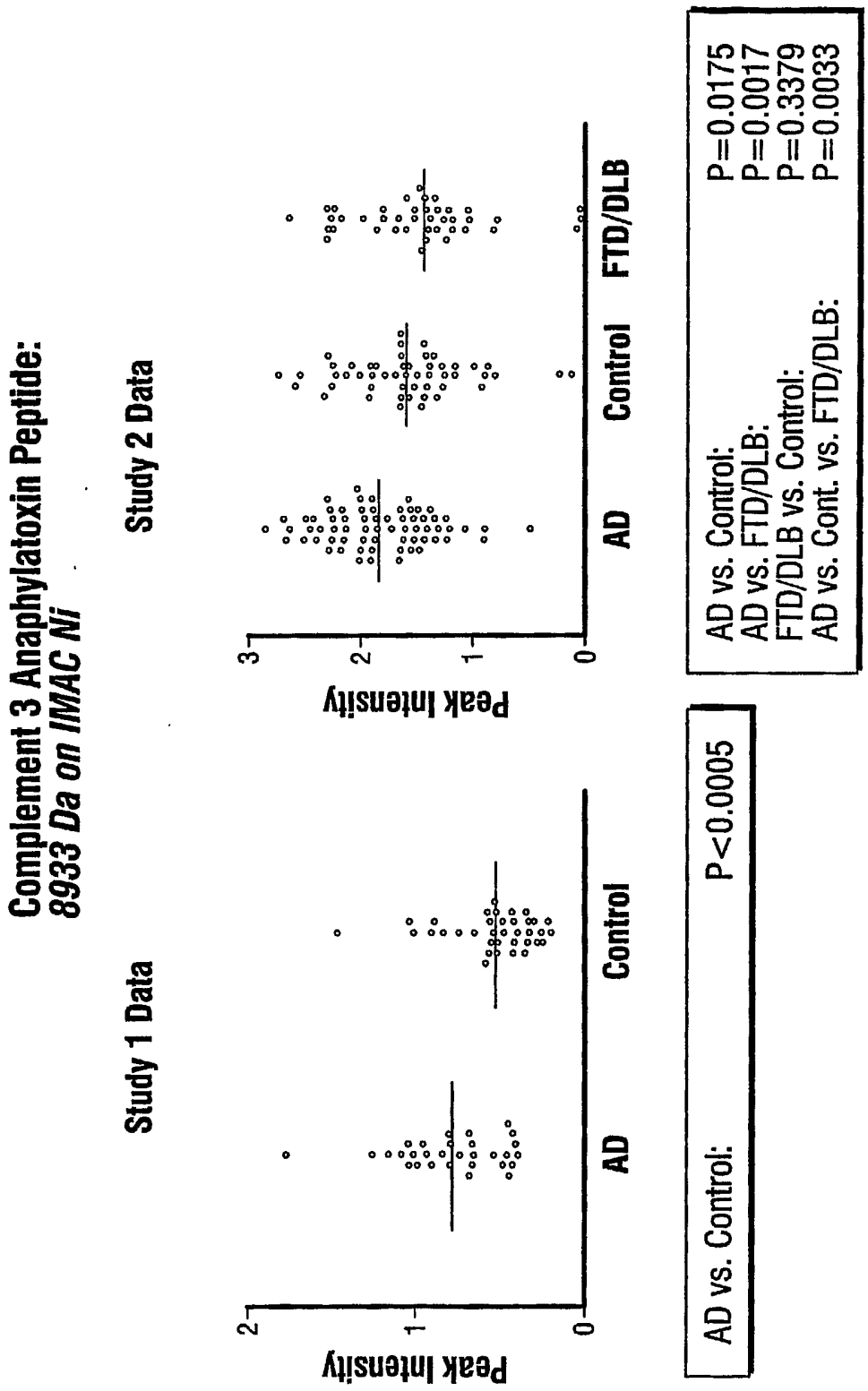

Complement 3 Anaphylatoxin Peptide:
8933 Da on IMAC Ni
Study 1 Data
Peak Intensity
0
1
2
AD
Control
AD vs. Control: P<0.0005
Study 2 Data
Peak Intensity
0
1
2
3
AD
Control
FTD/DLB
AD vs. Control: P=0.0175
AD vs. FTD/DLB: P=0.0017
FTD/DLB vs. Control: P=0.3379
AD vs. Cont. vs. FTD/DLB: P=0.0033

FIG. 4B

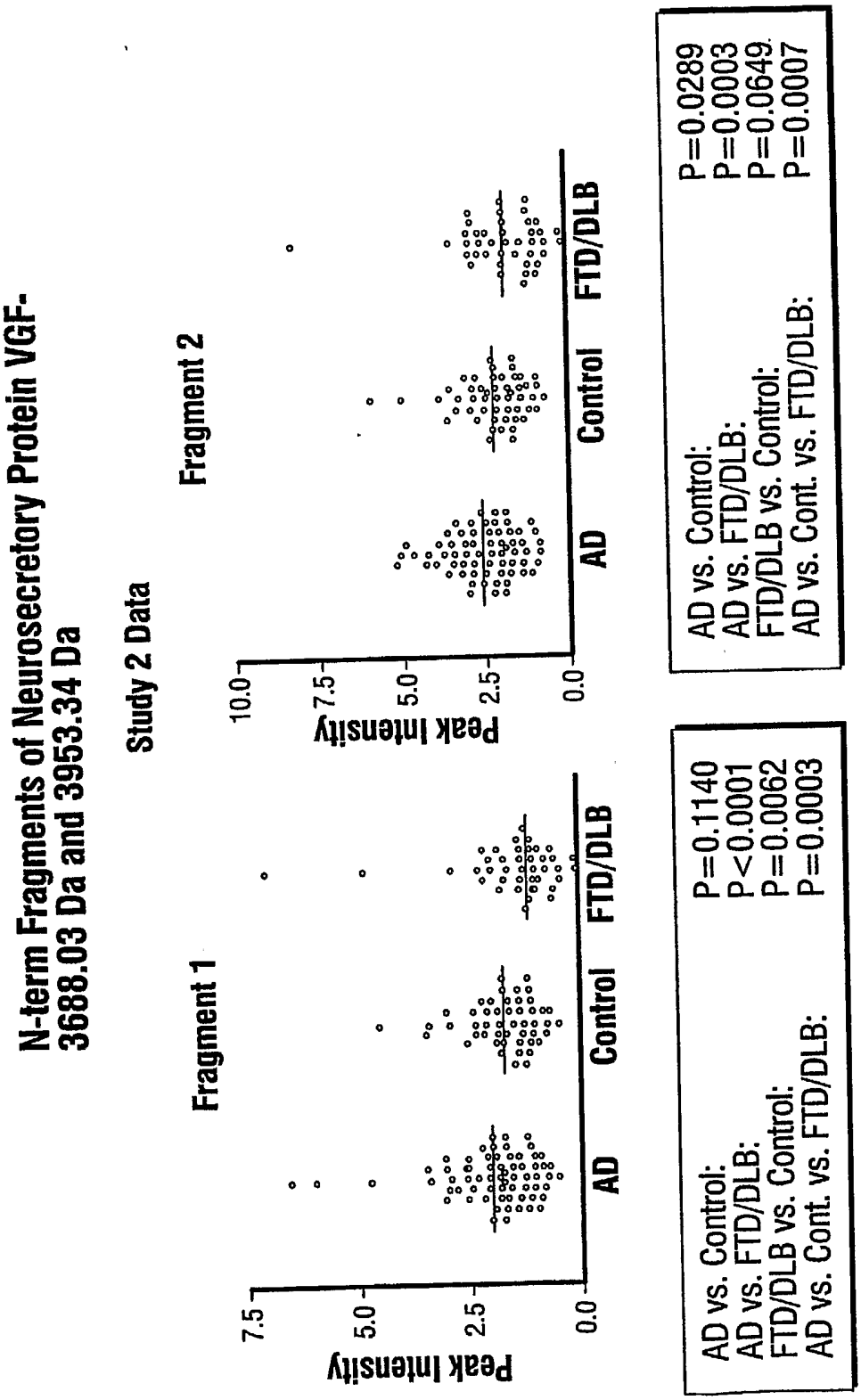

N-term Fragments of Neurosecretory Protein VGF-
3688.03 Da and 3953.34 Da
Study 2 Data
Fragment 1
Peak Intensity
7.5
5.0
2.5
0.0
AD
Control
FTD/DLB
AD vs. Control: P=0.1140
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P=0.0062
AD vs. Cont. vs. FTD/DLB: P=0.0003
Fragment 2
Peak Intensity
10.0
7.5
5.0
2.5
0.0
AD
Control
FTD/DLB
AD vs. Control: P=0.0289
AD vs. FTD/DLB: P=0.0003
FTD/DLB vs. Control: P=0.0649
AD vs. Cont. vs. FTD/DLB: P=0.0007

FIG. 4C

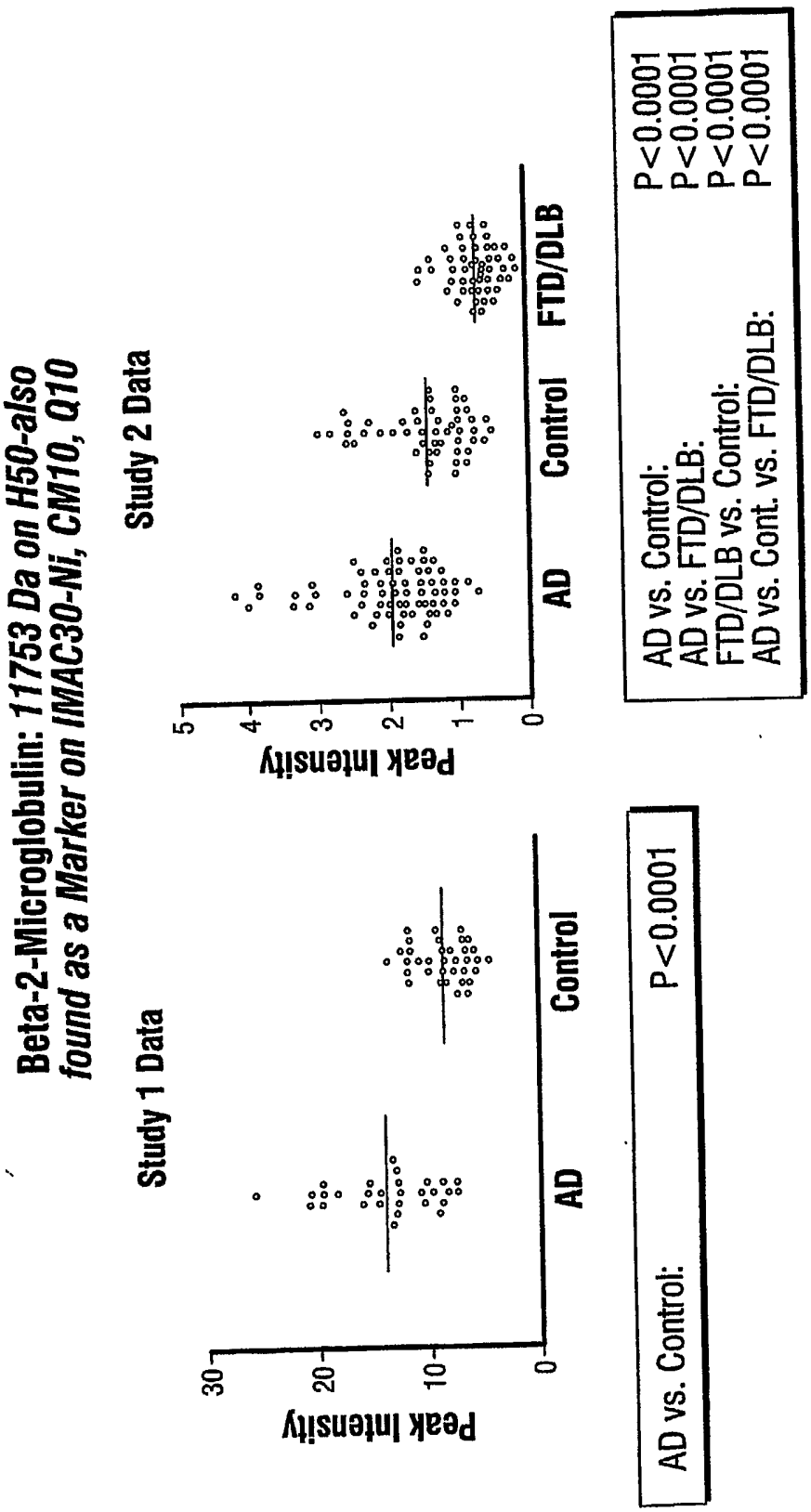

Beta-2-Microglobulin: 11753 Da on H50-also found as a Marker on IMAC30-Ni, CM10, Q10
Study 1 Data
Peak Intensity
30
20
10
0
AD
Control
AD vs. Control: P<0.0001
Study 2 Data
Peak Intensity
5
4
3
2
1
0
AD
Control
FTD/DLB
AD vs. Control: P<0.0001
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P<0.0001
AD vs. Cont. vs. FTD/DLB: P<0.0001

FIG. 4D

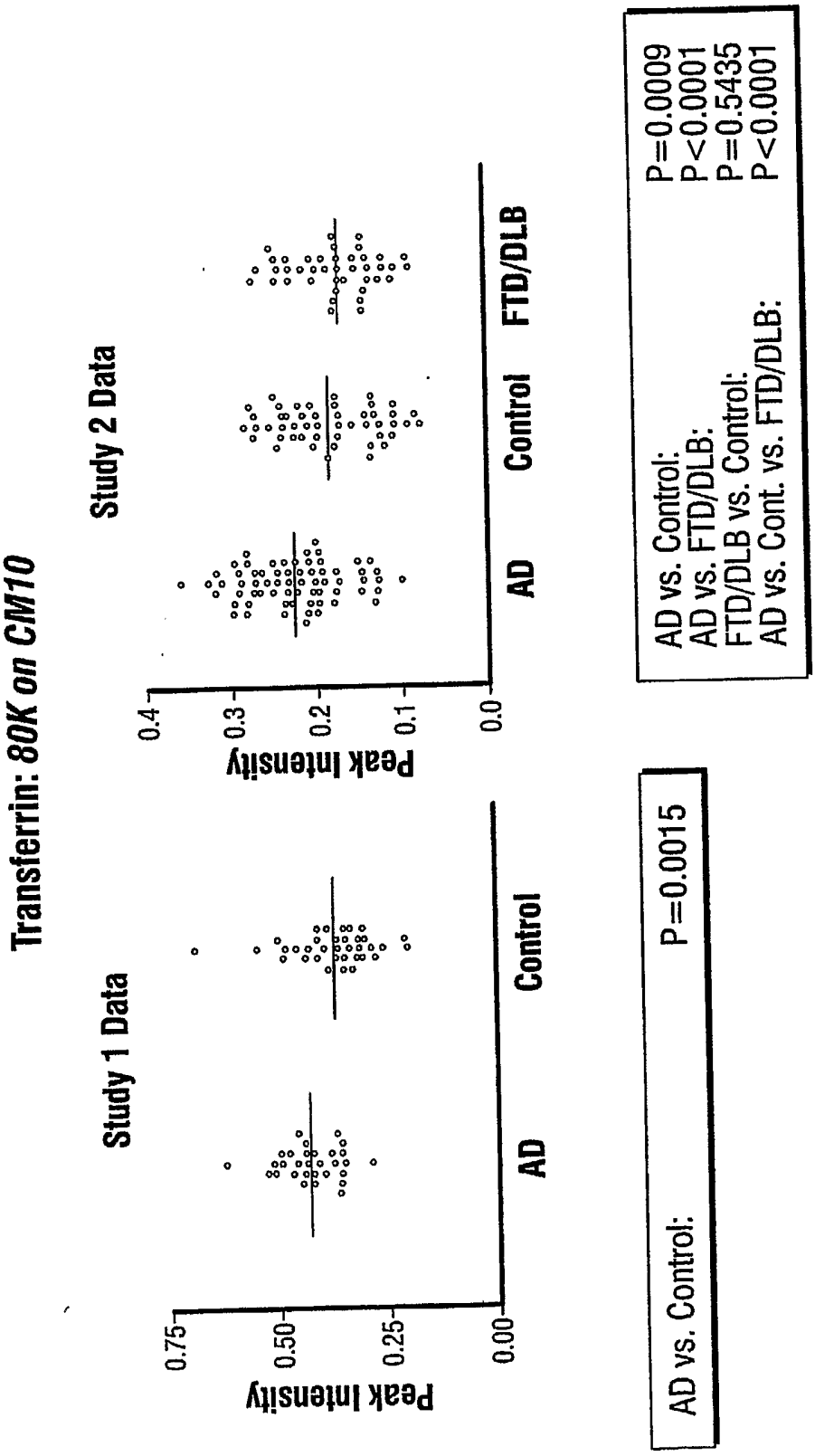

Transferrin: 80K on CM10
Study 1 Data
Peak Intensity
0.75
0.50
0.25
0.00
AD
Control
AD vs. Control: P=0.0015
Study 2 Data
Peak Intensity
0.4
0.3
0.2
0.1
0.0
AD
Control
FTD/DLB
AD vs. Control: P=0.0009
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P=0.5435
AD vs. Cont. vs. FTD/DLB: P<0.0001

