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(54) Title: METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE

(57) Abstract: The present invention relates to methods for the diagnosis of subjects that have or are at risk of having Alzheimer's disease (AD). In particular the present invention identifies individuals who have or are at risk of having AD through measurement of the levels of Afamin in combination with at least one other biomarker such as Alpha-1-antichymotrypsin, Alpha-2- macroglobulin, ApoB 100, Complement C5, Serine threonine protein kinase TBK1 or Complement C3 in a fluid sample taken from a subject. Furthermore, genotype (Apolipoprotein E or glutathione S-transferase Omega) may also be taken into consideration and used within classification algorithms to determine the probability of a subject having or being at risk of having AD.

Methods and compositions for the diagnosis of Alzheimer's disease

Background of the invention

Alzheimer's disease (AD) is a chronic neurodegenerative disease currently identified by progressive cognitive impairment and loss of memory leading to severe dementia. AD is typically a disease of the elderly, most prevalent in persons over the age of 65. It is the leading cause of dementia in the elderly and with an increasingly higher life expectancy, the prevalence in the population is only set to increase. AD is not typically life threatening, however as the disease progresses to severe dementia, patients are unable to care for themselves and usually require full time professional care.

There is currently no known cure for AD, but there are treatments that can slow the progression of the disease. Therefore a method that can identify patients with AD and potentially monitor their response to treatment would be an invaluable assay (tool for clinicians).

Current methods of diagnosis of AD involve mental assessment (such as MMSE), CT/MRI, measurement of cerebrospinal fluid for specific Tau or Beta-amyloid isoforms known in the art to be associated with AD or genotyping for genetic risk factors such as *Apolipoprotein E4 (ApoE4 variant)*; there are currently no clinically validated blood biomarkers of AD. Deficiencies of these methods can include a lack of specificity, they can be open to errors in interpretation, and may be highly invasive; generally a true diagnosis can only be made post mortem. There are currently no routinely used biomarker methods to assist the positive diagnosis for AD.

The pathogenesis of AD is not fully understood, but pathological investigations of patients revealed the presence of neurofibrillary tangles (caused by accumulation of Tau protein) and Beta-amyloid plaques. There is also widespread neuronal and synaptic loss, which is thought to underlie the reduced cognitive and mnemonic function. The formation of plaques has been shown to cause neurodegeneration, however the causes of plaque formation are unknown. Diagnostic tests that identify specific isoforms of these proteins have been the main focus in diagnostic assay development. However, the presence of these proteins may indicate that the disease has progressed past a therapeutically viable stage and therefore earlier risk markers may be more beneficial.

There have been several inventions describing methods for diagnosing AD using blood biomarkers, these include; EP 2293075 A2 and WO 2011/143597 A1. EP2293075 identified

several markers expressed in blood platelets using 2-D gel electrophoresis, which were differentially expressed between AD and control patients. These included variants of proteins which may correspond to a genetic susceptibility to AD. A further invention was described by these inventors (EP2507638) in which protein biomarkers were combined in an algorithm along with genotyping to improve the diagnostic model. In this algorithm patients whom were ApoE4 positive were more likely to have AD, as were patients whom were ApoE4 negative, but expressed two copies of the wild-type glutathione S-transferase 1 Omega (wtGSTO) gene. In the context of this previous invention, wtGSTO was defined as any GSTO gene which did not contain the rs4825 mutation (which encodes an Aspartic acid instead of an Alanine at residue 140 [A140D]). This invention highlights the effectiveness of combining blood-based biomarkers and genotyping to assist in the diagnosis of disease. WO 2011/143597 A1 identified multiple biomarkers that are differentially expressed between serum of AD and control patients using multiplexed assays. In this invention, greater accuracy of diagnosis is observed when using multiple combinations of biomarkers combined with clinical measurements and demographic variables using Random forests to develop a classification algorithm. However, these methods have not found clinical utility and there is an urgent need for a method that can be used routinely to aid the diagnosis of AD.

Summary of the Invention

The present invention relates to methods and compositions for the diagnosis of Alzheimer's disease.

The present invention identifies and describes proteins that are differentially expressed in the Alzheimer's disease state relative to their expression in the normal state.

According to the first aspect of the invention, there is provided a method of diagnosing Alzheimer's disease in a subject, comprising detecting two or more differentially expressed proteins chosen from Table 1 in a sample taken from the subject, whereby one of these is Afamin. More specifically, a method comprising detecting levels of Afamin and any of Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Apolipoprotein B100, complement C3 , Serine threonine kinase TBK-1, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG fc binding protein, hornerin, fibrinogen or complement C5 in a sample taken from a subject. Preferably the sample is serum or plasma.

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According to a further aspect of the invention, the relative levels of the differentially expressed proteins are used in conjunction with the ApoE or GST01 genotype or phenotype of a subject to increase the ability to differentiate between patients at risk of developing or having AD and those who are not at risk or do not have AD.

According to a further aspect of the invention, a method of detecting differentially expressed proteins chosen from Table 1 in a sample taken from a subject is provided wherein a specific probe for the protein is attached to the surface of a device. The respective levels of these proteins in a sample are calculated based on their ability to compete with biotinylated tracer substance. The tracer substance is modified plasma, where proteins contained have been conjugated to biotin.

According to a further aspect of the invention, a method for predicting the likelihood that a subject can be defined as suffering from or at risk of developing Alzheimer's disease, through developing a categorical prediction model using statistical modelling or machine learning methods. Such methods may include, but are not limited to; perceptron neural networks, support vector machines, logistic regression, decision trees and random forests.

According to an aspect of the invention, there is provided a method of diagnosing or monitoring a person at risk of developing or having Alzheimer's disease (AD) comprising obtaining a fluid sample from a person suspected of having or at risk of developing AD, measuring the concentration or relative level of the biomarker Afamin and Alpha-1 antichymotrypsin, and optionally at least one additional biomarker selected from Alpha-2-macroglobulin, Apolipoprotein B100, complement C3 , Serine threonine kinase TBK-1, vitamin D binding protein, alpha-1- B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5 and in the fluid sample, and establishing the significance of the concentrations or relative levels.

In certain embodiments, the measured concentration or relative level of Afamin and Alpha-1 antichymotrypsin, and optionally at least one of Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen and complement C5 is transformed into a ratio.

In certain embodiments, the ratio of afamin to alpha-1 antichymotrypsin is calculated.

In certain embodiments, the ratio of afamin to alpha-1 antichymotrypsin is calculated and the concentration or relative level of at least one additional biomarker selected from serine threonine protein kinase TBK1, alpha-2- macroglobulin, Apolipoprotein B100, complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2- antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, and complement C5 is also measured.

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In certain embodiments, one additional biomarker is serine threonine protein kinase TBK1.

In certain embodiments, one additional biomarker is complement C5.

In certain embodiments, one additional biomarker is alpha-2-macroglobulin.

In certain embodiments, one additional biomarker is Apolipoprotein B100.

In certain embodiments, one additional biomarker is complement C3.

In certain embodiments, the method further comprises determining the genotype of at least one of Apolipoprotein E and Glutathione S-Transferase 1 Omega of a person through identification of the nucleic acid sequence encoding the protein in the genome or through determining the form of protein produced in a fluid sample taken from the person.

In certain embodiments, the method further comprises using the measurements obtained in a classification method to calculate the probability of that person having or being at risk of developing AD.

In certain embodiments, the method of classification is at least one of artificial neural networks, logistic regression, decision trees, random forest, support vector machines or any other method developing classification models known in the art.

In certain embodiments, the fluid sample is plasma or serum.

According to a further aspect of the invention, there is provided a substrate comprising either: one or more probes specific for afamin and Alpha-1 antichymotrypsin and optionally one or more probes specific for one or more biomarkers selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, orwtGSTO; or one or more proteins and/or nucleic acid sequences consisting of afamin and Alpha-1 antichymotrypsin and optionally one or more proteins and/or nucleic acids sequences selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, or wtGSTO for use in a method of the invention.

According to a further aspect of the invention, there is provided a substrate comprising either: one or more probes specific for afamin and Alpha-1 antichymotrypsin and optionally one or more probes specific for one or more biomarkers selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG,

IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, orwtGSTO; or \one or more proteins and/or nucleic acid sequences consisting of afamin and Alpha-1 antichymotrypsin and optionally one or more proteins and/or nucleic acids sequences selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, or wtGSTO when used in a method of the invention.

In certain embodiments, the probe, protein or nucleic acid sequence is stabilised to a surface.

In certain embodiments, the one or more probes for Afamin, Alpha-1-antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, or complement C5 are monoclonal antibodies.

In certain embodiments, the substrate is a biochip.

Throughout the specification and the claims that follow, unless the context requires otherwise, the words “comprise” and “include” and variations such as “comprising” and “including” will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement of any form of suggestion that such prior art forms part of the common general knowledge.

It will be appreciated by those skilled in the art that the invention is not restricted in its use to the particular application described. Neither is the present invention restricted in its preferred embodiment with regard to the particular elements and/or features described or depicted herein. It will be appreciated that the invention is not limited to the embodiment or embodiments disclosed, but is capable of numerous rearrangements, modifications and substitutions without departing from the scope of the invention.

Description of Drawings

Figures 1-9, boxplots comparing relative levels of Afamin (BSI0268), Afamin (BSI0223), Afamin (BSI0220), Alpha-1-antichymotrypsin (BSI0221), Complement C5 (BSI0782), Not known (BSI0183), Not known (BSI0279), Complement C3 (BSI0243) and Alpha-1 B-glycoprotein (BSI0182) of control and AD patients.

Figure 10, ROC curve for use of Afamin (BSI0268) to discriminate between Control and AD Patients.

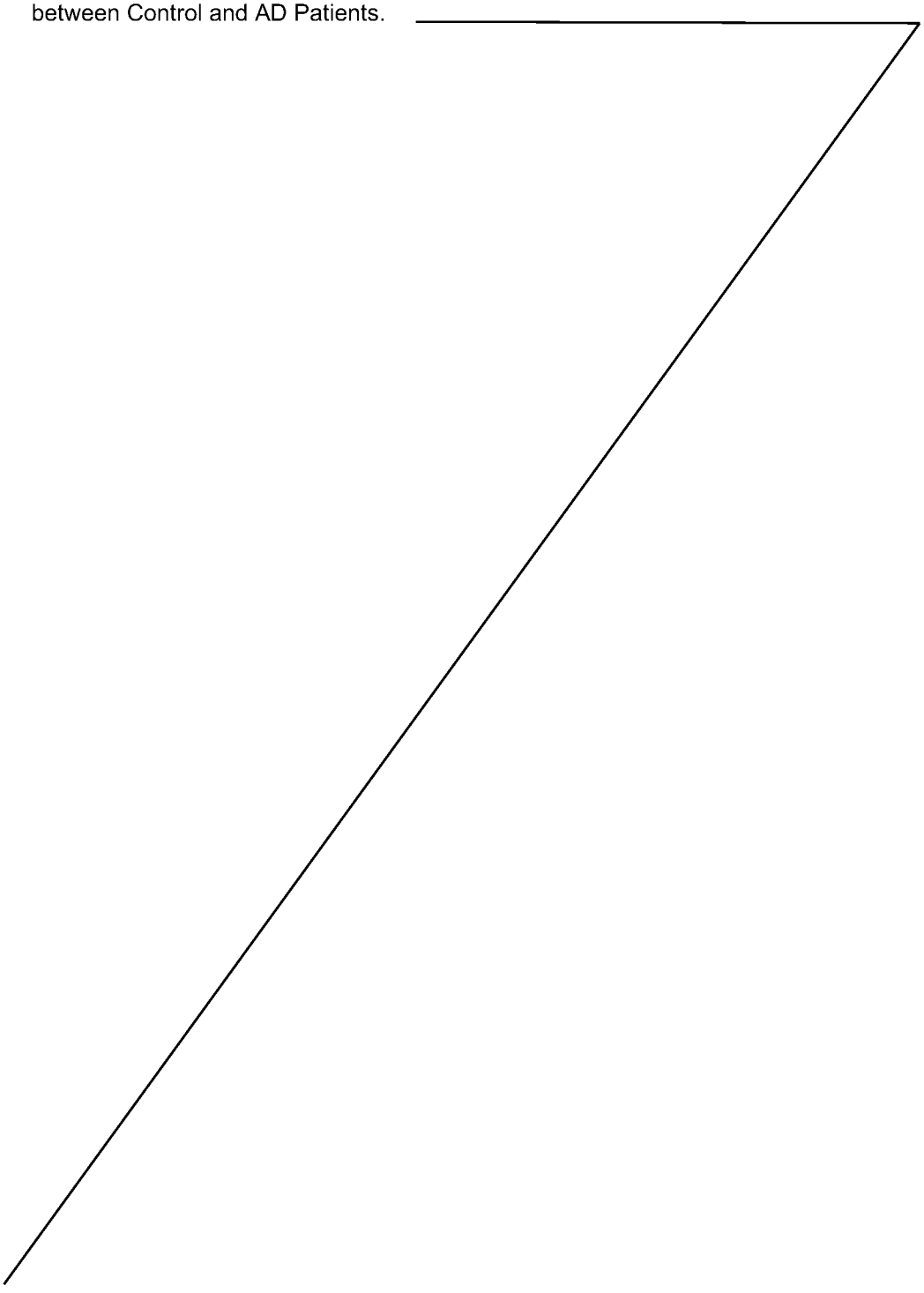
Figure 11, ROC curve for use of Alpha-1 antichymotrypsin (BSI0221) to discriminate

between Control and AD Patients.

Figure 12, ROC curve for use of a model comprising Afamin (BSI0268) and Alpha-1 antichymotrypsin (BSI0221) to discriminate between Control and AD Patients.

Figure 13, ROC curve for use of the ratio of Afamin (BSI0268) and Alpha-1 antichymotrypsin (BSI0221) to discriminate between Control and AD Patients.

Figure 14, ROC curve for use of a model comprising Afamin (BSI0268)/Alpha-1 antichymotrypsin (BSI0221) ratio and Complement C3 (BSI0217) to discriminate between Control and AD Patients.



In certain embodiments, one additional biomarker is serine threonine protein kinase TBK1.

In certain embodiments, one additional biomarker is complement C5.

