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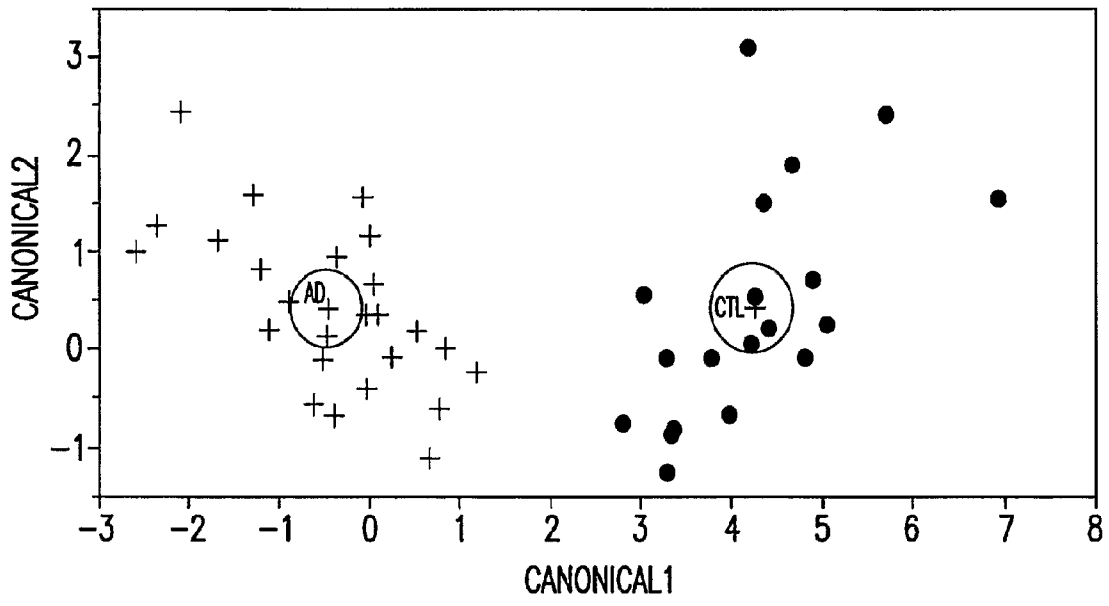
(54) Title: ASSAYS AND METHODS FOR THE DIAGNOSIS AND PROGRESSION OF ALZHEIMER'S DISEASE USING A MULTI-ANALYTE MARKER PANEL



(57) Abstract: The present invention provides a novel and sensitive means of monitoring Alzheimer's disease. The method consists of the construction of statistically relevant multi-analyte panels, through the use of shrunken centroids (SC), simulated annealing algorithm (SAA) and genetic algorithm (GA) within the framework of linear discriminant analysis (LDA) or through the use of random forest (RF) analysis of individual biomarkers, to more accurately and objectively assess the status of an individual for the purposes of disease classification and predicting cognitive endpoints such as MMSE, CAMCOG, or Learning Memory, a subscore of CAMCOG.

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TITLE OF THE INVENTION

ASSAYS AND METHODS FOR THE DIAGNOSIS AND PROGRESSION OF ALZHEIMER'S DISEASE USING A MULTI-ANALYTE MARKER PANEL

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/801,962, filed May 19, 2006 and U.S. Provisional Application No. 60/900,396, filed February 9, 2007, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the diagnosis and prognosis for therapy in the field of Alzheimer's disease. More specifically, it relates to biomarkers that can be used to diagnosis Alzheimer's disease or to determine the efficacy of drugs given to treat Alzheimer's disease.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a major neurodegenerative disease of unknown etiology that is characterized by the selective degeneration of basal forebrain cholinergic neurons. The degeneration of these cells leads to a secondary loss of neurons in the limbic system and cortex that control learning and memory. The consequent symptoms of the disease include a progressive loss of memory, the loss of the ability to communicate and the loss of other cognitive functions which occur over a course of approximately eight years. Over the course of this cognitive decline patients often become bedridden and completely unable to care for themselves. Although several symptomatic therapies have been approved to provide some compensation for the cholinergic deficit, for example, Aricept® (donepezil HCl, Eisai Co., Ltd. and Pfizer Inc.), the clinical effects of these are modest and none are able to significantly alter the course of the disease. Improving upon strategies for the treatment of AD has become a focus for the medical and scientific communities due to increases in the average age of the world population, the consequent increase in incidence and prevalence of age-related disorders such as AD, and the severe socioeconomic impact associated with supporting such cognitively impaired patients over the long term.

Requisite to improving the treatment of AD is improving the ability of clinicians to accurately diagnose the disease early in its course and to accurately monitor the progression of

the disease. Currently, a diagnosis of possible or probable AD is typically made based on clinical symptoms. A definitive diagnosis of AD can only be made post-mortem and requires a pathological examination of the affected brain tissue. The key pathological hallmarks of the disease are plaques consisting of deposited amyloid beta (Aβ) protein and tangles consisting of degenerated neuronal cells and their cytoskeletal elements (neurofibrillary tangles). There are currently no tests that, in and among themselves, have been validated to identify AD and differentiate it from other diseases affecting cognition. Compared to the pathological diagnosis, the pre-mortem clinical diagnosis can achieve an accuracy of approximately 80% to 90% at the very best of centers. However, this level of diagnostic accuracy more commonly occurs at well-experienced AD centers and for patients who have been manifesting clinical symptoms for several years (Rasmusson, D. X., et al., Alzheimer Dis. Assoc. Disord., 10(4): 180-188 (1996); Frank, R.A. et al., Proceedings of the Biological Markers Working Group: NIA Initiative on Neuroimaging in Alzheimer's Disease, Neurobiol. Ageing, 24: 521-536 (2003)). Following the clinical diagnosis, the progression of the disease is typically monitored through cognitive testing and assessment of everyday function. The course is often variable across patients and may be influenced by both organic and environmental elements.

The last decade has seen an increase in efforts to identify and validate AD-related biomarkers that might increase the sensitivity and specificity of diagnosis and provide a convenient and objective measure of disease progression (Regan Research Institute and National Institute of Ageing (NIA) Consensus Report of the Working Group on: 'Molecular and Biochemical Markers of Alzheimer's Disease,' Neurobiol. Ageing, 19(2): 109-116 (1998); Frank et al., 2003). Among the techniques that currently hold promise in this regard is the biochemical analysis of cerebrospinal fluid (CSF). The value of CSF analysis is based on the fact that the composition of this fluid may reflect brain biochemistry due to its direct contact with brain tissue.

The CSF proteins that have received the most attention are those thought to reflect key features of the disease pathogenesis, including Aβ deposition and neuronal degeneration. Studies have demonstrated reduced levels of the Aβ42 peptide in the CSF of clinically diagnosed AD patients compared to controls (Andreasen, N., et al., Arch. Neurol., 58: 373-379 (2001); NIA Consensus Report, 1998; Frank et al, 2003, Andreasen, N., et al., Clin. Neurol. Neurosurg. 107: 165-173 (2005)). Aβ42 is a cleavage product of the amyloid precursor protein (APP) and is thought to be a major constituent of the senile plaque. One theory of disease progression is that reduced CSF levels in AD patients may be due to increased deposition of the peptide in the brain.

In contrast, many studies have shown that the expression of the Aβ40 peptide, another APP cleavage product that is also a plaque component, may be similar in clinically diagnosed AD and control CSF (Frank et al, 2003).

The Tau protein is another CSF protein that has been studied for disease etiology. Tau is an axonal protein that, when hyperphosphorylated, assembles into the paired helical filaments that form neurofibrillary tangles. Whereas the presence of Tau in the CSF is thought to be a general reflection of axonal (i.e., neuronal) degeneration in the brain, the presence of phosphorylated Tau (p-Tau) may be a more specific indicator of AD-related pathology. CSF levels of both Tau and p-Tau in clinically diagnosed AD patients have been shown in many studies to be elevated compared to that in controls (Andreasen, 2001; and for review Consensus Report, 1998; Frank et al, 2003 and Andreasen, 2005).

A recent review article describes not only the status of biochemical biomarkers but also imaging biomarkers and their use in longitudinal clinical trials (Thal, L. J., et al., Alzheimer Dis. Assoc. Disord., 20(1): 6-15 (2006)).

SUMMARY OF THE INVENTION

The present invention relates to a method for classifying disease states in Alzheimer's disease comprising: (a) selecting a statistically relevant multi-analyte panel from human ante- mortem and healthy control fluid samples in which a plurality of biomarkers are differentially expressed to form a reference AD and control multi-analyte panel; (b) conducting a linear discriminate analysis on the multi-analyte data from step (a); (c) obtaining a test fluid sample from a patient; (d) conducting immunoassays on the test sample for the minimal number of analytes needed to specify the panel of step (a); (e) applying the results of step (d) to the linear discriminate analysis of step (b) to obtain an output; and (f) determining from the output of step (e) the classification of the disease state, where the output is either AD or control. In this method the multi-analyte panel is a plurality of biomarkers selected from the group consisting of Aβ40, Aβ42, Tau, pTau, sAPPα, sAPPβ, Aβx-42, Aβx-40, BACE activity, AF, Calcitonin, FABP, IFNγ, SCF, MCP-1, TBG and VEGF. In preferred embodiments of the invention, the multi-analyte panel is selected from the group consisting of (a) Aβ42, sAPPβ and BACE (b) Aβ40, Tau and pTau; (c) Aβ42, pTau and BACE; (d) Aβx-42, Tau and pTau; (e) Aβx-42, Tau, pTau, BACE; (f) Aβx-42, Tau7, pTau; and (g) Aβ42, sAPPβ.

In another embodiment, the invention relates to a method for predicting cognition scores for Alzheimer's disease ("AD") patients comprising: (a) selecting a statistically relevant

multi-analyte panel from human ante- mortem and healthy control fluid samples in which a plurality of biomarkers are differentially expressed to form a reference AD and control multi-analyte panel; (b) conducting a random forest analysis on the multi-analyte data from step (a); (c) obtaining a test fluid sample from a patient; (d) conducting immunoassays on the test sample for the minimal number of analytes needed to specify the panel of step (a); (e) applying the results of step (d) to the random forest analysis of step (b) to obtain an output; and (f) determining from the output of step (e) the cognition score, where the output is the assignment of the cognition score. In this method the predicted cognition score is selected from the group consisting of MMSE, Learning Memory and Total CAMCOG. In this method the multi-analyte panel is a plurality of biomarkers selected from the group consisting of Aβ40, Aβ42, Tau, pTau, sAPPα, sAPPβ, Aβx-42, Aβx-40, BACE activity, AF, Calcitonin, FABP, IFNγ, SCF, MCP-1, TBG and VEGF. In a preferred embodiment of the invention, the multi-analyte panel is selected from the group consisting of (a) Aβ42, sAPPβ and BACE (b) β40, Tau and pTau; (c) Aβ42, pTau and BACE; (d) Aβx-42, Tau and pTau; (e) Aβx-42, Tau, pTau, BACE; (f) Aβx-42, Tau7, pTau; and (g) Aβ42, sAPPβ.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the group means and confidence intervals for the expression of various analytes. Figure 1A shows Aβ42 (log-transformed), Figure 1B shows sAPPα (log-transformed), Figure 1C shows sAPPβ (log-transformed) and Figure 1D shows CSF BACE activity (log-transformed) in AD patients versus control subjects. Expression in AD patients is significantly lower than in control subjects in each case, while BACE activity in AD patients is significantly lower than in control subjects after adjusting for the baseline age difference in the ANOVA.

Figure 2 shows show a graphical output from a linear discriminant analysis (LDA) ("•", control; "+", definite AD) for the Expanded 9 marker panel. LDA analysis measures the distance from each point in the data set to each group's multivariate mean and classifies the point to the closest group. The distance measure used is the Mahalanobis distance, which takes into account the variance and covariance between the variables. Each multivariate mean is a labeled circle. The size of the circle corresponds to a 95% confidence limit for the mean. Groups that are significantly different tend to have non-intersecting circles. The markers considered in this analysis include Aβ42, Aβ40, Tau, pTau, sAPPα, and sAPPβ.

Figures 3A-3J show a graphical output from a random forest (RF) analysis for an Expanded 9 marker panel for MMSE. In Figure 3A the importance of the marker is scored and ranked with respect to its relative contribution to the prediction of cognition score. Aβ42 appears to be the most important predictor followed by Tau and pTau. In Figures 3B-3J the relative contribution of each marker is plotted after taking into account all of the other markers in the model. The partial residuals, plotted as the vertical axis of these graphs, can be considered as proportional to the MMSE scores and, thus, the plots provide an indication of the nature of contribution of each marker to the prediction of MMSE scores. The small vertical lines on the horizontal axis represent regions of particular importance as these are regions that are rich in data.

Figures 4A-4C show the group means and confidence intervals for the expression of various analytes. Figure 4A shows Fatty Acid Binding Protein ("FABP") (log-transformed), Figure 4B shows Stem Cell Factor ("SCF") (log-transformed) and Figure 4C shows Throxine Binding Globulin ("TBG") (log-transformed) in AD patients versus control subjects.

Figures 5A and 5B are canonical plots that provide a two-dimensional representation of the linear discriminant analysis (LDA) for an optimal 3-analyte panel (Figure 5A) and an optimal 6-analyte panel (Figure 5B). LDA measures the distance from each point in the data set to each group's multivariate mean (called a *centroid*) and classifies the point to the closest group. The distance measure used is the Mahalanobis distance which takes into account the variances and covariances between the variables. In this figure, each multivariate mean is a labeled circle. The size of the circle corresponds to a 95% confidence limit for the mean. As shown in these figures, separation of AD and control subjects is evident using these panels.

Figure 6 is a list of the 90 markers comprising the RBM panel. Among these markers, 27 markers (noted by an asterisk *) were measurable in fewer than five control and five AD subjects. Only markers from this panel that were measurable in at least five control and five AD subjects (63 markers) were used in all data analyses.

Figures 7A and 7B show the computer output from the R statistical software package. Figure 7A shows the software result indicating the sensitivity, specificity and overall classification accuracy of the composite three marker panel (Calcitonin, Fatty Acid Binding Protein, VEGF) as carried out with the SC algorithm (Table 4). Representative results from these computation runs are shown in Tables 3 and 4. Figure 7B shows the software result classifying ten unknown subjects as either AD or control based on their CSF measurements for the three marker panel of Figure 7A.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term "analyte" or "marker" or "biomarker" refers to one of the member proteins or protein states comprising the composite or multi-analyte panel, for example, Aβ42 or phosphorylated Tau at amino acid Threonine 181.

As used herein, the term "covariate" refers to variables such as the baseline age and sample storage time that are used as additional independent variables in the univariate analysis of variance (ANOVA).