FIG. 4E

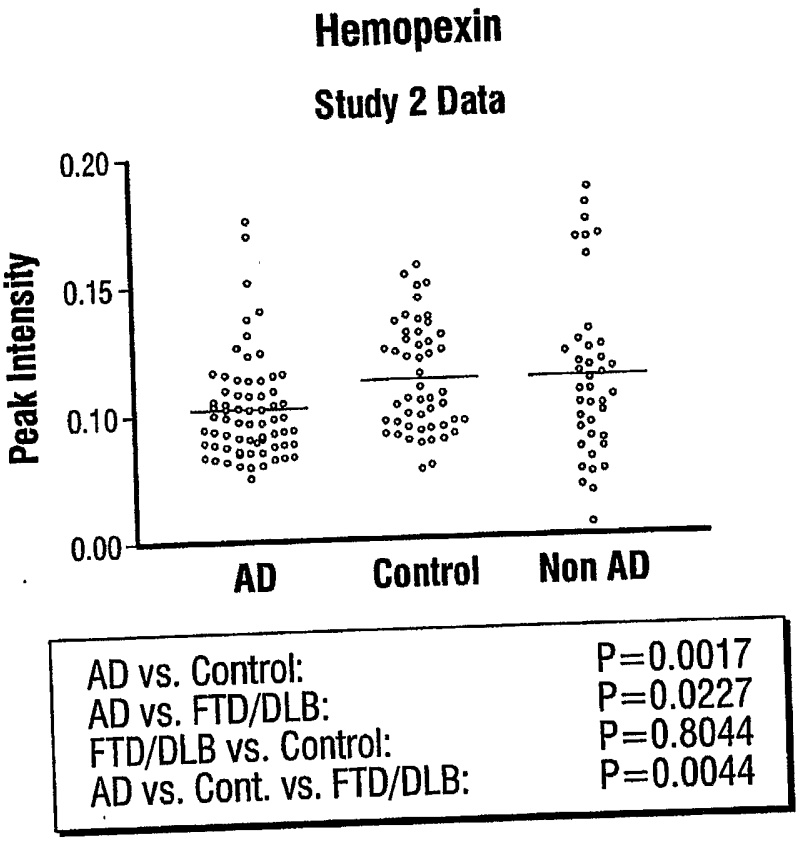

Hemopexin
Study 2 Data
Peak Intensity
0.20
0.15
0.10
0.00
AD
Control
Non AD
AD vs. Control: P=0.0017
AD vs. FTD/DLB: P=0.0227
FTD/DLB vs. Control: P=0.8044
AD vs. Cont. vs. FTD/DLB: P=0.0044

FIG. 4F

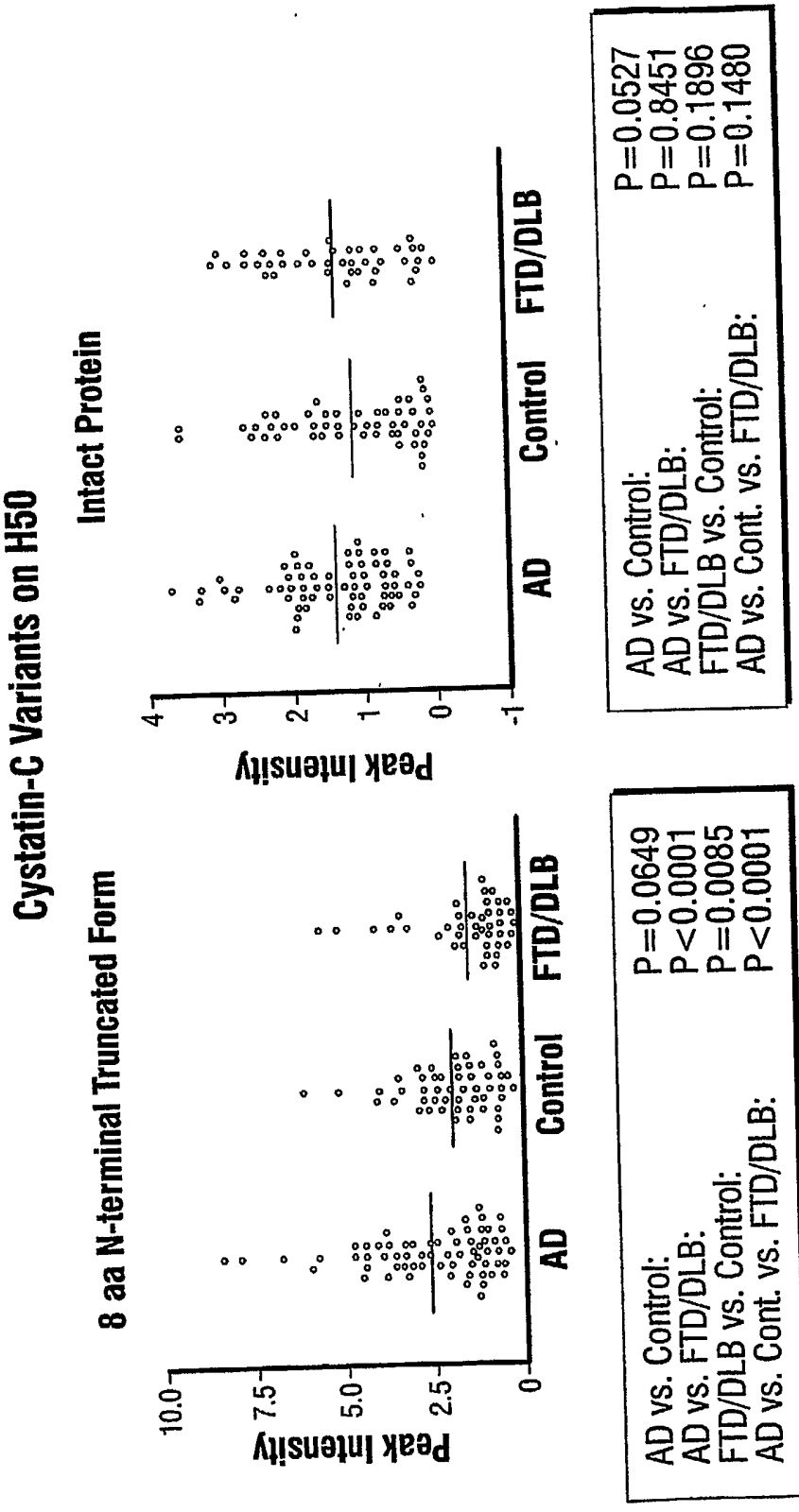

Cystatin-C Variants on H50
8 aa N-terminal Truncated Form
Peak Intensity
10.0
7.5
5.0
2.5
0
AD
Control
FTD/DLB
AD vs. Control: P=0.0649
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P=0.0085
AD vs. Cont. vs. FTD/DLB: P<0.0001
Intact Protein
Peak Intensity
4
3
2
1
0
-1
AD
Control
FTD/DLB
AD vs. Control: P=0.0527
AD vs. FTD/DLB: P=0.8451
FTD/DLB vs. Control: P=0.1896
AD vs. Cont. vs. FTD/DLB: P=0.1480

FIG. 4G

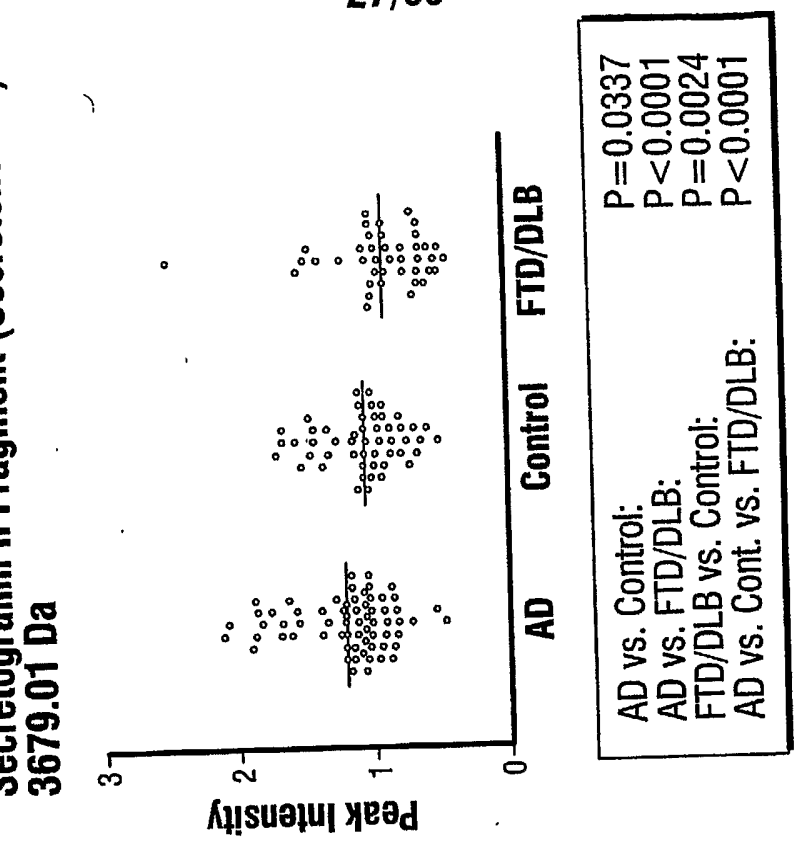

Secretogranin II Fragment (Secretoneurin)-
3679.01 Da
Peak Intensity
3
2
1
0
AD
Control
FTD/DLB
AD vs. Control: P=0.0337
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P=0.0024
AD vs. Cont. vs. FTD/DLB: P<0.0001

FIG. 4I

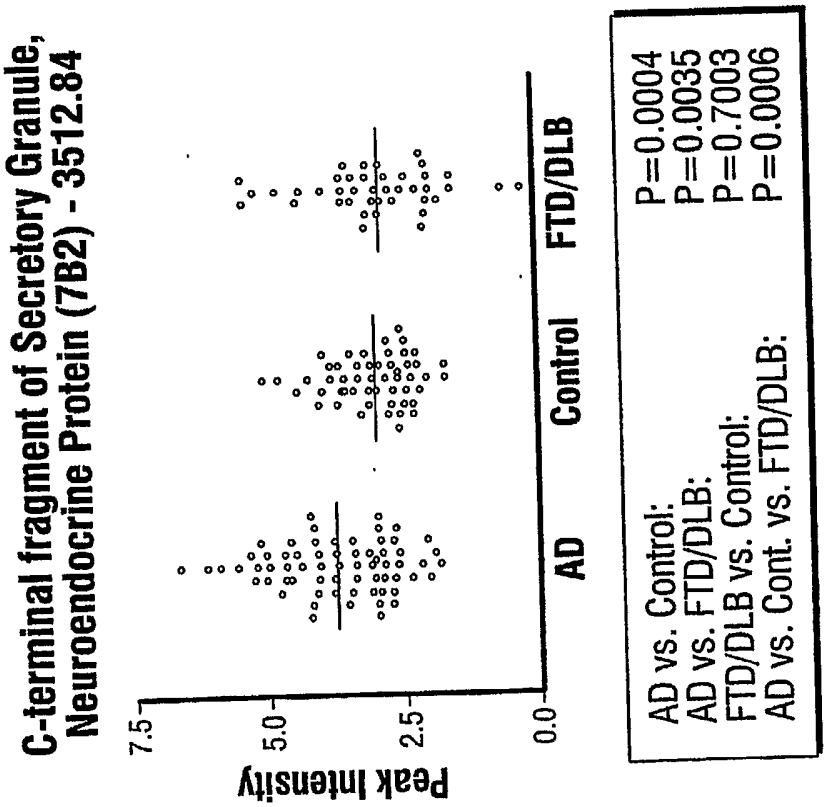

C-terminal fragment of Secretory Granule,
Neuroendocrine Protein (7B2) - 3512.84
Peak Intensity
7.5
5.0
2.5
0.0
AD
Control
FTD/DLB
AD vs. Control: P=0.0004
AD vs. FTD/DLB: P=0.0035
FTD/DLB vs. Control: P=0.7003
AD vs. Cont. vs. FTD/DLB: P=0.0006

FIG. 4H

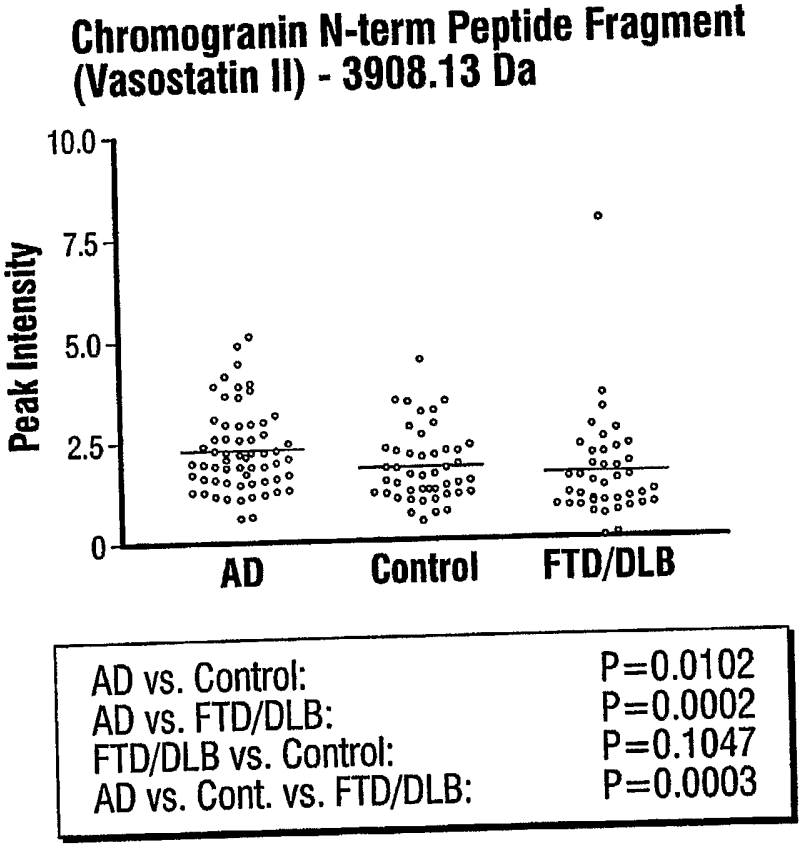

Chromogranin N-term Peptide Fragment
(Vasostatin II) - 3908.13 Da
Peak Intensity
10.0
7.5
5.0
2.5
0
AD
Control
FTD/DLB
AD vs. Control: P=0.0102
AD vs. FTD/DLB: P=0.0002
FTD/DLB vs. Control: P=0.1047
AD vs. Cont. vs. FTD/DLB: P=0.0003

FIG. 4J

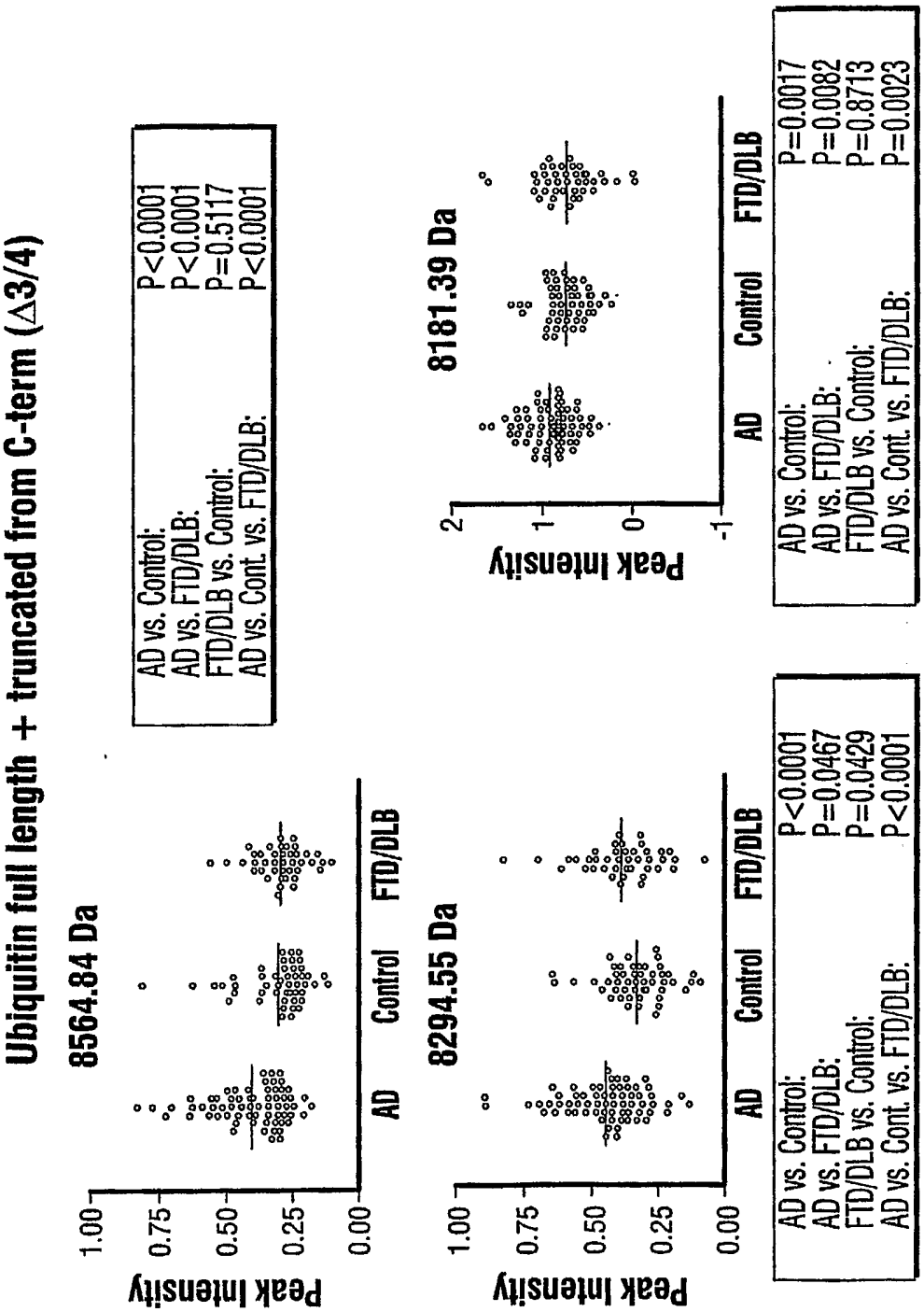

Ubiquitin full length + truncated from C-term (Δ3/4)
8564.84 Da
Peak Intensity
1.00
0.75
0.50
0.25
0.00
AD
Control
FTD/DLB
AD vs. Control: P<0.0001
AD vs. FTD/DLB: P<0.0001
FTD/DLB vs. Control: P=0.5117
AD vs. Cont. vs. FTD/DLB: P<0.0001
8181.39 Da
Peak Intensity
2
1
0
-1
AD
Control
FTD/DLB
AD vs. Control: P=0.0017
AD vs. FTD/DLB: P=0.0082
FTD/DLB vs. Control: P=0.8713
AD vs. Cont. vs. FTD/DLB: P=0.0023
8294.55 Da
Peak Intensity
1.00
0.75
0.50
0.25
0.00
AD
Control
FTD/DLB
AD vs. Control: P<0.0001
AD vs. FTD/DLB: P=0.0467
FTD/DLB vs. Control: P=0.0429
AD vs. Cont. vs. FTD/DLB: P<0.0001