In certain embodiments, one additional biomarker is alpha-2-macroglobulin.

In certain embodiments, one additional biomarker is Apolipoprotein B100.

In certain embodiments, one additional biomarker is complement C3.

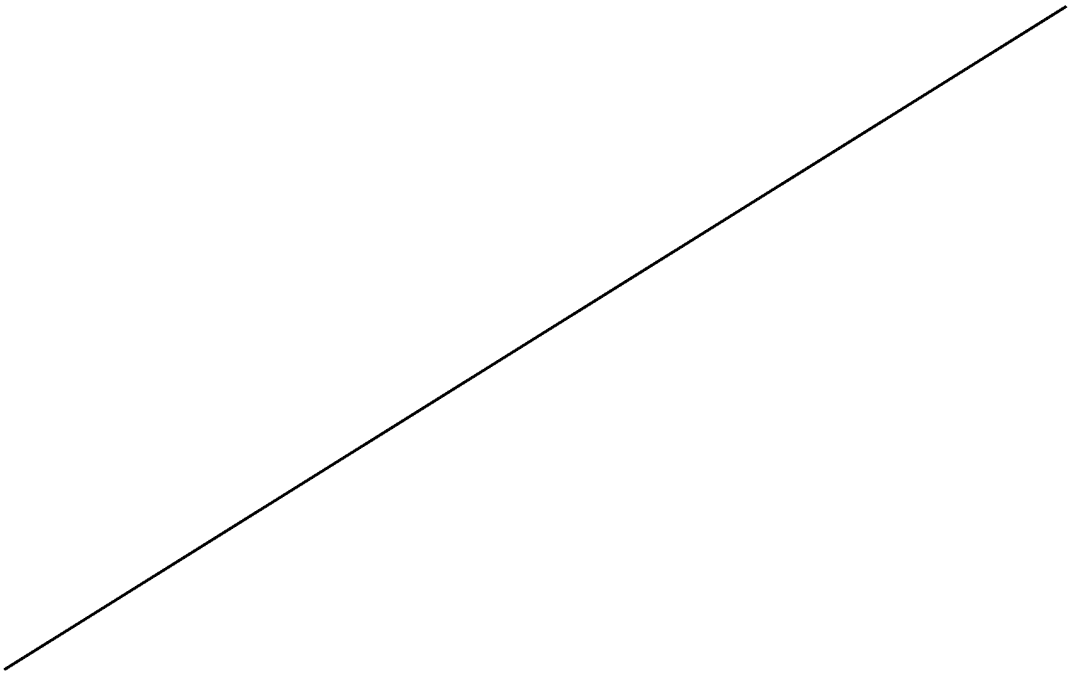
In certain embodiments, the method further comprises determining the genotype of at least one of Apolipoprotein E and Glutathione S-Transferase 1 Omega of a person through identification of the nucleic acid sequence encoding the protein in the genome or through determining the form of protein produced in a fluid sample taken from the person.

In certain embodiments, the method further comprises using the measurements obtained in a classification method to calculate the probability of that person having or being at risk of developing AD.

In certain embodiments, the method of classification is at least one of artificial neural networks, logistic regression, decision trees, random forest, support vector machines or any other method developing classification models known in the art.

In certain embodiments, the fluid sample is plasma or serum.

According to a further aspect of the invention, there is provided a substrate comprising either: one or more probes specific for afamin and Alpha-1 antichymotrypsin and optionally one or more probes specific for one or more biomarkers selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG,



in said biological fluid may be indicative of Alzheimer's disease. Such molecules may include peptides/proteins or nucleic acids and derivatives thereof; the term 'relative levels', in the context of the current invention refers to the light intensity or absorbance reading (However the invention is not restricted to measurement using these techniques, the skilled person will be aware of other methods for measuring biological molecules that do not utilise measuring the properties of visible light to determine a measurement) from a biological assay that results from comparing the levels of the biomarker in a given biological sample to a reference material with a known concentration (this concentration may be zero) of the biomarker or level by which the biomarker within a biological sample directly competes with a reference material known to contain said biomarker to bind to a specific probe for said biomarker, the latter method generates a level inversely related to the concentration of the biomarker; the term 'probe' in the context of the current invention, refers to a synthetic or biological molecule that specifically binds to a region of a biomarker; the term 'at risk of developing Alzheimer's disease', in the context of the current invention, refers to a patient that displays early clinical signs; such as mild cognitive impairment (MCI) or vascular dementia determined by methods known in the art (*such as MMSE*), has family history of Alzheimer's disease, has genetic prevalence for Alzheimer's disease or is classified 'at risk' due to lifestyle (e.g. age, diet, general health, occupation, geographical location); the term 'genetic prevalence' in the context of the current invention, can imply that the patients genome contains specific genotypes for certain proteins which are known in the art to be altered in patients who develop AD, such proteins include, but are not limited to, Apolipoprotein E (ApoE) and Glutathione S-Transferase Omega 1 (GSTO), this may be determined through genotyping or identifying the disease relevant form of the expressed protein in a biological fluid from the patient. More specifically, the number of alleles encoding ApoE4 and wild-type GSTO (wtGSTO) variants shall be determined. The term wtGSTO, in the context of the current invention, refers to any variant of GSTO that does not contain the rs4825 mutation in the genomic sequence, or an alanine to aspartic acid substitution at residue 140 of the protein sequence. The invention describes various biomarkers for use in diagnosing AD either alone or in combination with other diagnostic methods or as complementary biomarkers. A complementary biomarker in the current context implies a biomarker that can be used in conjunction with other biomarkers for AD.

A first aspect of the invention describes a method for diagnosing AD in a patient suspected of having, at risk of developing or of having AD which comprises taking an in vitro sample from the patient, determining the relative level or concentration of Afamin and one or more biomarkers chosen from Table 1 and establishing the significance of the relative level(s) or

concentration(s) of Afamin and one or more biomarkers. The significance of the relative level or concentration is gauged by comparing said relative level or concentration to a control value for the specific biomarker. The control value is derived from determining the relative level or concentration of said biomarker in a biological sample taken from an individual(s) who does not have AD, as determined by clinical assessment. For Afamin, the relative level or concentration in a patient with AD is reduced compared with a control value. A preferred embodiment of the invention utilises a method employing a combination of Afamin and at least one other biomarker chosen from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Apolipoprotein B100, complement C3, Serine threonine kinase TANK Binding Kinase-1 (TBK1), vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG fc binding protein, hornerin, fibrinogen or complement C5. A further preferred embodiment the invention uses a method whereby the relative level or concentration of Afamin is divided by the relative level or concentration of Alpha-1 antichymotrypsin to produce a ratio of Afamin/Alpha-1 antichymotrypsin. The term 'ratio' in the context of the current embodiment of the invention, relates to dividing the value of one biomarker by the other, this value should be the same for both biomarkers and can be represented as a weight or moles of biomarker in a given volume (concentration) or by a light intensity or absorbance level generated by means of an assay (relative level). A further embodiment of the invention utilises the value of the ratio of Afamin/Alpha-1 antichymotrypsin in combination with relative levels or concentration of one or more biomarkers chosen from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Apolipoprotein B100, complement C3 , Serine threonine kinase TBK-1, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG fc binding protein, hornerin, fibrinogen or complement C5. For example, a preferred combination of the current invention is the ratio of Afamin/Alpha-1 antichymotrypsin in combination with the relative level or concentration of Complement C3. Another preferred combination of the invention is the ratio of Afamin/Alpha-1 antichymotrypsin in combination with the relative level or concentration of serine threonine kinase TBK-1.

A further aspect of the invention is directed to the use of one or more of Afamin, Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Apolipoprotein B100, complement C3 , Serine threonine kinase TBK-1, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG fc binding protein, hornerin, fibrinogen or complement C5 as complementary biomarkers of AD. As complementary biomarkers they may be used for AD diagnosis in

conjunction with other clinical evidence such as mental state assessment (MMSE), neurological imaging, Beta-amyloid peptides, phosphorylated Tau, ApoE genotype, and wild-type GSTO 1 genotype (wtGSTO). In the context of the current invention, this clinical evidence may be added to the predictive model, based on the output measurement. For example, ApoE status of a patient may be determined through genotyping, by identifying the disease relevant form of protein that is expressed at the genetic level (DNA and/or RNA), or by detecting the presence of the specific expressed form of the protein from a fluid sample taken from the patient. In the context of the current invention, this output is expressed as either a dichotomised value, whereby the patient is either positive for the *ApoE4* gene or protein or not; or as an ordinal output for the number of ApoE4 alleles present in the patients genomic DNA (0-2), which can be calculated using relative levels of the gene or protein within a sample taken from the patient.

Biomarker relative levels or concentrations can be determined by contacting the sample with probes, preferably immobilised on a substrate, specific for each of the biomarkers included in the combination of biomarkers. Interactions between biomarker and its respective probe can be monitored and quantified using various techniques that are well-known in the art. An example of a suitable technique is an enzyme-linked immunosorbent assay (ELISA). Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

In a preferred embodiment of the current invention the 'sample' as referred to herein is serum or plasma, however it may be any sample from a patient from which biomarker levels or concentrations can be determined. These include but are not limited to whole blood, urine, saliva, cerebrospinal fluid and platelets.

The substrate comprises at least two, preferably three or four probes, each probe specific to an individual biomarker. As used herein, the term 'specific' means that the probe binds only to one of the biomarkers of the invention, with negligible binding to other biomarkers of the invention or to other analytes in the biological sample being analysed. This ensures that the integrity of the diagnostic assay and its result using the biomarkers of the invention is not compromised by additional binding events.

The substrate can be any surface able to support one or more probes, but is preferably a biochip. A "Biochip" is a general term for a reaction platform for hosting chemical, biochemical, proteomic or molecular tests, as may be required for medical diagnosis, drug detection, etc. Typically, a Biochip comprises an inert substrate, such as silicon, glass or ceramic (often of the order of about 1 cm^2 or less in surface area), on which one or a plurality of reaction sites is provided. The sites generally carry one or more ligands, for example, one or more antibodies, selected for the test (or "assay") to be performed, adsorbed to the surface of the chip for activation upon combination with a sample applied to the chip (e.g. a blood sample) and/or a reagent. The reactions can be detected using a number of alternative techniques, including detection of chemiluminescence generated by the reaction. Some biochips carry a very large number (hundreds or thousands) of such tests sites, typically arranged in a grid or array, making it possible to carry out numerous assays simultaneously, and using the same single specimen. When identifying the various biomarkers/proteins of the invention it will be apparent to the skilled person that as well as identifying the full length protein, the identification of a fragment or several fragments of a protein is possible, provided this allows accurate identification of the protein. Similarly, although a preferred probe of the invention is a polyclonal or monoclonal antibody, other probes such as aptamers, molecular imprinted polymers, phages, short chain antibody fragments and other antibody-based probes may be used. The invention also allows for nucleic acid sequence probes.

Preferably, a solid state device is used in the methods of the present invention, preferably the Biochip Array Technology system (BAT) (available from Randox Laboratories Limited). More preferably, the Evidence Evolution and Evidence Investigator apparatus (available from Randox Laboratories) may be used to determine the levels of biomarkers in the sample.

The accuracy of statistical methods used in accordance with the present invention can be best described by their receiver operating characteristics (ROC). The ROC curve addresses both the sensitivity, the number of true positives, and the specificity, the number of true

negatives, of the test. Therefore, sensitivity and specificity values for a given combination of biomarkers are an indication of the accuracy of the assay. For example, if a biomarker combination has sensitivity and specificity value of 80%, out of 100 patients, 80 will be correctly identified from the determination of the presence of the particular combination of biomarkers as positive for disease, while out of 100 patients who do not have disease 80 will accurately test negative for the disease.

If two or more biomarkers are to be used in the diagnostic method a suitable mathematical or machine learning classification model, such as logistic regression equation, can be derived. The logistic regression equation might include other variables such as age and gender of the patient. The ROC curve can be used to assess the accuracy of the logistic regression model. The logistic regression equation can be used independently or in an algorithm to aid clinical decision making. Although a logistic regression equation is a common mathematical/statistical procedure used in such cases and is preferred in the context of the present invention, other mathematical/statistical, decision trees or machine learning procedures can also be used.

By way of example, a logistic regression equation applicable to the present invention (at a classification cut-off value of 0.5) for the biomarker combination for indication of AD versus non-AD (control) in a patient suspected of having or being at risk of developing AD is calculated as follows;

$$Probability\ of\ AD = \frac{1}{1 + e^{-\left(3.1 + 9.4\left[\frac{\text{Afamin}}{\text{Alpha 1 antichymotrypsin}}\right] - 23.6[\text{Complement C5}]\right)}}$$

As further example, a decision tree may be grown where a decision branch is grown from each node (sub-population) to divide the population into classification groups. Figure 19 represents an example of a tree that was grown using the data described in this invention, which could correctly classify all AD patients with a relatively small error.

Methods

1. Normalised plasma/Quantiplasma™

Plasma normalisation was conducted as per US 2009/0136966. Briefly human plasma was normalised by removing high abundance proteins utilising the propriety method. Firstly, high abundance proteins were removed using Multiple Affinity Removal System (MARS) technology. The resultant plasma was then loaded on to a Multi-ImmunoAffinity

Normalisation (MIAN) column, where normalisation stringency was adjusted by altering the flow rate. The flow–throw and wash samples were combined to give a differentially normalised sample. Some of this normalised plasma was then ubiquitously biotinylated to provide a tracer substance, known as Quantiplasma™.

2. Antibody generation

Monoclonal antibodies were produced as per US 2009/0136966. Normalised plasma was used as an immunogen to generate polyclonal antibodies. B-cells were then isolated and monoclonal hybridomas were generated. Initial selection of hybridomas was done using an ELISA. Plates were coated with mouse Ig gamma-Fc specific GAM, and then incubated with the mAb hybridoma supernatant, following a wash step this complex was then incubated with the Quantiplasma™ and finally an enzyme-substrate reaction was induced to detect the binding of the biotinylated plasma (Quantiplasma™) to the mAb. This selection identified more than 1000 mAb. To identify the protein targets of monoclonal antibodies used in this study, western blotting, immunoprecipitation and mass spectrometry techniques were employed. There are, however, some antigens that could not be identified at this time, but as they are known to be present in the human plasma proteome they have been included.