As used herein, the term "composite" or "multi-analyte panel" or "multivariate panel" refers to any combination of two or more analytes or markers.

As used herein, the term "sensitivity" refers to the ability of an individual marker or a composite of markers to correctly identify patients with the disease, i.e. Alzheimer's disease, which is the probability that the test is positive for a patient with the disease. The current clinical criterion for patients who are deemed as probable for having AD is about 85% sensitive when compared to autopsy confirmed cases.

As used herein, the term "specificity" refers to the ability of an individual marker or a composite of markers to correctly identify patients that do not have the disease, that is, the probability that the test is negative for a patient without disease. The current clinical criterion is about 75% specific.

As used herein, the term "accuracy" or "overall classification accuracy" refers to the computed ability of an individual marker or a composite marker panel to correctly identify Both AD and control cases.

As used herein, the term "percent concordance (%C)" reflects the degree of agreement between the predicted results and their corresponding observed results. This metric is useful for assessing the accuracy of predicting quantitative endpoints such as the cognition scores.

As used herein, the term "prediction" or "prediction of cognitive scores" or "cognitive prediction" or "cognition prediction" refers to the translation or estimation of a cognitive score on a suitable scale from a set biochemical markers in a multi-analyte panel, that is, to assign an equivalent cognitive score based on where they fit within the statistically relevant multi-analyte panel. This can be done for MMSE based on a scale of 0 to 30, for CAMCOG based on a scale of 0 to 107 and for Learning Memory based on a scale of 0 to 17.

As used herein, the term "root mean squared error (RMSE)" is an estimate of the standard deviation of the predicted minus the observed results. Similar to percent concordance, it provides an alternative way to characterize the accuracy of the predictions of quantitative endpoints such as the cognition scores.

As used herein, the term "statistically relevant multi-analyte panel" refers to a multi-analyte panel that has been shown to meet an acceptable level of performance by those of ordinary skill in the art. For example, the 1998 National Institute of Ageing (NIA) criteria for classifying Alzheimer's disease from normal comprises sensitivity of greater than 85% and specificity of greater than 75%.

As used herein, the term "optimal multi-analyte panel" refers to a multi-analyte panel that represents the best subset of markers for that size panel using a specified algorithm.

As used herein, the term "Core 6" or "Core 6 marker panel" means the biomarker panel consisting of CSF Aβ40, Aβ2, pTau-181, tTau, sAPPα, and sAPPβ as defined in the examples.

As used herein, the term "Expanded 9" or "Expanded 9 marker panel" means the biomarker panel consisting of the Core 6 marker panel defined above (CSF Aβ40, Aβ42, pTau-181, tTau, sAPPα, and sAPPβ) plus the addition of CSF BACE activity, Aβx-40, Aβx-42 as defined in the examples.

As used herein, the term "RBM antigen panel" or "RBM panel" refers to the collection of markers, biomarkers or analytes comprising the proprietary human Multi-Analyte Profile (MAP®) (Figure 6) from Rules-Based Medicine, Inc., Austin, TX. MAPs, based on Luminex's xMAP bead based technology, consist of at least three pools of beads representing 90 antigens. While the total panel comprises 90 biomarkers, only 63 of these markers were measurable in at least five control and five AD subjects. Thus, in some instances only the 63 measurable markers were used in the data analysis. As a conservative approach the lowest calibrator concentration of the respective marker was used as the value for samples that fell below the lowest calibrator concentration in the standard curve. Designed originally for human plasma analysis of 100 µL samples, the Human MAP® has been analytically validated according to NACLES criteria and successfully used on human CSF when using 200 µL samples in manual mode and 250 µL samples in automated mode.

As used herein, the term "monitoring Alzheimer's disease" means both the ability to classify a subject as AD or control as well as the ability to predict the cognitive status of the individual, including MMSE, LM, and total CAMCOG.

As used herein, the term "classifying the disease state" means that a subject is classified as either having the Alzheimer's disease or as being normal.

As used herein, the term "tau" or "total tau" or "tTau" refer to the total Tau protein in a given sample or assay, regardless of phosphorylation state.

As used herein, the term "pTau" refers to the subset of Tau proteins which contain a phosphorylation site at a specified amino acid within the protein, in particular for the assays used herein, at amino acid position 181.

As used herein, the term "MMSE" refers to the Mini-Mental State Examination used in the cognitive assessment community.

As used herein, the term "total CAMCOG" or "CAMCOG" refers to the cognitive and self-contained part of the Cambridge Examination for Mental Disorders of the Elderly used in the cognitive assessment community.

As used herein, the term "Learning Memory" or "LM" refers to the Learning Memory component of the CAMCOG assessment.

As used herein, the term "CERAD" refers to the Consortium to Establish a Registry for Alzheimer's Disease used in the neuropathological community.

As used herein, the term "CSF" refers to cerebrospinal fluid.

The present invention provides a novel and sensitive means of monitoring Alzheimer's disease. The method comprises the construction of statistically relevant multi-analyte panels, through the use of linear discriminant analysis (LDA) or random forest (RF) analysis, of individual biomarkers to more accurately and objectively assess the status of an individual for the purposes of disease classification and predicting cognitive endpoints, such as MMSE, CAMCOG, or Learning Memory, a subscore of CAMCOG.

The National Institute of Aging (NIA) consensus white paper on AD biomarkers (Regan Research Institute and NIA Consensus Report of the working group on 'Molecular and Biochemical Markers of Alzheimer's Disease,' reported at Neurobiology of Aging, 19(2): 109-116 (1998)(hereinafter "1998 NIA Consensus") outlines several non-limiting uses of Alzheimer biomarkers. In particular, biomarkers of AD, either in the form of individual markers or multi-analyte panels can be used for multiple purposes: (1) to aid in the classification or diagnosis of the disease state of an individual to complement traditional clinical diagnosis with an objective measurement; (2) for epidemiological screening to select an enriched population or to characterize the prevalence of disease or demographics of any given epidemiological study; (3) for predictive testing or prognostic purposes of indicating who is susceptible to further

neurodegenerative and cognitive decline; (4) for studying brain-behavior relationships; and (5) for monitoring disease progression or response to treatment in clinical trials and clinical practice. In practice the latter purpose has two separate aspects, including, (A) to determine whether a treatment induces a measurable biochemical change and (B) to determine whether treatment changes the progression of the illness, using the biomarker or multi-analyte panel as an index of disease status or state. The 1998 NIA Consensus also stated that a proposed biomarker or multi-analyte panels should include as many of the features of an ideal marker, including: (1) be able to detect a fundamental feature of AD neuropathology; (2) be validated in neuropathologically confirmed AD cases; (3) be precise (ability to detect AD early in its course and distinguish it from other dementias); (4) be reliable; (5) be non-invasive; (6) be simple to perform; and lastly (7) be inexpensive. It has been acknowledged and remains the case that no known biomarker for Alzheimer's meets the 1998 NIA criteria indicated.

The 1998 NIA panel fully recognized the utility of multiple markers, when they specifically highlighted that, "[a] combination of markers may provide greater diagnostic accuracy than any single one individually. Critical evaluation of multiple simultaneous biomarkers should utilize the same principles outlined above, including sensitivity, specificity, prior probability, positive predictive value, and negative predictive value. Of these, high sensitivity and specificity are most important as they indicate the accuracy of the test."

With this guidance, the aim of the present invention was on those multi-analyte panels that meet the 1998 NIA panel criteria for sensitivity and specificity (sensitivity>85%, specificity>75%) and which were previously unknown to the AD biomarker community.

The literature describes other multi-analyte type analyses that have been conducted. For example, WO 2004/104597, "Method for Prediction, Diagnosis, and Differential Diagnosis of AD" describes methods of predicting disease status via an x/y ratio of Aβ peptides. WO 2005/047484, "Biomarkers for Alzheimer's Disease" describes a series of markers that can be used for the assessment of disease state and other scientifically interesting avenues. WO 2005/052592, "Methods and compositions for diagnosis, stratification, and monitoring of Alzheimer's disease and other neurological disorders in body fluids" teaches methods and markers gleaned from plasma for the monitoring of Alzheimer's disease.

Multi-analyte literature articles also include the measurement of 13 biological markers in CSF of Patients with AD and other Dementias (Blasko *et al.*, Dement Geriatr Cogn Disord. 21: 9-15, (2006). Carrette O, *et al.*, "A panel of cerebrospinal fluid potential biomarkers

for the diagnosis of Alzheimer's disease," Proteomics 3(8):1486-94 (2003), discusses use of CSF for potential biomarkers.

Sample Summary

Table 1A summarizes the key demographics of the cohort considered in Applicants' analysis. AD subjects are characterized according to the CERAD criteria and post-mortem confirmed. MMSE scores were obtained at the time of CSF sample collection.

Table 1A

		Control (n=29)	AD (n=27)
Age	Mean ± SD	69.3 ± 13.6	76.2 ± 7.8
	Range	35.9 to 94.3	56.7 to 87.9
Gender	Male	18	10
	Female	11	17
ApoE-e4	homozygote (4/4)	1	6
	heterozygote (2/4, 3/4)	(2, 6)	(1, 15)
	Other (2/3, 3/3)	(4, 16)	(0, 5)
MMSE	Mean	28.9 ± 1.4	9.4 ± 6.4
	Range	25 to 30	0 to 23

Table 1B shows the number of AD and control subjects by age: under 73 years of age (<73) and those individuals who are over 73 years of age (>73). Since this provided a roughly even split of the data (n = 26 in age<73 group, and n =30 in age > 30 group), the age groups were used as a baseline factor to reduce confounding in the comparison of AD and Control groups (ANOVA) for each marker.

Table 1B

Age < 73		Age > 73	
CTL	AD	CTL	AD
n=19	n=7	n=10	n=20

Table 1C lists the number of subjects for whom Applicants obtained measurable data for the biomarkers in the Expanded 9 marker panel. Data from all subjects were available for the first six markers, Aβ42, Aβ40, tTau, pTau, sAPPα, and sAPPβ. Due to limited CSF sample volume, data from some of the subjects were not available for Aβx-42, Aβx-40 and BACE Activity.

Table 1C

	Number of Subjects		
	AD	CTL	Total
Aβ42, Aβ40, tTau, pTau, sAPPα, sAPPβ	27	29	56
Aβx-42	26	23	49
Aβx-40	25	19	44
BACE Activity	26	26	52

Table 1D lists the number of subjects for whom Applicants obtained measurable data for the eight markers that were statistically significant ($p<0.05$) from the RBM panel. All of these markers except Calcitonin were measurable in all subjects. Calcitonin was not measurable for some subjects due to values falling below the lowest calibration concentration in the standard curve. As a conservative approach, the lowest calibrator concentration was used to impute the values for such samples. Only those markers from the RBM panel that were measurable in at least 5 AD and at least 5 CTL subjects were used in the data analyses. Sixty three (63) markers from the RBM panel met these criteria.

Table 1D

	Number of Subjects		
	AD	CTL	Total
AFP, FABP, IFN-γ, MCP-1, SCF, TBG, VEGF	27	29	56
Calcitonin	25	14	39

Univariate Marker Analysis for the Expanded 9 marker panel

Table 2 provides the descriptive univariate statistics for individual markers and the ratio of expression of each marker between AD and control (CTL) with respect to both observed (raw) data and the least squares (LS) means from ANOVA. The ratios with respect to

LS means derived from the ANOVA is generally considered to be a more accurate reflection of the effect as it takes into account the imbalance in the data and the effect of age as well. The p-values from the ANOVA are also provided, in addition to the False Discovery Rate q-values.

Covariates such as Age and Storage Time (ST) were included in the univariate analysis of variance (ANOVA) for each marker if the p-values corresponding to these covariates were less than 0.1. For each of the markers above, the fold change from AD to control using the raw data and from the least squares (LS) means from the ANOVA are reported, along with their respective false positive rate (FPR) represented by the p-value and their false discovery rate (FDR) represented by the q-value.

The 55% lower Aβ42 values obtained from the univariate analysis was consistent with many published studies to date literature. See, for example, Galasko *et al.*, Arch. Neurol. (1998); Motter *et al.*, Ann. Neurol., 38: 643-648 (1995); Tamaoka *et al.*, J. Neurol. Sci., 148: 41-45 (1997); Scheuner *et al.*, Nat. Med., 2: 864-870 (1996).

Similarly, consistent with the literature (Arai *et al.*, Alz. Res., 3: 211-213 (1997); Trojanowski *et al.*, Alzh. Dis. Review, 1: 77-83 (1996)), our observation was that both total Tau and pTau-181 are elevated in AD compared to control. See, for example, Kahle *et al.*, Neurology, 54: 1498(2000)). t-Tau levels are known to change with age, so age adjustment is required when t-Tau levels are diagnostically employed.

pTau generally has been measured at three different sites, Threonine 231, Serine 199, Threonine 181. Several studies indicate pTau levels may provide an early indicator useful for differential diagnosis.

A number of studies have shown that CSF Aβ40 levels are similar in AD and controls (Mecocci *et al.*, J. Neuroimmunol., 57:165-170 (1995); Tamoaka *et al.*, J. Neurol Sci., 148: 41-45 (1997)). Jensen *et al.*, Ann. Neurol., 45: 504-511 (1999), published a study in which a decrease in CSF Aβ40 was found with significant overlap between the groups, similar to our own results.

sAPP (including both sAPP□ and sAPP□) has been studied in the CSF of AD subjects by Olsson *et al.* (Experimental Neurology 183: 74-80 (2003), Table 2) where they saw no significant differences in either fragment.

Aβx-40 and Aβx-42 have been studied in the context of plaques immuno-reactivity and correlation to cognitive decline by Parvathy *et al.* (Arch. Neurol. 58: 2025-32 (2001)).

CSF BACE activity was originally published by Holsinger *et al.*, Ann. Neurol., 55: 898 (2004), and more recently followed up by Verheijen *et al.*, Clin. Chem., 52: E-published April 13, 2006.

