

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 April 2009 (16.04.2009)

PCT

(10) International Publication Number
WO 2009/048883 A1

(51) International Patent Classification:
G01N 33/53 (2006.01) **G01N 24/00** (2006.01)

(21) International Application Number:
PCT/US2008/079104

(22) International Filing Date: 7 October 2008 (07.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/978,321 8 October 2007 (08.10.2007) US

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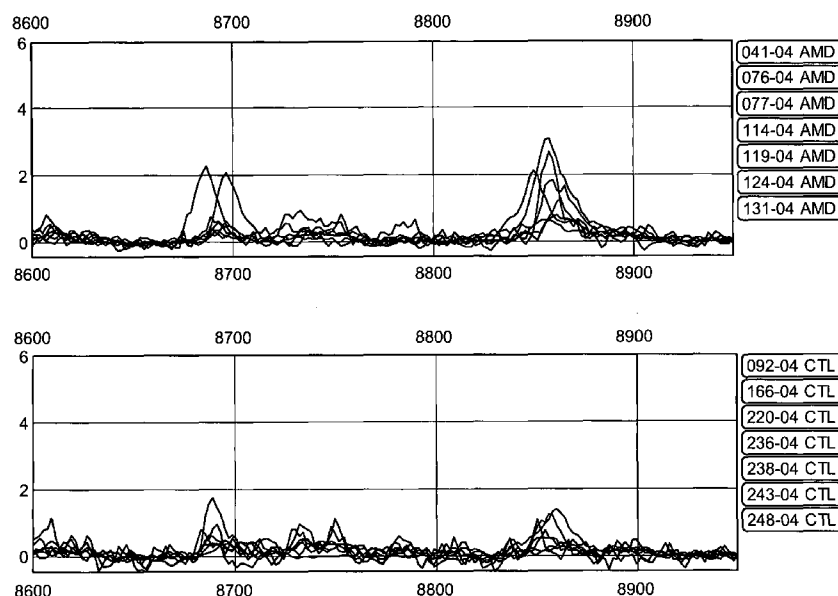
(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE,
EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID,
IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK,
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MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT,
RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,
NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG,
CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

(54) Title: DIAGNOSIS OF AGE-RELATED MACULAR DEGENERATION USING BIOMARKERS



(57) Abstract: The invention relates to proteins associated with age-related macular degeneration (AMD). These proteins, which are present in blood, are expressed in individuals with AMD at either elevated or reduced levels compared to healthy individuals. The invention provides methods for diagnosing AMD. The invention provides methods for assessing the efficacy of treatment of AMD. The invention provides methods for monitoring the progression of AMD.

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PATENT APPLICATION

DIAGNOSIS OF AGE-RELATED MACULAR DEGENERATION USING
BIOMARKERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application No. 60/978,321 filed October 8, 2007, the entire content of which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates to diagnosis of age-related macular degeneration and has application in biology and medicine.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER
FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0003] This invention was made with government support under NIH grant R01 EY11515. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0004] Pathological changes associated with many disease states are reflected in the protein profile of serum and plasma (because blood comes into contact with most of the tissues in the human body), as well as other body fluids, such as urine. Monitoring the levels (and changes in levels) of such proteins, or "biomarkers" is useful for diagnosis and prognosis of diseases. In addition, changes in levels of biomarker can serve as surrogate endpoints for assessing the effects and efficacy of therapeutic interventions.

[0005] Age-related macular degeneration (AMD) is a degenerative condition of a specialized region of the central retina called the macula. Early AMD is characterized by the thinning of the macula and formation of deposits called drusen in the macula. Most people with early AMD have good vision. Persons with drusen may develop advanced AMD, which is associated with profound vision loss. Advanced AMD has two forms: dry, which is a slow, degenerative process with gradual central vision loss due to loss of photoreceptors; and wet,

which is associated with sudden vision loss due to abnormal blood vessel growth (*i.e.*, choroidal neovascularization) under the macula.

[0006] AMD is the leading cause of blindness in adults over 60, affecting more than 50 million people worldwide (Klein *et al.*, *Am J Ophthalmol.* 137:486, 2004). Although some therapeutic options are available for patients with AMD, and others are being developed, there is a great need for additional treatments. Similarly, there is a great need for new methods for diagnosis of AMD, and for prognosis of AMD patients. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0007] The invention relates to proteins associated with age-related macular degeneration (AMD). These AMD-associated proteins (biomarkers) are present in the serum or other blood fraction, urine, or other body fluid of individuals with AMD at elevated or reduced levels compared to healthy individuals (*i.e.*, age-matched controls). The invention provides methods and kits for using the biomarkers for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD, in an individual. As used herein, diagnosing AMD includes detecting the very early stages of AMD, even prior to the individual showing any symptoms or signs of the disease.

[0008] The invention provides methods for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD by determining levels of biomarkers in a biological sample from an individual and comparing the levels of the biomarkers to earlier determined levels or reference levels of the biomarkers. Determination that a biomarker is at a level characteristic of a disease state in a subject suggests that the tested subject has or may be developing AMD, while determination that a biomarker is at a level characteristic of a non-disease state in a subject suggests that the tested subject does not have or is not developing the disease. Likewise, a change of biomarker levels over time to levels closer to that of a disease state suggests progression of AMD, while change of biomarker levels over time to levels closer to that of a non-disease state suggests regression of the disease (*e.g.*, due to therapeutic efficacy of a treatment).

[0009] In one embodiment, the methods for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD involve determining the levels of biomarkers in a biological sample from an individual and comparing the levels of the biomarkers to earlier determined levels and/or to reference levels of the biomarkers.

[0010] In one embodiment, the biological sample is from the serum of an individual. In another embodiment, the biological sample is from the plasma of an individual. In another embodiment, the biological sample is from the urine of an individual. In one embodiment, the biological sample is depleted of albumin and IgG.

[0011] As used herein, the biomarkers for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD in a biological sample from an individual are proteins, which may be identified and characterized by their mass-to-charge ratio as determined by mass spectrometry, as indicated in Tables 1-4.

[0012] The biomarkers of the invention are listed in Tables 1-4.

[0013] In certain embodiments, the levels of a combination of biomarkers (*i.e.*, a set of biomarkers) as described herein are determined, *e.g.*, the levels of 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 of the biomarkers listed in Tables 1-4.

[0014] In certain embodiments, the levels of a set of biomarkers as described herein are determined. The sets of biomarkers have shared properties, *e.g.*, presence at elevated levels in AMD patients compared to controls, presence at reduced levels in AMD patients compared to controls, ratio or difference in levels between AMD patients and controls (*e.g.*, between 1.25- and 2-fold, between 2- and 3-fold, between 3- and 5-fold, or at least 5-fold difference between levels in AMD patients compared to controls), source of the biological sample containing the biomarkers, method used to identify and characterize the biomarkers, function, or any combination of these properties.

[0015] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at different levels in the serum of individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed in Table 1.

[0016] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at different levels in albumin and IgG depleted serum of individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed in Table 2.

[0017] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at different levels in albumin and IgG depleted plasma of individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed in Table 3.

[0018] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at different levels in the urine of individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed in Table 4.

[0019] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at elevated levels in individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed as “↑” in Tables 1-4.

[0020] In one embodiment, the biomarkers in a set of biomarkers are selected from the biomarkers present at reduced levels in individuals diagnosed with AMD, compared to a control population, *e.g.*, biomarkers listed as “↓” in Tables 1-4.

[0021] In one embodiment, at least one biomarker is a biomarker present in serum at significantly different levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 1: 1287; 3329; 3350; 3452; 4627; 6233; 9452; 9593; and 9664.

[0022] In one embodiment, at least one biomarker is a biomarker present in serum at significantly elevated levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 1: 1287 and 6233.

[0023] In one embodiment, at least one biomarker is a biomarker present in serum at significantly reduced levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 1: 3329; 3350; 3452; 4627; 9452; 9593; and 9664.