3. Identification of clinical biomarkers using Quantiplasma™

Serum samples were obtained from 19 clinically confirmed Alzheimer's disease (AD) patients and 19 age/gender-matched control participants with normal cognitive function. These samples were frozen shortly after collection and stored at (-80°C) until analysis was performed. Additional clinical information was gathered for these subjects, this included basic personal and family medical history. Further to this, ApoE and GSTO genotype were determined through methods known in the art. For each patient, genomic DNA was isolated and the presence of DNA that encodes each of the 3 isoforms of ApoE (E2, E3, E4) or GSTO (wild-type, mutant A140 [rs4825]) were determined utilising polymerase chain reaction (PCR) techniques. Further analysis allowed the allelic frequency of each of the isoforms to be determined through methods known in the art.

A panel of 69 mAb antibodies (Table 1) were selected out of a catalogue of >1000 generated as per Section 2. Antibodies were then evaluated by competitive immunoassay. They were first immobilised on a biochip platform (9mm x 9mm), which was the substrate for the immunoreactions. The semi-automated bench top analyser Evidence Investigator was used (EV3602, Randox Laboratories Ltd., Crumlin, UK, patents-EP98307706, EP98307732, EP0902394, EP1227311, EP1434995 and EP1354623). The assay principle is based on

competition for binding sites of the monoclonal antibody between free antigen (the patient sample) and labelled tracer plasma (Quantiplasma™).

Sample and reagents are added to the biochip and incubated under controlled conditions. Following addition of substrate, a light signal is generated which is then detected using digital imaging technology. The system incorporates dedicated software to automatically process, report and archive the data generated. The level of a specific protein in the patient sample is determined by comparing the difference between the light signal (RLU) at the position of the respective antibody on a biochip containing sample and the tracer (test) and a biochip containing just the tracer (blank). A ratio between test and control samples is determined as;

$$relative\ level\ of\ biomarker = 1 - \frac{RLU(test)}{RLU(blank)}$$

with a high ratio indicating a relatively high level of the protein specific for its respective mAb present in the sample, and a low ratio indicating relatively little or none of the protein present in the sample. Ratios for AD patients and control patients for all mAbs were determined (Example Figures 1-9) and non-parametric analysis was conducted to identify those mAbs which could distinguish between AD and control patients (Table 2). Areas under the curve (AUC) of the receiver operator characteristic (ROC) curves were calculated for all mAbs, these are detailed in Table 3.

4. Disease classification model

As an example of how multiple markers identified by this study may be combined to provide a model to classify a patient whose disease state is unknown, we have used logistic regression as a method of model determination. Initial investigation showed that using the relative levels of Afamin (BSI0268) combined with that of Alpha-1-antichymotrypsin (BSI0221) generated a model with an AUC of 0.906 (Fig 10-13), a significant improvement on the predictive power of the individual measurements. In order to add further analytes to the model, without increasing the dimensions, a function of Afamin (BSI0268) as a proportion of Alpha-1-antichymotrypsin (BSI0221) was used as a single variant (AUC=0.875) and the effect of adding all other analytes systematically in to the model was analysed. The addition of Complement C3 (BSI0217), Alpha-2 macroglobulin (BSI0195), Serine threonine protein kinase TBK1 (BSI0112) or Complement C5 (BSI0782) to the model improved the model, AUC of 0.889, 0.906, 0.892 and 0.920 respectively (Fig 14-17). Further, improvements were identified when considering the ApoE genotype of the patients. A

categorical variable, whereby each patient was identified as having either no *Apoe4* alleles (0) or having one or more (1), was added to the analysis. This was further refined by identifying the number of *ApoE4* alleles each patient had (0, 1 or 2). In this study, 63% of the AD patients had at least one *Apoe4* allele, where only one out of the 19 control subjects (5%) was *Apoe4* positive. ApoE4 genotype in combination with the Afamin/Alpha-1 antichymotrypsin ratio produces an AUC of 0.925, this increases to 0.953 when taking into consideration the number of ApoE4 alleles. Furthermore, the AUC increases to 0.964 with the addition of Complement C5. These data suggest that a very accurate model may be generated using the current invention. These data are summarised in Table 4. The curse of dimensionality limits the number of variables that may be used in developing a model using this preliminary data set, but it is predicted that several markers included in this study may be combined to provide an optimal model. As well as logistic regression, other supervised learning models were generated using this data, such as; multi-layer perceptron neural networks, random forests, support vector machines and decision trees (Figure 21). These all provided models with similar accuracy as logistic regression and may be preferred as new analytes are added to the model.

Table 1 *mAb ID numbers and the respective protein that it has been found to bind to*

Probe ID	Protein ID
BSI0183	Not known
BSI0185	D vitamin binding protein
BSI0189	Complement C3b (shorter form)
BSI0198	Alpha-1-B glycoprotein
BSI0200	Alpha-2-macroglobulin
BSI0203	Ceruloplasmin
BSI0208	Apo B100
BSI0220	Afamin
BSI0221	Alpha-1-antichymotrypsin
BSI0197	Alpha-2-macroglobulin
BSI0201	Not known
BSI0214	ApoB100
BSI0190	Complement C3
BSI0191	Not known
BSI0195	Alpha-2-macroglobulin
BSI0223	Afamin
BSI0186	Alpha-1-antichymotrypsin
BSI0196	Alpha-1B-glycoprotein

BSI0279	Not known
BSI0281	Not known
BSI0217	Complement C3
BSI0289	ApoB100
BSI0311	Not known
BSI0144	Alpha-1-antichymotrypsin
BSI0112	Serine threonine protein kinase TBK1
BSI0022	Not known
BSI0002	Hemopexin
BSI0032	IgG Fc Binding protein
BSI0038	Alpha-2-macroglobulin
BSI0023	Not known
BSI0051	D-vitamin binding protein
BSI0095	Alpha-2-macroglobulin
BSI0097	Serum albumin
BSI0116	Alpha-1B-glycoprotein
BSI0099	Ceruloplasmin
BSI0136	ApoB100
BSI0172	Alpha-2-macroglobulin
BSI0177	Not known
BSI0142	Alpha-2-antiplasmin
BSI0173	Alpha-2-macroglobulin
BSI0179	Apolipoprotein A1
BSI0180	Not known
BSI0181	Not known
BSI0100	Serine threonine protein kinase TBK1
BSI0182	Alpha-1B-glycoprotein
BSI0040	ApoB100
BSI0314	Not known
BSI0243	Complement C3
BSI0348	Not known
BSI0257	D-vitamin binding protein
BSI0263	Complement C3
BSI0268	Afamin
BSI0355	Not known
BSI0660	Not known
BSI0670	ApoB100
BSI0747	Factor H
BSI0765	Complement C5
BSI0782	Complement C5
BSI0225	Not known (IgG)
BSI0239	Apolipoprotein A1
BSI0242	Complement C3
BSI0246	Not known
BSI0248	IgG
BSI0255	Hornerin

BSI0259	Not known
BSI0266	ApoB100
BSI0323	ApoB100
BSI0115	Alpha-2-antiplasmin
BSI0251	Fibrinogen

Table 2 *Summary statistics for all 69 mAbs for both Control (19) and AD (19) groups, p-value represents the significant difference between each group as determined by Mann-Whitney*

Protein ID (mAb ID)	Control			AD			
	Median	Min	Max	Median	Min	Max	p-value
Afamin (BSI0268)	.674	.441	.735	.532	.402	.683	.00021
Afamin (BSI0223)	.508	.319	.599	.405	.230	.477	.00384
Afamin (BSI0220)	.796	.485	.863	.690	.422	.826	.00506
Alpha-1-antichymotrypsin (BSI0221)	.613	.468	.722	.668	.388	.777	.01734
Complement C5 (BSI0782)	.592	.497	.656	.559	.429	.656	.02648
Not known (BSI0183)	.571	.240	.699	.475	.231	.614	.02853
Not known (BSI0279)	.484	.132	.702	.409	.210	.621	.03304
Complement C3 (BSI0243)	.625	.474	.826	.698	.336	.852	.04245
Alpha-1B-glycoprotein (BSI0182)	.504	.265	.737	.475	.238	.681	.05396
Alpha-1-antichymotrypsin (BSI0144)	.605	.342	.724	.652	.511	.744	.05584
ApoB100 (BSI0289)	.025	-1.623	.779	.291	-1.089	.819	.05969
Alpha-2-antiplasmin (BSI0115)	.302	-.298	.710	.209	-.131	.562	.07488
Factor H (BSI0747)	.409	.179	.535	.336	.083	.600	.08493
Serum albumin (BSI0097)	.725	.643	.756	.701	.583	.772	.08766
Apolipoprotein A1 (BSI0239)	.411	.275	.522	.382	.250	.478	.11827
Complement C3 (BSI0263)	.545	-.166	.915	.143	-.826	.850	.12534
Alpha-2-macroglobulin	.544	.168	.670	.444	.101	.660	.15249

(BSI0197)							
Complement C5 (BSI0765)	.309	.131	.947	.263	-.144	.865	.15254
ApoB100 (BSI0040)	.155	-.125	.560	.261	-.136	.697	.15666
Alpha-1B-glycoprotein (BSI0116)	.259	.109	.348	.227	.092	.335	.15679
Not known (BSI0023)	.481	.202	.825	.400	.028	.884	.16104
ApoB100 (BSI0670)	.644	.420	.776	.626	.408	.735	.16995
Serine threonine protein kinase TBK1 (BSI0112)	.576	-.207	.691	.464	-.275	.660	.17460
Not known (BSI0246)	.184	.031	.316	.136	-.006	.295	.17460
Not known (BSI0348)	.687	.416	.806	.646	.350	.781	.17924
Alpha-1-antichymotrypsin (BSI0186)	.478	-.195	.868	.365	-.691	.736	.18406
Complement C3 (BSI0217)	.141	-1.116	.788	.056	-.361	.716	.19389
ApoB100 (BSI0323)	.446	.236	.685	.394	.139	.607	.20409
Alpha-1B-glycoprotein (BSI0196)	.431	.239	.549	.453	.255	.569	.21469
Alpha-2-antiplasmin (BSI0142)	.643	.454	.712	.663	.585	.728	.21469
Ceruloplasmin (BSI0203)	.367	-.951	.865	.278	-1.956	.727	.22567
Fibrinogen (BSI0251)	.457	.222	.717	.426	.211	.608	.24281
Not known (BSI0022)	.530	.331	.895	.492	.256	.898	.26718
Alpha-1-B glycoprotein (BSI0198)	.723	.611	.791	.697	.376	.771	.29317
Alpha-2-macroglobulin (BSI0172)	.759	.642	.865	.737	.678	.887	.30001
D-vitamin binding protein (BSI0257)	.614	.014	.788	.601	.448	.722	.32087
Complement C3 (BSI0190)	.454	.268	.621	.513	.294	.646	.34271
Alpha-2-macroglobulin (BSI0038)	.636	.331	.738	.575	.246	.748	.35016

Not known (BSI0201)	.711	.495	.818	.688	.490	.867	.35776
Alpha-2-macroglobulin (BSI0195)	.940	.796	.974	.924	.423	.982	.37323
Apolipoprotein A1 (BSI0179)	.794	.328	.917	.761	.110	.937	.37323
ApoB100 (BSI0136)	.483	.155	.670	.522	.206	.637	.38910
Complement C3b (shorter form) (BSI0189)	.607	.487	.839	.585	.495	.747	.39711
Not known (BSI0660)	.945	-.223	.975	.947	-2.124	.968	.40535
Apo B100 (BSI0208)	.572	.202	.694	.595	-.250	.911	.40538
IgG (BSI0248)	.638	.400	.781	.556	.253	.699	.44774
Hemopexin (BSI0002)	.567	-2.587	.830	.708	-1.046	.890	.49266
Alpha-2-macroglobulin (BSI0095)	.761	.592	.924	.689	.645	.871	.49266
Not known (BSI0281)	.744	.511	.799	.740	.502	.837	.50189
Not known (BSI0355)	.423	.254	.686	.431	.097	.602	.51126
Not known (more candidates) (BSI0177)	.108	-.076	.216	.114	-.051	.296	.55927
Hornerin (BSI0255)	.513	.255	.824	.487	.146	.920	.61962
Alpha-2-macroglobulin (BSI0173)	.692	.482	.836	.661	.567	.833	.63001
D vitamin binding protein (BSI0185)	.613	.294	.743	.606	.381	.782	.65090
Not known (IgG) (BSI0225)	.390	.080	.651	.402	.054	.577	.65090
Not known (BSI0314)	.633	.014	.830	.625	.446	.764	.67206
ApoB100 (BSI0214)	.401	.073	.675	.386	-.090	.666	.69349
Not known (BSI0259)	.342	.047	.619	.307	.133	.507	.69349
Ceruloplasmin (BSI0099)	.590	.472	.734	.593	.321	.721	.77029
Not known (BSI0311)	.344	.020	.506	.351	-.031	.601	.82668
Complement C3 (BSI0242)	.446	.148	.653	.409	.277	.568	.82668
Not known (BSI0180)	.237	.032	.443	.210	.085	.521	.84949
ApoB100 (BSI0266)	.528	.303	.704	.516	.216	.648	.84949

D-vitamin binding protein (BSI0051)	.450	.035	.602	.472	.324	.629	.89548
Not known (BSI0181)	.502	.350	.708	.556	.227	.926	.90702
IgG Fc Binding protein (BSI0032)	.432	-1.988	.826	.431	-2.053	.928	.94182
Alpha-2-macroglobulin (BSI0200)	.577	.338	.660	.544	.309	.703	.96507
Serine threonine protein kinase TBK1 (BSI0100)	.767	.174	.839	.768	.680	.815	.96507
Not known (BSI0191)	.542	.328	.898	.583	.285	.902	1.00000

Table 3 *AUC for the ROC curve for each of the 69 mAb for distinguishing Control from AD*