FIG. 4K

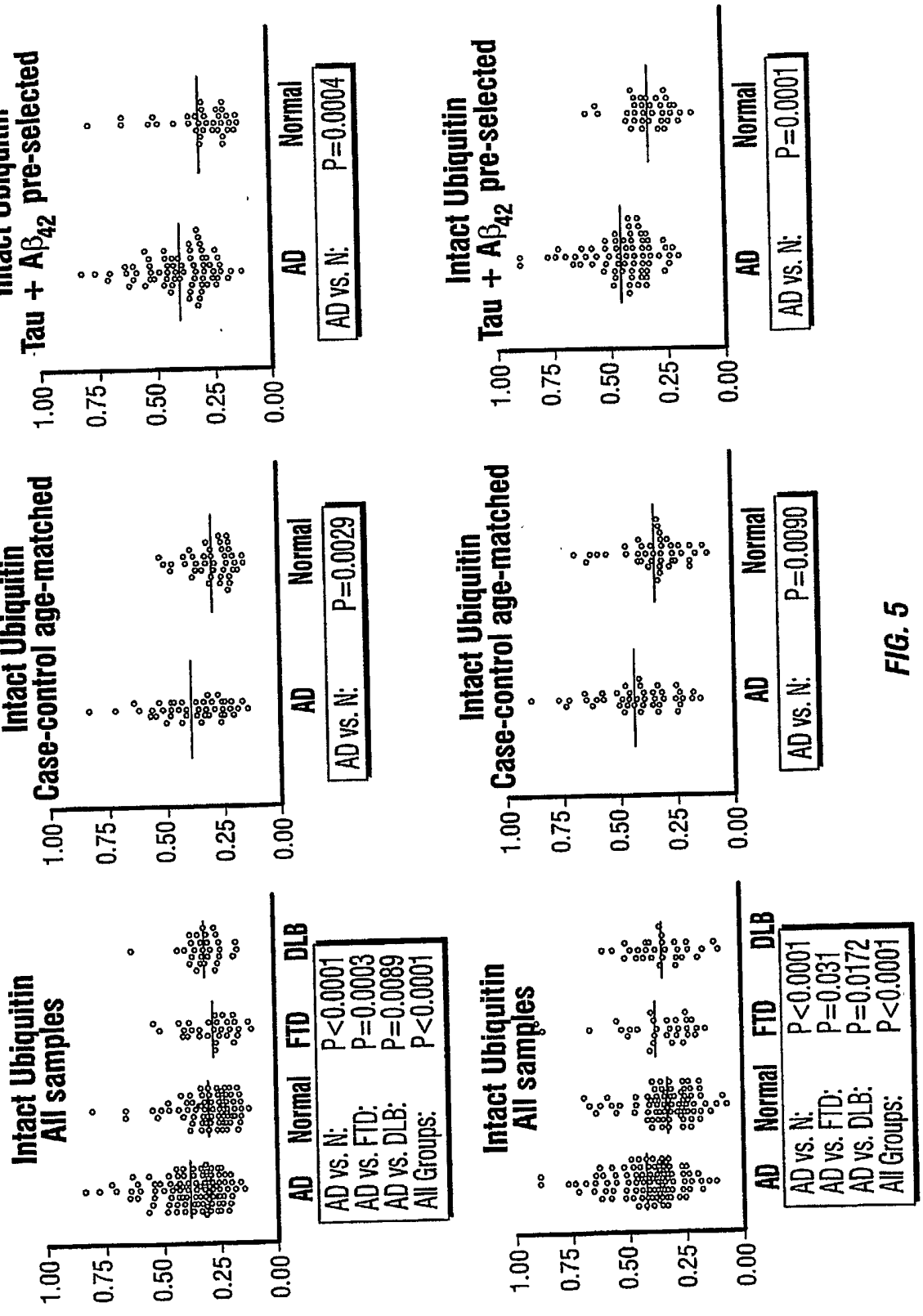

Intact Ubiquitin
All samples
AD Normal FTD DLB
AD vs. N: P<0.0001
AD vs. FTD: P=0.0003
AD vs. DLB: P=0.0089
All Groups: P<0.0001
Intact Ubiquitin
Case-control age-matched
AD Normal
AD vs. N: P=0.0029
Intact Ubiquitin
Tau + Aβ42 pre-selected
AD Normal
AD vs. N: P=0.0004
Intact Ubiquitin
All samples
AD Normal FTD DLB
AD vs. N: P<0.0001
AD vs. FTD: P=0.031
AD vs. DLB: P=0.0172
All Groups: P<0.0001
Intact Ubiquitin
Case-control age-matched
AD Normal
AD vs. N: P=0.0090
Intact Ubiquitin
Tau + Aβ42 pre-selected
AD Normal
AD vs. N: P=0.0001
1.00
0.75
0.50
0.25
0.00

FIG. 5

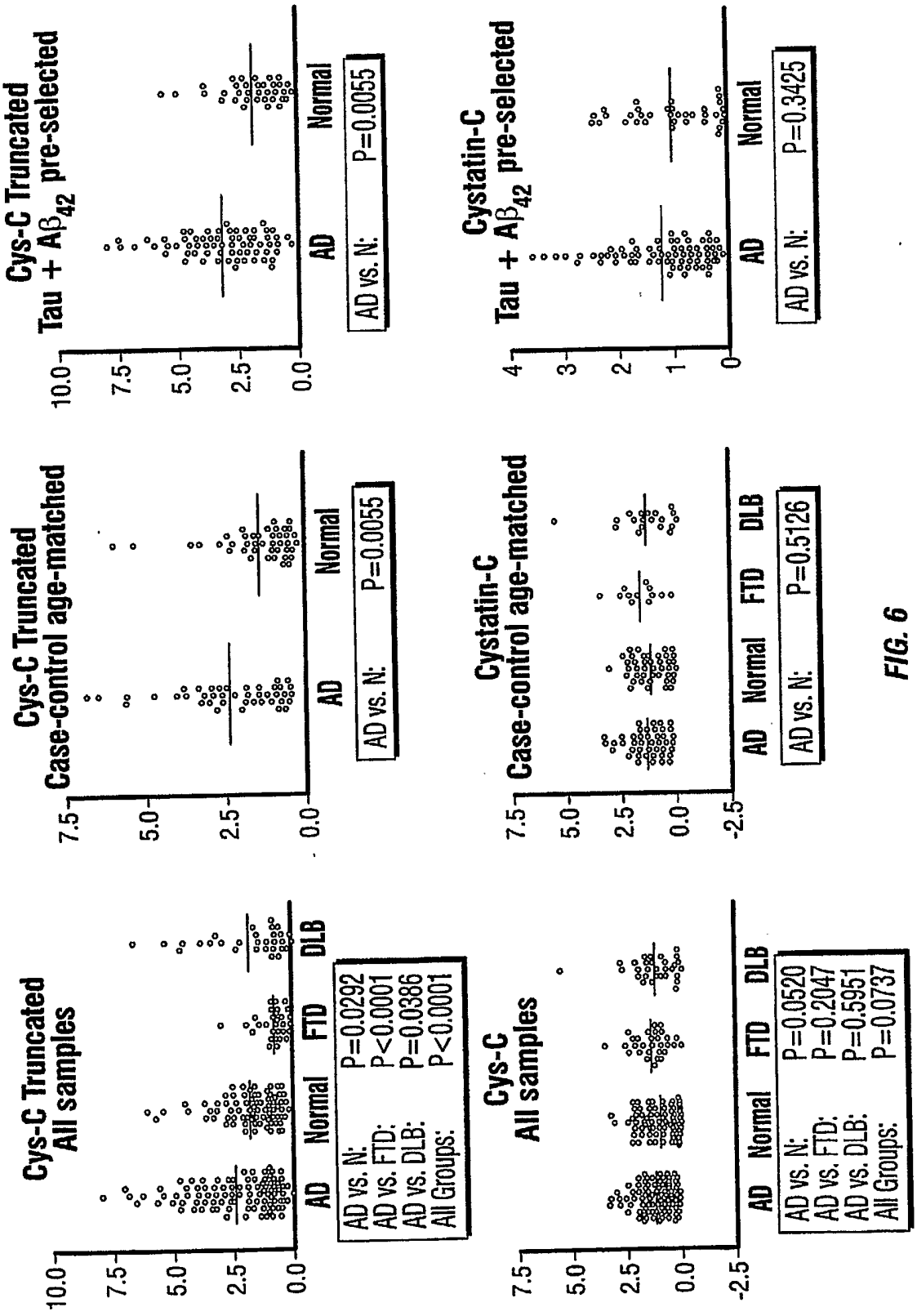

Cys-C Truncated
All samples
AD Normal FTD DLB
AD vs. N: P=0.0292
AD vs. FTD: P<0.0001
AD vs. DLB: P=0.0386
All Groups: P<0.0001
Cys-C Truncated
Case-control age-matched
AD Normal
AD vs. N: P=0.0055
Cys-C Truncated
Tau + Aβ42 pre-selected
AD Normal
AD vs. N: P=0.0055
Cys-C
All samples
AD Normal FTD DLB
AD vs. N: P=0.0520
AD vs. FTD: P=0.2047
AD vs. DLB: P=0.5951
All Groups: P=0.0737
Cystatin-C
Case-control age-matched
AD Normal FTD DLB
AD vs. N: P=0.5126
Cystatin-C
Tau + Aβ42 pre-selected
AD Normal
AD vs. N: P=0.3425

FIG. 6

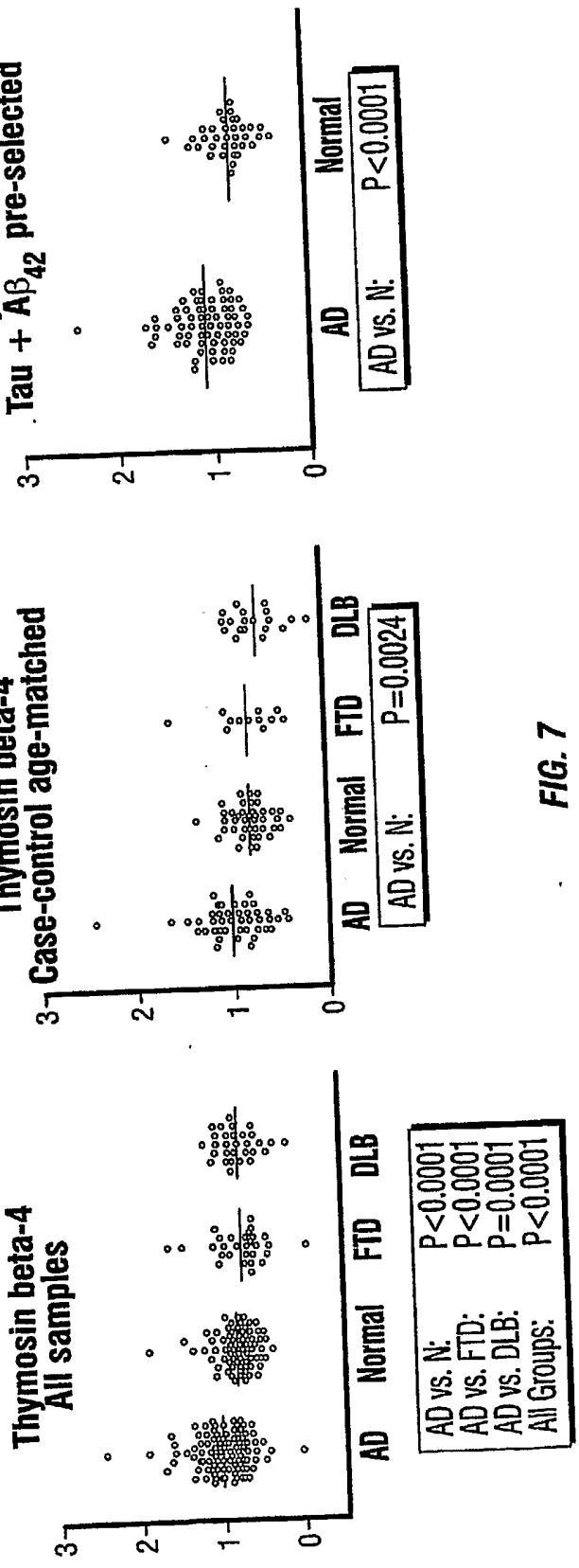

Thymosin beta-4
All samples
AD Normal FTD DLB
AD vs. N: P<0.0001
AD vs. FTD: P<0.0001
AD vs. DLB: P=0.0001
All Groups: P<0.0001
Thymosin beta-4
Case-control age-matched
AD Normal FTD DLB
AD vs. N: P=0.0024
Thymosin beta-4
Tau + Aβ42 pre-selected
AD Normal
AD vs. N: P<0.0001

FIG. 7

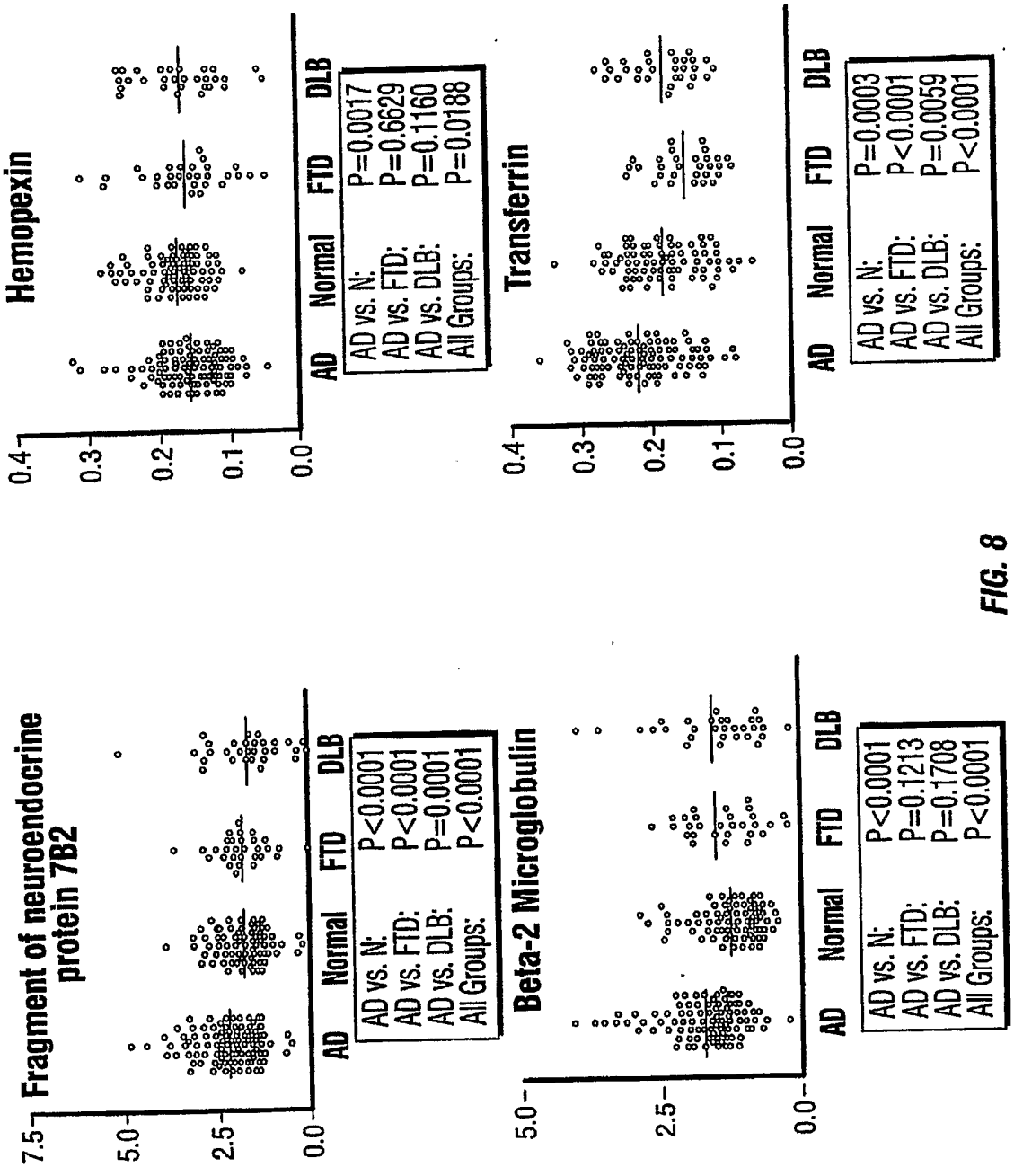

Hemopexin
0.4
0.3
0.2
0.1
0.0
AD Normal FTD DLB
AD vs. N: P=0.0017
AD vs. FTD: P=0.6629
AD vs. DLB: P=0.1160
All Groups: P=0.0188
Transferrin
0.4
0.3
0.2
0.1
0.0
AD Normal FTD DLB
AD vs. N: P=0.0003
AD vs. FTD: P<0.0001
AD vs. DLB: P=0.0059
All Groups: P<0.0001
Fragment of neuroendocrine protein 7B2
7.5
5.0
2.5
0.0
AD Normal FTD DLB
AD vs. N: P<0.0001
AD vs. FTD: P<0.0001
AD vs. DLB: P=0.0001
All Groups: P<0.0001
Beta-2 Microglobulin
5.0
2.5
0.0
AD Normal FTD DLB
AD vs. N: P<0.0001
AD vs. FTD: P=0.1213
AD vs. DLB: P=0.1708
All Groups: P<0.0001