[0024] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted serum at significantly different levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 2: 3070; 3216; 3402; 4951; 4976; 8858; 11834; 12577; 36008; 3003; 3061; 4144; 4490; 4603; 4775; 5816; and 6823.

[0025] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted serum at significantly elevated levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 2: 3070; 3216; 3402; 4951; 4976; 8858; 11834; 36008; 3003; 3061; 4144; 4490; and 4775.

[0026] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted serum at significantly reduced levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 2: 12577; 4603; 5816; and 6823.

[0027] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted plasma at significantly different levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 3: 3123;

3498; 3990; 4632; 5691; 5850; 6405; 11468; 3160; 3396; 3600; 3681; 3708; 3867; 3943; 3997; 6987; 33117; 145931; 58655; and 60449.

[0028] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted plasma at significantly elevated levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 3: 3123; 3498; 3990; 5691; 11468; 3160; 3396; 3600; 3681; 3708; 3867; 3997; 6987; 33117; 58655; and 60449.

[0029] In one embodiment, at least one biomarker is a biomarker present in albumin and IgG depleted plasma at significantly reduced levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 3: 4632; 5850; 6405; 3943; and 145931.

[0030] In one embodiment, at least one biomarker is a biomarker present in urine at significantly reduced levels in individuals with or developing AMD, compared to age-matched controls, selected from the following biomarkers in Table 4: 2564; 3027; 5916; 6189; 6316; 6864; 11724; and 11783.

[0031] In one embodiment, the biomarkers are measured by capturing the biomarker on an adsorbant of a surface enhanced laser desorption ionization (SELDI) probe and detecting the captured biomarkers by laser desorption-ionizing mass spectrometry. In one embodiment, the adsorbant is a cation exchange adsorbant, an anion exchange adsorbant, an immobilized metal affinity capture adsorbant, or a hydrophobic adsorbant. In one embodiment, the adsorbant is a biospecific adsorbant. In one embodiment, the biomarkers are measured using an immunoassay.

[0032] In one aspect, the invention provides a method for diagnosing AMD in an individual, by determining the levels of biomarkers in a biological sample from the individual, and comparing the levels of the biomarkers in the biological sample from the individual to reference levels of the biomarkers characteristic of a control population, where a difference in the levels of the biomarkers between the biological sample from the individual and the control population indicates that the individual is developing or has AMD. The methods may include the steps of obtaining a biological sample from the individual and determining the levels of the biomarkers. The methods may include determining the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above. The levels of certain biomarkers are significantly different in individuals with AMD than in healthy individuals. The levels of certain biomarkers are higher in individuals with

AMD than in healthy individuals. The levels of certain biomarkers are lower in individuals with AMD than in healthy individuals.

[0033] In one embodiment, a method for diagnosing AMD in an individual involves obtaining a biological sample from the individual and determining the levels of the biomarkers by separating and detecting proteins by surface enhanced laser desorption ionization (SELDI).

[0034] The biomarkers can be obtained in a biological sample, preferably a fluid sample, of the individual. The biological sample can also be a tissue sample, *e.g.*, a skin biopsy. The precise biological sample to be taken from an individual may vary, but the sampling is typically minimally invasive and is easily performed by conventional techniques known in the art. The biomarkers are preferentially obtained in a biological sample of the individual's blood, serum, plasma, urine, cerebral spinal fluid (CSF), or saliva. The biological sample can be depleted of albumin and IgG, if appropriate.

[0035] In another aspect, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, by determining the levels of biomarkers in a biological sample from the individual before treatment or at a first time point after treatment, determining the levels of biomarkers in the biological sample from the individual at a later time point during treatment or after treatment, and comparing the levels of the biomarkers at the two time points, where a difference in the levels of the biomarkers between the two determinations in which the levels of the biomarkers move closer to reference levels of the biomarkers characteristic of a control population indicates that the treatment is effective. The methods may include the steps of obtaining a biological sample from the individual and determining the levels of biomarkers as above. The methods may include determining the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above. The levels of certain biomarkers are higher in individuals with AMD than in healthy individuals. The levels of these biomarkers in individuals with AMD decrease (*i.e.*, moves to a more normal level) upon treatment with an agent effective to treat AMD. The levels of certain other biomarkers are lower in individuals with AMD than in healthy individuals. The levels of these biomarkers in individuals with AMD increase (*i.e.*, moves to a more normal level) upon treatment with an agent effective to treat AMD.

[0036] In one embodiment, a method for assessing the efficacy of treatment of AMD in an individual involves the individual being treated with an agent effective to treat the disease.

[0037] In one embodiment, a method for assessing the efficacy of treatment of AMD in an individual involves obtaining a biological sample from the individual and determining the levels of the biomarkers by separating and detecting proteins by SELDI.

[0038] In one embodiment, a method for assessing the efficacy of treatment of AMD in a individual involves obtaining a biological sample of blood, serum, plasma or urine from the individual and determining the level of the biomarkers.

[0039] For example, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, by determining the levels of biomarkers in a biological sample from the individual at a first time point during the course of treatment with an agent, determining the levels of the biomarkers in the biological sample from the individual at a later time point during or after treatment with the agent, and comparing the levels of the biomarkers at the two time points, where detection of more normal levels at a later time-point indicates regression of disease (*e.g.*, therapeutic efficacy) and detection of levels less like the normal level at a later time-point indicates progression of disease. The methods may include the steps of obtaining a biological sample from the individual and determining the levels of the biomarkers as above. The methods may include determining the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above.

[0040] In another aspect, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, comprising comparing levels of biomarkers in a biological sample from the individual after administration of an agent to levels of the biomarkers in the biological sample from the individual at an earlier time point and to reference levels of the biomarkers characteristic of a control population, where a reduced difference between the levels of the biomarkers in the individual after administration of the agent compared to the reference levels and the levels of the biomarkers in the individual taken at an earlier time point compared to the reference levels in which the levels of the biomarkers move closer to reference levels of the biomarkers characteristic of a control population indicates that the treatment is effective. The methods may include the steps of obtaining a biological sample from the individual and determining the levels of the biomarkers as above. The methods may include determining the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above.

[0041] In another aspect, the invention provides a method for monitoring the progression of AMD in an individual, comprising determining the levels of biomarkers in a biological sample from the individual, and comparing the levels of the biomarkers in the biological

sample from the individual to reference levels of the biomarkers characteristic of a control population. In a related aspect, the invention provides a method for monitoring the progression of AMD in an individual, comprising determining the levels of biomarkers in a biological sample from the individual before treatment or at a first time point after treatment, determining the levels of biomarkers in the biological sample from the individual at a later time point during treatment or after treatment, and comparing the levels of the biomarkers at the two time points. In one embodiment, the individual is being administered with an agent effective to treat or prevent AMD, and the levels of the biomarkers determine the future treatment regime for the individual. The methods may include the steps of obtaining a biological sample from the individual and determining the levels of the biomarkers as above. The methods may include determining the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above.

[0042] In one embodiment, a method for monitoring the progression of AMD involves obtaining a biological sample from the individual and determining the levels of the biomarkers by separating and detecting proteins by SELDI.

[0043] In one embodiment, a method for monitoring the progression of AMD involves obtaining a biological sample of blood, serum, plasma or urine from the individual and determining the level of the biomarkers.

[0044] In another aspect, the invention provides a kit comprising a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds at least two biomarkers selected from the biomarkers indicated in Tables 1-4, at least two biomarkers selected from the biomarkers indicated in Tables 1-4, and instructions for using the solid support to detect the biomarkers contained in the kit.

[0045] In one embodiment, the solid support comprising the capture reagent is a SELDI probe. In one embodiment, the adsorbant is a cation exchange adsorbant, an anion exchange adsorbant, an immobilized metal affinity capture adsorbant, or a hydrophobic adsorbant. In one embodiment, the adsorbant is a biospecific adsorbant.