Protein ID (Probe)	**AUC**
Afamin (BSI0268)	.852
Afamin (BSI0223)	.774
Afamin (BSI0220)	.766
Alpha-1-antichymotrypsin (BSI0221)	.726
Complement C5 (BSI0782)	.711
Not known (BSI0183)	.708
Not known (BSI0279)	.702
Complement C3 (BSI0243)	.693
Alpha-1B-glycoprotein (BSI0182)	.683
Alpha-1-antichymotrypsin (BSI0144)	.681
ApoB100 (BSI0289)	.679
Alpha-2-antiplasmin (BSI0115)	.669
Factor H (BSI0747)	.663
Serum albumin (BSI0097)	.662
Apolipoprotein A1 (BSI0239)	.648
Complement C3 (BSI0263)	.645
Alpha-2-macroglobulin (BSI0197)	.636
Complement C5 (BSI0765)	.636
ApoB100 (BSI0040)	.634
Alpha-1B-glycoprotein (BSI0116)	.634

Not known (BSI0023)	.633
ApoB100 (BSI0670)	.630
Serine threonine protein kinase TBK1 (BSI0112)	.629
Not known (BSI0246)	.629
Not known (BSI0348)	.627
Alpha-1-antichymotrypsin (BSI0186)	.626
Complement C3 (BSI0217)	.623
ApoB100 (BSI0323)	.620
Alpha-1B-glycoprotein (BSI0196)	.618
Alpha-2-antiplasmin (BSI0142)	.618
Ceruloplasmin (BSI0203)	.615
Fibrinogen (BSI0251)	.611
Not known (BSI0022)	.605
Alpha-1-B glycoprotein (BSI0198)	.600
Alpha-2-macroglobulin (BSI0172)	.598
D-vitamin binding protein (BSI0257)	.594
Complement C3 (BSI0190)	.590
Alpha-2-macroglobulin (BSI0038)	.589
Not known (BSI0201)	.587
Alpha-2-macroglobulin (BSI0195)	.584
Apolipoprotein A1 (BSI0179)	.584
ApoB100 (BSI0136)	.582
Complement C3b (shorter form) (BSI0189)	.580
Not known (BSI0660)	.579
Apo B100 (BSI0208)	.579
IgG (BSI0248)	.572
Hemopexin (BSI0002)	.565
Alpha-2-macroglobulin (BSI0095)	.565
Not known (BSI0281)	.564
Not known (BSI0355)	.562
Not known (BSI0177)	.555
Hornerin (BSI0255)	.547
Alpha-2-macroglobulin (BSI0173)	.546

D vitamin binding protein (BSI0185)	.543
Not known (IgG) (BSI0225)	.543
Not known (BSI0314)	.540
ApoB100 (BSI0214)	.537
Not known (BSI0259)	.537
Ceruloplasmin (BSI0099)	.528
Not known (BSI0311)	.521
Complement C3 (BSI0242)	.521
Not known (BSI0180)	.518
ApoB100 (BSI0266)	.518
D-vitamin binding protein (BSI0051)	.512
Not known (BSI0181)	.511
IgG Fc Binding protein (BSI0032)	.507
Alpha-2-macroglobulin (BSI0200)	.504
Serine threonine protein kinase TBK1 (BSI0100)	.504
Not known (BSI0191)	.500

Table 4 ***AUC for combinations of biomarkers***

Biomarker combination	Area	Std. Error	95% Confidence Interval	
			Lower	Upper
Afamin (BSI0268) and Alpha-1 antichymotrypsin (BSI0221)	.906	.052	.804	1.000
Afamin/Alpha-1 antichymotrypsin ratio*	.875	.063	.752	.998
Afamin/Alpha-1 antichymotrypsin Ratio, Complement C5 (BSI0782)	.920	.052	.819	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Complement C3 (BSI0243)	.889	.060	.771	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Alpha-2-macroglobulin (BSI0195)	.906	.052	.805	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Serine Threonine Kinase TBK1 (BSI0112)	.892	.057	.779	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, ApoE4 (presence/absence)	.925	.042	.844	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, ApoE4 (0,1 or 2 alleles)	.953	.032	.891	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, ApoB100 (BSI0289)	.875	.063	.752	.998
Afamin/Alpha-1 antichymotrypsin Ratio, Not Known (BSI0183)	.881	.064	.756	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Not Known (BSI0279)	.878	.064	.753	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Comp C5 (BSI0782), ApoE4 (presence/absence)	.958	.028	.903	1.000
Afamin/Alpha-1 antichymotrypsin Ratio, Comp C5 (BSI0782), ApoE4 (0,1 or 2 alleles)	.964	.028	.910	1.000

*For all Afamin/alpha-1 antichymotrypsin ratio combinations the probes BSI0268 and BSI0221 were used

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of diagnosing or monitoring a person at risk of developing or having Alzheimer's disease (AD) comprising obtaining a fluid sample from a person suspected of having or at risk of developing AD, measuring the concentration or relative level of the biomarker Afamin and Alpha-1 antichymotrypsin, and optionally at least one additional biomarker selected from Alpha-2-macroglobulin, Apolipoprotein B100, complement C3 , Serine threonine kinase TBK-1, vitamin D binding protein, alpha-1- B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5 and in the fluid sample, and establishing the significance of the concentrations or relative levels.

2. The method according to claim 1, wherein the measured concentration or relative level of Afamin and Alpha-1 antichymotrypsin, and optionally at least one of Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen and complement C5 is transformed into a ratio.

3. The method of claim 2, wherein the ratio of afamin to alpha-1 antichymotrypsin is calculated.

4. The method according to claim 3, wherein the ratio of afamin to alpha-1 antichymotrypsin is calculated and the concentration or relative level of at least one additional biomarker selected from serine threonine protein kinase TBK1, alpha-2- macroglobulin, Apolipoprotein B100, complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2- antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, and complement C5 is also measured.

5. The method of claim 4, wherein one additional biomarker is serine threonine protein kinase TBK1.

6. The method of claim 4, wherein one additional biomarker is complement C5.

7. The method of claim 4, wherein one additional biomarker is alpha-2-macroglobulin.

8. The method of claim 4, wherein one additional biomarker is Apolipoprotein B100.

9. The method of claim 4, wherein one additional biomarker is complement C3.

10. The method according to any one of the preceding claims, further comprising determining the genotype of at least one of Apolipoprotein E and Glutathione S-Transferase 1

Omega of a person through identification of the nucleic acid sequence encoding the protein in the genome or through determining the form of protein produced in a fluid sample taken from the person.

11. The method of any one of the preceding claims, further comprising using the measurements obtained in a classification method to calculate the probability of that person having or being at risk of developing AD.

12. The method according to claim 11, wherein the method of classification is at least one of artificial neural networks, logistic regression, decision trees, random forest, support vector machines or any other method developing classification models known in the art.

13. The method according to any one of the preceding claims, wherein the fluid sample is plasma or serum.

14. A substrate comprising either:

a. one or more probes specific for afamin and Alpha-1 antichymotrypsin and optionally one or more probes specific for one or more biomarkers selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, orwtGSTO; or

b. one or more proteins and/or nucleic acid sequences consisting of afamin and Alpha-1 antichymotrypsin and optionally one or more proteins and/or nucleic acids sequences selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, or wtGSTO

when used in a method according to any one of claims 1 to 13.

15. The substrate of claim 14, wherein the substrate comprises either:

a. one or more probes specific for afamin and Alpha-1 antichymotrypsin and additionally one or more probes specific for one or more biomarkers selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1,

complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, orwtGSTO; or

b. one or more proteins and/or nucleic acid sequences consisting of afamin and Alpha-1 antichymotrypsin and additionally one or more proteins and/or nucleic acids sequences selected from Alpha-1 antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, complement C5, ApoE4, or wtGSTO.

16. The substrate according to claim 14 or 15, wherein the probe, protein or nucleic acid sequence is stabilised to a surface.

17. The substrate according to any one of claims 14 to 16, wherein the one or more probes for Afamin, Alpha-1-antichymotrypsin, Alpha-2-macroglobulin, Serine threonine protein kinase TBK1, Apolipoprotein B100, Complement C3, vitamin D binding protein, alpha-1-B glycoprotein, hemopexin, serum albumin, ceruloplasmin, alpha-2-antiplasmin, apolipoprotein A1, complement factor H, IgG, IgG Fc binding protein, hornerin, fibrinogen, or complement C5 are monoclonal antibodies.