Table 2. Univariate Marker Analysis for the Expanded 9 marker panel plus the RBM panel

Markers	Covariates in ANOVA	AD/CTL (raw means)	AD/CTL (LS Means)	FPR (p-value)	FDR (q-value)
Aβ40	Age	0.66	0.63	<0.0001	<0.0001
Aβ42	None	0.46	0.45	<0.0001	<0.0001
Tau	None	2.40	2.38	<0.0001	<0.0001
pTau	Age	1.76	1.63	<0.0001	<0.0001
sAPPα	Age & ST	0.77	0.79	0.0682	0.1994
sAPPβ	Age & ST	0.72	0.74	0.0155	0.0819
BACE	Age	0.92	0.84	0.0383	0.1364
Aβx42	None	0.47	0.49	<0.0001	<0.0001
Aβx40	Age & ST	0.70	0.78	0.0112	0.0710
AFP	Age	1.19	1.16	0.0158	0.0819
Calcitonin	Age	2.43	3.17	0.0012	0.0102
FABP	Age	1.57	1.57	0.0097	0.0692
IFNγ	Age & ST	0.87	0.72	0.0236	0.1036
MCP-1	None	1.14	1.17	0.0312	0.1187
SCF	Age	0.92	0.83	0.0264	0.1077
TBG	None	1.38	1.33	0.0203	0.0965
VEGF	Age	0.83	0.77	0.0001	0.0011

Among the markers in the Expanded 9 marker panel, all except sAPPα were statistically significant at less than 5% false positive rate (FPR), i.e., at $p<0.05$. The effect of sAPPα was marginally significant with $p=0.0682$. Among these markers, Aβ40, Aβ42, Tau, pTau, and Aβx42 had the most robust effect at less than 5% false discovery rate (FDR), i.e., $q<0.05$. In addition, sAPPβ and Aβx40 had a robust effect at less than 10% false discovery rate ($q<0.1$).

Among the 63 measurable analytes in the RBM human MAP that were analyzed Alpha Fetoprotein (AFP) (16% elevated), Calcitonin (217% elevated), Fatty Acid Binding Protein (FABP) (57% elevated), Interferon–gamma (IFNγ (28% reduced), Monocyte Chemotactic Protein-1 (MCP-1) (17% elevated), Stem Cell Factor (SCF) (17% reduced), Thyroxine Binding Globulin (TBG) (33% elevated), and Vascular Endothelial Growth Factor

(VEGF) (23% reduced) percentages relative to control are significant with a false positive rate $p<0.05$. Among these, Calcitonin and VEGF have the most robust effect at less than 5% false discovery rate (FDR), i.e., $q<0.05$. In addition, AFP, FABP and TBG had a robust effect at less than 10% false discovery rate ($q<0.1$).

AFP is the major fetal plasma protein. The concentration of AFP peaks in the fetal bloodstream at 2-3 g/l around 12-14 weeks of gestation and then falls. AFP passes into the maternal bloodstream where it may be detected by assay. Fetal malformations such as neural tube defects seen in Down's syndrome elevate the maternal serum levels. Elevated AFP is seen most frequently in adults with germ cell tumours and hepatocellular carcinoma, but also in gastric, colon, biliary, pancreatic and lung cancers (~20% of patients). Swiss-Prot Accession Number: P02771.

Calcitonin is secreted by the parafollicular C-cells of the thyroid gland. Its primary physiological effect is to lower serum calcium levels. Elevated levels of calcitonin (>100 pg/mL) may be encountered in a variety of pathological conditions including leukemias and myeloproliferative disorders. The most notable condition expressing elevated calcitonin levels is medullary thyroid carcinoma (MTC). Swiss-Prot Accession Number: P01258.

FABP is typically a plasma marker of acute myocardial infarction (AMI). The plasma kinetics of FABP closely resemble those of myoglobin in that elevated plasma concentrations are found within 2 hours after AMI and return to normal generally within 18 to 24 hours. The concentration of FABP in skeletal muscle is 20 times lower than in cardiac tissue (for myoglobin the same content for cardiac and skeletal tissue). This makes FABP a useful biochemical marker for the early assessment or exclusion of AMI. FABP also appears to be a useful plasma marker for the estimation of cardiovascular risk. Swiss-Prot Accession Number: P05413.

IFNγ is secreted from T cells (cytotoxic and Th1) and Natural Killer cells. Its major functions are to activate macrophages and to increase the expression of class II MHC on APC. IFNγ stimulated macrophages are more phagocytic, they are more capable of killing intracellular pathogens and they have increased ability to present antigen. IFNγ secreted by Th1 cells has a cross regulatory role in controlling Th2 function, and will induce a class switch to IgG. It actually can inhibit the activities of the Th2 pathway by inducing IL-12 production by macrophages. This cytokine has a role in many different types of immune responses such as delayed type hypersensitivity, inflammation, antibody production and viral infection. Swiss-Prot Accession Number: P01579.

MCP-1 plays a role in the recruitment of monocytes to sites of injury and infection. MPC-1 has been found in the joints of people with rheumatoid arthritis where it may serve to recruit macrophages and perpetuate the inflammation in the joints. MPC-1 has also been found elevated in the urine of people with lupus as a sign warning of inflammation of the kidney. MCP-1 has also been called small inducible cytokine A2 (SCYA2) and monocyte chemotactic and activating factor (MCAF). Swiss-Prot Accession Number: P13500.

SCF is a stromal cell-derived cytokine synthesized by fibroblasts and other cell types. It is a glycoprotein that plays a key role in hematopoiesis acting both as a positive and negative regulator, often in synergy with other cytokines. It also plays a key role in mast cell development, gametogenesis, and melanogenesis. Swiss-Prot Accession Number: P21583.

TBG levels are particularly useful for cases in which total thyroid hormone levels do not correlate with the thyro-metabolic status, such as with pregnancy, the use of contraceptive steroids, or in patients with hereditary excesses or deficiencies of TBG. Swiss-Prot Accession Number: P05543

VEGF is important in the pathophysiology of neuronal and other tumors, probably functioning as a potent promoter of angiogenesis. It may be involved also in altering blood-brain-barrier functions under normal and pathological conditions. VEGF secreted from the stromal cells may be responsible for the endothelial cell proliferation in capillary hemangioblastomas which are composed of abundant microvasculature and primitive angiogenic elements represented by stromal cells. Swiss-Prot Accession Number: P15692.

Multi-analyte panels and models for the Expanded 9 markers

The present invention is focused on the determination and use of composite or multi-analyte panels for the monitoring of Alzheimer's disease. Unlike the published literature, which is generally focused on Aβ42, pTau, tTau, Applicants have extended the marker panel with six additional analytes and have discovered unexpectedly that some unique and novel composites can reach the level of performance that meet the criteria for sensitivity and specificity recommended by the 1998 NIA panel (sensitivity>85%, specificity>75%)..

In one embodiment of the invention, a composite or multi-analyte panel comprising Aβ42, sAPPβ and BACE is employed to monitor Alzheimer's disease. As shown in the Examples and Table 3, this multi-analyte panel performs quite well in post mortem confirmed AD cases. In preferred embodiments of the invention the multi-analyte panel is selected from the group consisting of (a) Aβ42, sAPPβ and BACE (b) Aβ40, Tau and pTau; (c)

Aβ42, pTau and BACE; (d) Aβx-42, Tau and pTau; (e) Aβx-42, Tau, pTau, BACE; (f) Aβx-42, Tau7, pTau; and (g) Aβ42, sAPPβ.

In another embodiment of the invention, the biomarker composites or multi-analyte panels can be used for classification of Alzheimer's disease. In this embodiment, reference samples are collected from at least 25 patients that have been characterized as post mortem confirmed AD and from at least 25 healthy subjects from a similar age group. The samples are then run in a specific multi-analyte panel to generate data. For example, using the Aβ42, sAPPβ and BACE panel each sample is measured in each assay. Those skilled in the art would understand that each assay of the multi-analyte analyte panel needs to undergo fit-for-purpose assay validation, which includes the assessment of key issues such as freeze-thaw stability, dilution linearity, precision, sensitivity, etc. One then builds a statistical model on the composite biomarker using linear discriminant analysis (LDA). This can be performed using a contributed program library within the R language/environment (A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2005, ISBN 3-900051-07-0, Reference for the contributed programs within R for performing LDA: Venables, W. N. and Ripley, B. D., Modern Applied Statistics with S. Fourth Edition. Springer, New York (2002) ISBN 0-387-95457-0). The LDA is carried out on this reference dataset, thus creating a model on the specific composite in the R environment.

New samples are collected in the clinic from prospective patients that are awaiting diagnosis. These samples are run in the minimal assays necessary for the specific composite. The data generated from the multi-analyte panel are then inputted into the LDA model/algorithm that was built using the reference dataset as described above with the composite in place in the R environment. The output from this process gives a determination of AD or control for the new subjects to aid in the diagnosis. This classification is useful for doctors and caregivers to aid in determining whether a patient is suffering from Alzheimer's disease as compared to other forms of cognitive decline.

In still another embodiment of the invention, the biomarker composites or multi-analyte panels can be used for predicting cognition scores such as MMSE, Total CAMCOG of Learning Memory, a subscore of CAMCOG. In this embodiment, reference samples are collected from at least 25 patients that have been characterized as post mortem AD and from at least 25 healthy subjects from a similar age group. The samples are then run in the specific multi-analyte panel to generate data. As noted above, those skilled in the art would understand that each assay of the multi-analyte analyte panel needs to undergo fit-for-purpose assay

validation. To build a statistical model on the composite biomarker, random forest analysis is performed using a contributed program library within the R language/environment as described above. The random forest analysis is carried out on this reference dataset, thus creating a model on the specific composite in the R environment.

New samples are collected in the clinic from prospective patients that are awaiting assessment of their cognitive ability in terms of MMSE, Learning Memory, Total CAMCOG. The samples are run in the minimal assays necessary for the specific composite. The data generated from this multi-analyte panel are then input into the random forest model/algorithm that was built using the reference dataset as described above with the composite in place in the R environment. The output from this process provides an estimate of the cognitive scores of interest: MMSE, Learning Memory, Total CAMCOG. This analysis is useful for doctors and caregivers to aid in determining if someone has a low cognitive score attributable to Alzheimer's disease as compared to other forms of cognitive impairment.

Table 3 provides a selected list of various individual markers and composites from the Expanded 9 marker panel that were considered in the LDA to determine their utility for correctly classifying AD from control. While the invention described herein may comprise composites other than those shown, one skilled in the art would choose analytes such that the composites would have sensitivities greater than 85% and specificities greater than 75% to meet the 1998 NIA criteria for sensitivity and specificity. Preferred composites from those listed in Table 3 are ones meeting the 1998 NIA criteria for sensitivity and specificity.

One skilled in the art would recognize that the addition of a marker to a composite may sometimes have the undesired effect of lowering the sensitivity and/or specificity. For example, the inclusion of sAPPα in the composite of Aβ42, Tau and pTau lowers the specificity from 95% to 90%. Similarly, the inclusion of sAPPβ in the composite of Aβ40, BACE and sAPPα lowers the specificity from 81% to 73%. On the other hand, adding a marker such as Aβ42 to a composite of Tau and pTau may improve the sensitivity and specificity. For example, the inclusion of BACE Activity to the composite of Aβ40 and sAPPα improved the sensitivity from 74% to 83% and specificity from 75 to 81%. It should be noted that data for BACE was not available from four subjects and these subjects were excluded in the analysis for composites that were of special interest for comparing with and without BACE Activity.

Table 3

Analyte or multi-analyte panels	% Accuracy	% Sensitivity	% Specificity
Aβ42	91	93	90
Aβ40	74	74	74
Tau	75	57	93
pTau	74	56	92
sAPPα	66	66	66
sAPPβ	68	69	67
Aβx-42	82	96	66
Aβx-40	80	86	72
BACE	43	51	35
Tau, pTau	75	35	75
Aβ42, Tau	94	96	92
Aβ42, pTau	96	100	93
Aβ42, Tau, pTau	97	100	95
Aβ42, Tau, pTau, sAPPα	95	100	90
Aβ40, Tau, pTau	95	90	99
Aβ40, Aβ42, Tau, pTau	96	97	96
Aβ42, Tau, pTau*	99	100	99
Aβ42, Tau, pTau, BACE	99	100	99
Aβ42, pTau, BACE	98	100	96
Aβ40, sAPPα *	75	74	75
Aβ40, sAPPα, BACE	82	83	81
Aβ40, sAPPα, sAPPβ, BACE	79	84	73
Aβ40, sAPPβ *	76	75	77
Aβ40, sAPPβ, BACE	81	85	76
Aβ42, sAPPβ *	92	95	90
Aβ42, sAPPβ, BACE	92	96	88
Aβx-40, Aβ42, Tau, pTau	98	99	97
Aβx-42, Tau, pTau	96	96	95
Aβx-42, Aβ42, Tau, pTau	100	100	100
Aβx-42, Aβ42, Tau, pTau, BACE	99	99	100
Aβx-42, Tau, pTau, BACE	96	94	98
Aβx-42, Tau, pTau*	95	96	95

* excludes 4 subjects with missing BACE activity data

Multi-analyte panels and models for the RBM panel

The present invention is focused on the determination and use of composites or multi-analyte panels for the monitoring of Alzheimer's disease. Applicants have further extended the multi-analyte panels or composites based on the Expanded 9 marker panel presented above with the addition of 63 measurable analytes that were measurable from the RBM panel. From the collection of measurable markers, Applicants have discovered unexpectedly that several composites can achieve a level of performance that meet the criteria for sensitivity and specificity recommended by the 1998 NIA panel (sensitivity>85%, specificity>75%).

In various embodiments of the invention, optimal panels of varying sizes have been identified for monitoring Alzheimer's disease. In one embodiment the composite or multi-analyte panel is made up of three analytes/biomarkers, for example, Calcitonin, FABP and VEGF, used to monitor Alzheimer's disease. In other embodiments, composites with additional markers, such as the six marker multi-analyte panel comprising Calcitonin, FABP, MMP-3, Myoglobin, SCFactor and VEGF, provide improved ability to monitor Alzheimer's disease.

Representative optimal panels or composites of varying sizes are shown in Table 4 which can be used for classification of Alzheimer's disease. As is done with the multi-analyte panels for the Expanded 9 markers above, reference samples are collected and analyzed from at least 25 patients that have been characterized as post mortem confirmed AD and from at least 25 healthy subjects from a similar age group. For example, one could select a composite that utilizes Calcitonin, FABP, MMP-3, Myoglobin, SCF and VEGF for a six analyte panel for use with the SAA algorithm and then measure each sample for these anlaytes. As is done with the Expanded 9 marker panels above, one then builds a statistical model for the selected composite analytes using LDA as described above, creating a model of the specific composite in the R environment.

New samples are then collected in the clinic from prospective patients awaiting diagnosis. These samples are run in the minimum number of assays necessary for the specific composite. The data generated from this multi-analyte panel are then inputted into the LDA model/algorithm that was built using the reference dataset. The output from this process gives the determination of AD or control for the new subjects to aid in the diagnosis. This classification is useful for doctors and caregivers to aid in determining whether a patient is suffering from Alzheimer's disease as compared to other forms of cognitive decline.