[0046] In one embodiment, the kit provides at least two biomarkers selected from the biomarkers indicated in Tables 1-4. In various embodiments, the kit contains a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030] above.

[0047] In one embodiment, the kit provides instructions for using the solid support to detect the biomarkers contained in the kit.

BRIEF DESCRIPTION OF THE DRAWINGS

[0048] Figure 1 shows a representative mass spectra displaying proteins present in serum from 7 individuals diagnosed with AMD (top panel) and 7 age-matched controls (bottom panel). The potential biomarkers are detected using an IMAC-Cu array, a 50% SPA matrix, and low laser for data acquisition, according to the methods described in Example 5. The figure shows the mass-to-charge ratio (X-axis) and relative peak intensity (Y-axis) for a portion of the spectra.

DETAILED DESCRIPTION OF THE INVENTION

[0049] The invention relates to biomarkers associated with age-related macular degeneration (AMD). The biomarkers are proteins present in the serum or other blood fraction, urine, or other body fluid of individuals with AMD at elevated or reduced levels compared to healthy individuals (*i.e.*, age-matched controls). Certain of these biomarkers are present at elevated levels in individuals with AMD compared to controls. Certain other of these biomarkers are present at reduced levels in individuals with AMD compared to controls.

[0050] In one aspect, the invention provides methods for diagnosing AMD by determining the levels of at least two biomarkers in an individual and comparing the levels of the at least two biomarkers with reference levels characteristic of a control, healthy population. In these methods, the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030], are determined. In one aspect, the invention provides methods for assessing the efficacy of treatment of AMD by determining the levels of at least two biomarkers in an individual with AMD being treated for the disease and comparing the levels of the at least two biomarkers to earlier determined levels or reference levels of the biomarker. In these methods, the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030], are determined. In one aspect, the invention provides methods for monitoring the progression of AMD by determining the levels of at least two biomarkers in an individual with AMD and comparing the levels of the at least two biomarkers with reference levels characteristic of a control, healthy population. In a related aspect, the invention provides methods for monitoring the progression of AMD by determining the levels of at least two biomarkers in an individual with AMD being treated for the disease and comparing the levels of the at least two biomarkers to earlier determined

levels or reference levels of the biomarker. In these methods, the levels of a combination or set of biomarkers, for example, as described in paragraphs [0021] to [0030], are determined.

I. DEFINITIONS

[0051] The following definitions are provided to aid in understanding the invention.

Unless otherwise defined, all terms of art, notations and other scientific or medical terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the arts of medicine and molecular biology. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not be assumed to represent a substantial difference over what is generally understood in the art.

[0052] "Biomarkers" are proteins present at different, *i.e.*, reduced or elevated, levels in a biological fluid or tissue sample from individuals diagnosed with AMD compared to age-matched control individuals.

[0053] "Biological sample" refers to a fluid or tissue sample obtained from an individual that contains the biomarkers of the invention. The biological fluid sample can be, for example, a sample of an individual's blood, serum, plasma, urine, CSF or saliva. The biological tissue sample can be, for example, a skin biopsy. The biological sample can also be depleted of particular proteins, for example, albumin and IgG, if appropriate.

[0054] "Level" refers to the amount of a biomarker in a biological sample obtained from an individual. The level(s) of a biomarker(s) can be determined as described below for a single biomarker or for a "set" of biomarkers. A set of biomarkers refers to a group or combination of more than one biomarker that have been grouped together by a shared property such as for example and not for limitation their presence at elevated levels in AMD patients compared to controls, by their presence at reduced levels in AMD patients compared to controls, by their ratio or difference in levels between AMD patients and controls (*e.g.*, between 1.25- and 2-fold, between 2- and 3-fold, between 3- and 5-fold, or at least 5-fold difference between levels in AMD patients compared to controls), by the source of the biological sample containing the biomarkers, by the method used to identify and characterize the biomarkers, by function, or by any combination of these properties.

[0055] The level of the biomarker can be determined by any method known in the art and will depend in part on the nature of the biomarker. Methods for determining the level of a biomarker include surface enhanced laser desorption ionization (SELDI) mass spectrometry,

electrophoresis (including capillary electrophoresis, 1- and 2-dimensional electrophoresis, 2-dimensional difference gel electrophoresis DIGE followed by MALDI-ToF mass spectrometry), chromatographic methods (such as high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography), mass spectrometry (MS), various immunological methods (such as fluid or gel precipitin reactions, single or double immunodiffusion, immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbant assays (ELISA), immunofluorescent assays, and Western blotting), and assays for an activity of a biomarker. It is understood that the level of the biomarker need not be determined in absolute terms, but can be determined in relative terms. For example, the level of the biomarker may be expressed as its concentration in a biological sample, as the concentration of an antibody that binds to the biomarker, or as the functional activity (*i.e.*, binding or enzymatic activity) of the biomarker.

[0056] "Difference" as it relates to the level of a biomarker of the invention refers to a difference that is statistically different. A difference is statistically different, for example and not for limitation, if the expectation is < 0.05 , *i.e.*, the p value determined using the Student's t-test is < 0.05 . The difference in level of a biomarker between an individual with AMD and a control individual or population can be, for example and not for limitation, at least 10% different (1.10 fold), at least 25% different (1.25-fold), at least 50% different (1.5-fold), at least 100% different (2-fold), at least 200% different (3-fold), at least 400% different (5-fold), at least 10-fold different, at least 20-fold different, at least 50-fold different, at least 100-fold different, at least 150-fold different, or at least 200-fold different.

[0057] "Reference" as it relates to a biomarker of the invention refers to an amount of a biomarker in a healthy individual or control population. The reference level or amount may be determined by obtaining a biological sample and detecting the biomarker in a healthy individual, or may be determined by taking the level or amount known or readily determined from a control population.

[0058] "Control" refers to an individual who has not been diagnosed as having AMD and who has not displayed upon examination any symptoms characteristic of AMD, or a group of such individuals. An exemplary control population are age-matched individuals who have not been diagnosed as having AMD.

[0059] "Treating" or "treatment" refers to the treatment of a disease or condition in a mammal, preferably a human, in which the disease or condition has been diagnosed as AMD involving thinning of the macula and formation of drusen in the macula. Treating or

treatment includes inhibiting the disease or condition (*i.e.*, arresting progression), relieving or ameliorating the disease or condition (*i.e.*, causing regression), or preventing or delaying progression of the disease or condition. Treating or treatment can involve a course of treatment in which an individual with AMD is administered an agent more than once periodically over time that is expected to be effective in inhibiting, relieving or ameliorating, preventing or delaying progression of the disease.

[0060] "Agent" refers to a drug or drug candidate. An agent may be a naturally occurring molecule or may be a synthetic compound, including, for example and not for limitation, a small molecule (*e.g.*, a molecule having a molecular weight < 1000 Daltons), a peptide, a protein, an antibody, or a nucleic acid, used to treat an individual with AMD or other disease of the eye.

[0061] "Progression" refers to an increase in symptoms of AMD, including, for example and not for limitation, increased drusen in the macula or decreased visual acuity for an individual with AMD undergoing treatment for the disease.

II. BIOMARKERS

[0062] Biomolecules present in the blood, plasma, serum, urine or other body fluid, or in a tissue sample, may be present at different levels in individuals with a disease or condition as compared to otherwise healthy individuals or a control population. The inventor has discovered that particular proteins are present in the serum, plasma or urine of individuals with AMD at elevated or reduced levels compared to age-matched control individuals.

[0063] In one aspect, the invention relates to biomolecules, in particular, proteins, that are differentially present in serum, plasma or urine from individuals with AMD as compared to age-matched control individuals (*i.e.*, individuals without the disease). These proteins are therefore associated with AMD and are termed AMD-associated proteins (biomarkers). These biomarkers are present in individuals with AMD at either elevated or reduced levels compared to healthy individuals. Exemplary biomarkers shown to be present in individuals with AMD at different levels compared to age-matched control individuals are provided in Tables 1-4, as described in Examples 5-7.