18. The substrate of any one of claims 14 to 17, which is a biochip.

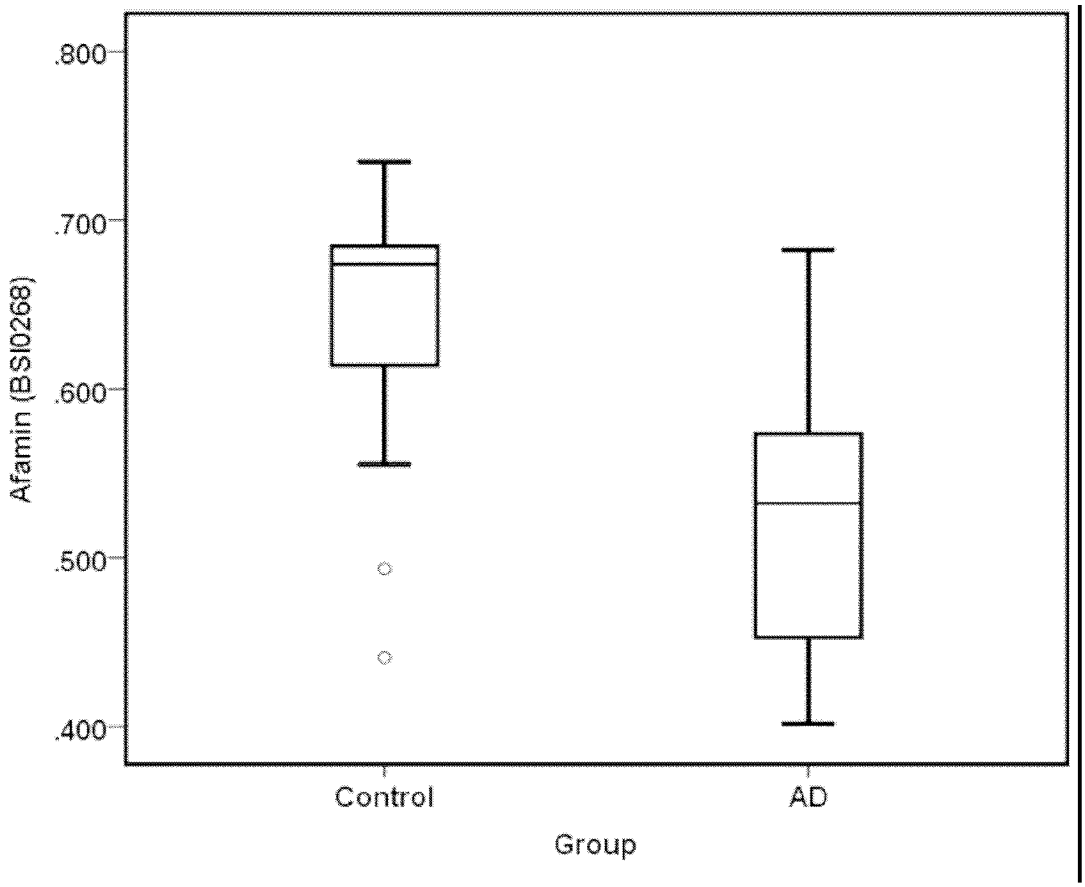


Figure 1

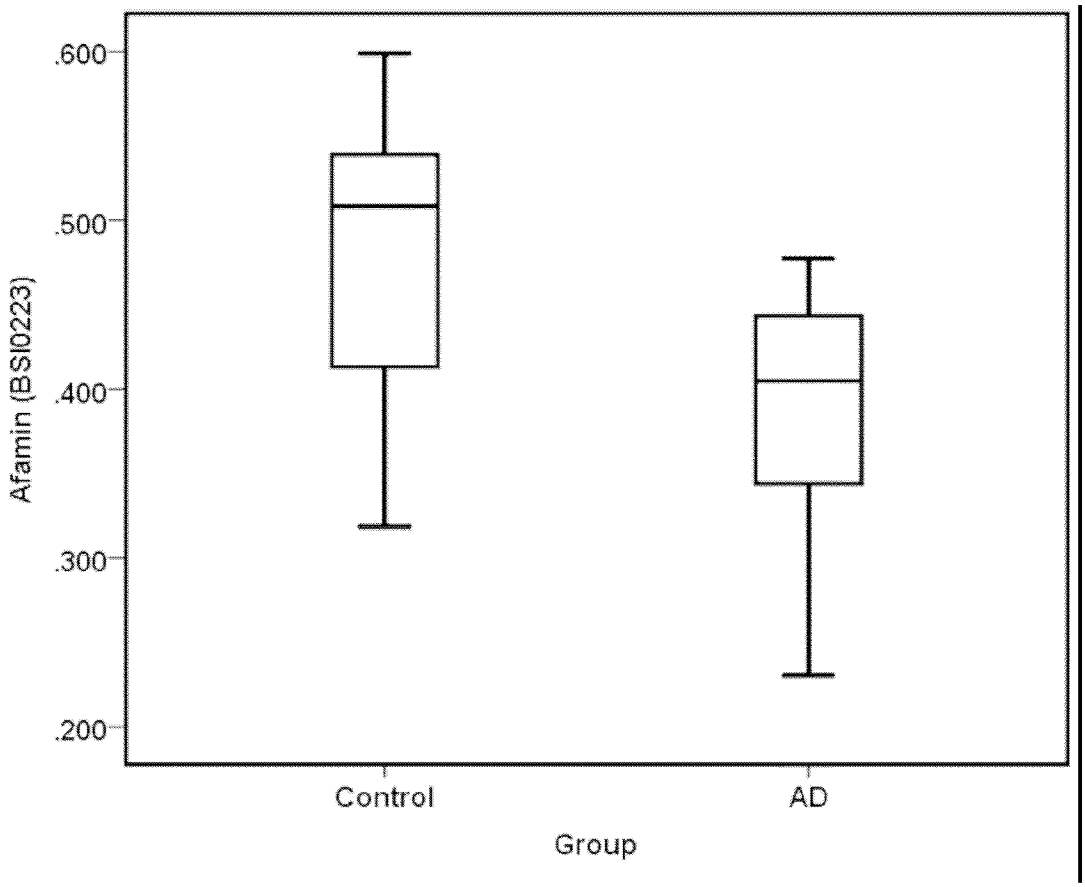


Figure 2

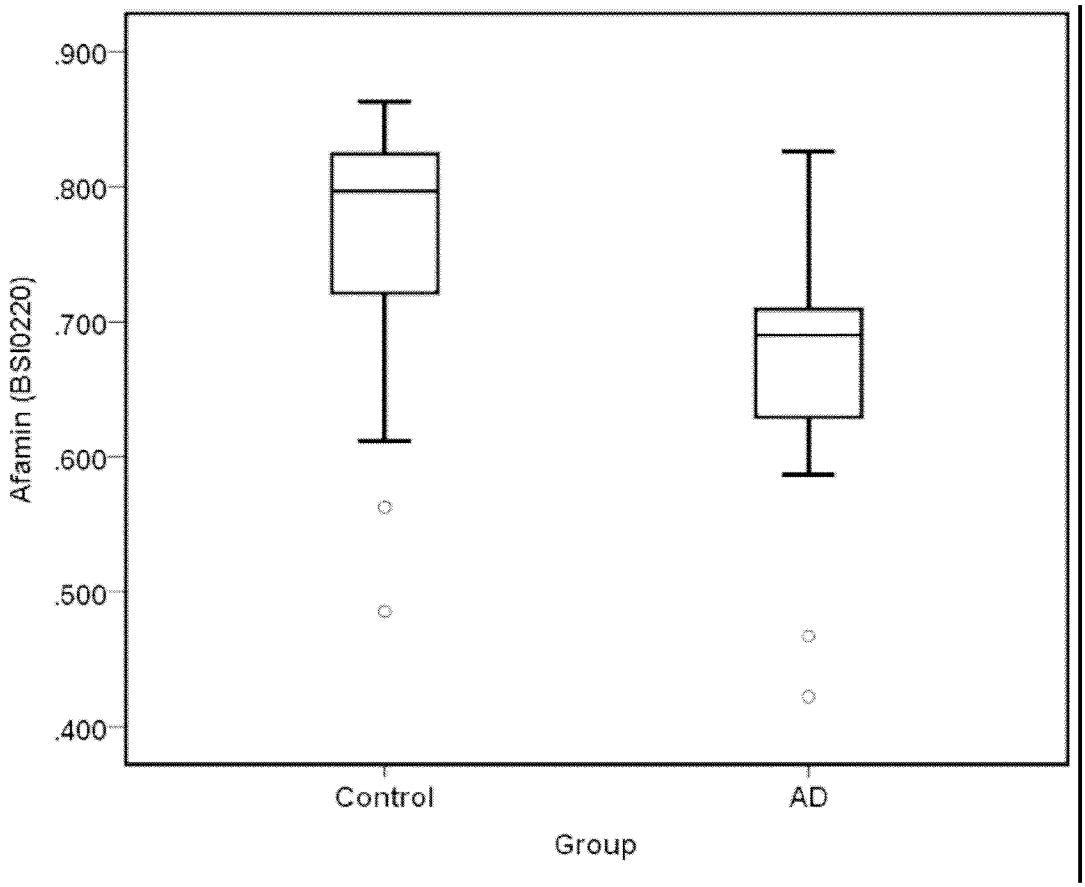


Figure 3

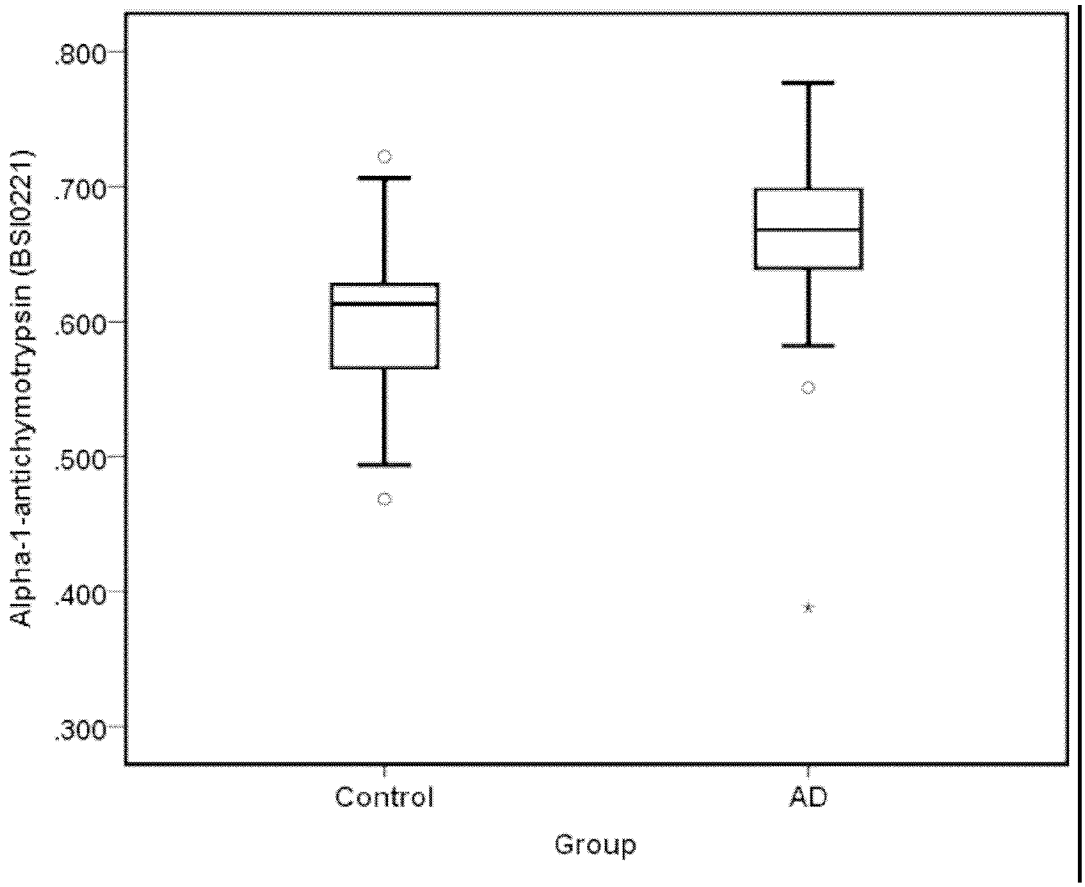

.800
.700
.600
.500
.400
.300
Alpha-1-antichymotrypsin (BSI0221)
Control
AD
Group