Multivariate analysis was performed using LDA to examine the collective role of subsets of analytes that distinguish CSF samples from the AD and control subjects. Multi-

analyte panels that provide the best predictive performance for each panel size were determined from the collection of measurable analytes in the RBM panel. For each of the panel sizes ranging from three to twelve analytes, optimal multi-analyte panels were determined using the Simulated Annealing Algorithm (SAA) and the Genetic Algorithm (GA) within the framework of the LDA method (Duarte Silva, A.P., Efficient Variable Screening for Multivariate Analysis, J. Multivariate Analysis, 76: 35-62 (2001)). In addition, an optimal panel of three markers from the Shrunken Centroid (SC) method (Tibshirani RJ, Hastie T, Narasimhan B, and G. Chu., Diagnosis of Multiple Cancer Types by Shrunken Centroids of Gene Expression, P.N.A.S.,U.S.A., 99(10): 6567-6572 (2002)) was also derived. These representative optimal multi-analyte panels are listed in Table 4, along with their predictive performance determined from the aggregate of 50 replicates of 10-fold cross-validation.

In a preferred embodiment of the invention, the composite or multi-analyte panel comprises at least three biomarkers and the composite or multi-analyte panel meets the 1998 NIA criteria for sensitivity (>85%) and specificity (>85%). In a more preferred embodiment, the multi-analyte panel comprises three to six biomarkers which meet this criteria and, in an even more preferred embodiment, the multi-analyte panel comprises six biomarkers which meet this criteria. In addition to the composite of three biomarkers derived using the SC method, all composites comprising at least four biomarkers listed in Table 4 meet the 1998 NIA criteria for sensitivity and specificity. The % sensitivity and % specificity metrics reported in this table are within 0.4% standard error. Those skilled in the art would recognize that other composites could be derived that meet the desired criteria and, as such, the invention described herein is not limited to the representative optimal composites listed in Table 4.

Table 4: Optimal multi-analyte panels using markers from the RBM panel

Model	Panel Size	Optimal Panel Search Algorithm	Multi-Analyte Panel	% Accuracy	% Sensitivity	% Specificity
LDA	3	SAA	AFP, TNF-RII, VEGF	84	84	85
		GA	AFP, TNF-RII, VEGF	84	84	85
		SC	Calcitonin, FABP, VEGF	86	85	87
	4	SAA	Calcitonin, FABP, MMP-3, VEGF	91	89	92
		GA	Calcitonin, FABP, MMP-3, VEGF	91	89	92
	5	SAA	Calcitonin, FABP, IgE, MMP-3, VEGF	90	89	91
		GA	Calcitonin, SHBG, TNF-RII, Thyroid Stimulating Hormone (TSH), VEGF	86	80	92
	6	SAA	Calcitonin, FABP, MMP-3, Myoglobin, SCF, VEGF	94	93	95
		GA	AFP, MIP-1β, MMP-3, SCF, TNF-RII, VEGF	88	88	88
	7	SAA	Calcitonin, CD40, IgE, SHBG, TNF-RII, THS, VEGF	89	86	92
		GA	Calcitonin, FABP IgE, MMP-3, Myoglobin, SCF, VEGF	94	93	95
	8	SAA	Calcitonin, CD40, Creatine Kinase MB (CK-MB), FABP, IgE, SCF TSH, VEGF	91	90	91
		GA	AFP, MIP-1β, Prostatic Acid Phosphatase (PAP), SCF, SHBG, TNF-RII, TSH, VEGF	88	88	89
	9	SAA	Calcitonin, CD40, FABP, IgE, MMP-3, Myoglobin, SCF, TSH, VEGF	94	91	96
		GA	Calcitonin, FABP, IgE, MMP-3, Myoglobin, PAP, SCF, SGOT, VEGF	93	91	93
	10	SAA	β-2 Microglobulin (β2M), Calcitonin, FABP, IgE, IL-10, MMP-3, Myoglobin, PAP, SCF, SGOT	95	95	96
		GA	β-2 M, Calcitonin, FABP, IgE, IL-10, MMP-3, Myoglobin, PAP, SCF, SGOT	95	95	96
	11	manual	β-2 M, Calcitonin, FABP, IgE, IL-10, MMP-3, Myoglobin, PAP, SCF, SGOT, VEGF	96	96	96
	12	SAA	β-2 M, Calcitonin, FABP, GM.CSF, IgE, IL-16, MMP-3, Myoglobin, PAP, SCF, SGOT, VEGF	94	92	96

In order to graphically illustrate the performance of these panels, canonical plots from a LDA for an optimal panel for a 3-analyte panel as determined from the Shrunken

Centroids (SC) algorithm and an optimal panel for a 6-analyte panel as determined from the Simulated Annealing Algorithm (SAA) are shown in Figures 5A and 5B, respectively. The 3-analyte panel, Calcitonin, FABP and VEGF, provides 86% classification accuracy. The percent accuracy improves to 94% with the addition of MMP-3, Myoglobin and SCF to form the 6-analyte panel.

Those skilled in the art would recognize and understand that the present invention is not limited to either the composites from the Expanded 9 marker panel (Table 3) or the composites from the RBM panel (Table 4), but would also include composites derived using markers from both panels. It is notable that several markers emerged from the multivariate LDA that were not individually statistically significant, but that when combined become important markers in a multi-analyte composites. Examples of analytes that were individually not statistically significant but that emerged as part of an optimal composite include the following:

TNF RII (Tumor Necrosis Factor receptor type 2) is a soluble form of the TNF receptor. Two types of soluble TNF receptors have been identified in human serum and urine that neutralize the biological activities of TNF-α and TNF-β. These binding proteins represent truncated forms of the two types of high-affinity cell surface receptors for TNF (TNFR-p60 Type B and TNFR-p80 Type A). Soluble TNF RI corresponds to TNFR-p60 Type B. Soluble TNF RII corresponds to TNFR-p80 Type A. In the TNF superfamily nomenclature, TNF RI and TNF RII are referred to as TNFRSF1A and TNFRSF1B, respectively. These apparent soluble forms of the receptors appear to arise as a result of shedding of the extracellular domains of the membrane-bound receptors. Swiss-Prot Accession Number: Q92956

MMP-3 (Matrix Metalloproteinase 3), or stromelysin, can degrade numerous extracellular matrix (ECM) substrates, such as collagen. It can also release cell surface molecules such as heparin-binding EGF-like growth factor and TNF-alpha, and it can activate other MMPs, including MMP-9 and collagenases. It can also inactivate several serine proteinase inhibitors. Swiss-Prot Accession Number: P08254

IgE (Immunoglobulin E) levels of circulating IgE in serum are extremely low compared to the other immunoglobulins. Levels at birth are almost non-detectable, but increase with age. IgE has been linked to atopic disease and there is a strong correlation between increased total serum or plasma IgE levels and allergy. The determination of total IgE levels has been found to be useful in the assessment of atopic diseases such as allergic rhinitis, extrinsic asthma, urticaria, and atopic eczema. Patients with pulmonary aspergillosis, parasitic

infestations and some immunodeficiencies have also been found to have increased amounts of IgE.

SHBG (Sex Hormone Binding Globulin) is a glycoprotein that binds to sex hormones, specifically testosterone and estradiol. These sex hormones circulate in the bloodstream, bound mostly to SHBG and to some degree bound to albumin. Only a small fraction is unbound, or "free," and thus biologically active and able to enter a cell and activate its receptor. Thus, bioavailability of sex hormones is influenced by the level of SHBG. SHBG levels are controlled by a delicate balance of enhancing and inhibiting factors. Its level is decreased by high levels of insulin and insulin-like growth factor-I (IGF-I). High androgen levels decrease SHBG, while high estrogen and thyroxine levels increase it. Conditions with low SHBG include polycystic ovary syndrome, diabetes, and hypothyroidism. Conditions with high SHBG include pregnancy, hyperthyroidism, and anorexia nervosa. Swiss-Prot Accession Number: P04278.

TSH (Thyroid Stimulating Hormone), or thyrotropin, is a glycoprotein synthesized and secreted by the pituitary gland. It stimulates synthesis and secretion of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3). Secretion of TSH is stimulated by thyrotropin-releasing hormone (TRH), a hypothalamic tripeptide. TSH synthesis and release are regulated via a negative feedback mechanism by the level of thyroid hormones. Increased serum levels of free T4 and T3 depress TSH secretion (hyperthyroidism), while decreased serum levels of free T4 and T3 result in excess TSH secretion (primary hypothyroidism). Serum TSH concentration is inversely proportional to the free T4 (FT4) concentration in a log/linear relationship, making TSH a sensitive marker for monitoring thyroid hormone replacement therapy. Swiss-Prot Accession Number: Alpha P01215; Beta P01222

Myoglobin is a monomeric heme protein that is structurally related to hemoglobin. Very little free myoglobin circulates. It is synthesized and found predominantly in skeletal and cardiac muscle. During the course of a myocardial infarction (MI), myoglobin escapes from the ischemic cardiac muscle and can reach levels 5-10 times normal during the first 5-18 hours. A wide variety of pathological processes damage skeletal muscles, causing release of myoglobin into the circulation. Muscle damage resulting in high levels of myoglobinuria is clinically referred to as rhabdomyolysis. Measurement of myoglobin in rhabdomyolysis may be useful to determine the likelihood of significant renal toxicity. A high serum myoglobin level associated with a low urine myoglobin clearance rate indicates high risk for renal failure. A high serum myoglobin level with high myoglobin clearance rate indicates low risk for renal failure. A

relatively low serum myoglobin level indicates minimal risk for renal failure. Swiss-Prot Accession Number: P02144.

MIP-1 β (Macrophage Inflammatory Protein 1 beta) Swiss-Prot Accession Number: P13236. Like, MIP-1 α (Macrophage Inflammatory Protein 1 alpha): the two MIP proteins are the major factors produced by macrophages following their stimulation with bacterial endotoxins. Both proteins are involved in the cell activation of human granulocytes (neutrophils, eosinophils, and basophils) and appear to be involved in acute neutrophilic inflammation. Both forms of MIP-1 stimulate the production of reactive oxygen species in neutrophils and the release of lysosomal enzymes. They also induce the synthesis of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF in fibroblasts and macrophages. Swiss-Prot Accession Number: P10147.

CD40 is a receptor molecule on the cell surface of all mature B cells (B lymphocytes), most B-cell malignancies, and monocytes, dendritic cells (in the nervous system), endothelial cells (within blood vessels), and epithelial cells. CD40 is a member of the tumor necrosis factor superfamily. Together with CD40 ligand, the molecule that binds to it, CD40 is an important contributor to the inflammatory processes that lead to atherosclerosis and thrombosis (clotting). Swiss-Prot Accession Number: Q6P2H9.

CK-MB (Creatine Kinase MB) is released by damaged cardiac tissue 2-6 hours following infarction. CK-MB values peak at 12-24 hours after infarction and return to normal within 24-48 hours. Certain diseases of skeletal muscle result in an increased amount of CK-MB. The various causes of rhabdomyolysis, including the muscular dystrophies, commonly result in an abnormal increase in serum CK-MB activity. Swiss-Prot Accession Number: Brain P12277; Muscle P06732.

PAP (Prostatic Acid Phosphatase) is richly produced in the prostate gland in men.. It normally contributes a small amount to the serum concentration. The clinical use of this prostate-specific fraction is in cases of prostatic adenocarcinoma, where it is elevated most commonly in men with metastatic disease (about 60% of the cases). It is a much less sensitive test in men with localized disease (10-40% depending upon clinical grade). PAP will be transiently elevated following prostatic massage, needle biopsy, cystoscopy, and infarction. PAP is not unique to the prostate, but is detected in numerous body tissues. Swiss-Prot Accession Number: P15309.

SGOT Ag (Serum Glutamic Oxaloacetic Transaminase Antigen) is an enzyme that is normally present in liver and heart cells. SGOT is released into blood when the liver or

heart is damaged. The blood SGOT levels are thus elevated with liver damage or with an insult to the heart. Some medications can also raise SGOT levels. SGOT is also called aspartate aminotransferase (AST). It is an acute phase reactant suggestive of inflammation. Swiss-Prot Accession Number: P17174.

β-2 M (Beta 2-Microglobulin) is a protein found on the surfaces of all nucleated cells and is shed into the blood, particularly by tumor cells and lymphocytes. Due to its small size, it passes through the glomerular membrane, but normally less than 1% is excreted due to reabsorption in the proximal tubules of the kidney. Therefore, high plasma levels occur in renal failure, inflammation, and neoplasms, especially those associated with B-lymphocytes. Swiss-Prot Accession Number: P01884.

EXAMPLE 1

Selection of Patients and CSF Samples

OPTIMA (Oxford Project To Investigate Memory and Ageing) is a highly defined longitudinal cohort of community volunteers with interest in the periodic assessment of their memory and cognitive status who have been studied serially since 1988 and includes controls, AD and other dementias. There are over four hundred subjects and over 300 controls that undergo neuropsychological tests, CT and SPECT scans and various biochemical tests on their blood at regular intervals. Cerebrospinal fluid is obtained from a subset of patients who have consented specifically for this procedure. After death, autopsy is performed and the brains are examined by a neuropathologist to define brain pathology. To date, the autopsy rate has been 94%. All of the information and samples of the OPTIMA cohort are stored at the Radcliff Infirmary in Oxford, UK.

In a pilot study, Applicants have analyzed for biomarker expression CSF specimens obtained ante-mortem from 56 subjects: 29 clinical controls and 27 pathologically confirmed amyloid AD. Pathological data was available for all AD patients and for 13 of the control subjects. The remaining control subjects are to date either still living or did not provide consent for postmortem examination. In the AD group, all 27 subjects were considered by neuropathology to have CERAD definite AD. As a preliminary study, samples were arbitrarily chosen based on sample volume available which led to a mismatch in gender between the control and AD groups. Since gender plays a minimal role in the incidence and progression of AD this difference was not considered significant.

The demographic characteristics of the pilot population at the time of CSF collection are listed in the Table 1A. The control and confirmed AD groups were similar in age and marital status, but showed differences in gender distribution as described above.. The confirmed AD group reported a greater family history of AD and had moderate to severe disease.

EXAMPLE 2

Aβ40 Expression

Aβ40 was measured in the CSF with a human Aβ 1-40 Colorimetric solid phase sandwich Enzyme Linked Immuno-Sorbent Assay (ELISA) kit (catalogue # KHB3482, BioSource International, Camarillo, CA) following the manufacturer's recommendations. A standard sandwich immunoassay was performed wherein the analyte, Aβ40, was first captured with an antibody specific for the N-terminal half of Aβ and then detected with a second detection antibody specific for the Aβ40 neo-epitope. This sandwich immunoassay can be performed using any suitable antibody pair that measures Aβ40 or its truncated equivalents. The detection antibody consisted of rabbit anti-Aβ40 and a secondary anti-rabbit IgG:horse radish peroxidase (HRP) conjugate. HRP catalyzes the formation of a chromophore, tetramethylbenzidine (TMB), which was quantitatively measured at 450 nm to provide readout of Aβ40 concentration. This procedure was carried out according to the BioSource kit instructions. A blocking buffer was used to minimize non-specific interactions. Standards were used as received in the kit. Determinations of unknowns were made using a four parameter logistic fit to the standards measured in duplicate wells. Quality controls samples (low, mid, and high) were run on all plates to insure valid results consistent with previous measurements.