[0064] The biomarkers of the invention are proteins identified and characterized by their body fluid source, their binding characteristics to adsorbant surfaces of a SELDI probe, their mass-to-charge ratio as determined by mass spectrometry, and the shape of the spectral peak in time-of-flight mass spectrometry (ToF-MS). These characteristics provide one method of

uniquely identifying biomolecules and determining whether a biomolecule is a biomarker of the invention. These characteristics represent inherent properties of biomolecules and not process limitations in the manner in which the biomolecules are discriminated.

[0065] As discussed in detail in the Examples, the biomarkers of the invention were identified using SELDI technology employing PROTEINCHIP arrays from Ciphergen Biosystems, Inc. (Fremont, CA). Serum, plasma or urine samples were collected from individuals diagnosed with AMD and from age-matched control individuals not diagnosed with AMD. In some circumstances, the serum, plasma or urine samples were pre-fractionated by albumin and IgG depletion (see, *e.g.*, Examples 2 and 6, *infra*). In other circumstances, the serum, plasma or urine samples were used without prior fractionation. Samples, either fractionated or not, were applied to SELDI biochips and the spectra of proteins in the samples that bound to the biochips were generated by ToF-MS on a Ciphergen PBSII mass spectrometer. The spectra were analyzed by Ciphergen Express Data Manager Software with BIOMARKER WIZARD and Biomarker Pattern Software from Ciphergen Biosystems, Inc. The mass spectra for each group were subjected to scatter plot analysis. A Student's t-test analysis was employed to compare AMD and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly (p -value < 0.05 , or, in some cases, < 0.1) between the two groups. This method is described in more detail in Example 5.

[0066] In the practice of the invention biomarkers can be obtained in a biological sample, preferably a fluid sample, of the individual. The biomarkers are preferentially obtained in a sample of the individual's blood, serum, plasma, urine, CSF or saliva. The biological sample can also be a tissue sample, *e.g.*, a skin biopsy. The biological sample can be depleted of particular proteins, for example, albumin and IgG, if appropriate.

[0067] In one embodiment, a method for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD, in an individual involves obtaining a sample of blood, serum or plasma from the individual and determining the levels of at least two biomarkers. As an example, the biomarkers of the invention were obtained from the serum, plasma and blood of individuals with AMD and age-matched control individuals, as described in Examples 5-7.

[0068] Examples of identified and characterized biomarkers for diagnosing AMD, assessing the efficacy of treatment of AMD, or monitoring the progression of AMD are presented in Tables 1-4. The "Mass" column refers to the mass-to-charge ratio in Daltons

(Da) as determined by mass spectrometry. The "Assay" column refers to the type of SELDI biochip used bind the biomarker, the chromatographic fraction and/or wash condition used, if applicable, and the mass spectrometry condition (*i.e.*, type of matrix and laser setting), as described in detail in the Examples. The "P-value" column refers to the statistical significance reached for the difference in the level of the indicated biomarker between samples from individuals diagnosed with AMD and control individuals. The "Up/Down" column specifies whether the level of the indicated biomarker is elevated or reduced in individuals diagnosed with AMD as compared to control individuals.

[0069] The biomarkers of the invention are identified and characterized by their mass-to-charge ratio as determined by mass spectrometry. The mass-to-charge ratio of each biomarker is provided in the "Mass" column in Tables 1-4. For example, the mass-to-charge ratio of protein #1 in Table 1 is 1287. The mass-to-charge ratios were determined by mass spectra generated on a Ciphergen Biosystems, Inc. PBS II mass spectrometer. This instrument has a mass accuracy of about ± 0.15 percent (*e.g.*, for a 5000 Da protein, the error is ± 7.5 Da). Thus, the biomarkers herein which are referred to by a measured apparent mass are not expected to provide precisely the same apparent mass every time their presence is detected in a given sample. Additionally, the PBS II mass spectrometer has a mass resolution of about 400 to 1000 m/dm, where m is mass and dm is the mass spectral peak width at 0.5 peak height. The mass-to-charge ratio of the biomarkers was determined using BIOMARKER WIZARD software (Ciphergen Biosystems, Inc.). BIOMARKER WIZARD assigns a mass-to-charge ratio to a biomarker by clustering the mass-to-charge ratios of the same peaks from all the spectra analyzed, as determined by the PBS II mass spectrometer, taking the maximum and minimum mass-to-charge-ratio in the cluster, and dividing by two. Accordingly, the masses provided reflect these specifications.

[0070] The biomarkers of the invention are also characterized by the shape of their spectral peak in ToF-MS. A representative example of mass spectra showing peaks representing potential biomarkers of the invention is presented in Figure 1.

[0071] The biomarkers of the invention are also characterized by the source of biological sample and chromatographic fraction, if appropriate, in which the biomarker is found. Examples of biological samples containing the biomarkers of the invention include, for example and not for limitation, serum, plasma and urine. Examples of chromatographic fractions containing the biomarkers of the invention include, for example and not for

limitation, anion exchange chromatography fraction, cation exchange chromatography fraction, and size exclusion chromatographic fraction.

[0072] The biomarkers of the invention are also characterized by their binding properties on chromatographic surfaces. The biomarkers of the invention bind to cation exchange adsorbants (*e.g.*, CM10 or WCX2 PROTEINCHIP array from CIPHERGEN Biosystems, Inc.), anion exchange adsorbants (*e.g.*, SAX2 or Q10 PROTEINCHIP array from CIPHERGEN Biosystems, Inc.), hydrophobic exchange adsorbants (*e.g.*, H4 or H50 PROTEINCHIP array from CIPHERGEN Biosystems, Inc.), hydrophilic exchange adsorbants (*e.g.*, NP20 PROTEINCHIP from CIPHERGEN Biosystems, Inc.) and/or immobilized metal affinity capture (IMAC) adsorbants (*e.g.*, IMAC3 or IMAC30 PROTEINCHIP array from CIPHERGEN Biosystems, Inc.).

[0073] The biomarkers of this invention are characterized by their charge-to-mass ratio, the shape of their spectral peaks, the source of biological sample and chromatographic fraction, and their binding properties on chromatographic surfaces. Thus, a biomarker of the invention can be uniquely identified without knowledge of its specific molecular identity. However, if desired, the specific molecular identity of a biomarker of the invention can be determined by, for example, determining the amino acid sequence of the protein, *e.g.*, by peptide mapping or sequencing. For example, a biomarker can be peptide mapped using a number of proteases, such as trypsin or V8 protease, and the molecular weights of the resultant peptide digestion fragments can be used to search databases for sequences that match the molecular weights of the digestion fragments generated by the various proteases. Alternatively, biomarkers can be sequenced using tandem MS. In this method, the protein is isolated, for example, by gel electrophoresis. The protein is excised from the gel and subjected to proteolytic digestion. Individual peptide fragments are separated by MS, subjected to collision-induced cooling, further fragmenting the peptides and producing a polypeptide ladder, which is then analyzed by MS. The difference in mass of the members of the polypeptide ladder identifies the amino acids in the sequence. This method can be used to determine the entire protein sequence, or to use a sequenced peptide fragment to search databases for matching sequences.

[0074] Once the sequence of a biomarker of the invention is determined, its presence in a biological sample from an individual can be measured by methods known in the art, including for example and not for limitation methods described in paragraph [0046] above.

[0075] The biomarkers of the invention can be detected in the serum of an individual. The biomarkers of the invention can be detected in other blood fractions, *i.e.*, plasma, or in urine.

Many of the biomarkers of the invention can be found in both serum and plasma, and in urine. The biological sample can be depleted of albumin and IgG, if appropriate.