Figure 4

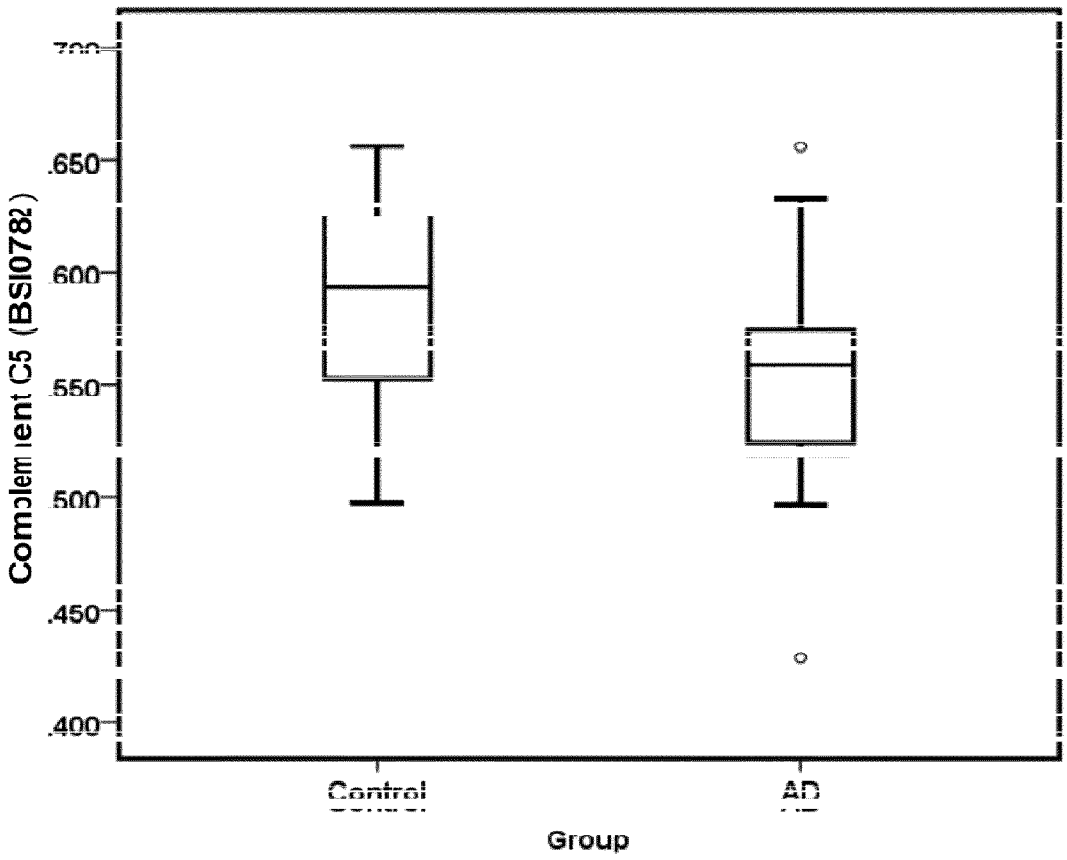

Complement C5 (BSI0782)
.650
.600
.550
.500
.450
.400
Control
AD
Group

Figure 5

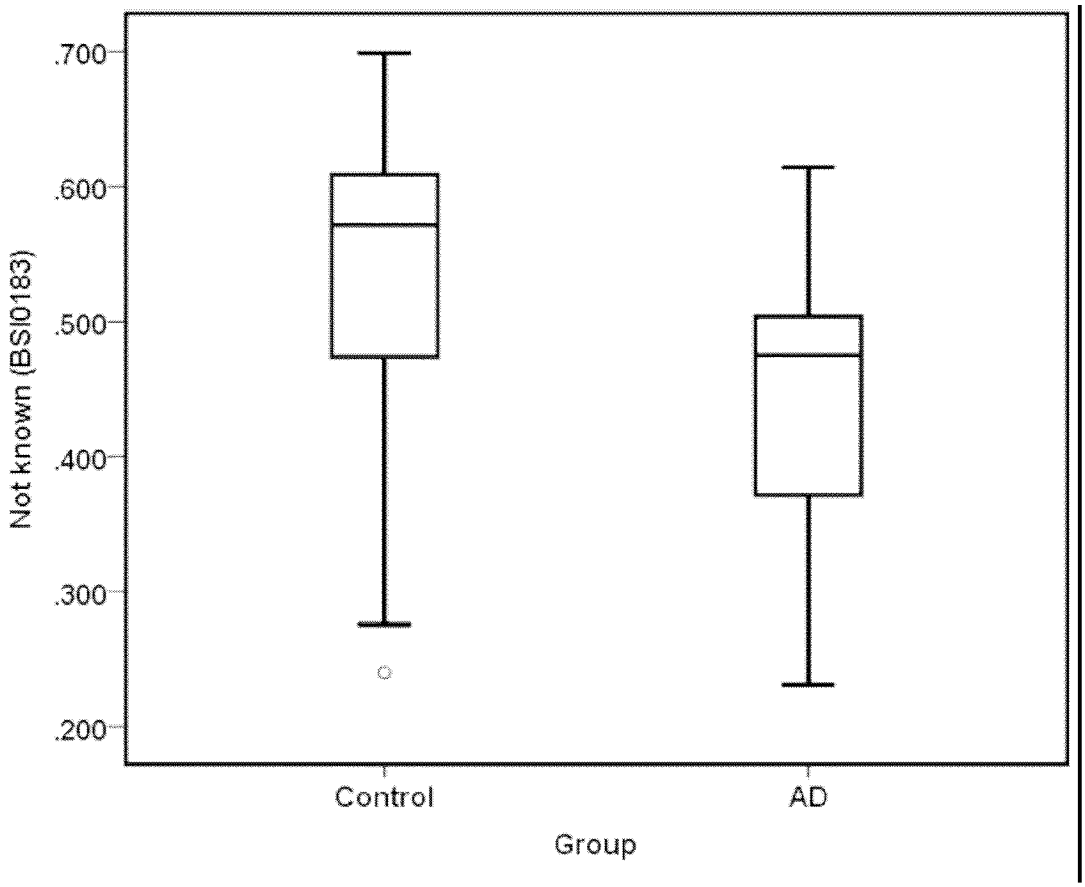


Figure 6

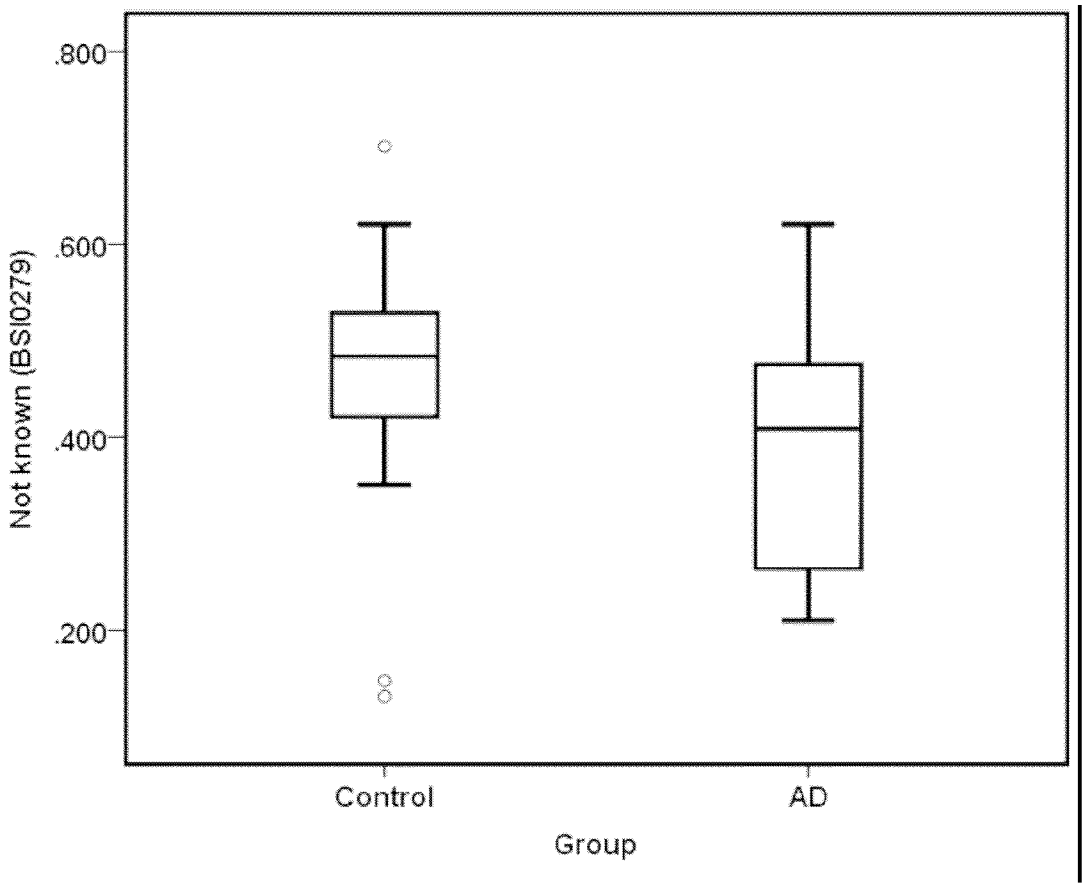


Figure 7

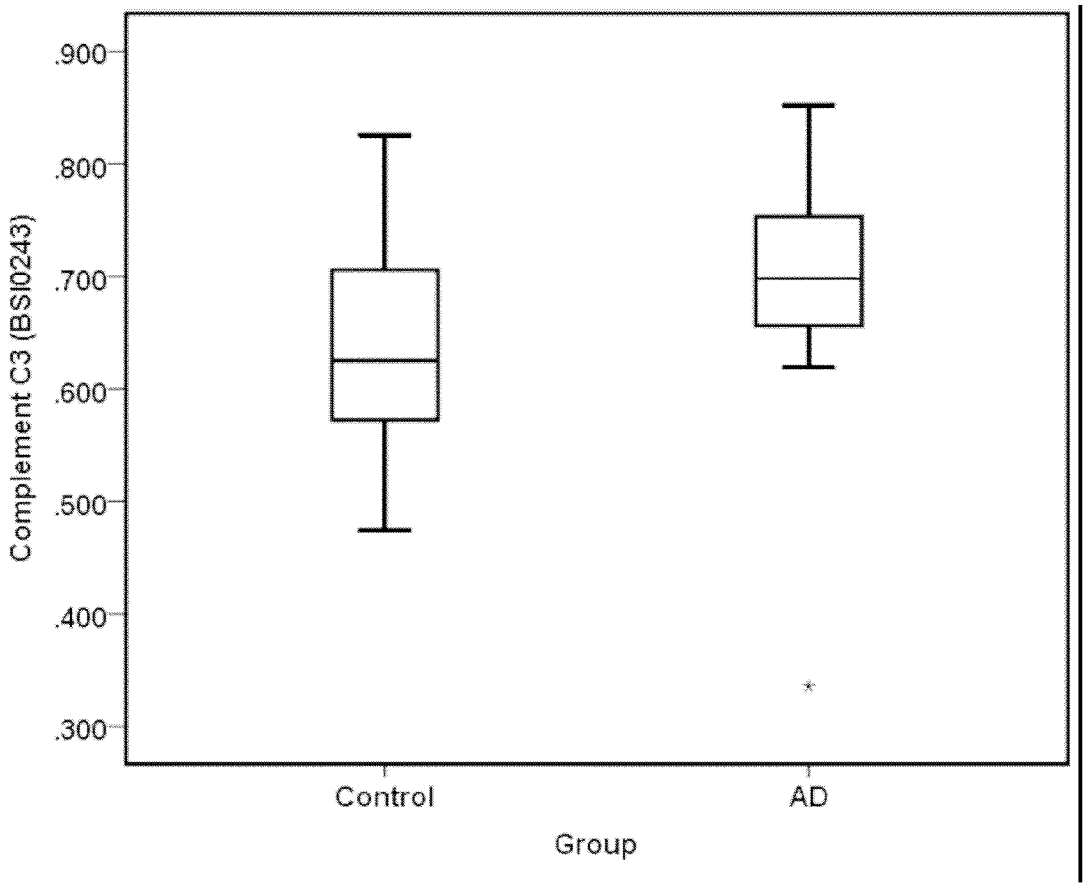

.900
.800
.700
.600
.500
.400
.300
Complement C3 (BSI0243)
Control
AD
Group

Figure 8

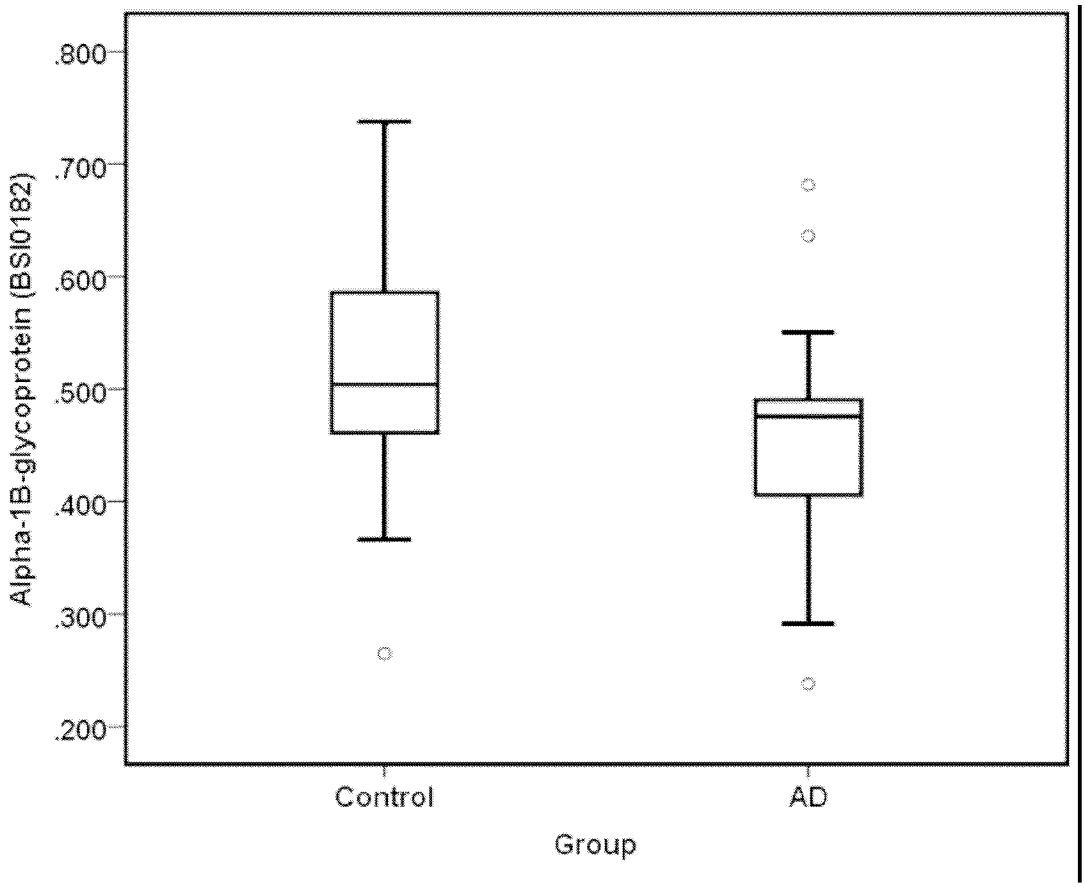

.800
.700
.600
.500
.400
.300
.200
Alpha-1B-glycoprotein (BSI0182)
Control
AD
Group

Figure 9

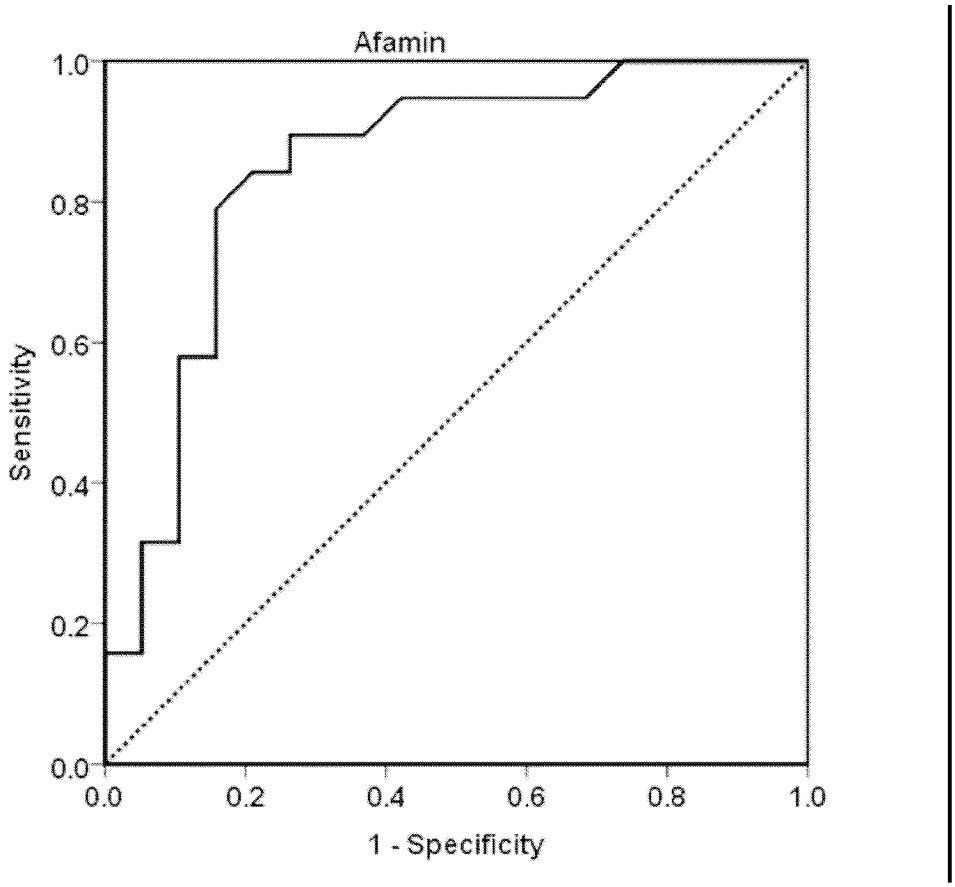

Afamin
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 10

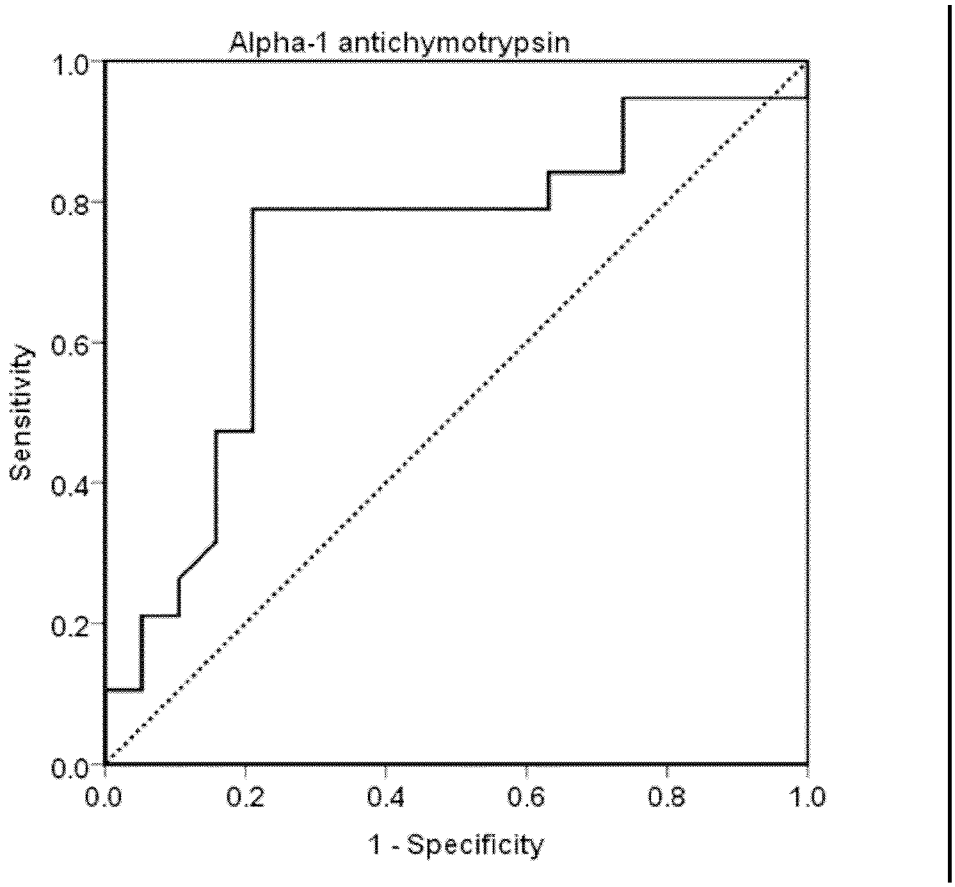

Alpha-1 antichymotrypsin
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Figure 11