FIG. 8

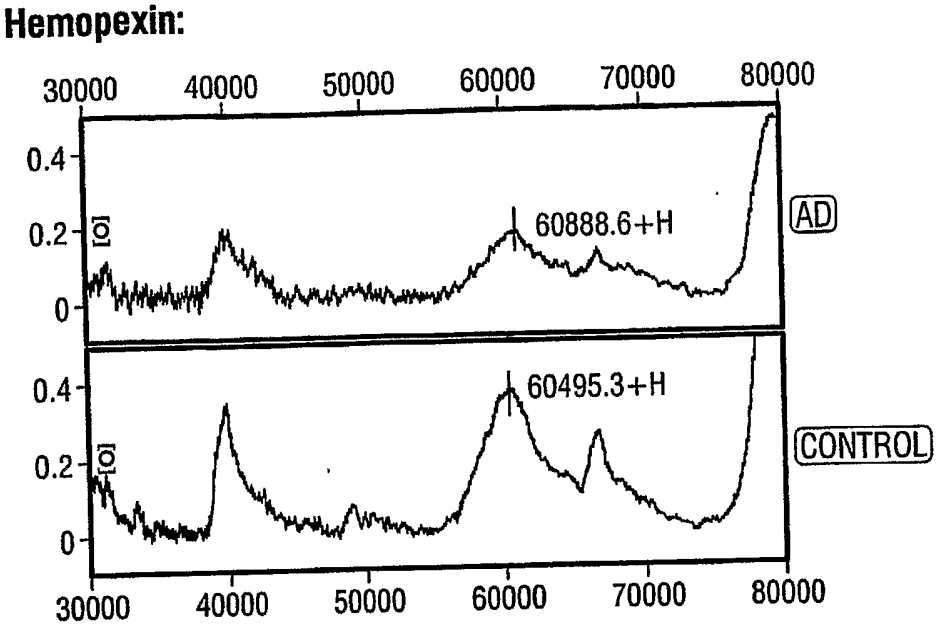

Hemopexin:
30000
40000
50000
60000
70000
80000
0.4
0.2
0
[o]
60888.6+H
AD
60495.3+H
CONTROL

FIG. 9A

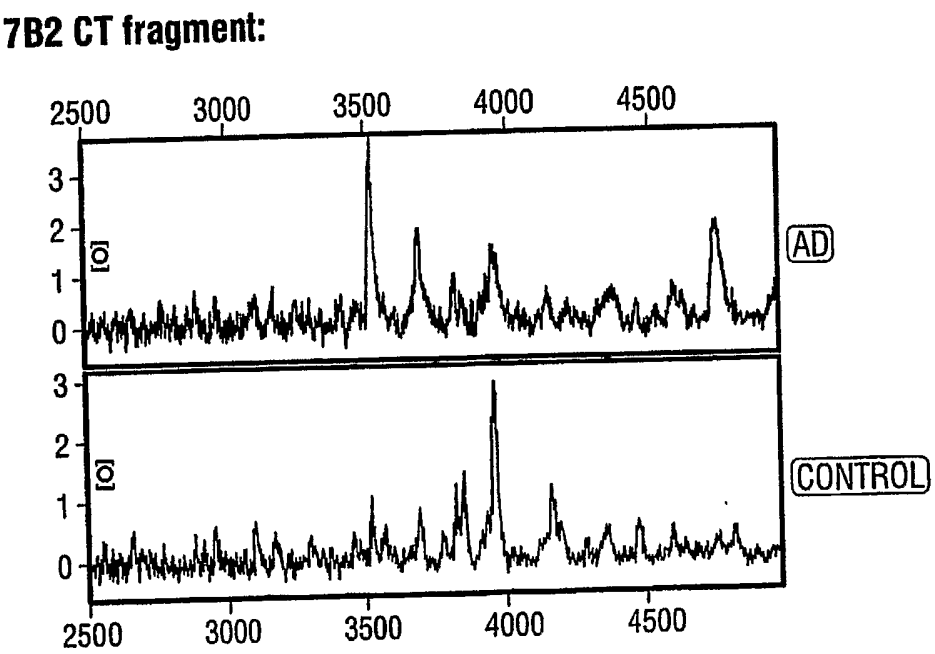

7B2 CT fragment:
2500
3000
3500
4000
4500
3
2
1
0
[o]
AD
CONTROL

FIG. 9B

Ubiquitin -3aa from CT:

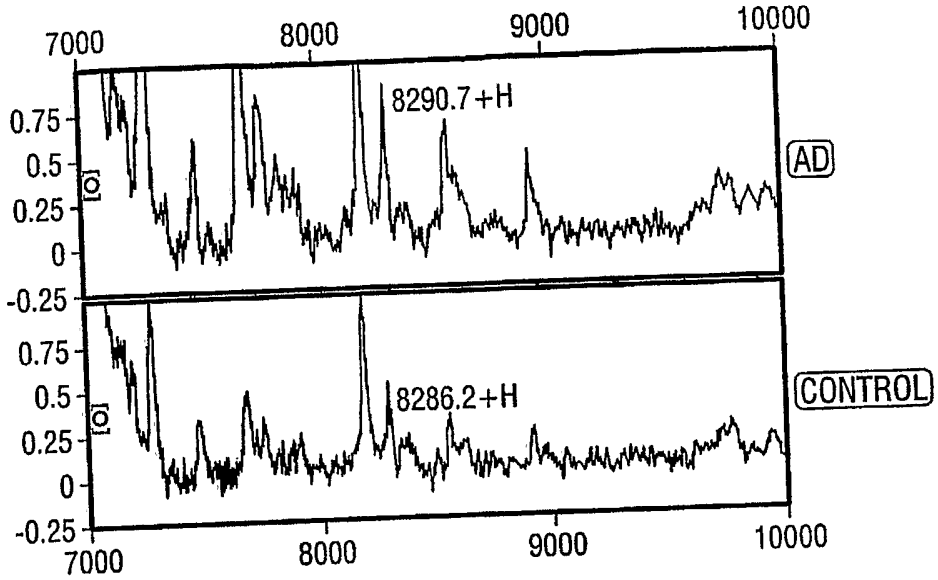


FIG. 9C

Beta-2-microglobulin:

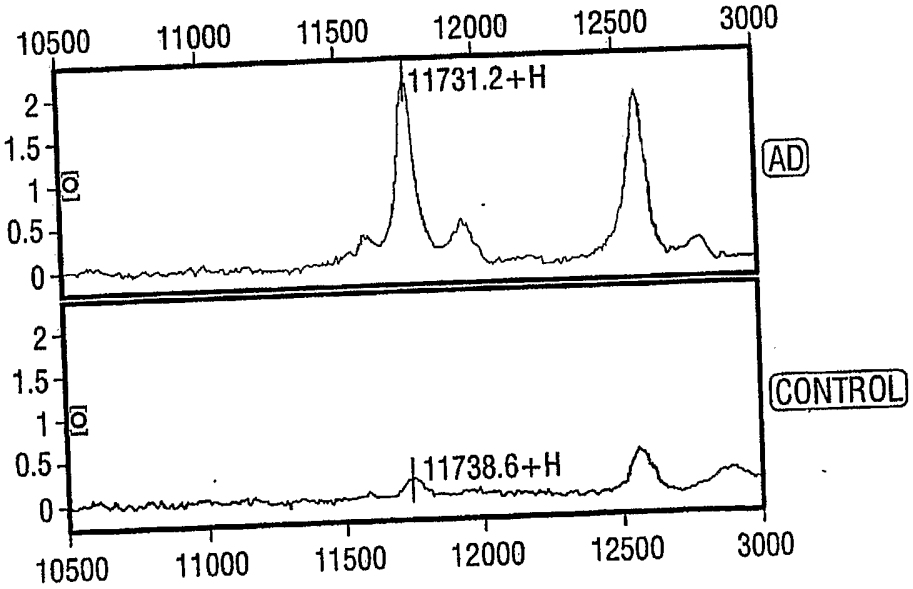


FIG. 9D

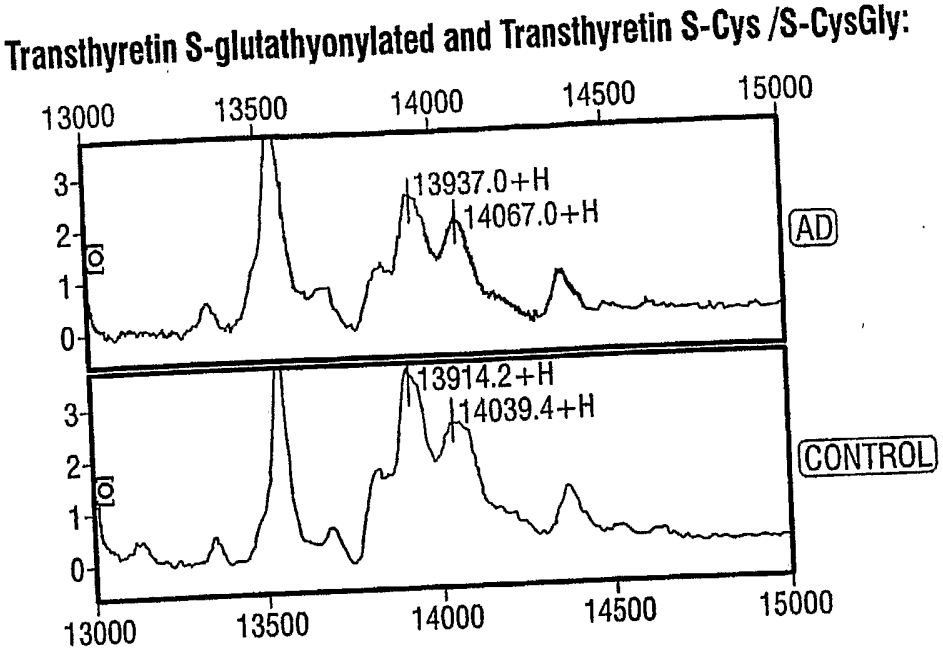

Transthyretin S-glutathyonylated and Transthyretin S-Cys /S-CysGly:
13000
13500
14000
14500
15000
13937.0+H
14067.0+H
AD
13914.2+H
14039.4+H
CONTROL
[o]

FIG. 9E

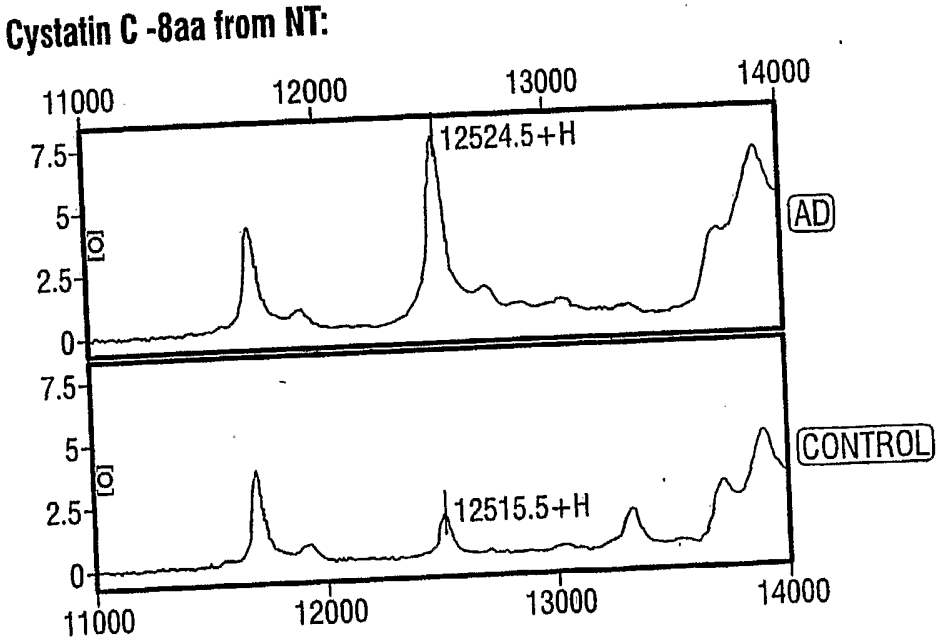

Cystatin C -8aa from NT:
11000
12000
13000
14000
12524.5+H
AD
12515.5+H
CONTROL
[o]

FIG. 9F

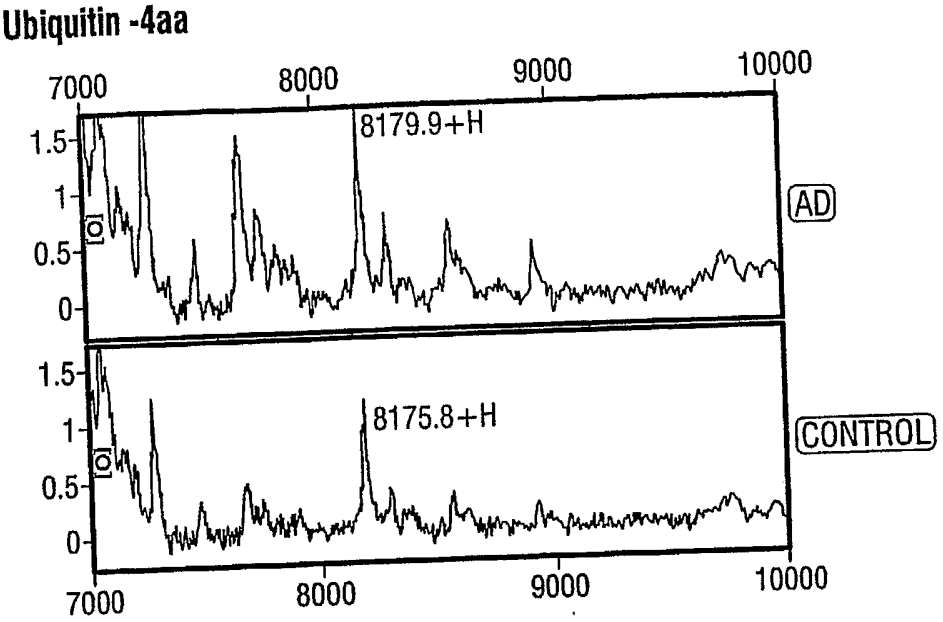

Ubiquitin -4aa
7000
8000
9000
10000
8179.9+H
AD
8175.8+H
CONTROL
1.5
1
0.5
0
[o]

FIG. 9G

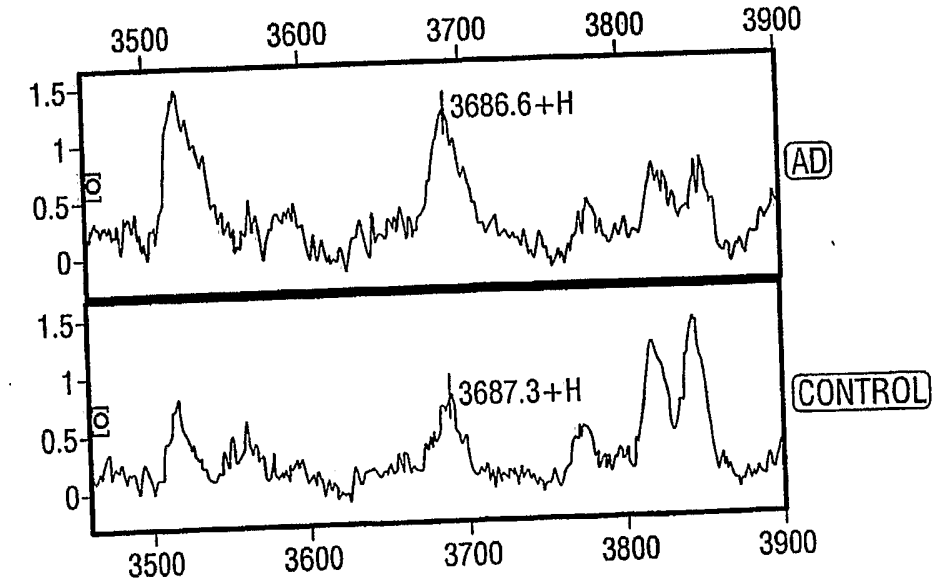

Secretoneurin:
3500
3600
3700
3800
3900
3686.6+H
AD
3687.3+H
CONTROL
1.5
1
0.5
0
[o]

FIG. 9H

Secretoneurin:

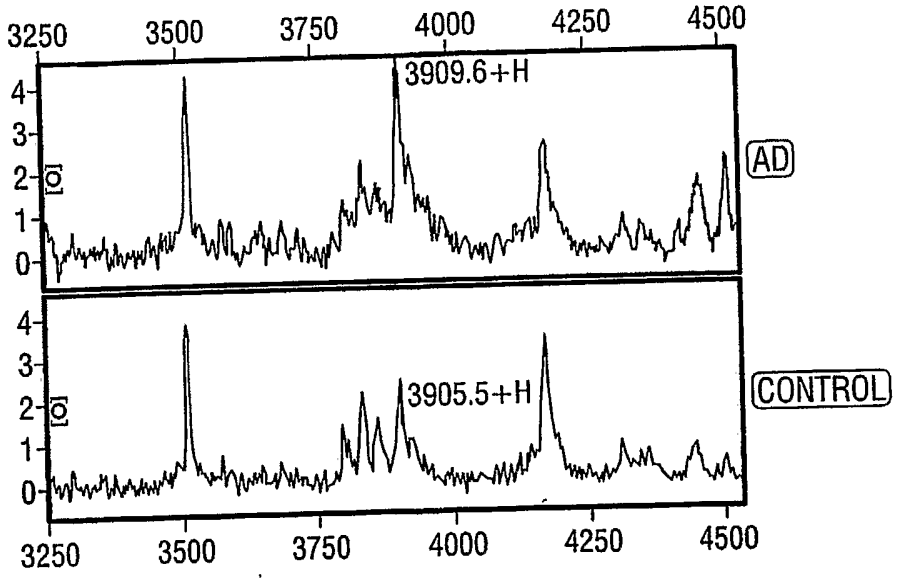


FIG. 9I

Transferrin:

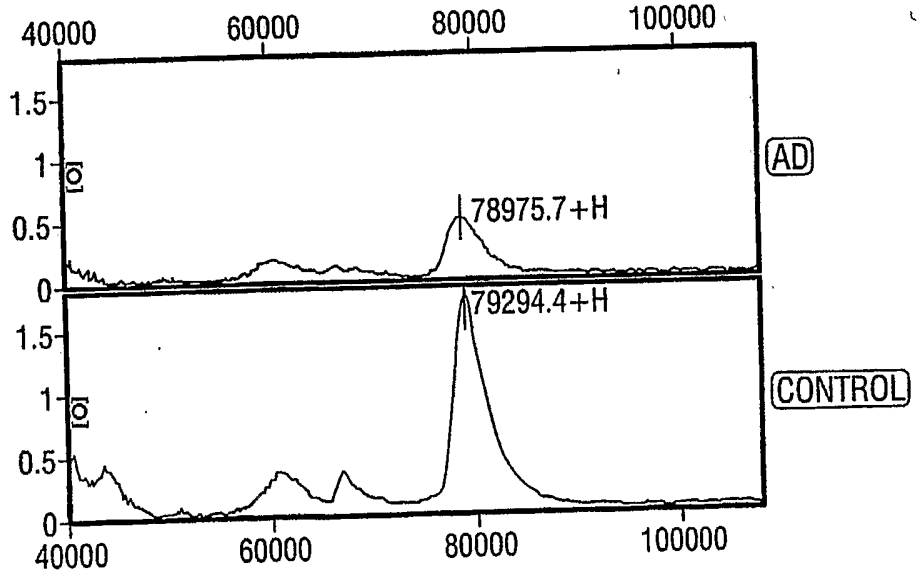


FIG. 9J

Chromogranin B peptide 6.5 kDa:

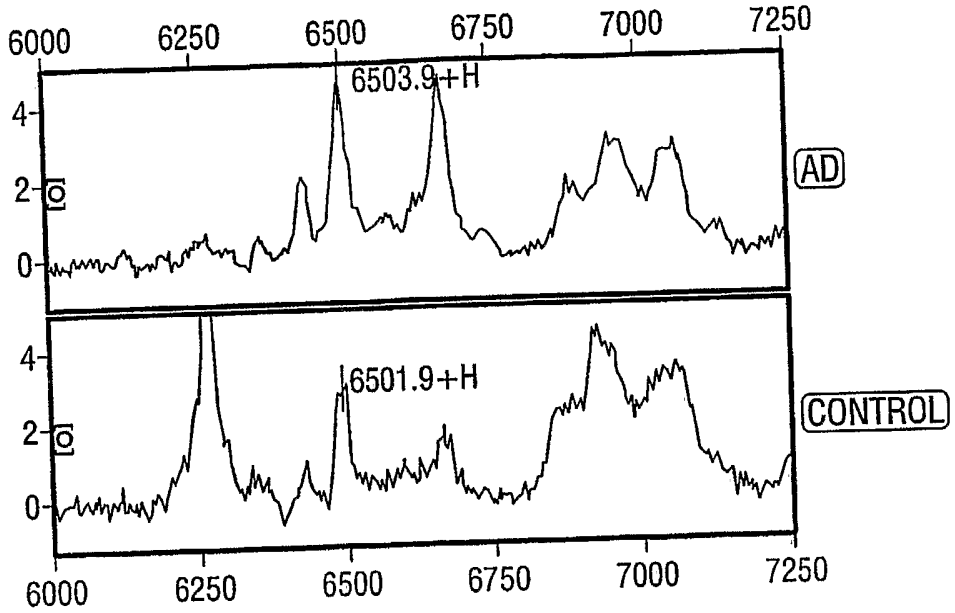


FIG. 9K

A-beta 1-40:

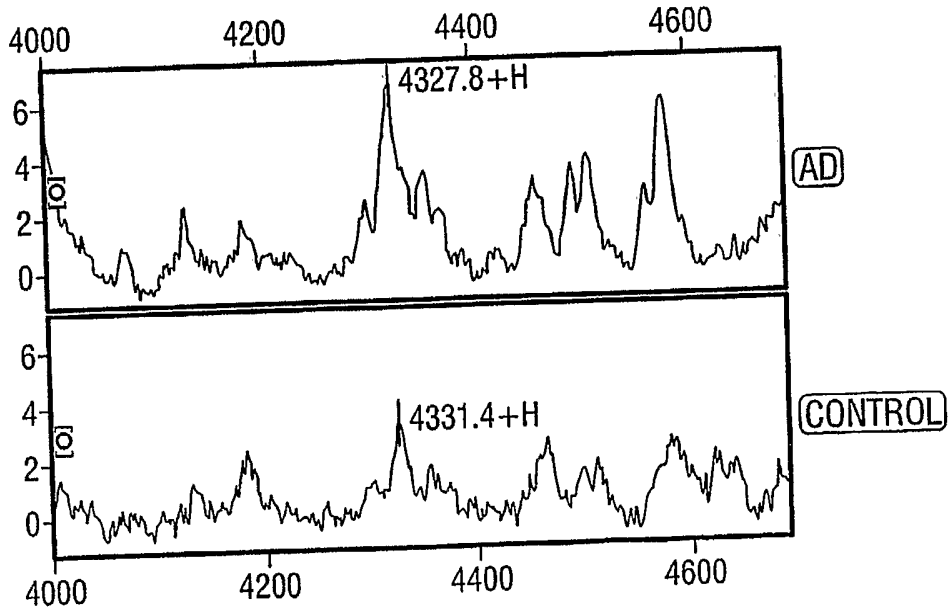


FIG. 9L

Chromogranin B peptide 7.2 kDa:

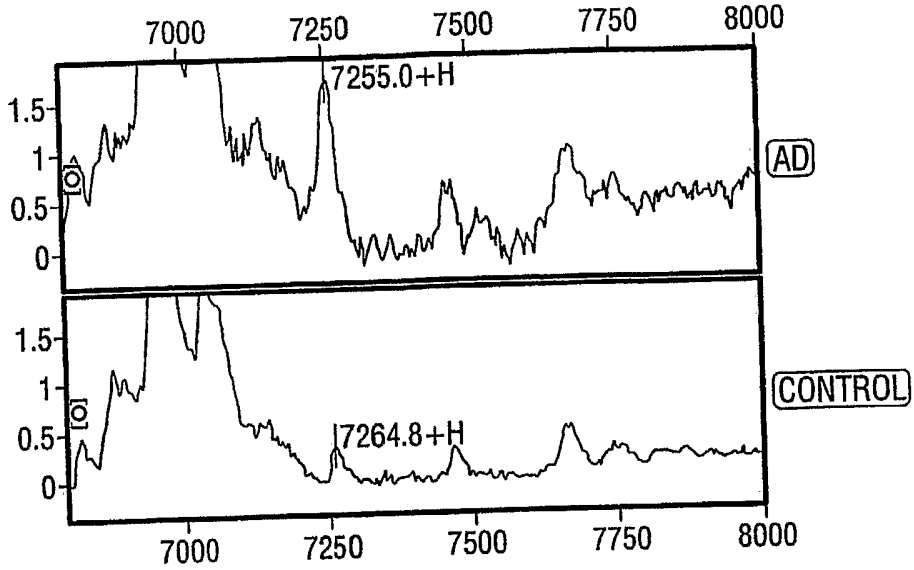


FIG. 9M

Apo A-II dimmer:

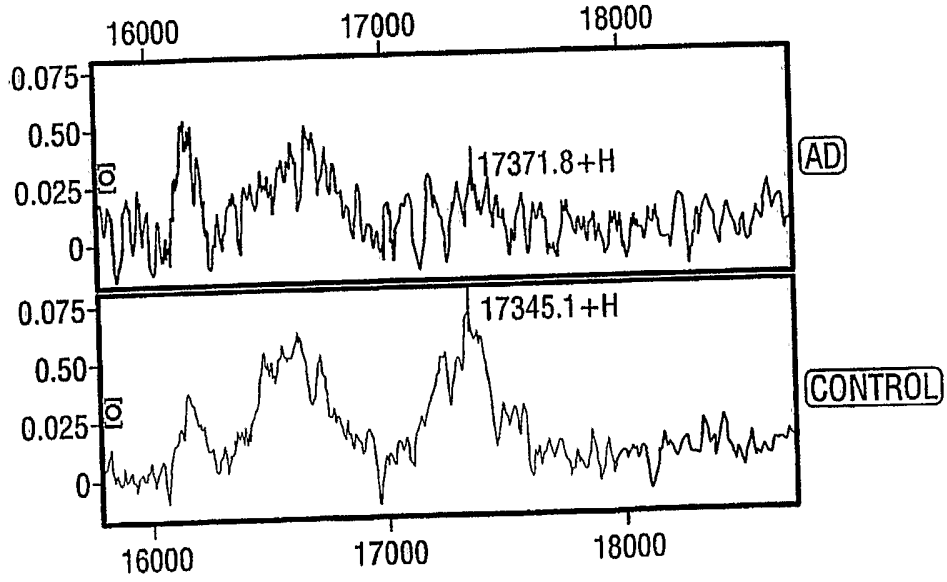


FIG. 9N

C3a des-Arg:

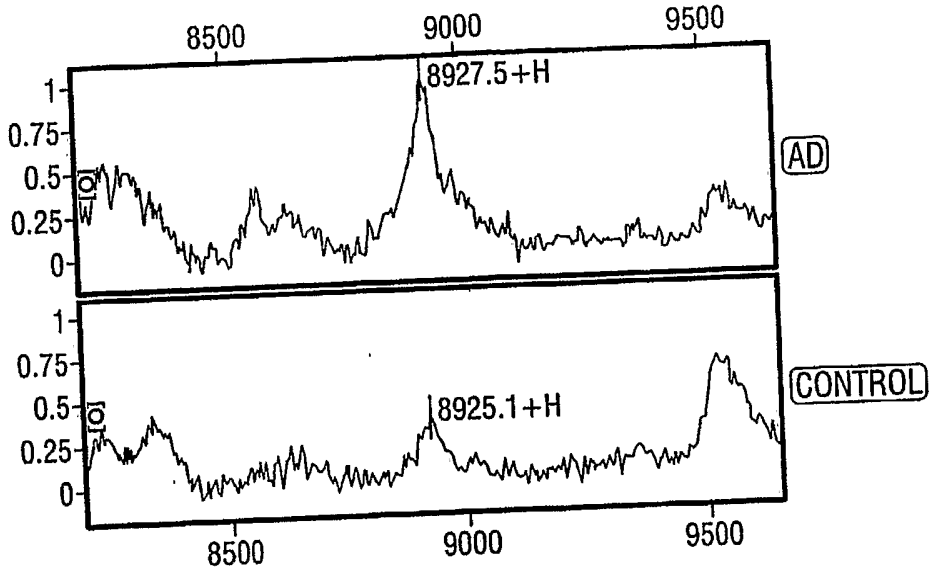


FIG. 90

Cys C:

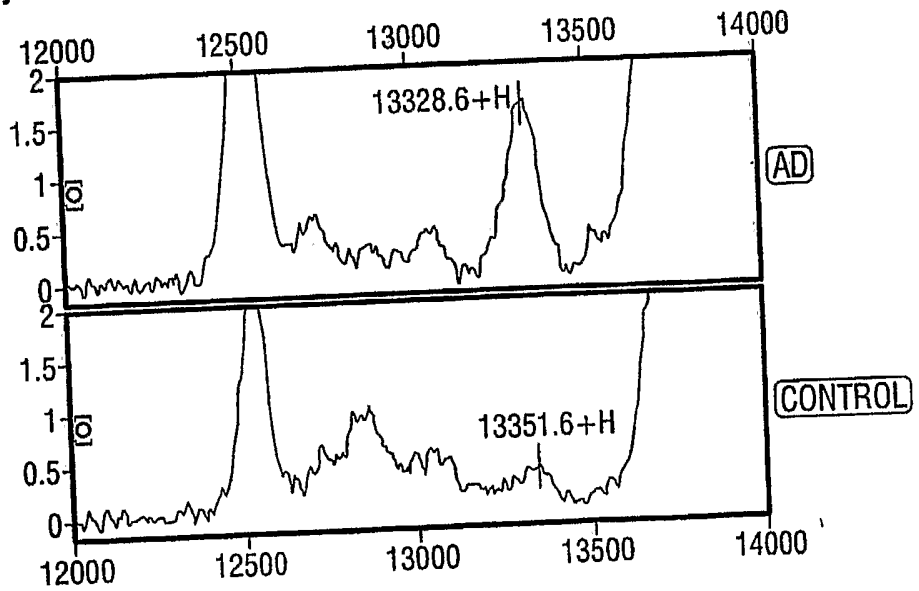


FIG. 9P

Prostagladin-D snythase:

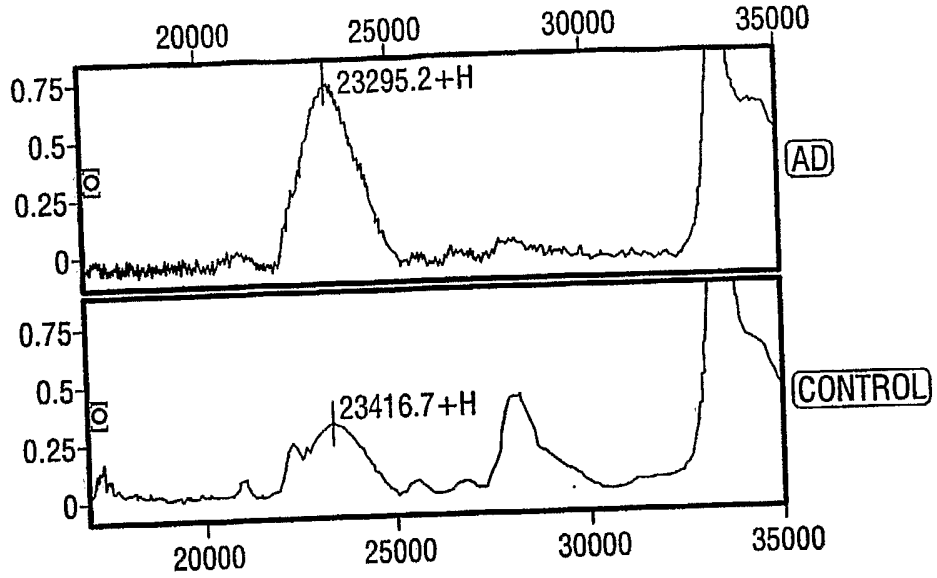


FIG. 9Q

Alpha-1-antichymotrypsin CT fragment:

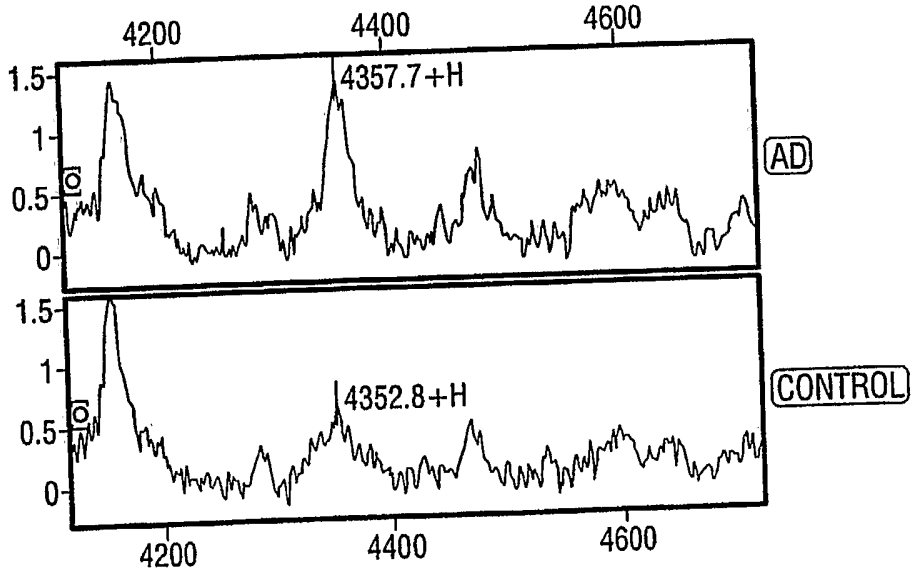


FIG. 9R

Osteopontin CT fragment:

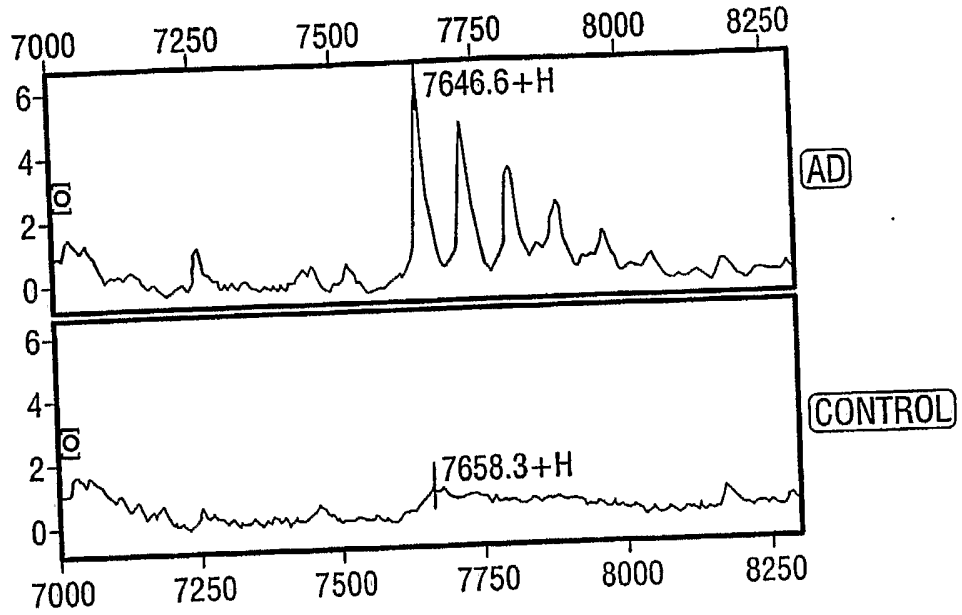


FIG. 9S

VGF (NCBI) peptide:

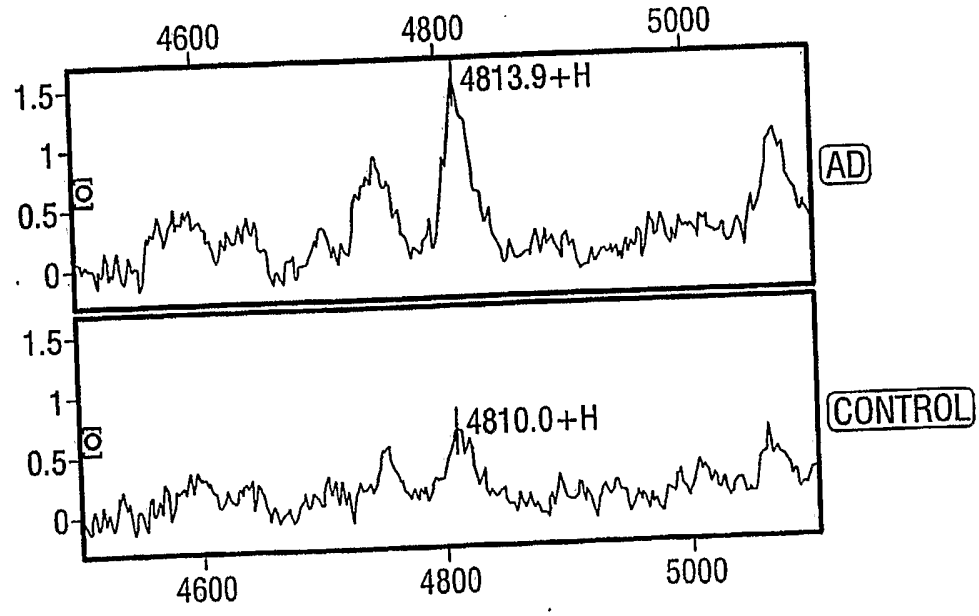


FIG. 9T

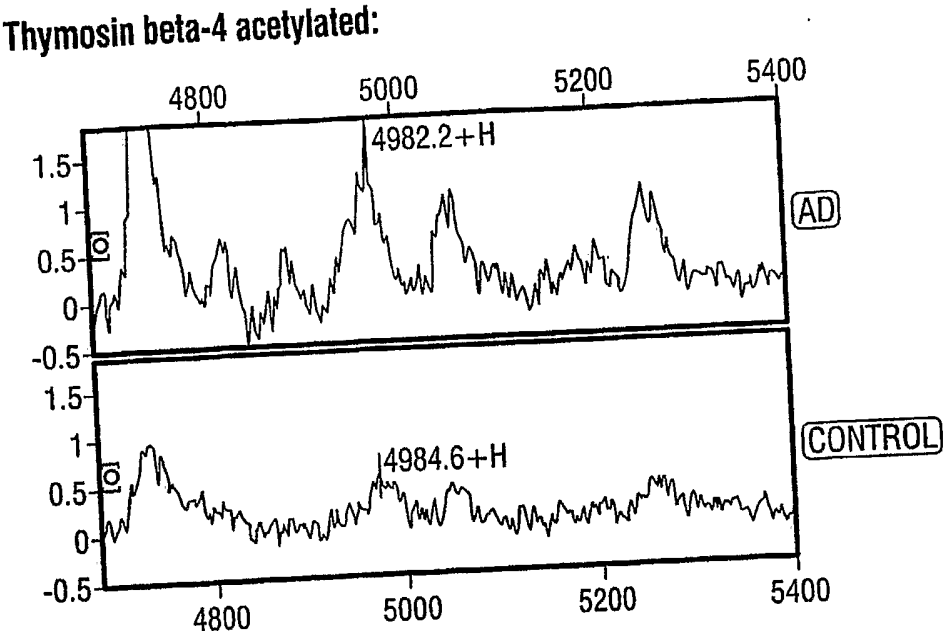

Thymosin beta-4 acetylated:
4800
5000
5200
5400
4982.2+H
1.5
1
0.5
0
-0.5
101
AD
CONTROL
4984.6+H
4800
5000
5200
5400

FIG. 9U

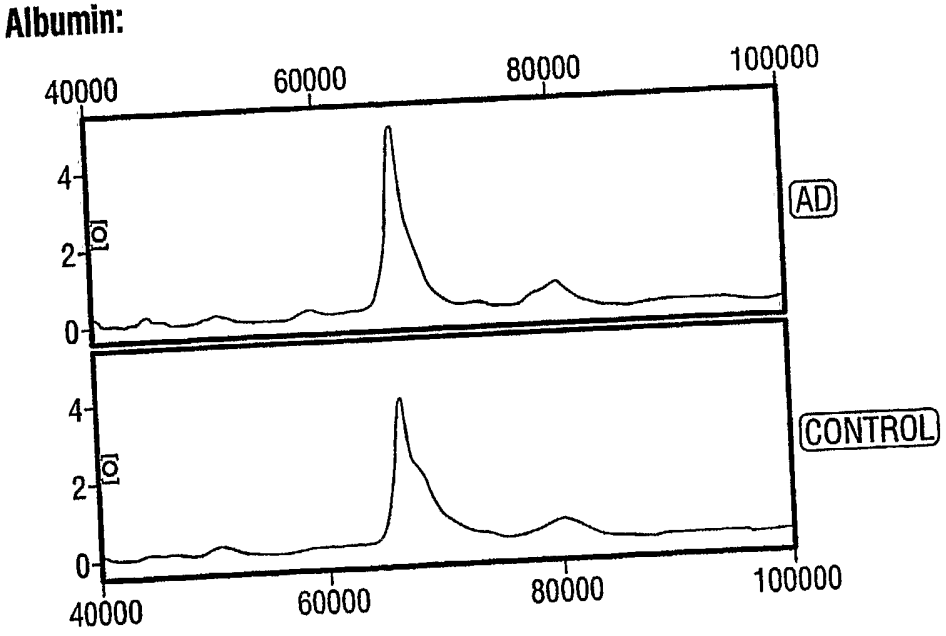

Albumin:
40000
60000
80000
100000
4
2
0
101
AD
CONTROL

FIG. 9V

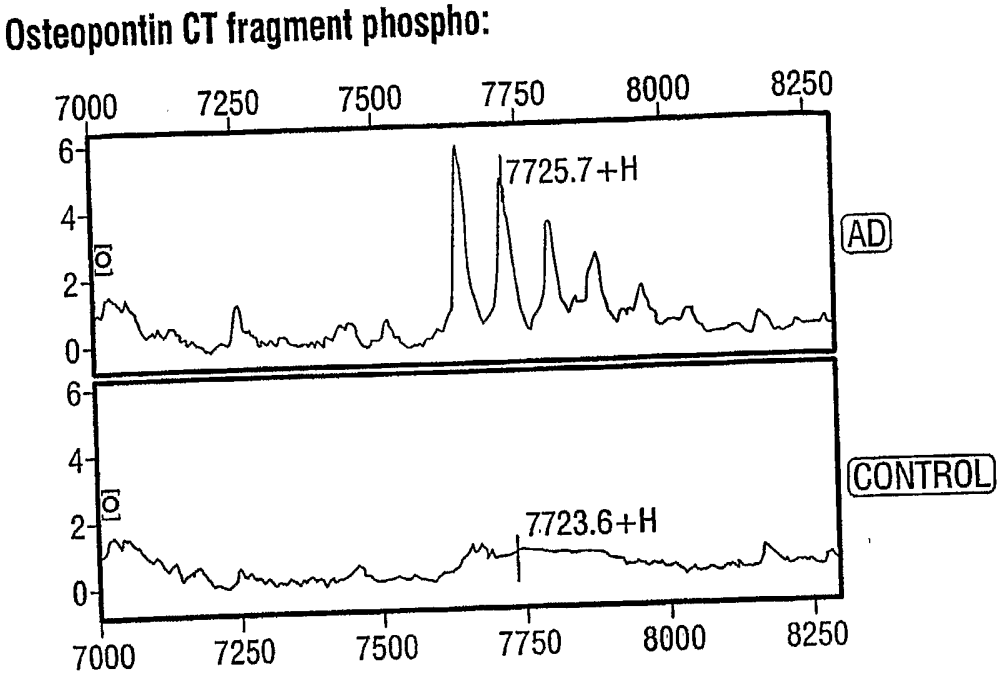

Osteopontin CT fragment phospho:
7000
7250
7500
7750
8000
8250
6
4
2
0
[o]
7725.7+H
AD
CONTROL
7723.6+H

FIG. 9W

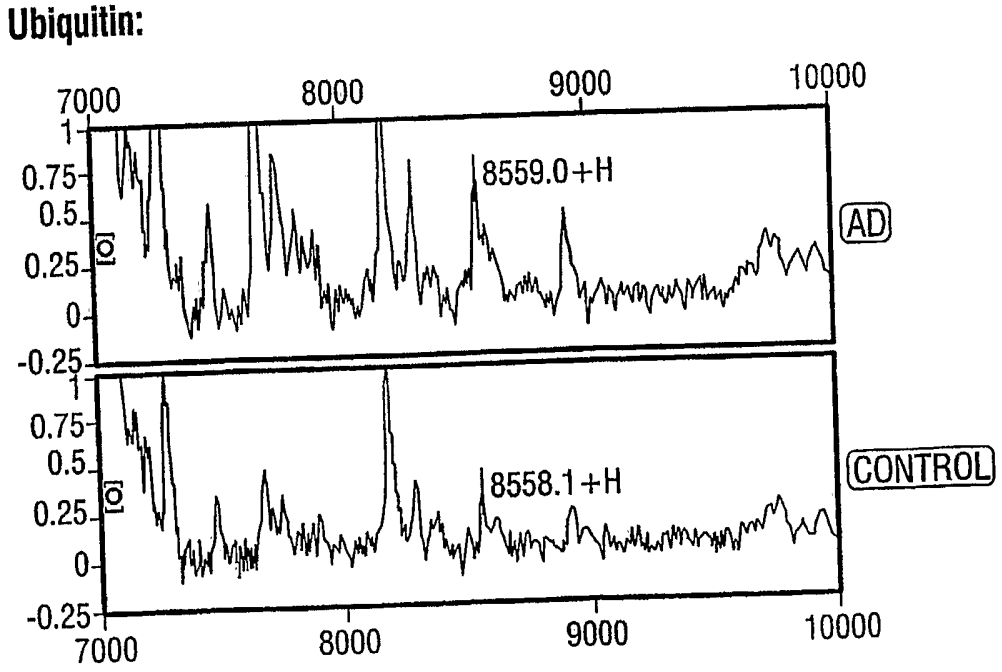

Ubiquitin:
7000
8000
9000
10000
1
0.75
0.5
0.25
0
-0.25
[o]
8559.0+H
AD
8558.1+H
CONTROL

FIG. 9X

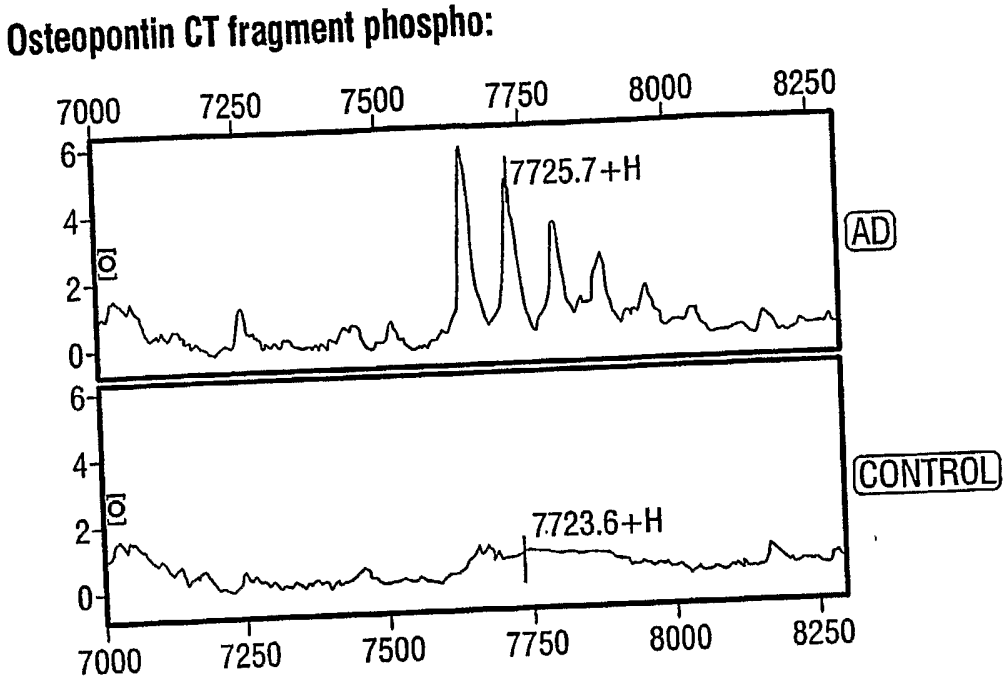

Osteopontin CT fragment phospho:
7000
7250
7500
7750
8000
8250
6
4
2
0
[o]
7725.7+H
AD
CONTROL
7723.6+H

FIG. 9W

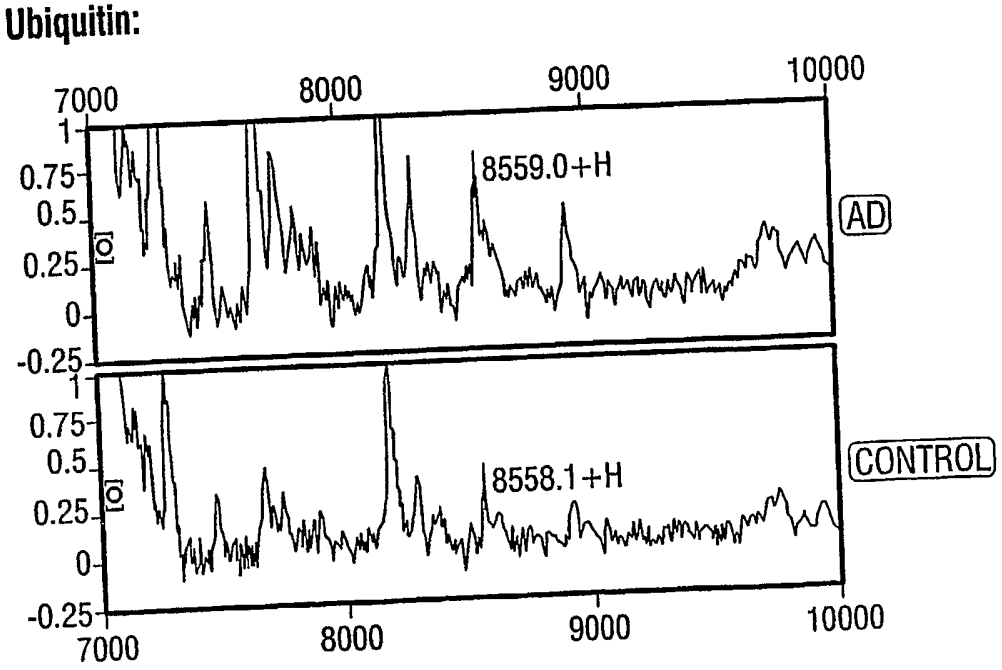

Ubiquitin:
7000
8000
9000
10000
1
0.75
0.5
0.25
0
-0.25
[o]
8559.0+H
AD
CONTROL
8558.1+H

FIG. 9X

5.0 kDa:

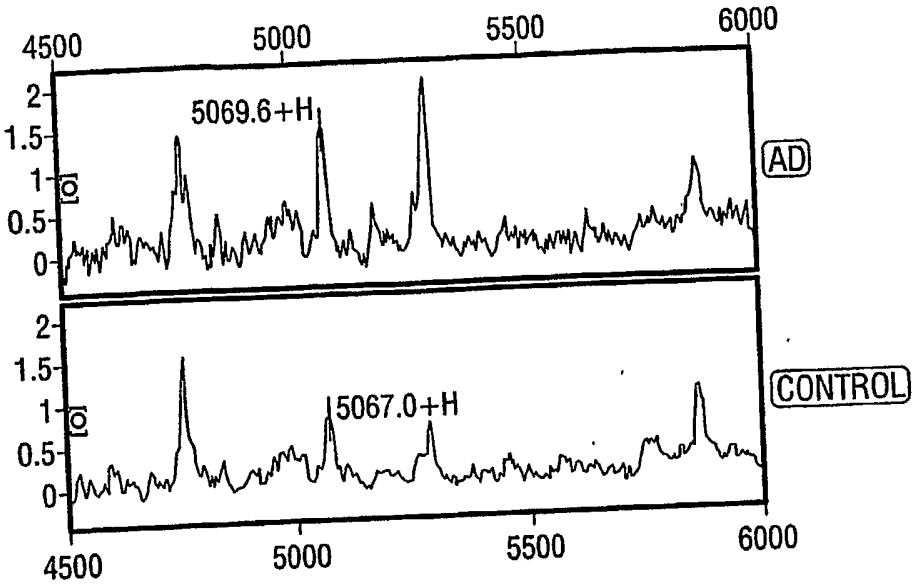


FIG. 9Y

9.7, 9.9, 10.2 and 10.3 kDa:

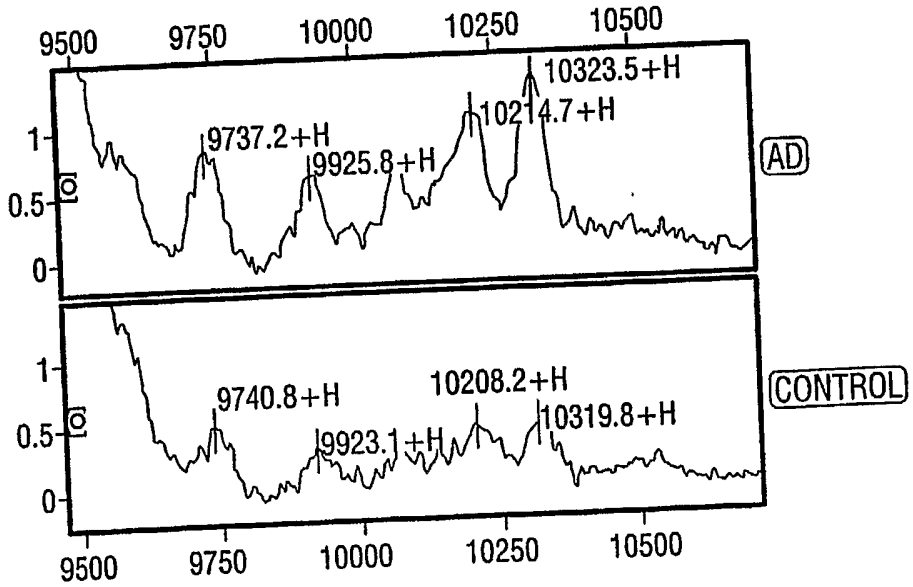


FIG. 9Z

EA-92 Chromogranin A peptide:

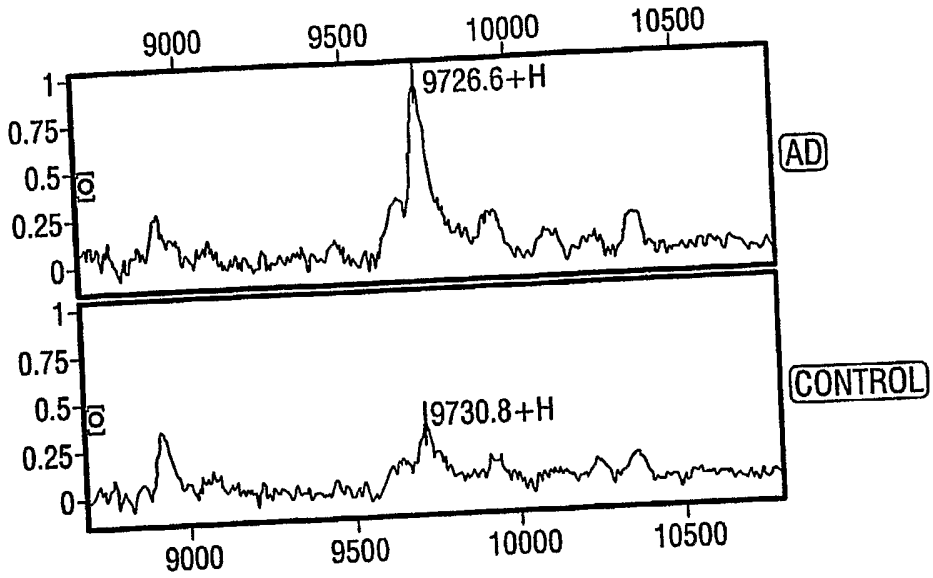


FIG. 9AA

Pancreatic ribonuclease:

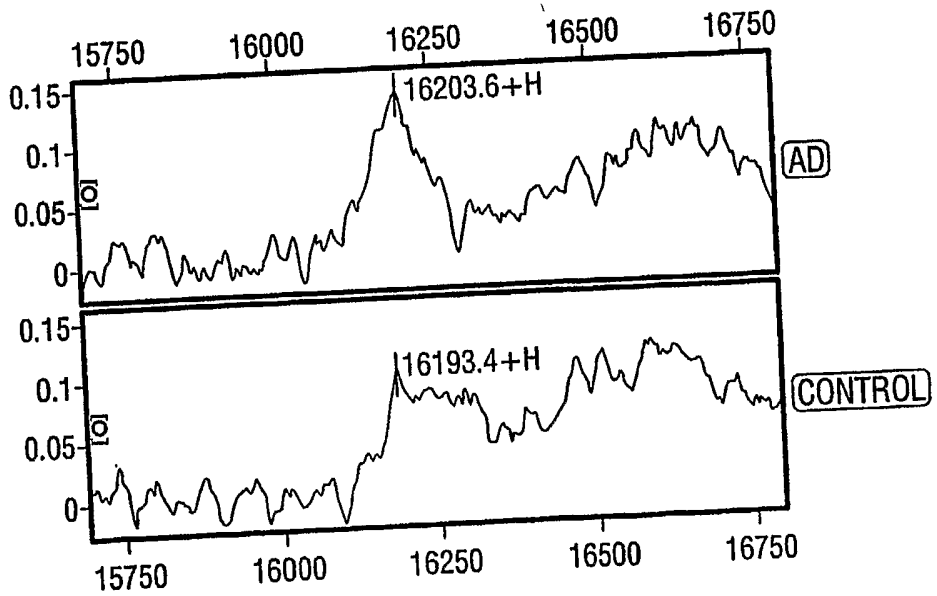


FIG. 9BB

5.2 kDa:

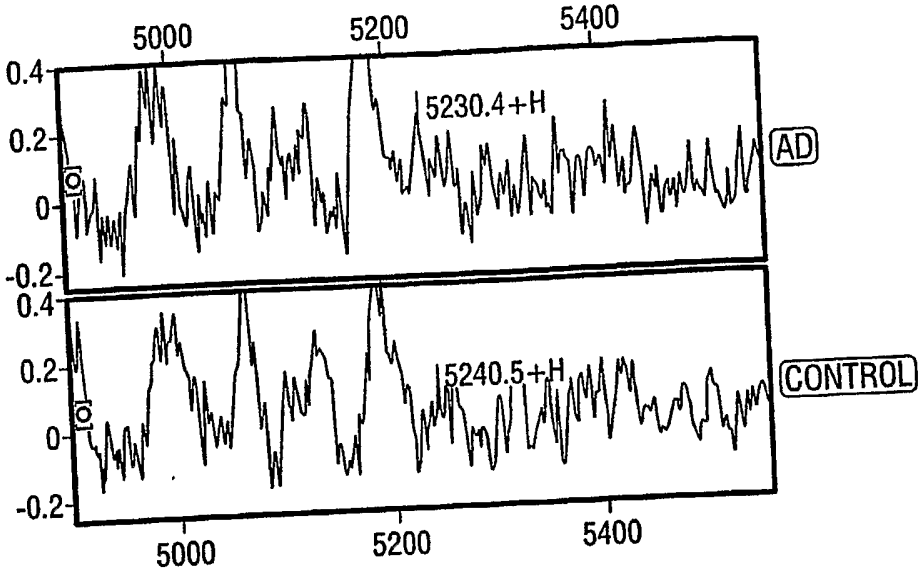


FIG. 9CC

3.8 kDa:

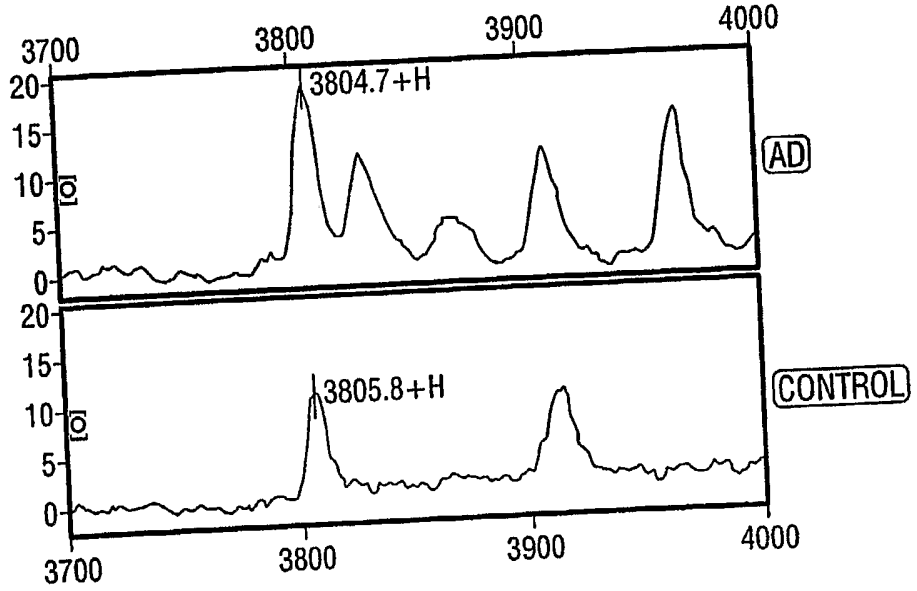


FIG. 9DD

8.9 kDa:

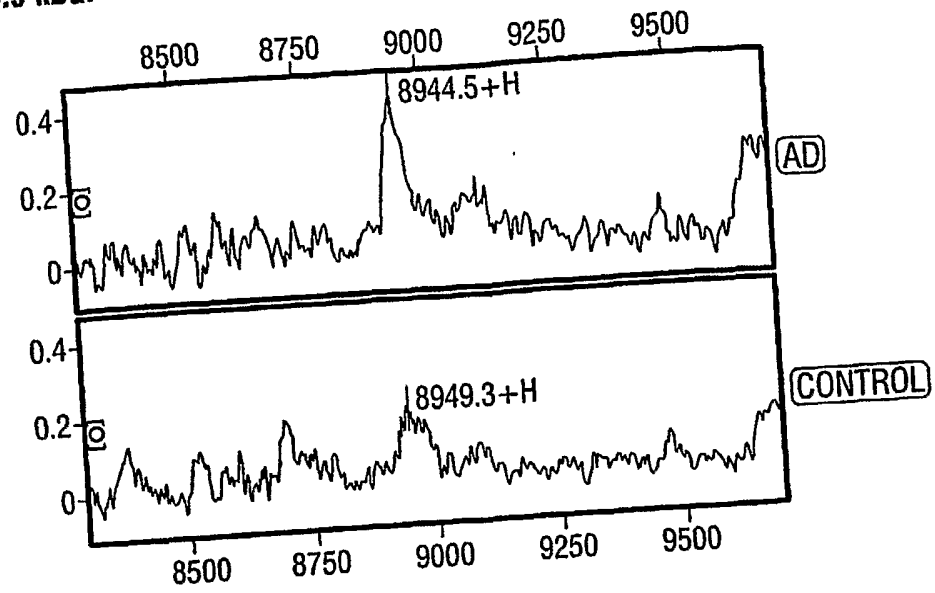


FIG. 9EE

5.2 kDa:

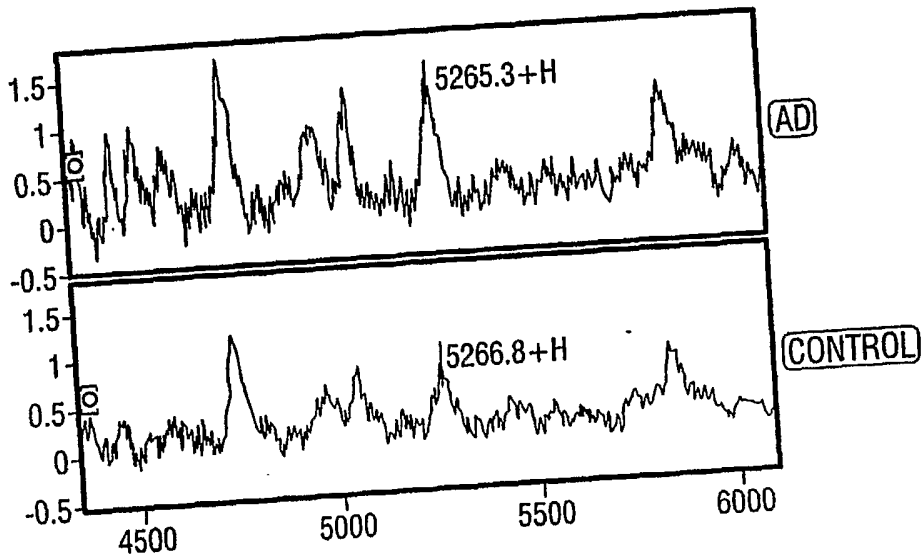


FIG. 9FF

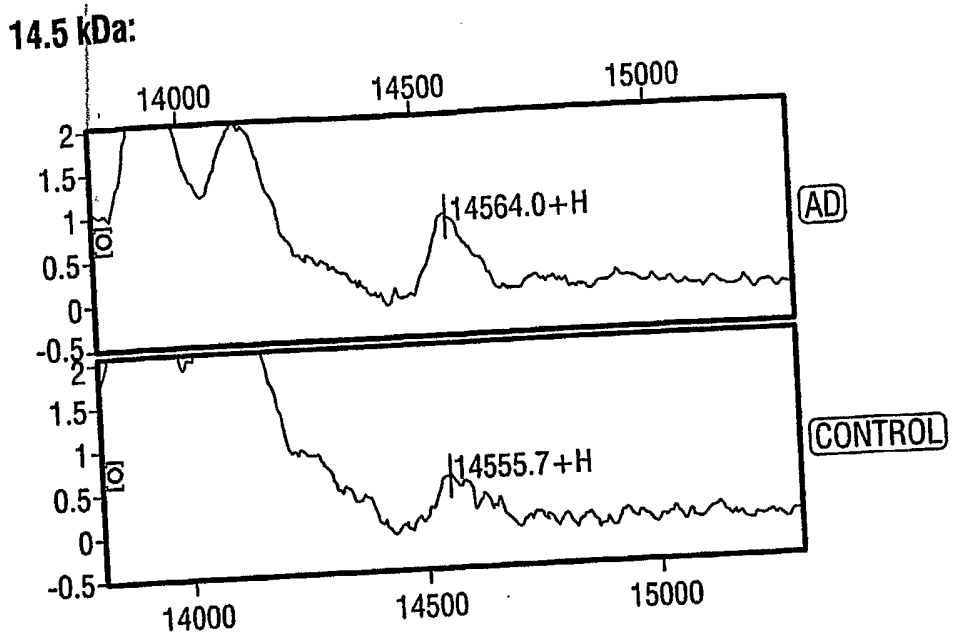


FIG. 9GG

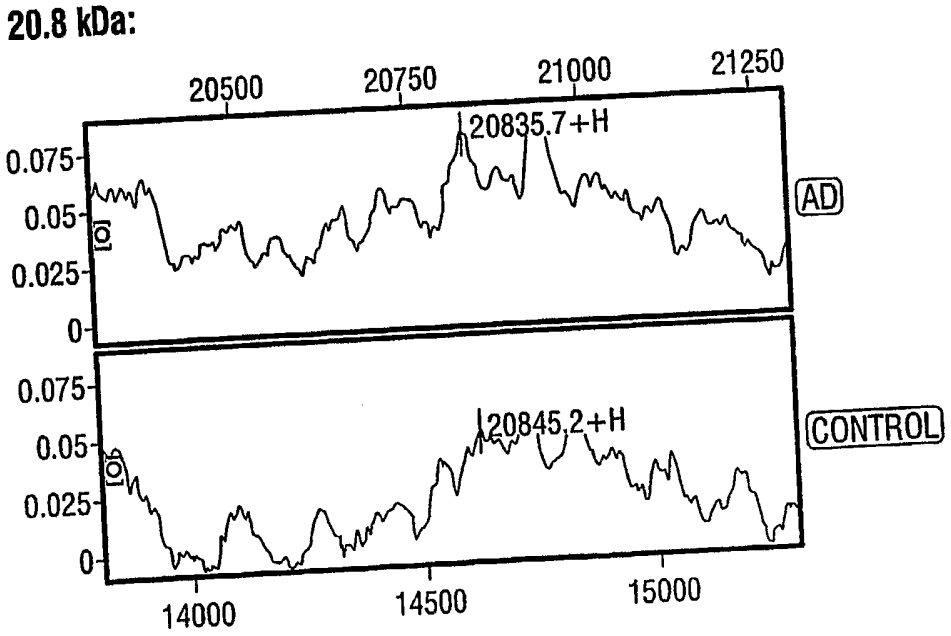


FIG. 9HH

6.6 kDa:

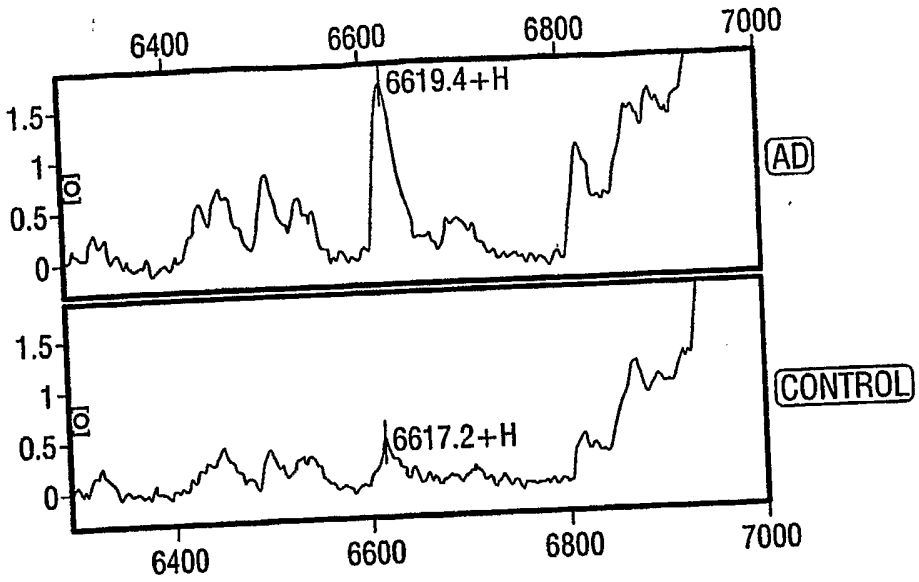


FIG. 9II

7.8 kDa:

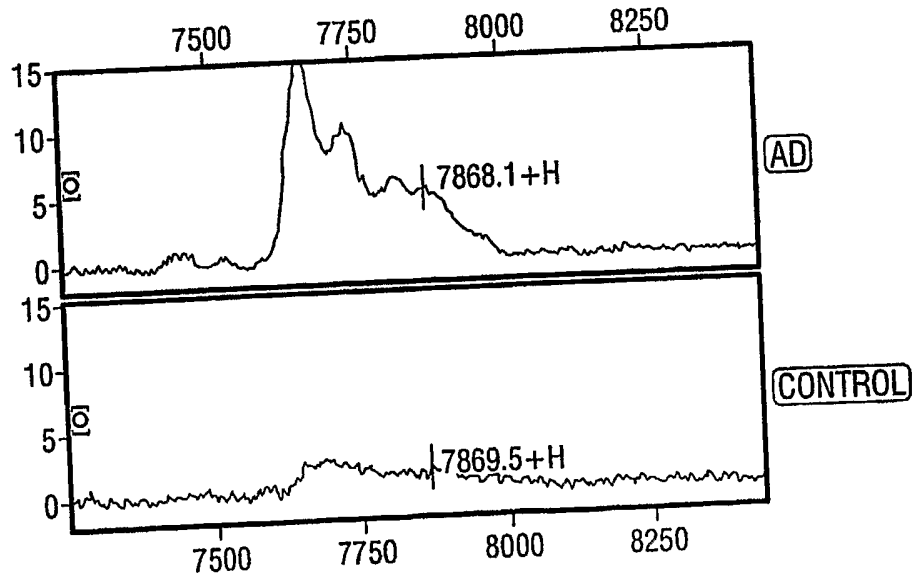


FIG. 9JJ

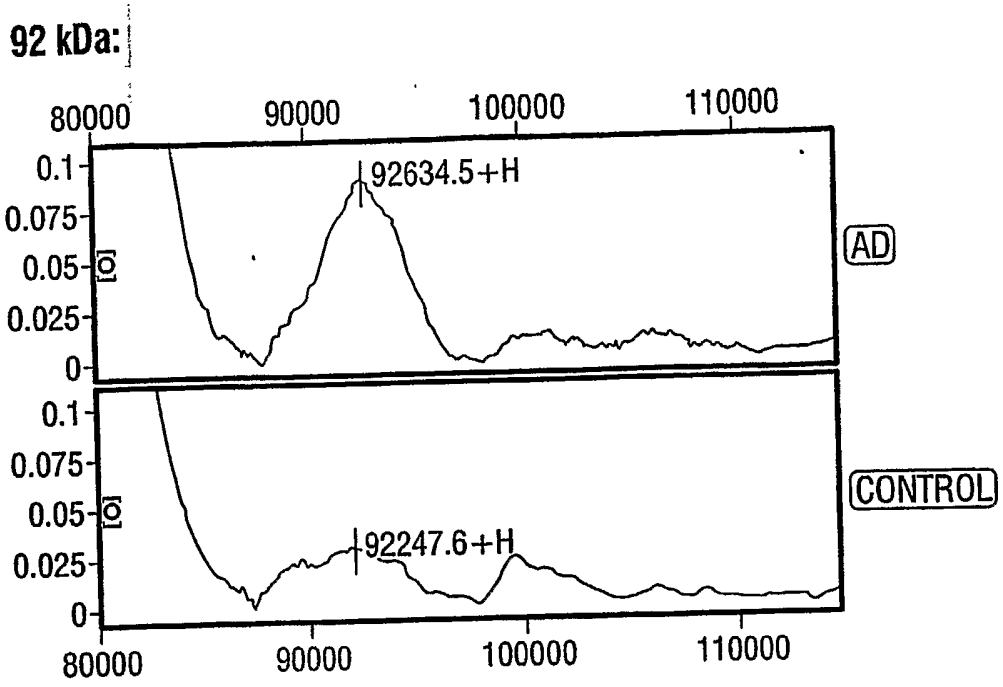


FIG. 9KK

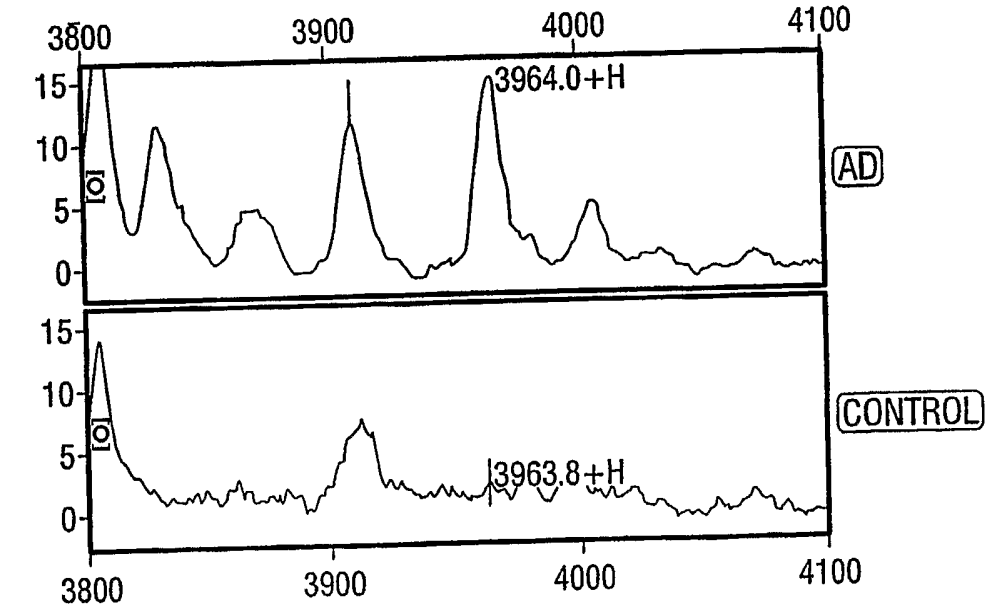


FIG. 9LL

89 kDa:

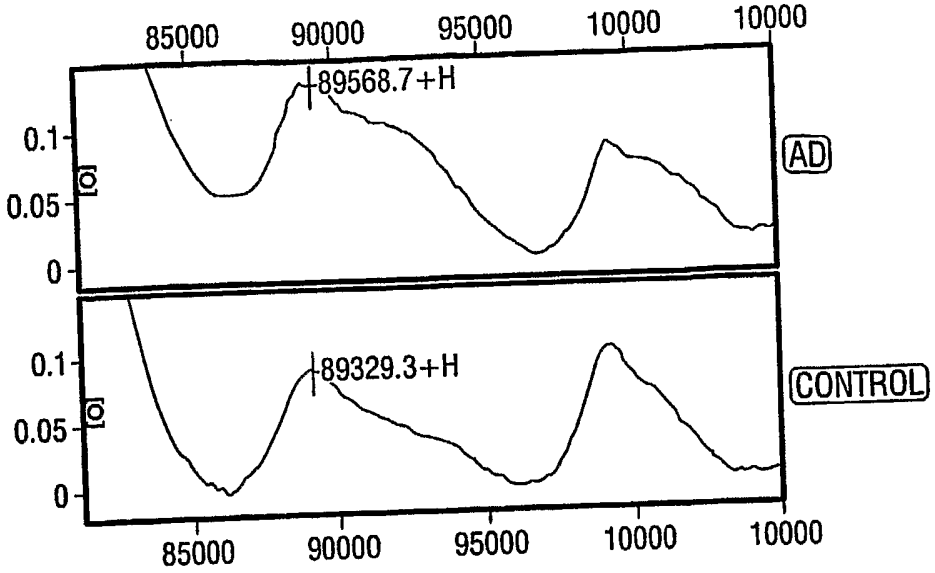


FIG. 9MM

11.5 kDa:

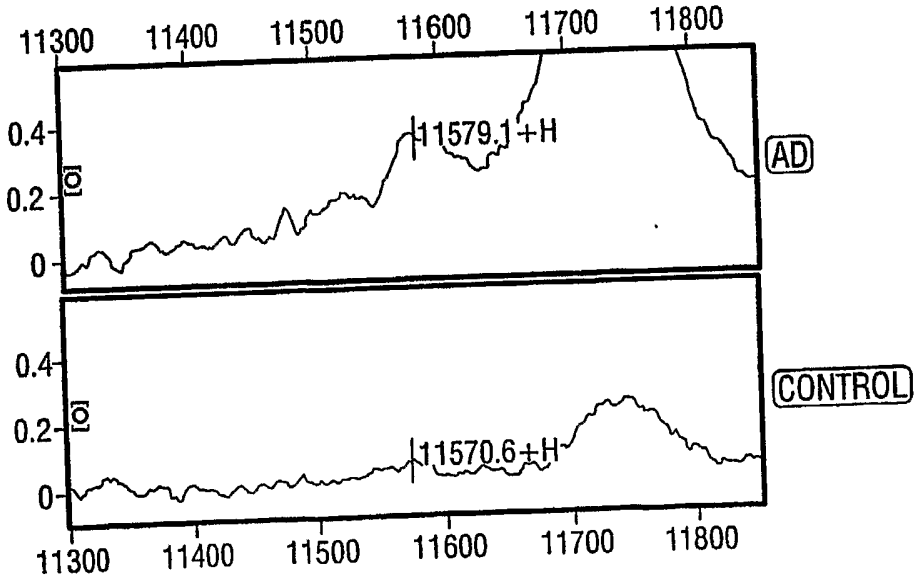


FIG. 9NN

4.4 kDa:

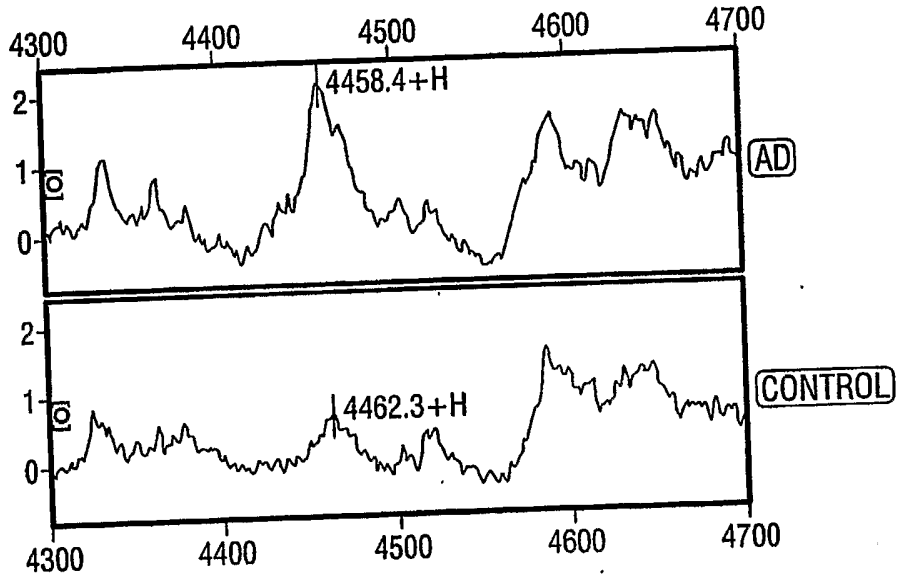


FIG. 900

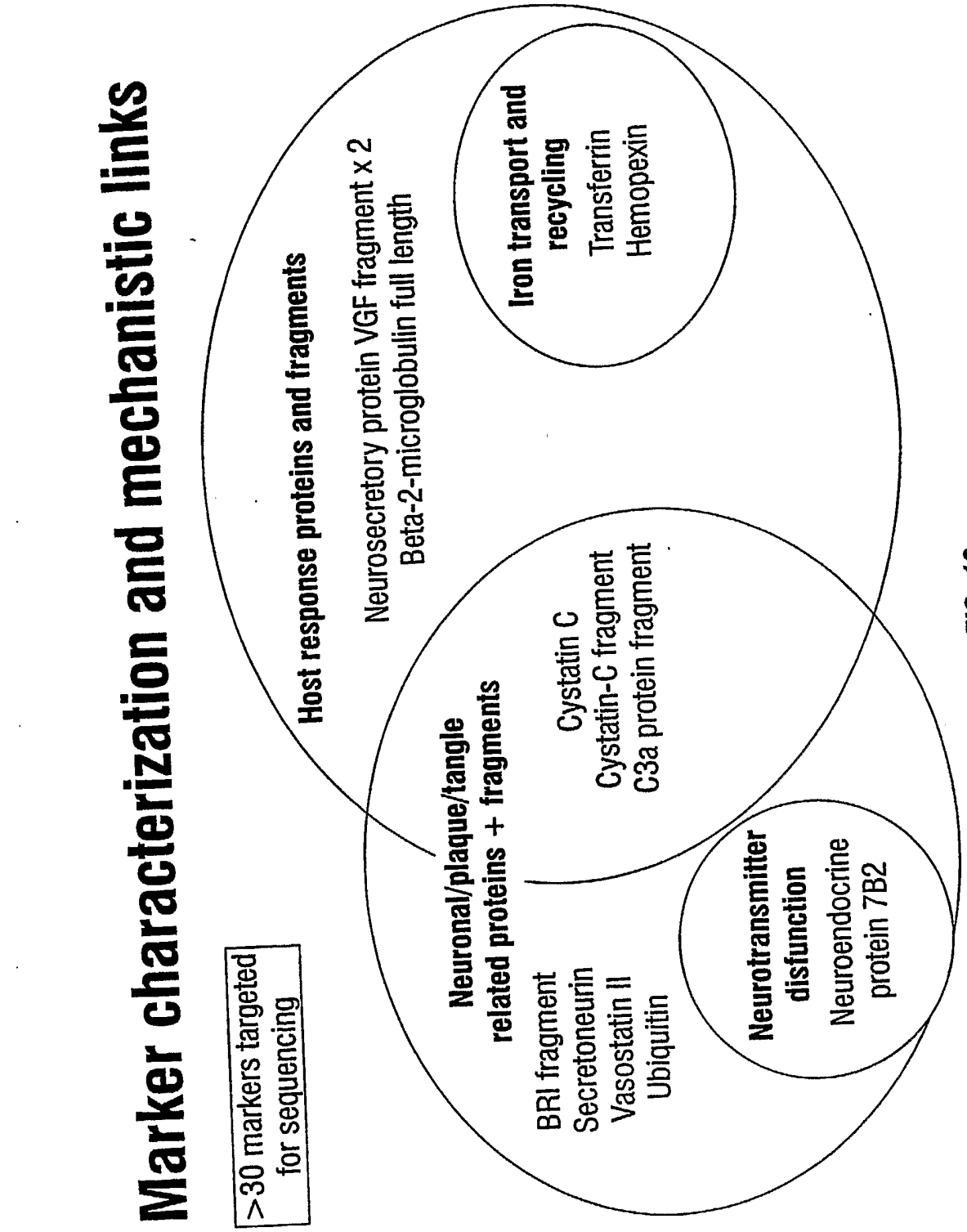
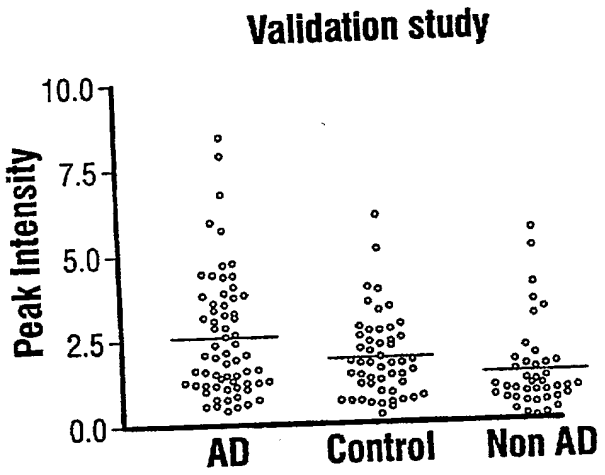

Marker characterization and mechanistic links
>30 markers targeted for sequencing
Host response proteins and fragments
Neurosecretory protein VGF fragment x 2
Beta-2-microglobulin full length
Iron transport and recycling
Transferrin
Hemopexin
Neuronal/plaque/tangle related proteins + fragments
BRI fragment
Secretoneurin
Vasostatin II
Ubiquitin
Cystatin C
Cystatin-C fragment
C3a protein fragment
Neurotransmitter disfunction
Neuroendocrine protein 7B2

FIG. 10

Marker validation

12583.8 Da marker

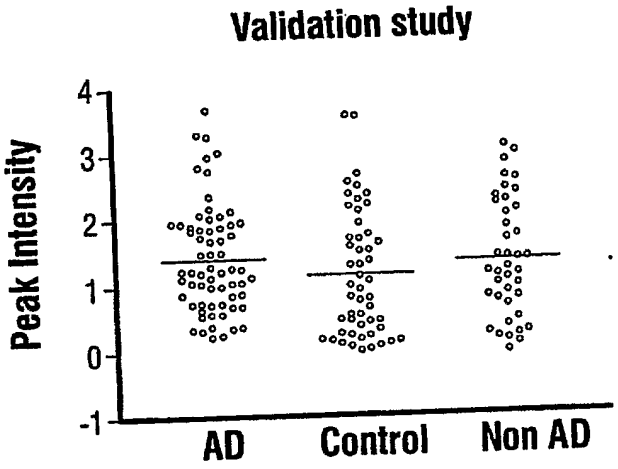
Kruskal Wallis p value	Mann Whitney p value		
A/C/N	A/C	A-N	C-N
0.0001	0.0649	<0.0001	0.0085



P = 0.0001

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



P = 0.148

FIG. 11

SEQUENCE LISTING

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SEQ ID NO:2

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 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

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  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

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  1 MAGPLRAPLL LLAILAVALA VSPAAGSSPG KPPRLVGGPM DASVEEEGVR RALDFAVGEY  60
 61 NKASNDMYHS RALQVVRARK QIVAGVNYFL DVELGRTTCT KTQPNLDNCP FHDQPHLKRK 120
121 AFCSFQIYAV PWQGTMTLSK STCQDA
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SEQ ID NO:5

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  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
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361 AREEEEAEQE RRGGEERVGE EDEEAAEAAE AEADEAERAR QNALLFAEEE DGEAGAEDKR 420
421 SQEETPGHRR KEAEGTEEGG EEEDDEEMDP QTIDSLIELS TKLHLPADDV VSIIEEVEEK 480
481 RNRKKKAPPE PVPPPRAAPA PTHVRSPQPP PPPPSARDEL PDWNEVLPPW DREEDEVYPP 540
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601 QEELENYIEH VLLRRP
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SEQ ID NO:6

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 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

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   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: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
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SEQ ID NO:14

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  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
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SEQ ID NO: 16

TLLVFEVQQPFLFVLWDQQHKFPVFMGRVYDPRA