[0076] The biomarkers of the invention can exist in a biological sample from an individual in various forms with different mass-to-charges ratios. For example, different forms of biomarkers can result from either pre- or post-translational modification, or both. Pre-translational modifications include, for example, allelic variants and splice variants. Post-translation modifications include, for example, proteolytic cleavage, glycosylation, phosphorylation, lipidation, oxidation, methylation, cystinylation, sulphonation and acetylation. Once the sequence of a biomarker of the invention is determined, the presence and level of various forms of the biomarker in a biological sample from an individual can be determined by methods known in the art, as described above. In certain cases, a modified form of the biomarker may have a more pronounced difference in expression between individuals diagnosed with AMD and control individuals than its unmodified form.

III. DETECTION OF BIOMARKERS ASSOCIATED WITH AMD

[0077] AMD biomarkers can be separated and detected using any of a number of methods including immunological assays (*e.g.*, ELISA), separation-based methods (*e.g.*, gel electrophoresis), protein-based methods (*e.g.*, mass spectroscopy), function-based methods (*e.g.*, enzymatic or binding activity), and the like. Other methods will be known to those of skill in the art guided by this specification. The method used for detecting the biomarkers and determining their levels will depend, in part, on the identity and nature of the biomarker protein. Suitable methods for detecting the biomarkers of the invention include, for example and not for limitation, optical methods, including confocal and non-confocal microscopy, and detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (*e.g.*, surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry), electrochemical methods (*e.g.*, voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods (*e.g.*, multipolar resonance spectroscopy).

[0078] In one embodiment, the method for separating, detecting and determining the levels of at least two biomarkers of the invention involves obtaining a biological sample from an individual, separating the proteins by chromatography, if appropriate, capturing the proteins

on a biochip (*i.e.*, an adsorbent of a SELDI probe), and detecting and determining the levels of the captured biomarkers by mass spectrometry (*i.e.*, ToF-MS).

[0079] A biochip generally comprises a solid substrate and has a generally planar surface to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound thereto.

[0080] A “protein biochip” refers to a biochip adapted for the capture of proteins. Protein biochips are known in the art, including, for example, those produced by Ciphergen Biosystems, Inc. (Fremont, CA), Packard BioScience Company (Meriden, CT), Zyomyx (Hayward, CA), Phylos (Lexington, MA) and Biacore (Uppsala, Sweden). Examples of such protein biochips are described in, *e.g.*, U.S. Patent Nos. 6,225,047, 6,329,209 and 5,242,828, and PCT Publication Nos. WO 99/51773 and WO 00/56934.

[0081] In one embodiment, the biomarkers of the invention are detected by mass spectrometry (MS) methods. Examples of mass spectrometers are time-of-flight (ToF), magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer, and hybrids of these.

[0082] In one embodiment, the mass spectrometer is a laser desorption/ionization mass spectrometer. In laser desorption/ionization mass spectrometry, the analytes (*i.e.*, proteins) are placed on the surface of a MS probe, which engages a probe interface of the mass spectrometer and presents an analyte to ionizing energy for ionization and introduction into the mass spectrometer. A laser desorption mass spectrometer employs laser energy, typically from an ultraviolet laser, but also from an infrared laser, which desorbs the analytes from the surface, and volatilizes and ionizes the analytes, thereby making them available to the ion optics of the mass spectrometer.

[0083] A mass spectrometry method for use in the invention is “Surface Enhanced Laser Desorption and Ionization” or “SELDI,” as described, for example, in U.S. Patents Nos. 5,719,060 and 6,225,047. SELDI refers to a method of desorption/ionization gas phase ion spectrometry in which the analyte (*i.e.*, at least two of the biomarkers) is captured on the surface of a SELDI MS probe. There are several versions of SELDI, including “affinity capture mass spectrometry,” “Surface-Enhanced Affinity Capture” or “SEAC,” “Surface-Enhanced Neat Desorption” or “SEND,” and “Surface-Enhanced Photolabile Attachment and Release” or “SEPAR”.

[0084] SEAC involves the use of probes having a material on the probe surface that captures analytes (*i.e.*, proteins) through non-covalent affinity interactions (*i.e.*, adsorption) between the material and the analyte. The material is variously called an “adsorbent,” a “capture reagent,” an “affinity reagent” or a “binding moiety.” Such probes are called “affinity capture probes” having “adsorbent surfaces.” The capture reagent can be any material capable of binding an analyte. The capture reagent may be attached directly to the substrate of the selective surface, or the substrate may have a reactive surface that carries a reactive moiety capable of binding the capture reagent, *e.g.*, through a reaction forming a covalent or coordinate covalent bond. Epoxide and carbodiimidazole are useful reactive moieties to covalently bind protein capture reagents, such as antibodies or cellular receptors. Nitriloacetic acid and iminodiacetic acid are useful reactive moieties that function as chelating agents to bind metal ions that interact non-covalently with histidine containing peptides. Adsorbents are generally classified as either chromatographic adsorbents or biospecific adsorbents.

[0085] A “chromatographic adsorbent” refers to an adsorbent material typically used in chromatography. Chromatographic adsorbents include, for example, anion and cation exchange materials, metal chelators (*e.g.*, nitriloacetic acid or iminodiacetic acid), immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, simple biomolecules (*e.g.*, nucleotides, amino acids, simple sugars and fatty acids) and mixed mode adsorbents (*e.g.*, hydrophobic attraction/electrostatic repulsion adsorbents).

[0086] A “biospecific adsorbent” refers to an adsorbent comprising a biomolecule, *e.g.*, a nucleic acid molecule (*e.g.*, an aptamer), a polypeptide, a polysaccharide, a lipid, a steroid or a conjugate of these (*e.g.*, a glycoprotein, a lipoprotein, a glycolipid, or a nucleic acid (*e.g.*, DNA)-protein conjugate). In certain instances, the biospecific adsorbent can be a macromolecular structure, such as a multi-protein complex, a biological membrane or a virus. Examples of biospecific adsorbents include antibodies, receptor proteins and nucleic acids. Typically, biospecific adsorbents have higher specificity for a target analyte than chromatographic adsorbents. Further examples of adsorbents for use in SELDI can be found in U.S. Patent No. 6,225,047. A “bioselective adsorbent” refers to an adsorbent that binds to an analyte with an affinity typically of at least 10^{-8} M.

[0087] Protein biochips produced by CIPHERGEN Biosystems, Inc. comprise surfaces having chromatographic or biospecific adsorbents attached thereto at addressable locations.

Ciphergen PROTEINCHIP arrays include NP20 (hydrophilic); H4 and H50 (hydrophobic); SAX2, Q10 and LSAX30 (anion exchange); WCX2, CM10 and LWCX30 (cation exchange); IMAC3, IMAC30 and IMAC40 (metal chelate); and PS10, PS20 (reactive surface with carboimidazole, epoxide) and PG20 (protein G coupled through carboimidazole).

Hydrophobic PROTEINCHIP arrays have isopropyl or nonylphenoxy-poly(ethylene glycol)methacrylate functionalities. Anion exchange PROTEINCHIP arrays have quaternary ammonium functionalities. Cation exchange PROTEINCHIP arrays have carboxylate functionalities. Immobilized metal chelate PROTEINCHIP arrays have nitriloacetic acid functionalities that adsorb transition metal ions, such as copper, nickel, zinc, and gallium, by chelation. Preactivated PROTEINCHIP arrays have carboimidazole or epoxide functional groups that can react with groups on proteins for covalent binding.

[0088] Protein biochips are further described in U.S. Patent Nos. 6,579,719 and 6,555,813, PCT Publication Nos. WO 00/66265 and WO 03/040700, U.S. Patent Application Nos. US 20030032043 A1, US 20030218130 A1 and US 20050059086 A1.