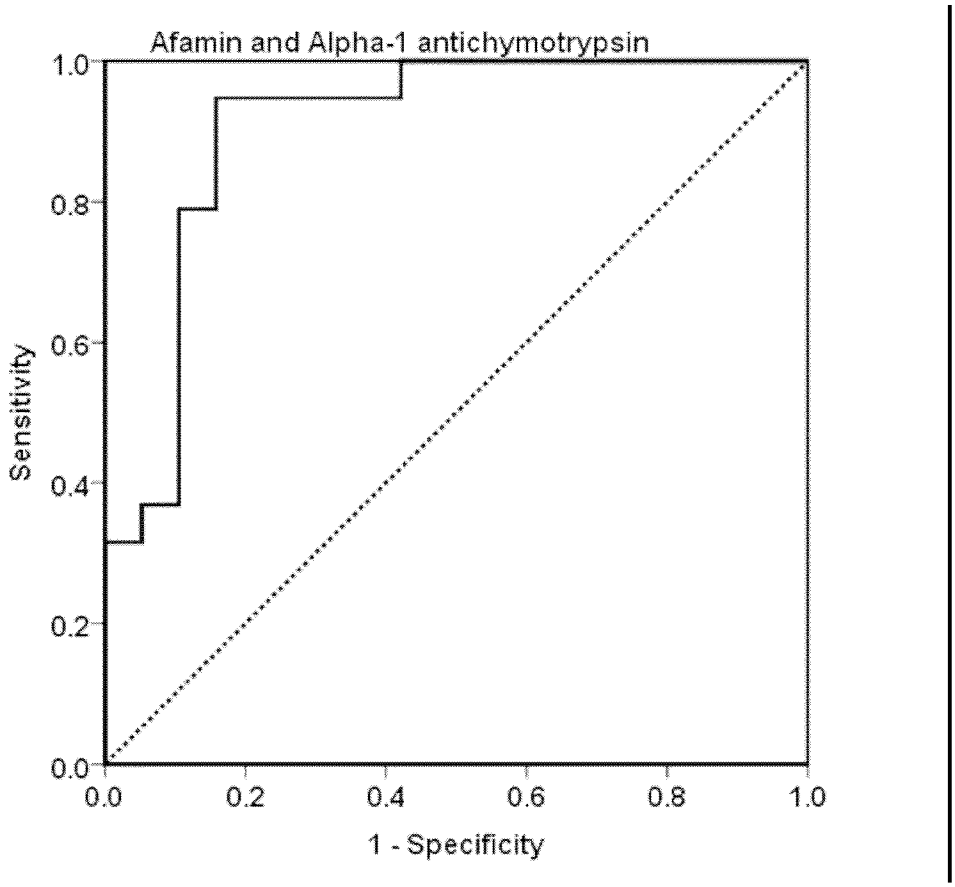

Afamin and Alpha-1 antichymotrypsin
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 12

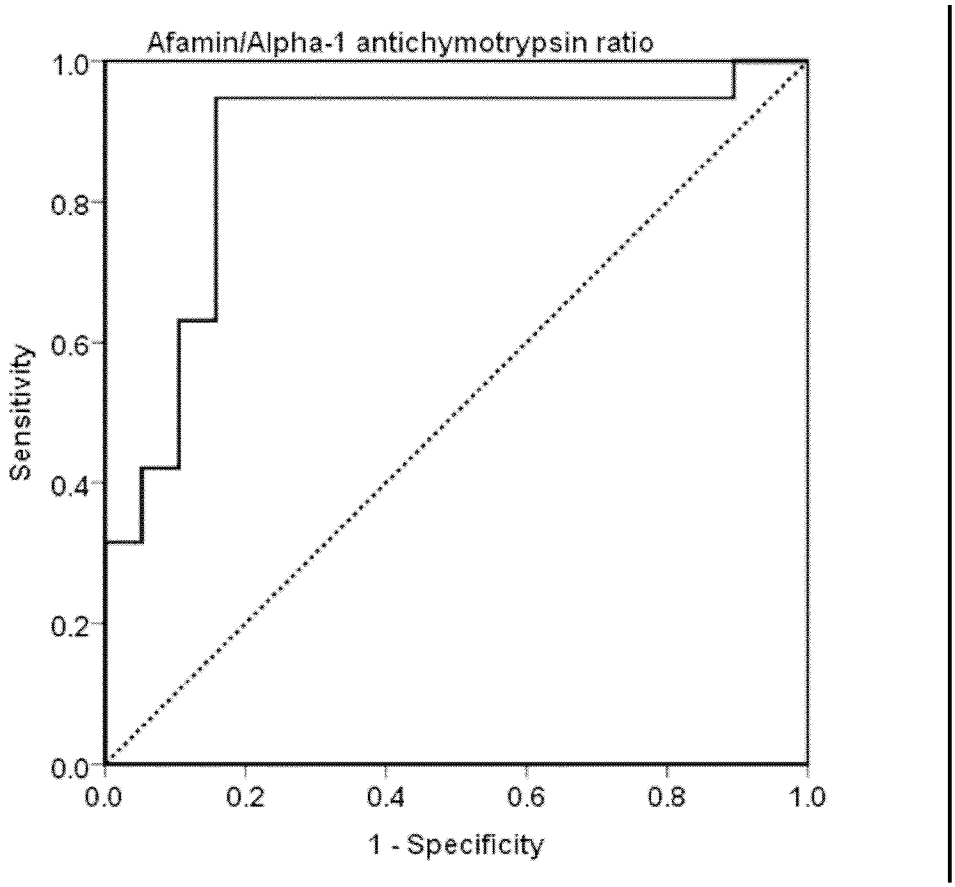

Afamin/Alpha-1 antichymotrypsin ratio
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Figure 13

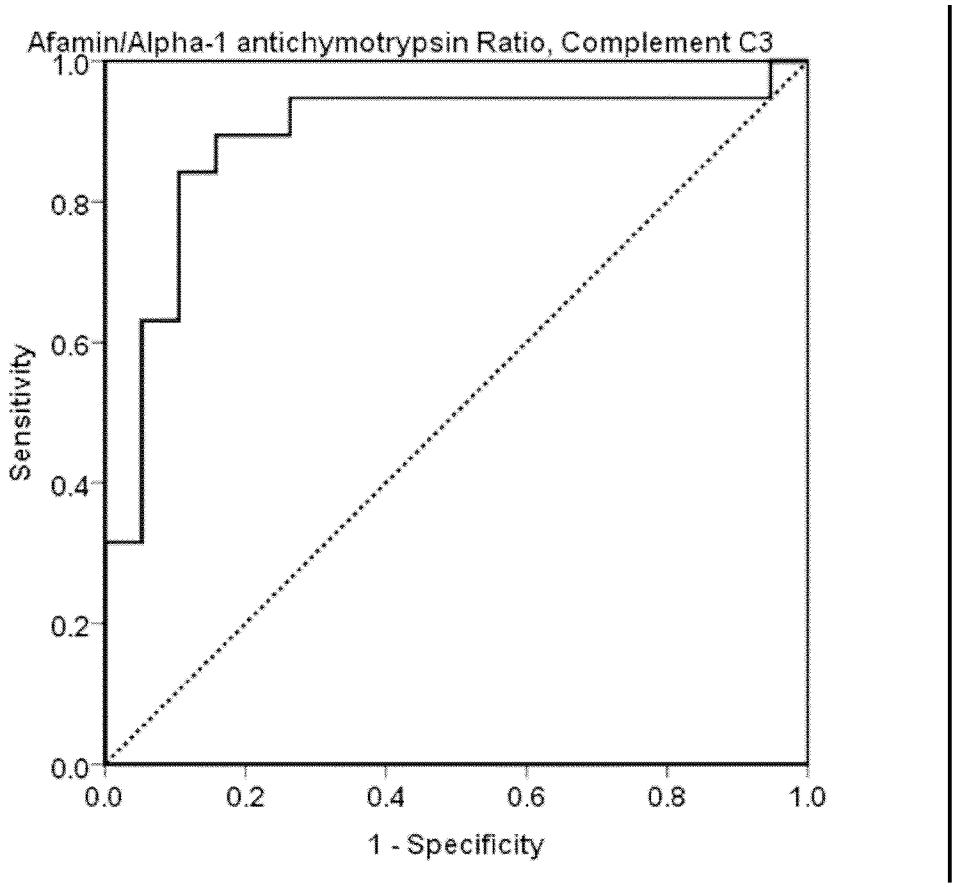

Afamin/Alpha-1 antichymotrypsin Ratio, Complement C3
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 14

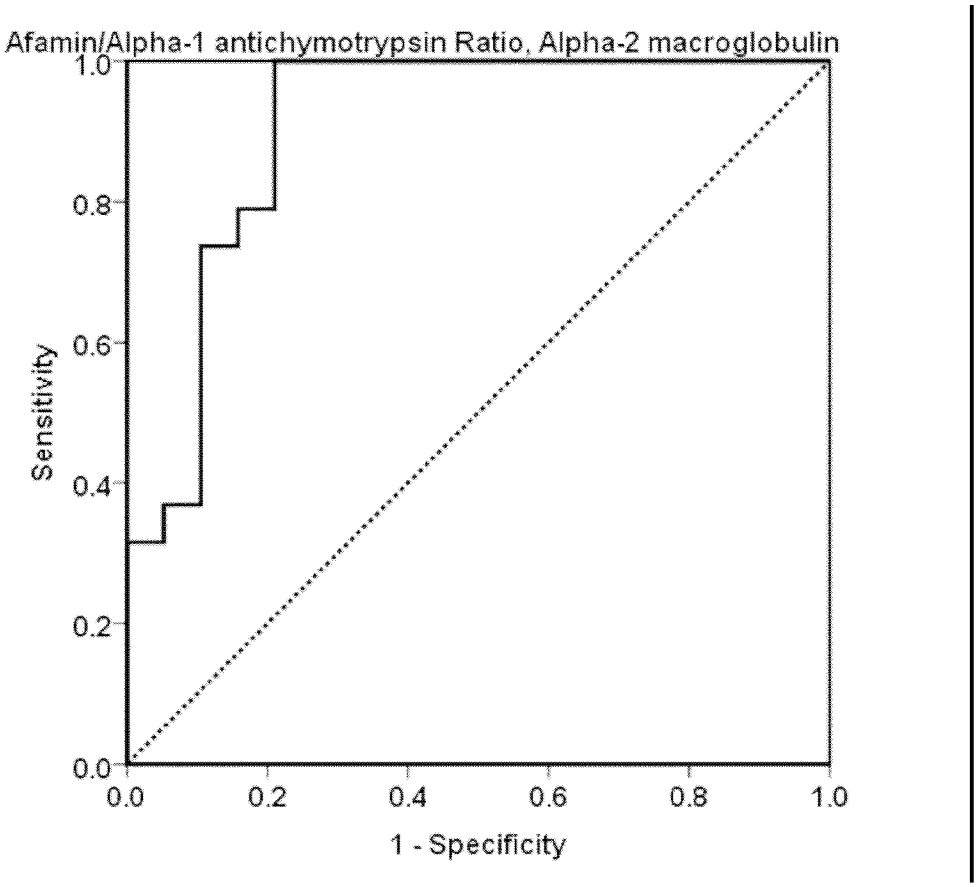

Afamin/Alpha-1 antichymotrypsin Ratio, Alpha-2 macroglobulin
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 15

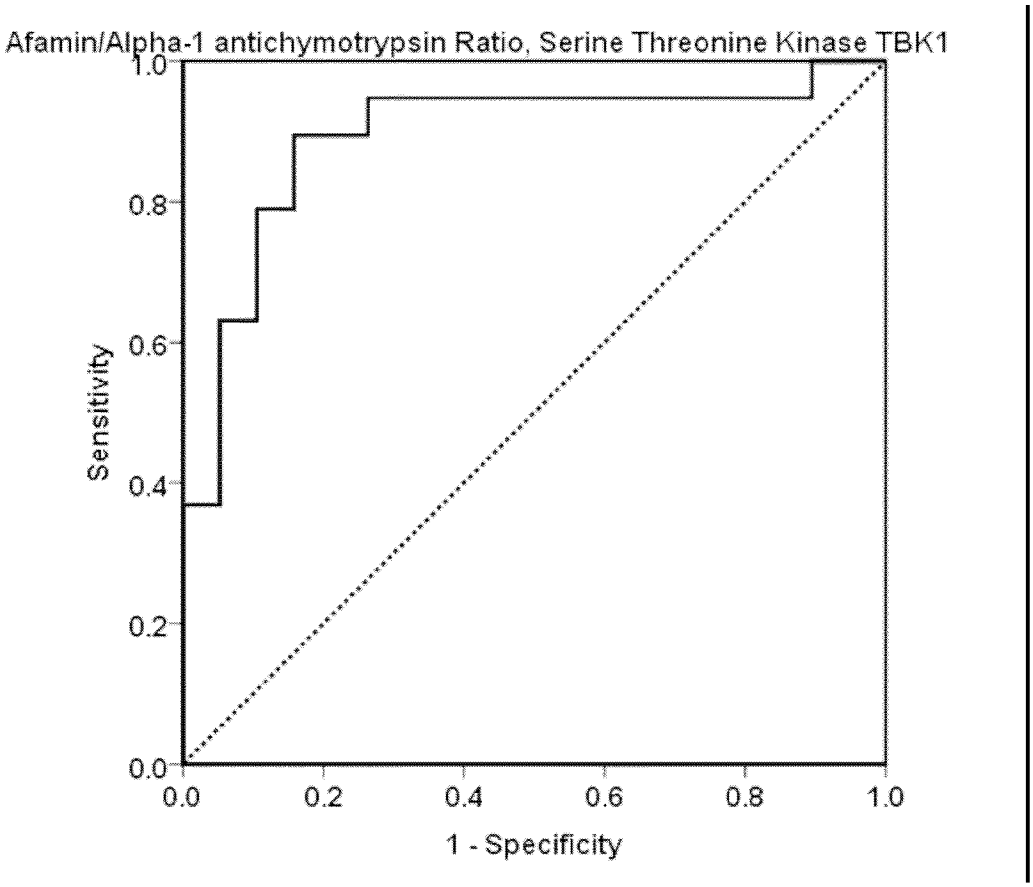

Afamin/Alpha-1 antichymotrypsin Ratio, Serine Threonine Kinase TBK1
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Figure 16