The results of the Aβ40 analysis in the pilot cohort of control and confirmed AD human CSF samples are summarized in Table 2 and Table 3. One skilled in the art would recognize that there was a clear reduction of Aβ40 in the AD CSF compared to control; this result is consistent with some published findings and inconsistent with others. In this instance Applicants obtained information regarding the amount of Aβ40 processed by β- and γ-secretase from APP and found that there was enhanced APP processing in AD brain compared to normal age match controls.

EXAMPLE 3

Aβ42 Expression

Aβ42 was measured with Innotest™ Aβ42 ELISA kit (Innogenetics Inc., Cat. #80040, Ghent, Belgium) following the manufacturer's recommendations with modifications as follows. Similar to the Aβ40 assay above, a standard sandwich immunoassay was performed wherein the analyte, Aβ42, was first captured with an antibody specific for the N-terminal half of Aβ (3D6) and then detected with a second detection antibody (21F12) specific for the Aβ42 neo-epitope. The assay utilized a mouse monoclonal capture antibody specific for the C-terminus of Aβ42. The detection system employed an N-terminal specific biotinylated mouse monoclonal antibody and a secondary conjugate made of horse radish peroxidase (HRP) labeled strepavidin. The HRP was used to convert tetramethyl benzidine to a chromophore which was quantitatively measured at 450 nm to provide readout of AB42 concentration. This sandwich immunoassay can be performed using any suitable antibody pair that measures Aβ42 or its truncated equivalents. A blocking buffer was used to minimize non-specific interactions. After detection of the amount of bound detection antibody with a substrate for a conjugated enzyme to the detection antibody, the amount of analyte was determined against a standard curve generated from a known master stock In an attempt to reduce variability between kit lot numbers, Applicants deviated from the standard manufacturer's protocol by creating a concentrated solution of amino acid analyzed Aβ42 (0.778 mg/mL in DMSO). This was used across different kit lots instead of the standard material supplied by the manufacturer. The range of standards used for sample analysis was 5.45 to 350 pg/mL. Quality controls samples (low, mid, and high) were run on all plates to insure valid results consistent with previous measurements.

The results of the Aβ42 analysis in the pilot cohort of control and confirmed AD human CSF samples are shown in Figure 1A. The data have been log-transformed in order to ensure approximate symmetry in the distribution. One skilled in the art would recognize that there was a clear reduction of Aβ42 in the AD CSF compared to control, a result that is consistent with most of the published findings in the literature. Applicants obtained information regarding the amount of Aβ42 processed by β- and γ-secretase from APP and found that there was enhanced APP processing in the AD brain compared to normal age match controls.

EXAMPLE 4

t-Tau Expression

Total Tau (t-Tau) expression was measured with a human Tau (hTAU AG Innotest™) ELISA kit (Innogenetics Inc., catalogue number 80226, Ghent, Belgium) following the manufacturer's recommendations. Similar to the Aβ assays in Examples 2 and 3 above, a standard sandwich immunoassay was performed wherein the analyte, total tau protein independent of phoshorylation state, was first captured with a monoclonal antibody specific for all isoforms of Tau and then subsequently bound by two biotinylated tau-specific antibodies. The final detection was performed by peroxidase-labeled streptavidin. This sandwich immunoassay can be performed using any suitable antibody pair that measures all Tau species, including truncated equivalents. A blocking buffer was used to minimize non-specific interactions. After detection of the amount of bound detection antibody with a substrate for a conjugated enzyme to the detection antibody the amount of analyte was determined against a standard curve generated from a known master stock Quality control samples (low, mid, and high) were run on all plates to insure valid results consistent with previous total Tau measurements.

The results of the total Tau analysis in a pilot cohort of control and confirmed AD human CSF samples are summarized in Table 2 and Table 3. There was a clear increase in total Tau protein in the CSF of AD subjects compared to controls, a result that was consistent with most of the published findings in the literature. Applicants obtained information regarding the amount of total Tau protein, information which some have speculated is a marker of the degree of neurodegeneration which is or has occurred in the brain, and found that there was enhanced levels of total Tau protein in the AD brain compared to normal age match controls.

EXAMPLE 5

p-Tau-181 Expression

Phosphorylated Tau-181 (pTau-181) was measured with the Phospho-TAU ($_{181P}$) Innotest™ ELISA kit (Innogenetics Inc., catalogue number 80062, Ghent, Belgium), following the manufacturer's recommendations. Similar to the total Tau assay above, a standard sandwich immunoassay was performed wherein the analyte, tau protein phosporylated at amino acid 181, was first captured with an antibody specific for all isoforms of Tau and then detected with a second detection antibody which specifically detected Tau molecules phosphorylated at threonine 181 (phospho-tau-181). This sandwich immunoassay can be performed using any suitable

antibody pair that measures specific phopho-181 Tau species, including truncated equivalents. A blocking buffer was used to minimize non-specific interactions. After detection of the amount of bound detection antibody with a substrate for a conjugated enzyme to the detection antibody the amount of analyte was determined against a standard curve generated from a known master stock Quality controls samples (low, mid, and high) were run on all plates to insure valid results consistent with previous total Tau measurements.

The results of the phosphor-181-Tau analysis in the pilot cohort of control and confirmed AD human CSF samples are summarized in Table 2 and Table 3. There was a clear increase in pT-181 tau protein in the CSF of AD subjects compared to controls, a result that was consistent with most of the published findings in the literature. Applicants obtained information regarding the amount of Tau protein phosphorylated at a specific threonine at position 181, information which some have speculated is a marker of the degree of neurodegeneration which is or has occurred in the AD brain, and found that there was enhanced abundance of pTau-181 protein in the AD brain compared to normal age match controls.

EXAMPLE 6

sAPPα and sAPPβ Expression

When APP is processed by either α-secretase or β-secretase, it is cleaved into two fragments, of which the amino terminal fragment has been called the secreted APPα or β fragment, respectively. These two cleavage products of APP, sAPPα and sAPPβ, were measured with the MSD® sAPPα/sAPPβ Multiplex kit (MesoScale Discovery Cat #N41CB-1, Gaithersburg, MD), following the manufacturer's recommendations. Unlike the previous Examples above, this assay was run in a duplex format whereby two signals were read from a single well of a 96 well plate enabling simultaneous determinations of both sAPPα and sAPPβ. A standard sandwich immunoassay was performed wherein the analyte, either of the sAPPs, was first captured with an antibody specific for the C -terminal region of sAPPα or the sAPPβ C-terminal neo-epitope and then detected with a second detection antibody directed towards an N-terminal region of APP. This sandwich immunoassay can be performed using any suitable antibody pair that measures these analytes specifically, however, Applicants have assessed several antibodies in the literature and found that most have poor immunoreactivity to the naturally occurring isoforms and post-translational modifications of sAPP found in human CSF. A blocking buffer was used to minimize non-specific interactions. After detection of the amount

of bound detection antibody using the MSD TPA buffer solution as a substrate for a Ruthinium conjugated enzyme as detection antibody the amount of analyte was determined against a standard curve generated from a known master stock Quality controls samples (low, mid, and high) were run on all plates to insure valid results consistent with previous measurements.

The results of the sAPPα and sAPPβ analysis in the pilot cohort of control and confirmed AD human CSF samples are shown in Figures 1B and 1C, respectively. The data have been log-transformed in order to ensure approximate symmetry in the distribution. Unexpectedly there was a clear reduction of both sAPPα and sAPPβ in the AD CSF compared to controls, a result that Applicants believe has not been found in published findings in the literature. Applicants obtained information regarding the amount of sAPP processed by β- and α-secretase from APP, and found that there was enhanced APP processing in the AD brain compared to normal age match controls.

EXAMPLE 7

BACE Activity

β-APP Cleaving Enzyme (BACE) (also known as, memapsin or aspartle protease-2 (Asp2)) activity in CSF has been measured using a two-step method. In the first step, cleavage of a biotinylated peptide substrate was accomplished using CSF as the source of BACE enzyme. In the second step, the extent of enzymatic cleavage of substrate was detected using an avidin-biotin complex and enzyme linked immunosorbent assay (ELISA).

In the peptide substrate cleavage step, 25 µl of either purified recombinant baculovirus expressed BACE (amino acid residues 1-460) at a range of concentration from 0.8 pM to 100 pM or human CSF was added to a 96 well assay plate (Costar, Cat#3365, Corning, NY). To each of these wells, 25 µl of reaction buffer containing 50 mM NaOAc, 0.01% BSA, 15 mM EDTA, 0.2% CHAPS (Pierce, Cat#28300, Rockford, IL), 1 mM Deferoxamine Mesylate (Sigma, Cat# D9533) and 10 µM pepstatin A (Calbiochem, Cat #516481) at pH 4.5 was added. The plate was gently agitated on a shaker for 15 minutes in order to block any non-BACE aspartyl protease activity by pepstatin A. Finally, 100 µl of 200 nM substrate (biotin-KTEEISEVNF-EVEFR, SEQ ID NO.; 1) prepared in reaction buffer with 10 µM pepstatin A was added. The plate was sealed tightly and incubated at 37°C and agitated at 40 rpm for 2.5 hours. The enzymatic reaction was then arrested by adding 50 µl of 1M Tris (pH 8.0).

In the second step, the product of BACE enzymatic cleavage from the above reaction "biotin-KTEEISEVNF" (SEQ ID NO.:2) was measured by ELISA. The above reaction

mixture was transferred onto a streptavidin coated black plate (High binding capacity, Pierce, Cat# 15503, Rockford, IL) and incubated overnight at 4°C. The following day, the plate was washed three times with phosphate buffered saline with 0.1% Tween-20 (PBST) at pH 7.4. This was followed by addition of 100 µl of NF C-terminal neo-epitope rabbit polyclonal antibody at 1:30,000 dilution in 0.1% Tween-20 in Superblock PBS (Pierce, Cat#37515, Rockford, IL), and incubated for 1 hour at room temperature. After this incubation the plate was washed three times with PBST. Then 100 µl of Donkey anti-rabbit IgG-HRP or goat anti-rabbit IgG-AP (Bio-Rad, cat# 170-6518) at 1:30,000 dilution in 0.1% Tween 20-superblock was added and incubated for 1 hour at room temperature. The plate was then washed five times with PBST. The reaction was finally developed using 100 µl/well of CDP-Star ready-to-use with HRP substrate (TMB) or Sapphire-II Enhancer substrate (Applied Biosystems, Cat# T2214) for 30 minutes at room temperature. Absorbance or Luminescence counts were measured in LJL-Analyst (Molecular Devices Inc.). The counts from individual CSF samples were converted to BACE concentration using coefficients determined by a quadratic fit to the baculo-BACE standard curve.

The results of the CSF BACE activity analysis in a pilot cohort of control and confirmed AD human CSF samples are shown in Figures 1D. The data have been log-transformed in order to ensure approximate symmetry in the distribution. From the ANOVA on the log transformed BACE data, after adjusting for the baseline age differences by including it as a factor in the model, it was found that there was a meaningful reduction of CSF BACE activity in AD patients compared to controls, which was inconsistent with the literature whereby initial results indicated that BACE activity in the CSF of AD subjects was elevated compared to age matched controls.

EXAMPLE 8

Aβx-40 and Aβx-42 Expression

Human CSF Aβx-40 and Aβx-42 was measured using a sandwich ELISA consisting of analyte capture with mouse monoclonal antibody 4G8 (epitope Aβ17-24) followed by detection with alkaline phosphatase conjugated neo-epitope antibodies specific for Aβ40 (G210) and Aβ42 (4D7A3), respectively. The 4G8 antibody was purchased from Signet Inc., while G210 was licensed from University of Heidelberg (Heidelberg, Germany) and 4D7A3 was received from Innogenetics Inc. (Ghent, Belgium).

The Aβx-40 and Aβx-42 assays consisted of coating black 96 well costar plates (Costar #3365) with capture antibody 4G8 at 2 μg/ml, in carbonate-bicarbonate buffer (Pierce #28382) at pH of about 9.4. After overnight incubation the plates are washed with PBS and then blocked with 0.1% Tween20 in Superblock (Pierce #37515). CSF samples are diluted 1:8 in 0.1% Tween 20-superblock buffer. Pooled human immunodepleted CSF at a 1:8 dilution was used to prepare standard curves with Aβ40 and Aβ42 respectively. 50 μL of standards, QC's and samples, respectively, were added to wells in duplicate followed by 50 μL of detection antibodies G210-AP and 4D7A3-AP at 1:3000 dilution in 0.3%Tween20/Superblock. Isotype control mouse IgG-AP was used at 1:3000 for background subtraction. The plates are incubated overnight at 4°C. The plates are then washed three times with 0.05% PBST and then plates are thoroughly dried. Plates are then washed with Activation buffer (20 mM Tris-HCl, 1 mM MgCl2, pH 9.8) and then dried. 100 μL alkaline phosphatase substrate (Applied Biosystem #T2214) is added all wells and incubated at RT for 30 minutes. Luminescence counts are measured on the LJL Analyst system (Molecular Devices Inc.). All counts are corrected using the mouse isotype control counts. A third order spline fit to the standards on each plate was used to determine the coefficients that were used to calculate of actual Aβ concentrations for the individual samples.

The results of the CSF Aβx-40 and Aβx-42 analysis in the pilot cohort of control and confirmed AD human CSF samples are summarized in Table 2 and Table 3. Consistent with the literature there was a meaningful difference between the AD CSF compared to controls.

EXAMPLE 9

Multivariate analysis of marker panels -

Expanded 9 markers for the classification AD versus control

The combined ability of the expanded set of nine CSF markers to differentiate AD from control was assessed using Linear Discriminant Analysis (LDA). This analysis measured the distance from each point in the data set to each group's multivariate mean (called a *centroid*) and classified the point to the closest group. The distance measure used was the Mahalanobis distance which takes into account the variances and covariances between the variables. In Figure 2 each multivariate mean is a labeled circle. The size of the circle corresponds to a 95% confidence limit for the mean. Groups that are significantly different tend to have non-intersecting circles.