[0089] In general, a probe with an adsorbent surface is contacted with the sample for a period of time sufficient to allow proteins present in the sample to bind to the adsorbent. After the incubation period, the substrate is washed to remove unbound material. Any suitable washing solutions can be used; preferably, aqueous solutions are employed. The extent to which proteins remain bound to the adsorbent can be manipulated by adjusting the stringency of the wash. The elution characteristics of a wash solution can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength, temperature, and the like. Unless the probe has both SEAC and SEND properties (as described herein), an energy absorbing molecule is then applied to the substrate with the bound proteins.

[0090] The biomarkers bound to the substrates are detected in a gas phase ion spectrometer such as a ToF mass spectrometer. The biomarkers are ionized by an ionization source such as a laser, the generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. The detector then translates information of the detected ions into mass-to-charge ratios. Detection of a biomarker typically involves detection of signal intensity. Thus, both the quantity and mass of the biomarker can be determined.

[0091] SEND involves the use of probes comprising energy absorbing molecules that are chemically bound to the probe surface ("SEND probe"). The phrase "energy absorbing

molecules” (EAM) denotes molecules that are capable of absorbing energy from a laser desorption/ionization source and, thereafter, contribute to desorption and ionization of analyte molecules in contact therewith. The EAM category includes molecules used in MALDI, frequently referred to as “matrix,” and is exemplified by cinnamic acid derivatives, sinapinic acid (SPA), cyano-hydroxy-cinnamic acid (CHCA) and dihydroxybenzoic acid, ferulic acid, and hydroxyaceto-phenone derivatives. In certain embodiments, the EAM are incorporated into a linear or cross-linked polymer, *e.g.*, a polymethacrylate. For example, the composition can be a co-polymer of α -cyano-4-methacryloyloxy-cinnamic acid and acrylate. In another embodiment, the composition is a co-polymer of α -cyano-4-methacryloyloxy-cinnamic acid, acrylate and 3-(tri-ethoxy)silyl propyl methacrylate. In another embodiment, the composition is a co-polymer of α -cyano-4-methacryloyloxy-cinnamic acid and octadecylmethacrylate (“C18 SEND”). SEND is further described in U.S. Patent No. 6,124,137 and PCT Publication No. WO 03/64594.

[0092] SEAC/SEND is a version of SELDI in which both a capture reagent and an EAM are attached to the sample presenting surface. SEAC/SEND probes therefore allow the capture of analytes through affinity capture and ionization/desorption without the need to apply an external matrix. The C18 SEND biochip is a version of SEAC/SEND, comprising a C18 moiety which functions as a capture reagent, and a CHCA moiety which functions as an EAM.

[0093] SEPAR involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, *e.g.*, to laser light (*see* U.S. Patent No. 5,719,060). SEPAR and other forms of SELDI are readily adapted to detecting a biomarker or biomarker profile, pursuant to the present invention.

[0094] In another MS method, the biomarkers are first captured on a resin having chromatographic properties that bind biomarkers. In the examples herein, this could include a variety of methods. For example, one could capture the biomarkers on a cation exchange resin, such as CM CERAMIC HYPERD F resin, wash the resin, elute the biomarkers and detect them by MALDI. Alternatively, this method could be preceded by fractionating the sample on an anion exchange resin, such as Q CERAMIC HYPERD F resin, before application to the cation exchange resin. In another alternative, one could fractionate the sample on an anion exchange resin and detect by MALDI directly. In yet another method, one could capture the biomarkers on an immuno-chromatographic resin comprising

antibodies that bind particular biomarkers, wash the resin to remove unbound material, elute the biomarkers from the resin and detect the eluted biomarkers by MALDI or by SELDI.

[0095] Analysis of analytes by ToF-MS generates a time-of-flight spectrum. The time-of-flight spectrum ultimately analyzed typically does not represent the signal from a single pulse of ionizing energy against a sample, but rather the sum of signals from a number of pulses. This reduces noise and increases dynamic range. This time-of-flight data is then subject to data processing using Ciphergen's PROTEINCHIP software, or any equivalent data processing software. Data processing typically includes TOF-to-M/Z transformation to generate a mass spectrum, baseline subtraction to eliminate instrument offsets and high frequency noise filtering to reduce high frequency noise.

[0096] Data generated by desorption and detection of biomarkers can be analyzed with the use of a programmable digital computer. The computer program analyzes the data to indicate the number of biomarkers detected, and optionally the strength of the signal and the determined molecular mass for each biomarker detected. Data analysis can include steps of determining signal strength of a biomarker and removing data deviating from a predetermined statistical distribution. For example, the observed peaks can be normalized, by calculating the height of each peak relative to some reference. The reference can be background noise generated by the instrument and chemicals such as the energy absorbing molecule which is set at zero in the scale.

[0097] The computer can transform the resulting data into various formats for display. The standard spectrum can be displayed, but in one useful format only the peak height and mass-to-charge information are retained from the spectrum view, yielding a cleaner image and enabling biomarkers with nearly identical molecular weights to be more easily seen. In another useful format, two or more spectra are compared, conveniently highlighting unique biomarkers and biomarkers that are up- or down-regulated between samples. Using any of these formats, one can readily determine whether a particular biomarker is present in a sample.

[0098] Analysis generally involves the identification of peaks in the spectrum that represent signal from an analyte. Peak selection can be done visually, but software is available, for example, as part of Ciphergen's PROTEINCHIP software package, which can automate the detection of peaks. In general, this software functions by identifying signals having a signal-to-noise ratio above a selected threshold and labeling the mass of the peak at the centroid of the peak signal. In one useful application, many spectra are compared to identify identical

peaks present in some selected percentage of the mass spectra. One version of this software clusters all peaks appearing in the various spectra within a defined mass range, and assigns a mass (M/Z) to all the peaks that are near the mid-point of the mass (M/Z) cluster.

[0099] Software used to analyze the data can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a peak in a signal that corresponds to a biomarker according to the present invention. The software also can subject the data regarding observed biomarker peaks to classification tree or ANN analysis, to determine whether a biomarker peak or combination of biomarker peaks is present that indicates the status of the particular clinical parameter under examination. Analysis of the data may be “keyed” to a variety of parameters that are obtained, either directly or indirectly, from the mass spectrometric analysis of the sample. These parameters include, but are not limited to, the presence or absence of at least two peaks, the shape of a peak or group of peaks, the height of at least two peaks, the log of the height of at least two peaks, and other arithmetic manipulations of peak height data.

[0100] A general protocol for the detection of biomarkers of the invention is as follows. The biological sample to be tested is obtained from consenting individuals diagnosed with AMD and control individuals, depleted of albumin and IgG or pre-fractionated on an anion exchange chromatographic resin or other chromatographic resin, as appropriate, and then contacted with an affinity capture SELDI probe comprising a cation exchange adsorbant (*e.g.*, CM10 or WCX2 PROTEINCHIP array from Ciphergen Systems, Inc.), an anion exchange adsorbant (*e.g.*, Q10 PROTEINCHIP array from Ciphergen Systems, Inc.), a hydrophobic exchange adsorbant (*e.g.*, H50 PROTEINCHIP array from Ciphergen Systems, Inc.), or an IMAC adsorbant (*e.g.*, IMAC3 or IMAC30 PROTEINCHIP array from Ciphergen Systems, Inc.). The SELDI probe is washed with a suitable buffer that retains the biomarkers of the invention, while washing away unbound biomolecules. Examples of suitable buffers are described in Examples 2-3. The biomarkers specifically retained on the SELDI probe are then detected by laser desorption/ionization mass spectrometry.

[0101] The biological sample, *e.g.*, serum, plasma or urine, can be depleted of albumin and IgG or subjected to pre-fractionation before binding to a SELDI probe. One method of pre-fractionation involves contacting the biological sample with an anion exchange chromatographic resin. The bound biomolecules are then subjected to stepwise pH elution using buffers at various pH, as described in the Examples. Various fractions containing biomolecules are collected and subjected to binding to a SELDI probe.