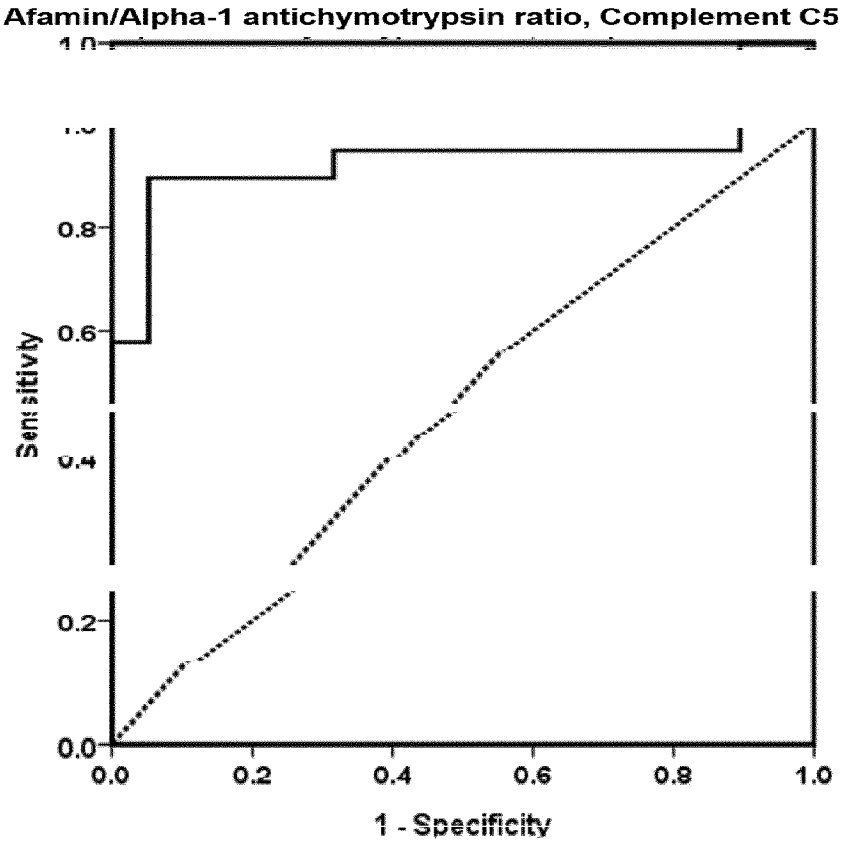

Afamin/Alpha-1 antichymotrypsin ratio, Complement C5
Sensitivity
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 17

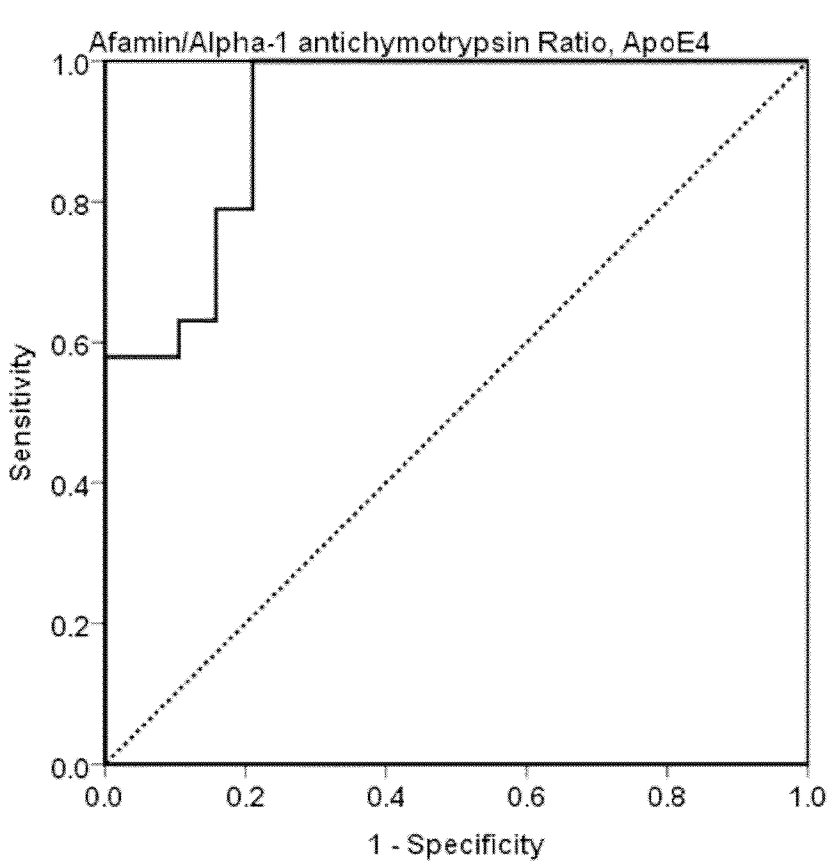

Afamin/Alpha-1 antichymotrypsin Ratio, ApoE4
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 18

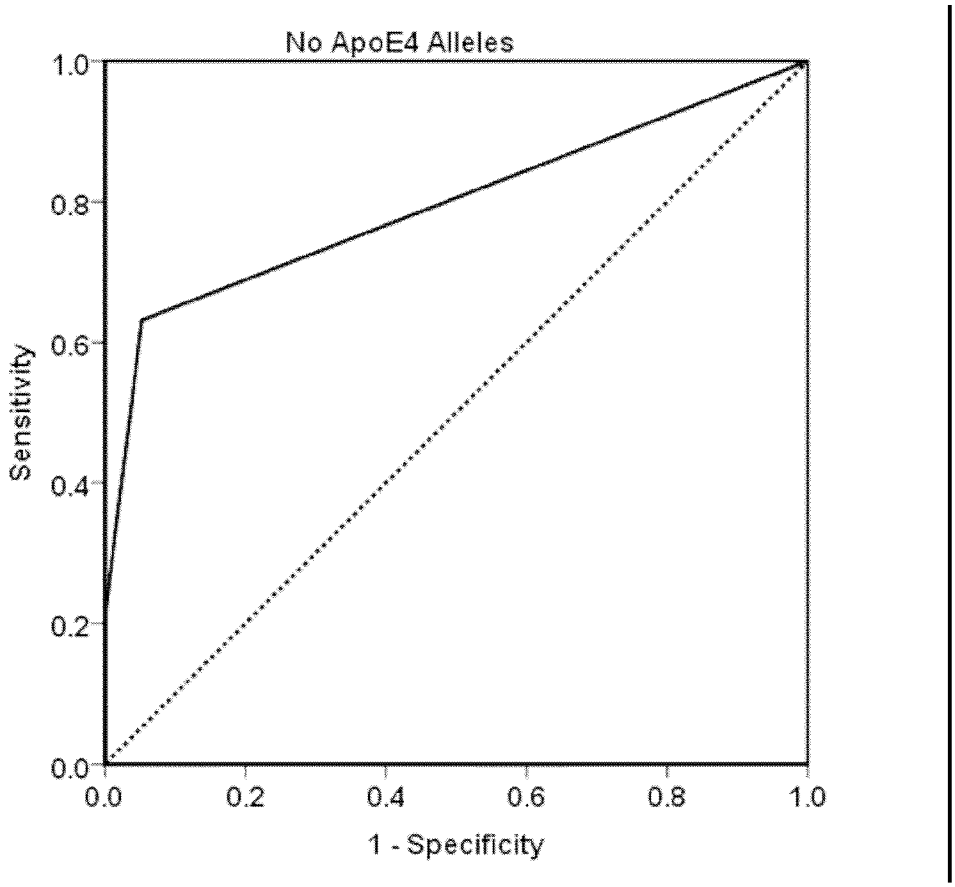

No ApoE4 Alleles
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 19

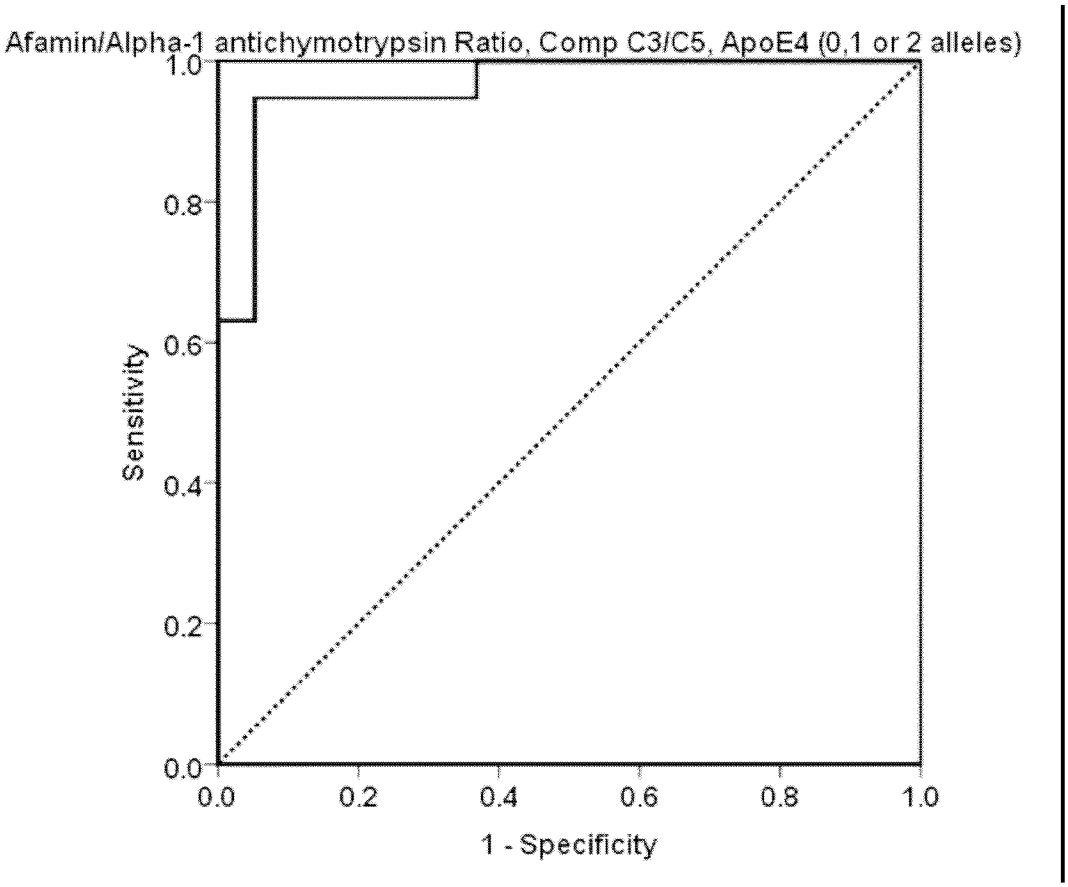
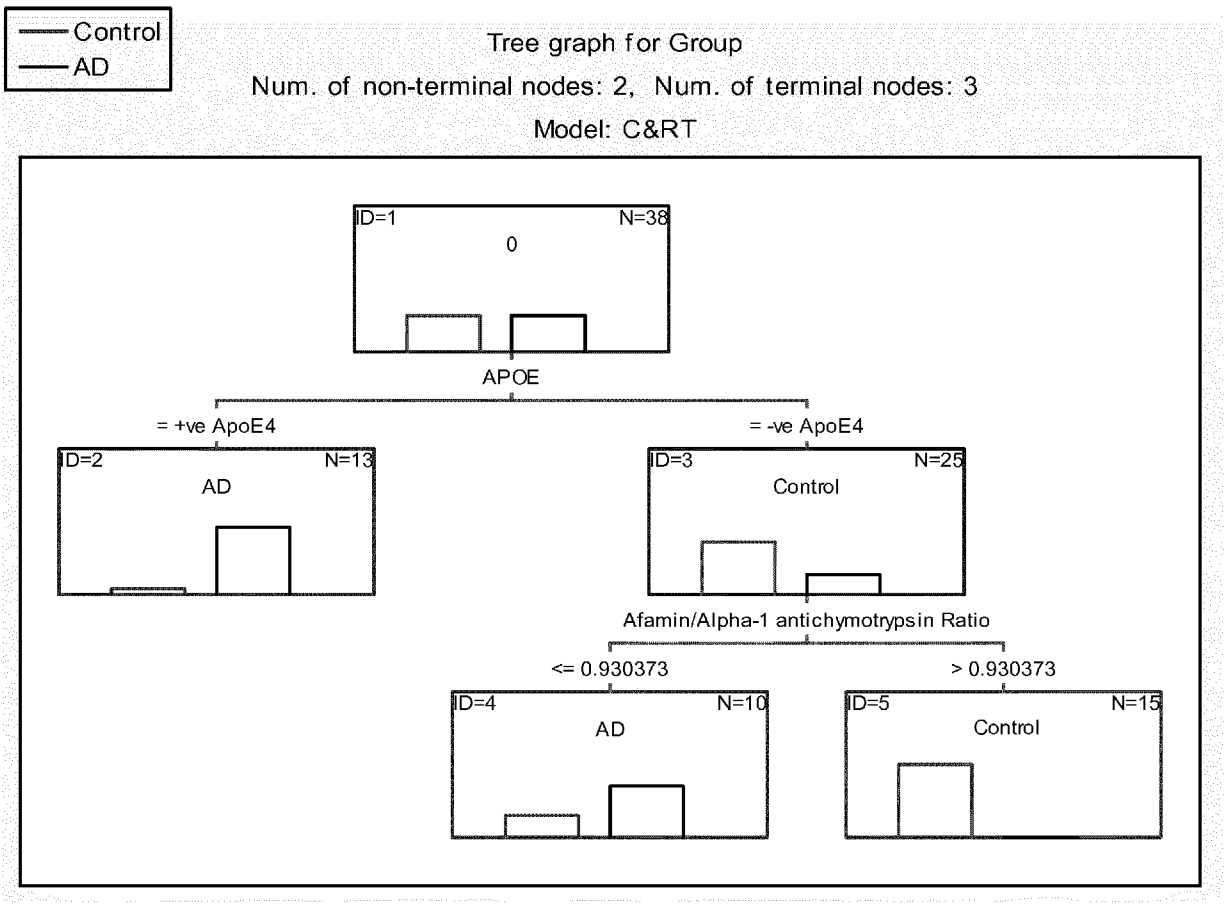

Afamin/Alpha-1 antichymotrypsin Ratio, Comp C3/C5, ApoE4 (0,1 or 2 alleles)
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Figure 20


Control
AD
Tree graph for Group
Num. of non-terminal nodes: 2, Num. of terminal nodes: 3
Model: C&RT
ID=1
N=38
0
APOE
= +ve ApoE4
= -ve ApoE4
ID=2
N=13
AD
ID=3
N=25
Control
Afamin/Alpha-1 antichymotrypsin Ratio
<= 0.930373
> 0.930373
ID=4
N=10
AD
ID=5
N=15
Control