The assessment of relative importance of the biomarkers with respect to their ability to discriminate AD from control subjects was done within the framework of a random forest analysis (Breiman, 2001). In each tree of the forest (i.e., large collection of trees derived from many simulated samples from the original data), data was permuted in one biomarker at a time and predictions on the permuted data from the random forest method were obtained. These were compared to the predictions from the unpermuted data and the loss in accuracy is assessed. A large loss in accuracy, represented as the percent of mean decrease in accuracy ("MeanDecreaseAccuracy"), indicates the relative importance of the corresponding marker.

With respect to LDA, the predictive ability of these markers to separate AD from control for hold-out datasets was investigated using fifty replicates of 10-fold cross validation. This analysis entailed dividing up the data randomly into ten subgroups, using the fitted model from nine of these subgroups to predict the disease classification in the tenth subgroup, repeating this for all ten groups and then averaging the results across all ten repetitions. This analysis was repeated fifty times to generate a reliable estimate of the overall accuracy along with the sensitivity and specificity of the biomarker composites for separating AD from normal, i.e. control, subjects.

These analyses were based on 29 controls and 27 AD subjects. The LDA graph in Figure 2 was generated using JMP software, v5.0.1 from SAS Institute (Cary, NC). All other analyses were performed using R (R: A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2005, ISBN 3-900051-07-0).

LDA for assessing the performance of the various composites of markers was performed using a contributed library within R (Venables, W. N. and Ripley, B. D., Modern Applied Statistics with S., Fourth Edition, Springer, New York (2002) ISBN 0-387-95457-0). The random forest analysis was performed using a contributed library within R (Andy Liaw and Matthew Wiener, Classification and Regression by Random Forest, R News, 2 (3): 18-22 (2002)). The 10-fold cross-validation for obtaining reliable estimates of the performance metrics of the biomarker composites was performed using a contributed library within R (Andrea Peters and Torsten Hothorn, Improved Predictors, R package version 0.8-3(2004)).

Table 4 lists representative composites of biomarkers from the LDA analysis that met the 1998 NIA criteria on sensitivity and specificity (sensitivity>85%, specificity>75%). For example, the composite of Aβ42, Tau, pTau and BACE activity provide 99.35% overall accuracy, 99.62% sensitivity and 99.08% specificity. One skilled in the art would understand

that the addition of a marker to a composite may not improve the performance. For example, inclusion of sAPPβ in the composite of Aβ40, BACE and sAPPα reduced the specificity from 81% to 73%. Conversely, adding a marker to a composite, such as the addition of BACE activity to the composite of Aβ40 and sAPPα, improved the sensitivity from 74% to 83% and specificity from 75 to 81%.

EXAMPLE 10

Multivariate analysis of marker panels –

Expanded 9 markers for cognition prediction (MMSE)

The relationship between CSF markers and the Mini Mental State Exam (MMSE) cognition scores (MMSE) was analyzed using the RF method (Breiman, 2001). This multivariate analysis method entailed an aggregation of results from a forest of decision tree models that were built to ensure maximum prediction accuracy and minimum effect from the correlation (colinearity) between biomarkers. Several (typically 500) bootstrap samples (random samples of the same size as the data set drawn with replacement) were drawn from the original dataset and a decision tree of maximum size was built from each bootstrap dataset resulting in trees with low bias but high imprecision. Only a random subset of a few biomarkers was considered at each tree node for determining the optimal split, helping reduce the effect of correlation/colinearity between the predictor variables. The predictions were obtained from each tree in the forest and then aggregated in the form of a majority vote for a class/event in the classification model (categorical response) or in the form of the average prediction in a regression model (continuous/quantitative response). The high imprecision from a single tree was reduced by aggregating across the large number of trees in the forest. This method of aggregating the results across a forest of several trees, by maintaining high accuracy and minimizing the correlation effect among biomarkers, results in the optimal predictions.

The assessment of relative importance of the biomarkers with respect to their ability to predict the MMSE scores was done within the framework of the RF analysis (Breiman, 2001). In each tree of the forest (i.e., large collection of trees derived from many simulated samples from the original data), data were permuted in one biomarker at a time and predictions on the permuted data from the random forest method were obtained. These were compared to the predictions from the unpermuted data and the increase in the variability of the predictions was assessed. A large increase in the prediction variability, represented as the percent increase in MSE ("%IncMSE"), indicated the relative importance of the corresponding biomarker. Figure

3A shows the ranking of the relative importance of the six markers for cognition prediction. Applicants observed that Tau, Aβ42, and pTau were the top three markers for cognition prediction.

The predictive power of the CSF markers from the RF algorithm was assessed using fifty replicates of 10-fold cross validation. Data were divided into ten random parts, each part was used as the hold-out (test) set on which the predictions were obtained from the model fit to the rest of the data and this was repeated for all ten parts. This analysis was replicated fifty times and the prediction errors, such as the root mean squared error (RMSE) and the percent concordance (%C), between the predicted and observed results from the hold-out data were averaged across these repetitions. The composites considered from the nine markers provided RMSE of 7 and %C of 73.6% agreement between the observed and predicted MMSE scores from fifty replicates of 10-fold cross validation as described above.

All of these analyses were carried out using R (R: A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2005, ISBN 3-900051-07-0). The random forest analysis was performed using a contributed library within R (Andy Liaw and Matthew Wiener, Classification and Regression by Random Forest, R News 2 (3):18-22 (2002)).

The 10-fold cross-validation for obtaining reliable estimates of the performance metrics of the biomarker composites was performed using a contributed library within R (Improved Predictors, Andrea Peters and Torsten Hothorn, R package version 0.8-3 (2004)).

EXAMPLE 11

Analysis of samples in the Rules-Based Medicine human Multi-Analyte Profile (MAP)

CSF samples of AD and control were sent to Rules-Based Medicine, Inc. (Austin, TX) for analysis in their proprietary human Multi-Analyte Profile (MAP®) under a fee for service agreement. MAP is a Luminex bead based multi-antigen profile consisting of at least three pools of beads representing some 90 total antigens (Figure 6). Designed originally for human plasma analysis of 100 µL samples, the human MAP has been analytically validated according to NACLES criteria and successfully used on human CSF when using 200 µL samples in manual mode and 250 µL samples in automated mode.

The human CSF samples responded well in the plasma based antigen panel, producing nearly 50 analytes with good measurements in the AD CSF samples. 63 analytes had measurable levels in at least five AD and five control CSF samples.

EXAMPLE 12

Univariate analysis of the Expanded 9 plus markers from the RBM panel

The ability of each marker from the Expanded 9 marker panel and the 63 measurable markers as measured from the RBM panel (total of 72 markers) to separate disease from normal (AD versus control) was assessed using analysis of variance (ANOVA) with the disease group as fixed effect. The baseline age group and sample storage time were included as covariates in the ANOVA of each marker if their p-values were less than 0.1. The age groups used in the model was defined by those that are less than and greater than 73 years old at baseline. The analysis was carried out after applying logarithmic transformation (base 10) on the markers to ensure approximate symmetry. The p-values (false positive rate) and q-values (false discovery rates) from this analysis on the 72 markers were determined. False Positive Rate (FPR) or p-value estimates the proportion of false positives among all the proteins that in reality did not change. False discovery rate (FDR) or q-value estimates the proportion of significant changes that are false positives. Analytes from the RBM panel that had $p < 0.05$ are reported in this document, with special reference to those that had a more robust effect at $q < 0.1$. Summary statistics obtained from these analyses for each of the markers included the ratio of AD to control using the observed data and also using the least squares means from the ANOVA after adjusting for baseline age factor and sample storage time (if they were significant at $p<0.1$), the false positive rate (p-value) and the false discovery rate (q value) of the AD versus control comparison. Analysis was carried out using R version 2.4 (R: A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2005, ISBN 3-900051-07-0). Figures 4A, 4B and 4C were generated using JMP v5.0.1 from the SAS Institute (Cary, NC). The false discovery rate (q values) for each marker was derived using the method proposed by Benjamini & Hockberg (Benjamini & Hockberg, The adaptive control of the false discovery rate in multiple hypotheses testing with independent statistics, *J. Behav. Educ. Statist.* 25: 60–83 (2000)) and determined using a contributed library within R (Strimmer, Estimation and Control of (Local) False Discovery Rates, fdrtool package, version 1, August 8, 2006).

As shown in Table 2 all markers in the Expanded 9 panel significantly differ between AD and control ($p<0.05$) with the exception of sAPPα that is marginally significant ($p=0.0682$). Aβ40, Aβ42, Tau, pTau and Aβx42 have the most robust effect at less than 5%

false discovery rate (FDR), i.e., $q<0.05$. In addition, sAPPβ and Aβx40 have a robust effect at less than 10% false discovery rate ($q<0.1$).

Among the measurable markers from the RBM panel, eight markers were statistically significant at $p < 0.05$. These include Alpha Fetoprotein (AF), Calcitonin, Fatty Acid Binding Protein (FABP), Interferon gamma (IFN□, Monocyte Chemotactic Protein-1 (MCP-1), Stem Cell Factor (SCF), Thyroxine Binding Globulin (TBG), and Vascular Endothelial Growth Factor (VEGF). Among these markers, Calcitonin and VEGF have the most robust effect at less than 5% false discovery rate (FDR), i.e., $q<0.05$. In addition, AF, FABP and TBG had a robust effect at less than 10% false discovery rate ($q<0.1$).

EXAMPLE 13

Multivariate results using RBM panel for classifying AD versus control using LDA

Multi-analyte panels that provide the best predictive performance were determined from the collection of measurable analytes in the RBM panel. For each of the panel sizes ranging from three to twelve markers representative optimal multi-analyte panels were determined using the Simulated Annealing Algorithm (SAA) and the Genetic Algorithm (GA) within the framework of the LDA method (Duarte Silva, A.P., Efficient Variable Screening for Multivariate Analysis, Journal of Multivariate Analysis, 76: 35-62 (2001)). In addition, an optimal panel comprising three markers sing the Shrunken Centroid (SC) method (Tibshirani, R.J., Hastie, T., Narasimhan, B. and Chu, G., Diagnosis of Multiple Cancer Types by Shrunken Centroids of Gene Expression. P.N.A.S., USA, 99(10):6567-6572 (2002)) was also derived.

Representative optimal multi-analyte panels are shown in Table 4 along with their predictive performance determined from the aggregate of fifty replicates of 10-fold cross-validation. This analysis entailed dividing up the data randomly into ten subgroups, using the fitted model from nine of these subgroups to predict the disease classification in the tenth subgroup, repeating this for all ten groups and averaging the results across all repetitions. This analysis was repeated fifty times to generate a reliable estimate and standard error of the overall accuracy along with the sensitivity and specificity of the biomarker composites for separating AD from control, i.e. normal subjects.

In addition to the three-marker composite derived from the SC method, all multi-analyte composites of four markers or greater, shown in Table 4, that were derived using the SAA and the GA algorithms met the 1998 NIA criteria for sensitivity and specificity

(sensitivity>85%, specificity>75%). The percent sensitivity and percent specificity metrics reported in this table are within 0.4% standard error.

In order to graphically illustrate the performance of these panels, canonical plots for an optimal three-analyte panel determined from the SC method and an optimal six-analyte panel determined from the SAA method are shown in Figures 5A and 5B. The three-analyte panel, Calcitonin, FABP and VEGF, provides 86% classification accuracy. This classification accuracy improves to 96% with the addition of MMP-3, Myoglobin and SCF to form the six-analyte panel.

These analyses were based on 29 controls and 27 AD subjects. The LDA graphs in Figures 5A and 5B was generated using JMP software, v5.0.1 from SAS Institute (Cary, NC). All data analyses were performed using R (R: A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2005, ISBN 3-900051-07-0).

The linear discriminant analysis for assessing the performance of the various composites of markers was performed using a contributed library within R (Venables, W. N. and Ripley, B. D., Modern Applied Statistics with S., Fourth Edition. Springer, New York (2002) ISBN 0-387-95457-0). The SC method was implemented using a contributed library within R (Hastie, Tibshirani, Narasimhan, Chu, PAM: Prediction Analysis of Microarrays, PAMr package, version 1.25, March 31, 2006). The Simulated Annealing Algorithm was implemented using a contributed library within R (Cerdeira, Duarte-Silva, Cadima and Minhoto, Selecting Variable Subsets, The subselect Package, version 0.9-99, (June 8, 2006)). The 10-fold cross-validation for obtaining reliable estimates of the performance metrics of the biomarker composites was performed using a contributed library within R (Improved Predictors, Andrea Peters and Torsten Hothorn, R package version 0.8-3 (2004)).

WHAT IS CLAIMED:

1. A method for classifying disease states in Alzheimer's disease ("AD") comprising:

a. selecting a statistically relevant multi-analyte panel from human ante-mortem and healthy control fluid samples in which a plurality of biomarkers are differentially expressed to form a reference AD and control multi-analyte panel;

b. conducting a linear discriminate analysis on the multi-analyte data from step (a);

c. obtaining a test fluid sample from a patient;

d. conducting immunoassays on the test sample for the minimal number of analytes needed to specify the panel of step (a);

e. applying the results of step (d) to the linear discriminate analysis of step (b) to obtain an output; and

f. determining from the output of step (e) the classification of the disease state, where the output is either AD or control.

2. The method of claim 1 wherein the multi-analyte panel comprises a plurality of at least three biomarkers selected from the group consisting of Aβ40, Aβ42, Tau, pTau, sAPPα, sAPPβ, Aβx-42, Aβx-40, BACE activity, AF, Calcitonin, FABP, IFNγ, SCF, MCP-1, TBG and VEGF.

3. The method of claim 2 wherein the multi-analyte panel results in a composite having >85% sensitivity and >75% specificity.

4. The method of claim 3 wherein the multi-analyte panel comprises an optimal panel as set forth in Table 5.

5. A method for predicting cognition scores for Alzheimer's disease ("AD") patients comprising:

a. selecting a statistically relevant multi-analyte panel from human ante-mortem and healthy control fluid samples in which a plurality of biomarkers are differentially expressed to form a reference AD and control multi-analyte panel;

b. conducting a random forest analysis on the multi-analyte data from step (a);

c. obtaining a test fluid sample from a patient;

d. conducting immunoassays on the test sample for the minimal number of analytes needed to specify the panel of step (a);

e. applying the results of step (d) to the random forest analysis of step (b) to obtain an output; and

f. determining from the output of step (e), where the output is the assignment of the cognition score.

6. The method of claim 5 wherein the predicted cognition score is selected from the group consisting of MMSE, Learning Memory and Total CAMCOG.

7. The method of claim 6 wherein the multi-analyte panel comprises a plurality of at least three biomarkers selected from the group consisting of Aβ40, Aβ42, Tau, pTau, sAPPα, sAPPβ, Aβx-42, Aβx-40, BACE activity, AF, Calcitonin, FABP, IFNγ, SCF, MCP-1, TBG and VEGF.