[0102] Alternatively, if analysis of particular proteins and various forms thereof is desired, antibodies which recognize specific proteins can be attached to the surface of a SELDI probe (e.g., pre-activated PS10 or PS20 PROTEINCHIP array from Ciphergen Systems, Inc.). The antibodies capture the target proteins from a biological sample onto the SELDI probe. The captured proteins are then detected by, e.g., laser desorption/ionization mass spectrometry. The antibodies can also capture the target proteins on immobilized support, and the target proteins can be eluted and captured on a SELDI probe and detected as described above.

[0103] Antibodies to target proteins are either commercially available or can be produced by methods known in the art, e.g., by immunizing animals with the target proteins isolated by standard purification techniques or with synthetic peptides of the target proteins.

[0104] In some cases it will be desirable to establish normal or baseline values (or ranges) for biomarker expression levels. Normal levels can be determined for any particular population, subpopulation, or group of humans according to standard methods well known to those of skill in the art. Generally, baseline (normal) levels of biomarkers are determined by quantifying the level of a biomarker in biological samples (e.g., fluids, cells or tissues) obtained from normal (healthy) subjects. Application of standard statistical methods used in medicine permits determination of baseline levels of expression, as well as significant deviations from such baseline levels.

[0105] In carrying out the diagnostic and prognostic methods of the invention, as described above, it will sometimes be useful to refer to "diagnostic" and "prognostic" values. As used herein, "diagnostic value" refers to a value that is determined for the biomarker gene product detected in a sample which, when compared to a normal (or "baseline") range of the biomarker gene product is indicative of the presence of a disease. "Prognostic value" refers to an amount of the biomarker that is consistent with a particular diagnosis and prognosis for the disease. The amount of the biomarker gene product detected in a sample is compared to the prognostic value for the biomarker such that the relative comparison of the values indicates the presence of disease or the likely outcome of the disease progression. In one embodiment, for example, to assess AMD prognosis, data are collected to obtain a statistically significant correlation of biomarker levels with different AMD classes or grades. A predetermined range of biomarker levels is established from subjects having known clinical outcomes. A sufficient number of measurements is made to produce a statistically significant value (or range of values) to which a comparison will be made.

[0106] It will be appreciated that the assay methods do not necessarily require measurement of absolute values of a biomarker, unless it is so desired, because relative values are sufficient for many applications of the methods of the present invention. Where quantification is desirable, the present invention provides reagents such that virtually any known method for quantifying gene products can be used.

IV. DIAGNOSIS OF AMD

[0107] In a first aspect, the invention provides a method for diagnosing AMD in a individual, by determining the levels of at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 biomarkers in a biological sample from the individual, and comparing the levels of the biomarkers in the biological sample from the individual to reference levels of the biomarkers characteristic of a control population, where a difference in the levels of the biomarkers between the sample from the individual and the control population indicates that the individual has AMD. The methods include obtaining a biological sample from the individual and determining the levels of the biomarkers. The levels of certain biomarkers are significantly different in individuals with AMD than in healthy individuals. The levels of certain biomarkers are higher in individuals with AMD than in healthy individuals. The levels of certain biomarkers are lower in individuals with AMD than in healthy individuals. The biomarkers can be obtained in a biological sample, and the levels of the biomarkers can be determined, by any suitable method, as described above.

[0108] As used herein, the term "diagnosis" is not limited to a definitive or near definitive determination that an individual has a disease, but also includes determining that an individual has an increased likelihood or propensity of having or developing the disease, compared to healthy individuals or to the general population. For example, a patient with very early asymptomatic disease or disease antecedents may be identified. The methods of the invention may be used for screening.

[0109] In one embodiment, a method for diagnosing AMD in a individual involves obtaining a biological sample from the individual and determining the levels of the at least two biomarkers by pre-fractionating the biomarkers in the biological sample on an anion exchange chromatographic resin, binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0110] In one embodiment, a method for diagnosing AMD in a individual involves obtaining a biological sample from the individual and determining the levels of the at least two biomarkers by binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0111] In one embodiment, a method for diagnosing AMD in a individual involves obtaining a biological sample of blood, serum, plasma or urine from the individual and determining the levels of the at least two biomarkers. The biological sample can be depleted of albumin and IgG, if appropriate.

[0112] In one embodiment, the method for diagnosing AMD involves determining the level of one biomarker. An example of a single biomarker that may be used is protein #1 (mass-to-charge ratio 1287) as shown in Table 1 in Example 5.

[0113] In one embodiment, the method for diagnosing AMD involves determining the levels of more than one biomarker. Examples of combinations or sets of biomarkers are described in paragraphs [0021] to [0030].

[0114] In one embodiment, the method for diagnosing AMD involves determining the levels of a set of biomarkers (*i.e.*, more than one biomarker). The biomarkers in a particular set may be related or grouped in a number of ways. By measuring multiple biomarkers, conclusions can be reached that are more precise and with higher confidence. The biomarkers in a set may be related by the magnitude of the difference in their levels between individuals with AMD and control individuals. For example, in one embodiment the levels of biomarkers in controls compared to AMD patients differs by a factor of at least 1.5-fold, sometime at least 2-fold and sometimes at least 2.5-fold. Other sets include biomarkers having an at least 1.25-fold, at least 3 -fold, at least 4-fold, at least 5-fold, or at least 10-fold difference between AMD patients and control individuals. In one embodiment, biomarkers in a set are related by the direction of change in AMD patients compared to controls, *i.e.*, at elevated or reduced levels, indicated as "Up" or "Down", respectively, in Tables 1-4.

[0115] In one embodiment, the method for diagnosing AMD involves determining the levels of a set of biomarkers (*i.e.*, more than one biomarker) in which all of the biomarkers in the set are present at elevated levels in individuals with AMD as compared to control individuals. Examples of such a set of biomarkers are provided in Tables 1-4 below (biomarkers indicated as "Up" in the "Up/Down" column). In one embodiment, the set of biomarkers can comprise at least 2, at least 3, at least 4, or at least 5 of the biomarkers listed as "Up" in Tables 1-4.

[0116] In one embodiment, the method for diagnosing AMD involves determining the levels of a set of biomarkers (*i.e.*, more than one biomarker) in which all of the biomarkers in the set are present at reduced levels in individuals with AMD as compared to control individuals. An example of such a set of biomarkers is provided in Tables 1-4 below (biomarkers indicated as "Down" in the "Up/Down" column). In one embodiment, the set of biomarkers can comprise at least 2, at least 3, at least 4, or at least 5 of the biomarkers listed as "Down" in Tables 1-4.

[0117] In one embodiment, the method for diagnosing AMD involves determining the levels of a set of biomarkers such as, without limitation, those sets described in Section VIII below.

V. BIOMARKERS AS SURROGATE ENDPOINTS FOR ASSESSING THE EFFICACY OF TREATMENT OF AMD

[0118] In a first aspect, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, comprising determining the levels of at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 biomarkers in a biological sample from the individual before treatment or at a first time point after treatment, and determining the levels of the biomarkers in the individual at a later time point or time points during treatment or after treatment, and comparing the levels of the biomarkers at the two or more time points. A change from a level characteristic of AMD to a more normal level is an indication of efficacy of the treatment. The methods include obtaining a biological sample from the individual and determining the levels of the biomarkers. The levels of certain biomarkers are higher in individuals with AMD than in healthy individuals. The levels of these biomarkers in an individual with AMD decrease upon treatment with an agent effective to treat AMD. The levels of certain other biomarkers are lower in individuals with AMD than in healthy individuals. The levels of these biomarkers in an individual with AMD increase upon treatment with an agent effective to treat AMD. The methods include obtaining a biological sample from the individual and determining the levels of the at least two biomarkers as above.

[0119] In one embodiment, a method for assessing the efficacy of treatment of AMD in an individual involves the individual being treated with an agent effective to treat the disease.