8. The method of claim 7 wherein the multi-analyte panel results in a composite having >85% sensitivity and >75% specificity.

9. The method of claim 8 wherein the multi-analyte panel comprises an optimal panel as set forth in Table 5.

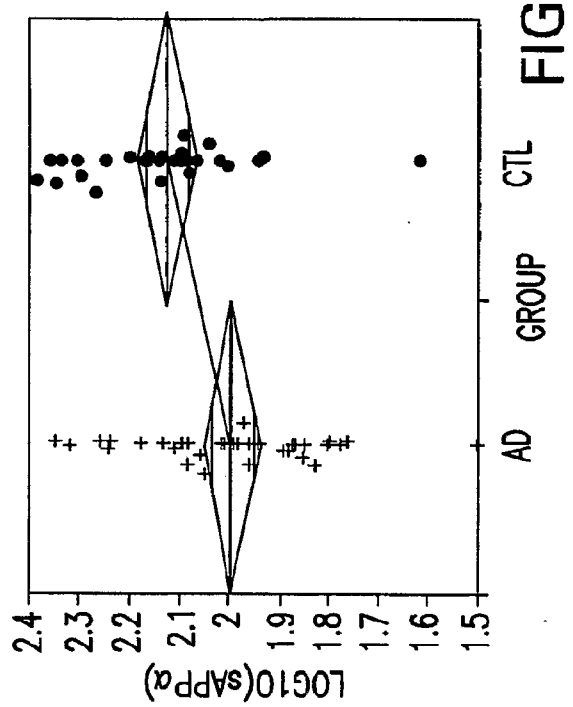


FIG. 1B

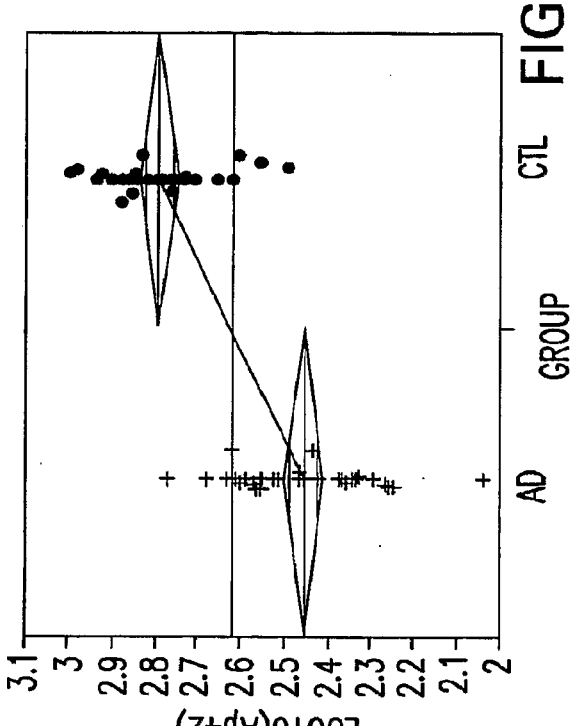


FIG. 1A

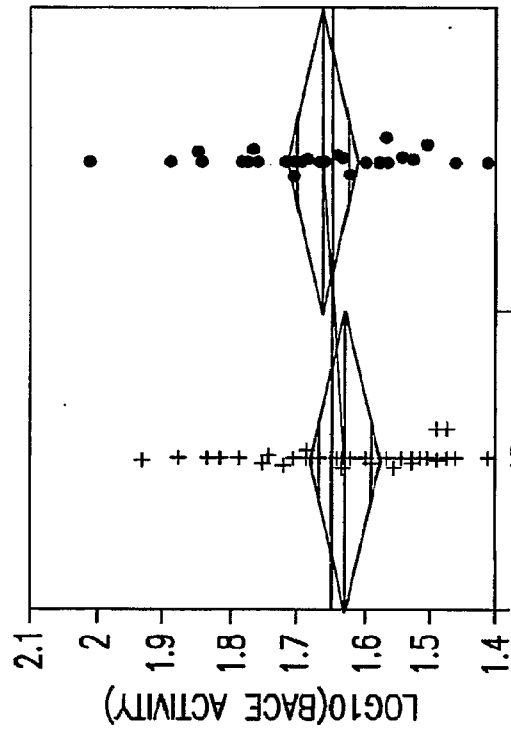


FIG. 1D

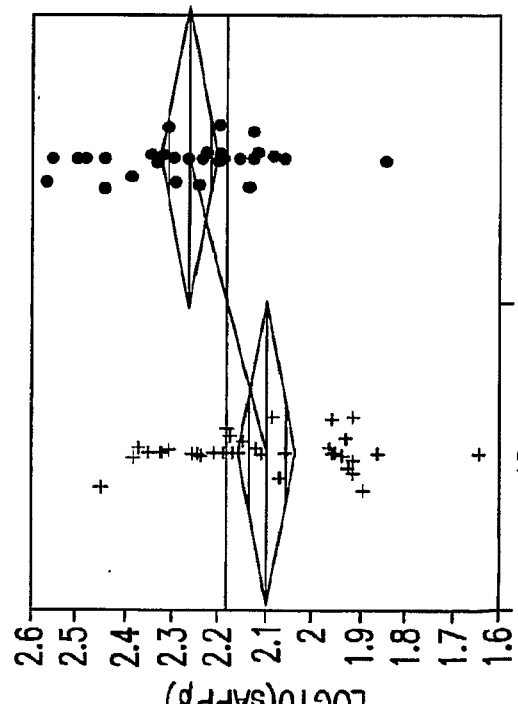


FIG. 1C

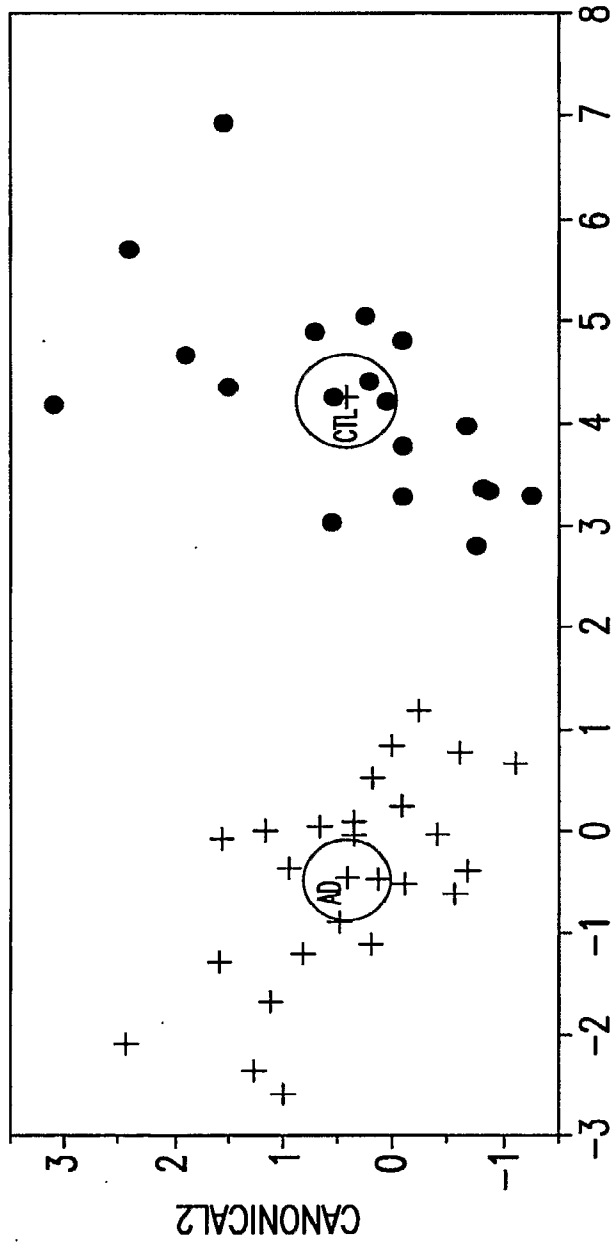
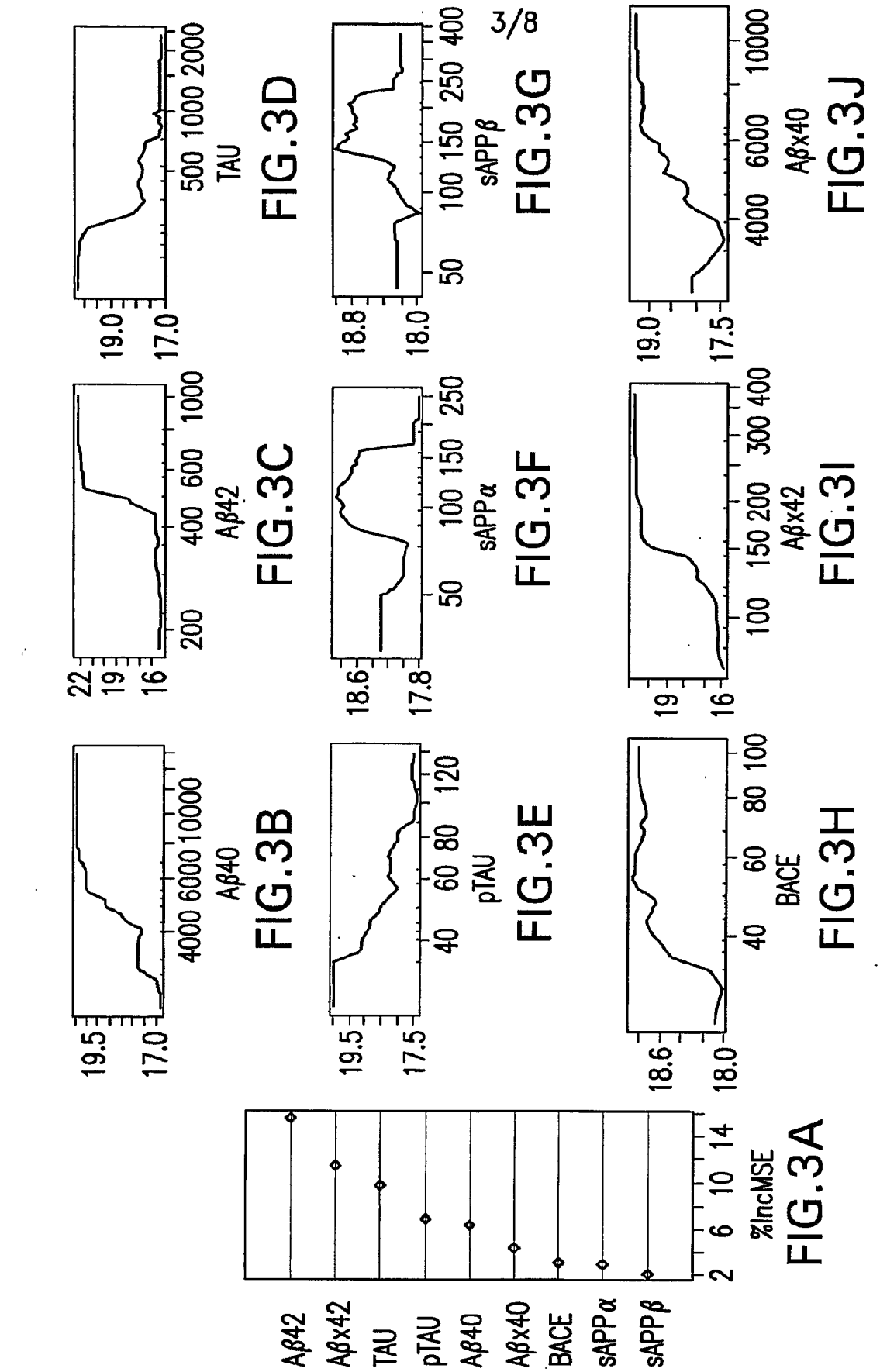


FIG.2


Aβ42
Aβx42
TAU
pTAU
Aβ40
Aβx40
BACE
sAPPα
sAPPβ
2 6 10 14
%IncMSE
FIG.3A
19.5
17.0
4000 6000 10000
Aβ40
FIG.3B
22
19
16
200 400 600 1000
Aβ42
FIG.3C
19.0
17.0
500 1000 2000
TAU
FIG.3D
19.5
17.5
40 60 80 120
pTAU
FIG.3E
18.6
17.8
50 100 150 250
sAPPα
FIG.3F
18.8
18.0
50 100 150 250 400
sAPPβ
FIG.3G
18.6
18.0
40 60 80 100
BACE
FIG.3H
19
16
100 150 200 300 400
Aβx42
FIG.3I
19.0
17.5
4000 6000 10000
Aβx40
FIG.3J

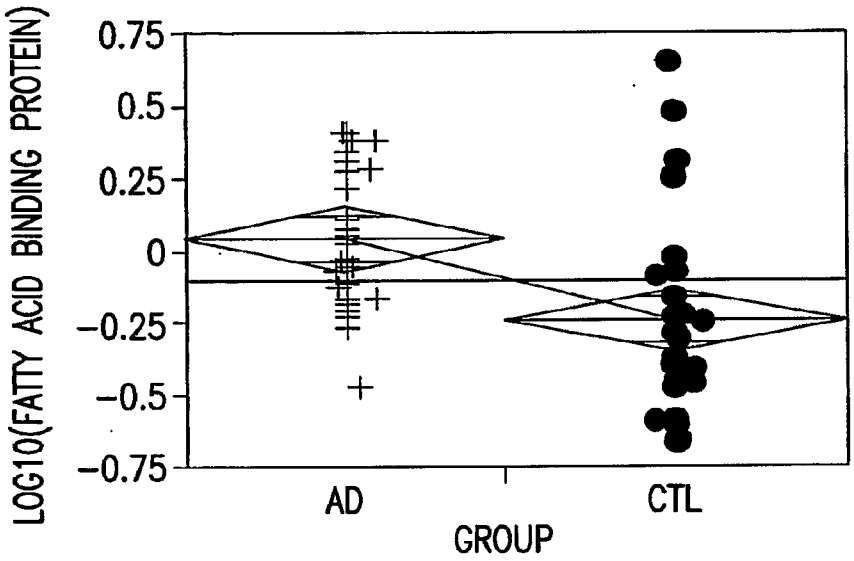


FIG.4A

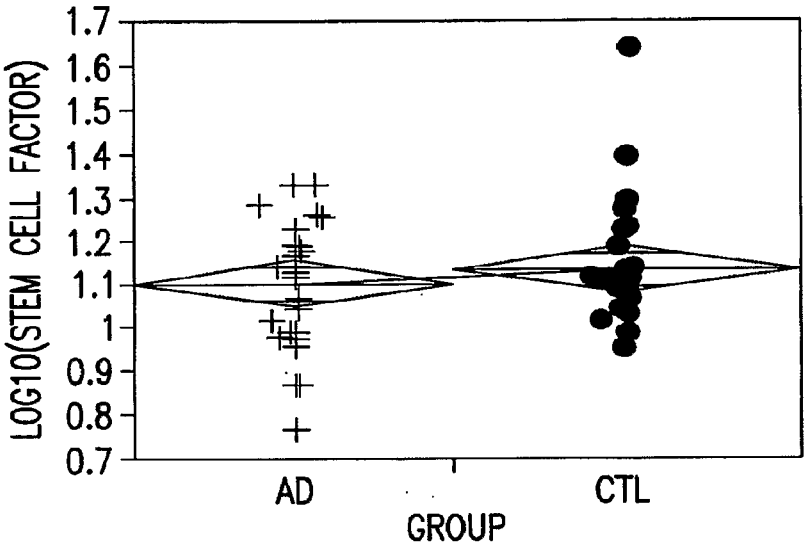


FIG.4B

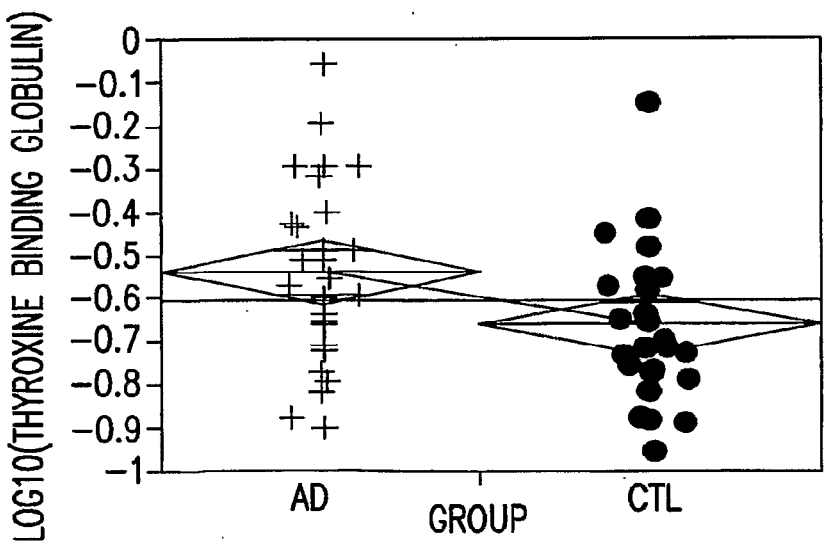


FIG.4C

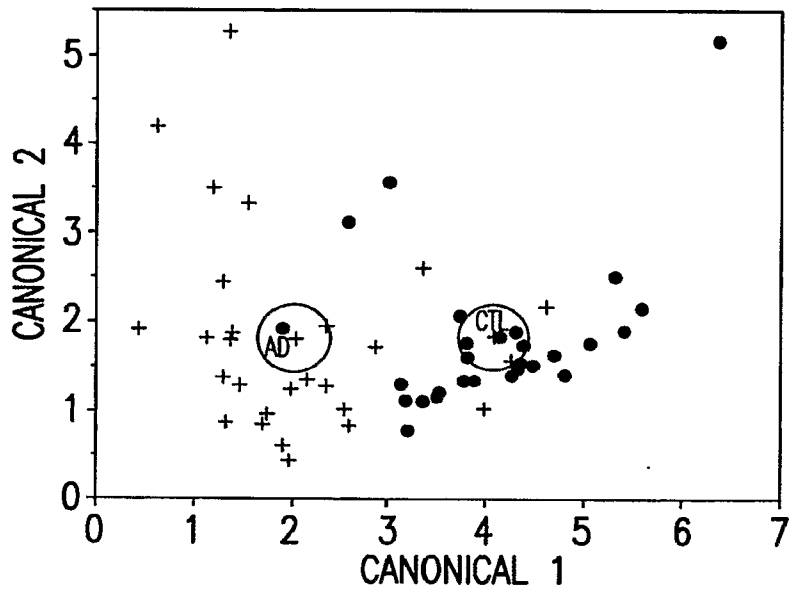

CANONICAL 2
CANONICAL 1
AD
CTL
0
1
2
3
4
5
6
7

FIG.5A

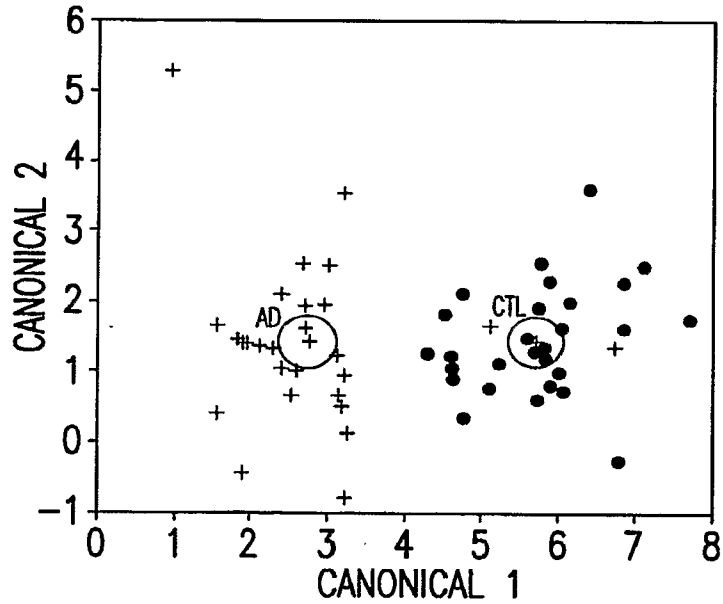

CANONICAL 2
CANONICAL 1
AD
CTL
−1
0
1
2
3
4
5
6
7
8

FIG.5B

	RBM PANEL OF 90 MARKERS
1	ALPHA-1 ANTITRYPSIN
2	ADIPONECTIN
3	ALPHA-2 MACROGLOBULIN
4	ALPHA-FETOPROTEIN
5	APOLIPOPROTEIN A1
6	APOLIPOPROTEIN CIII
7	APOLIPOPROTEIN H
8	BETA-2 MICROGLOBULIN
9*	BRAIN-DERIVED NEUTROPHIC FACTOR
10	COMPLEMENT 3
11*	CANCER ANTIGEN 125
12*	CANCER ANTIGEN 19-9
13	CALCITONIN
14	CD40
15	CD40 LIGAND
16*	CARCINOEMBRYONIC ANTIGEN
17	CREATINE KINASE-MB
18	C REACTIVE PROTEIN
19*	EGF
20*	ENA-78
21	ENDOTHELIN-1
22	EN-RAGE
23	EOTAXIN
24*	ERYTHROPOIETIN
25	FATTY ACID BINDING PROTEIN
26*	FACTOR VII
27	FERRITIN
28*	FGF BASIC
29	FIBRINOGEN
30*	G-CSF
31*	GROWTH HORMONE
32	GM-CSF
33*	GLUTATHIONE S-TRANSFERASE
34	HAPTOGLOBIN
35	ICAM-1
36	IFN-GAMMA
37	IgA
38	IgE
39*	IGF-1
40	IgM
41	IL-10
42*	IL-12p40
43*	IL-12p70
44	IL-13

FIG.6

45*	IL-15
46	IL-16
47*	IL-18
48	IL-1ALPHA
49*	IL-1BETA
50	IL-1RA
51*	IL-2
52*	IL-3
53*	IL-4
54	IL-5
55	IL-6
56	IL-7
57	IL-8
58*	INSULIN
59	LEPTIN
60	LIPOPROTEIN (a)
61*	LYMPHOTACTIN
62	MCP-1
63*	MDC
64	MIP-1ALPHA
65	MIP-1BETA
66	MMP-2
67	MMP-3
68*	MMP-9
69*	MYELOPEROXIDASE
70	MYOGLOBIN
71	PAI-1
72	PROSTATIC ACID PHOSPHATASE
73	PAPP-A
74	PROSTATE SPECIFIC ANTIGEN, FREE
75	RANTES
76	SERUM AMYLOID P
77	STEM CELL FACTOR
78	SGOT
79	SHBG
80	THYROXINE BINDING GLOBULIN
81	TISSUE FACTOR
82	TIMP-1
83	TNF RII
84	TNF-ALPHA
85*	TNF-BETA
86	THROMBOPOIETIN
87	THYROID STIMULATING HORMONE
88	VCAM-1
89	VEGF
90	VON WILLEBRAND FACTOR

FIG.6-1

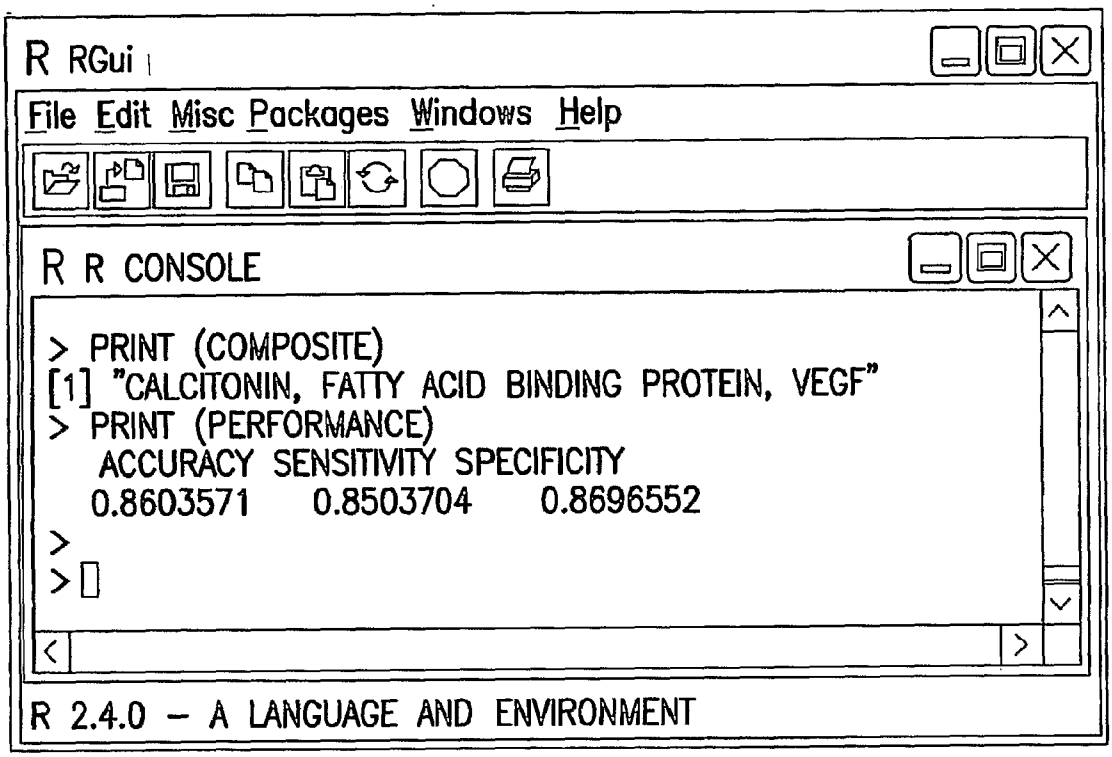

R RGui
File Edit Misc Packages Windows Help
R R CONSOLE
> PRINT (COMPOSITE)
[1] "CALCITONIN, FATTY ACID BINDING PROTEIN, VEGF"
> PRINT (PERFORMANCE)
ACCURACY SENSITIVITY SPECIFICITY
0.8603571 0.8503704 0.8696552
>
>
R 2.4.0 – A LANGUAGE AND ENVIRONMENT

FIG.7A

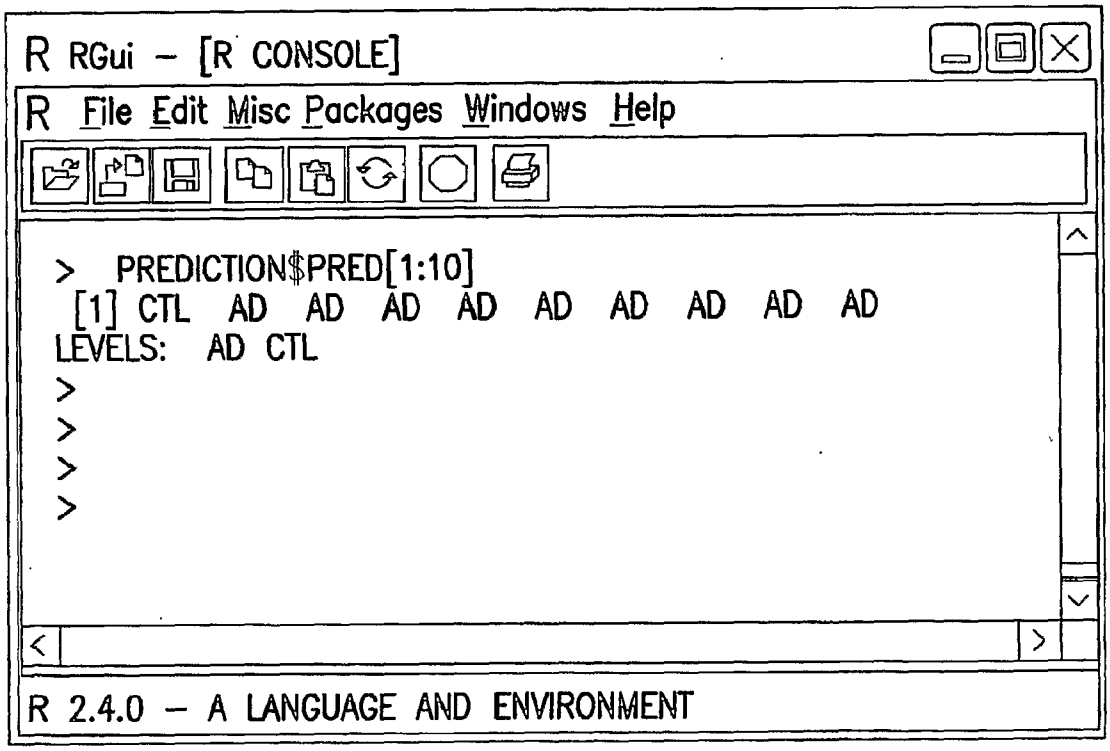

R RGui – [R CONSOLE]
R File Edit Misc Packages Windows Help
> PREDICTION$PRED[1:10]
[1] CTL AD AD AD AD AD AD AD AD AD
LEVELS: AD CTL
>
>
>
>
R 2.4.0 – A LANGUAGE AND ENVIRONMENT

FIG.7B