[0120] In one embodiment, a method for assessing the efficacy of treatment of AMD in a individual involves obtaining a biological sample from the individual and determining the

levels of the at least two biomarkers by fractionating the biomarkers in the biological sample on an anion exchange chromatographic resin, binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0121] In one embodiment, a method for assessing the efficacy of treatment of AMD in a individual involves obtaining a biological sample from the individual and determining the levels of the at least two biomarkers by binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0122] In one embodiment, a method for assessing the efficacy of treatment of AMD in a individual involves obtaining a sample of blood, serum, plasma or urine from the individual and determining the levels of the at least two biomarkers. The biological sample can be depleted of albumin and IgG, if appropriate.

[0123] In one embodiment, the method for assessing the efficacy of treatment of AMD involves determining the level of one biomarker. An example of a single biomarker that may be used is protein #1 (mass-to-charge ratio 1287) as shown in Table 1 in Example 5.

[0124] In one embodiment, the method for assessing the efficacy of treatment of AMD involves determining the levels of more than one biomarker. Examples of combinations or sets of biomarkers are described in paragraphs [0021] to [0030].

[0125] In one embodiment, the method for assessing the efficacy of treatment of AMD involves determining the levels of a set of biomarkers (*i.e.*, more than one biomarker). Sets may be defined, for example, as described above.

[0126] In a second aspect, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, comprising first determining the levels of at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 biomarkers in a biological sample from the individual at a first time point during the course of treatment with an agent, and determining the levels of the at least two biomarkers in a biological sample from the individual at multiple later time points during or after treatment with the agent, and second comparing the levels of the at least two biomarkers at the first time point and the later time point. The methods include obtaining a sample from the individual and determining the levels of the at least two biomarkers as above.

[0127] In a third aspect, the invention provides a method for assessing the efficacy of treatment of AMD in an individual, comprising comparing the levels of at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 biomarkers in a biological sample from the individual after administration of an agent to the levels of the

biomarkers in a biological sample from the same individual taken at an earlier time point and to reference levels of the biomarkers characteristic of a control population, wherein a reduced difference between the levels of the biomarkers in the individual after administration of the agent compared to the reference levels and the levels of the biomarkers in the individual taken at an earlier time point compared to the reference levels indicates that the treatment is effective. Methods to determine the reference levels of the biomarkers characteristic of a control population are well-known in the art. The methods can include obtaining a sample from the individual and determining the levels of the biomarkers as above.

[0128] In one embodiment, the method for assessing the efficacy of treatment of AMD involves determining the levels of a set of biomarkers such as, without limitation, those sets described in Section VIII below.

VI. MONITORING PROGRESSION OF AMD

[0129] In one aspect, the invention provides a method for monitoring the progression of AMD in an individual, comprising determining the levels of at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 biomarkers in a biological sample. In one embodiment, the individual is not yet under treatment for the disease. In one embodiment, the individual is under treatment with an agent effective to treat or prevent AMD, and the levels of the at least two biomarkers determine the future treatment regime for the individual. The methods include obtaining a sample from the individual and determining the levels of the at least two biomarkers as above.

[0130] In one embodiment, a method for monitoring the progression of AMD in an individual involves obtaining a biological sample from the individual and determining the levels of the at least two biomarkers by fractionating the biomarkers in the biological sample on an anion exchange chromatographic resin, binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0131] In one embodiment, a method for monitoring the progression of AMD in an individual involves obtaining a biological sample from the individual and determining the levels of the at least two biomarkers by binding the biomarkers to a SELDI probe, and detecting the bound biomarkers by laser desorption/ionization mass spectrometry.

[0132] In one embodiment, a method for monitoring the progression of AMD in an individual involves obtaining a sample of blood, serum, plasma or urine from the individual

and determining the levels of the at least two biomarkers. The biological sample can be depleted of albumin and IgG, if appropriate.

[0133] In one embodiment, the method for monitoring the progression of AMD involves determining the levels of one biomarker. An example of a single biomarker that may be used is protein #1 (mass-to-charge ratio 1287, as shown in Table 1 in Example 5).

[0134] In one embodiment, the method for monitoring the progression of AMD involves determining the levels of more than one biomarker. Examples of combinations or sets of biomarkers are described in paragraphs [0021] to [0030].

[0135] In one embodiment, the method for monitoring the progression of AMD involves determining the levels of a set of biomarkers (*i.e.*, more than one biomarker). Sets may be defined, for example, as described above.

[0136] In one embodiment, the method for monitoring the progression of AMD involves determining the levels of a set of biomarkers such as, without limitation, those sets described in Section VIII below.

VII. KITS

[0137] In one aspect, the invention provides a kit comprising a solid support comprising at least one capture reagent attached thereto, wherein the capture reagent binds at least two biomarkers selected from the biomarkers indicated in Tables 1-4, at least two biomarkers selected from the biomarkers indicated in Tables 1-4, and instructions for using the solid support to detect the biomarkers selected from the biomarkers indicated in Tables 1-4.

[0138] In various embodiments, the kit contains a combination of biomarkers, for example, as described in paragraphs [0021] to [0030] above.

VIII. EXEMPLARY SETS OF BIOMARKERS

[0139] The invention provides methods for diagnosing age-related macular degeneration (AMD) (see Section IV, *supra*), for assessing the efficacy of treatment of AMD (see Section V, *supra*), and for monitoring the progression of AMD (see Section VI, *supra*), in an individual. The invention also provides kits useful for diagnosing AMD, assessing the efficacy of treatment of AMD, and monitoring the progression of AMD (see Section VII, *supra*). The methods and kits use at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 biomarkers that are associated with AMD listed in Tables 1-4.

[0140] The experiments described in the Examples below were used to identify and characterize the biomarkers of the invention.

EXAMPLE 1

Collection and Preparation of Serum, Plasma and Urine Samples

[0141] The following protocols are used to collect and prepare the biological samples obtained from individuals to identify and characterize the biomarkers of the invention.

[0142] *Serum and Plasma.* Blood is drawn from individuals into one tube each of Grey Top (sodium fluoride/potassium oxalate) and Red Top (empty) to prepare plasma and serum, respectively. The tubes are stored upright in a refrigerator until ready for processing. A preferred storage time is less than 1 hour. Blood in the Red Top tubes is allowed to coagulate for 1 hour at room temperature (RT), and then is centrifuged at 1500g for 10 min at RT. The supernatant is aspirated into separate tubes and centrifuged again at 3000g for 10 min at RT. The resulting supernatant is divided into following aliquots: 4 x 25 μ L, 2 x 100 μ L, and 2 x 250 μ L in Eppendorf tubes. The remaining supernatant is divided in 500 μ L aliquots. This aliquot scheme can be modified depending on whether native serum or plasma is used, or if depleted or pre-fractionated serum or plasma is used. All tubes are labeled, flash frozen in LN₂ and stored at -80°C.

[0143] *Urine.* Urine is obtained from individuals and centrifuged at 16,000g for 10 min at 4°C, and the supernatant is aliquoted into 10 x 500 μ L Eppendorf tubes. The remainder of the supernatant is divided into 15 mL culture tubes. All tubes are frozen at -80°C until analysis.

EXAMPLE 2

Processing of Serum, Plasma and Urine Samples

[0144] The following protocols are used to process the serum, plasma and urine samples obtained from individuals to identify and characterize the biomarkers of the invention.

[0145] The required number of aliquots are thawed at RT and centrifuged at 10,000g for 2 min at RT. The supernatant is aspirated into separate tubes for further processing.

[0146] *Native Serum or Plasma.* Serum or plasma samples are denatured by diluting 1:5 in extraction buffer (9M Urea, 2% CHAPS, 2.3% DTT, 50mM Tris-HCl pH 9) (10 μ L serum or plasma + 40 μ L extraction buffer) and incubating for 30 minutes at RT with shaking.

Alternatively, serum or plasma samples are denatured by diluting 1:5 in Hepes buffer (10 μ L

serum + 40 μ L buffer). A portion of the diluted, denatured serum or plasma samples are further diluted 1:20 in Hepes buffer (5 μ L of 1:5 dilution + 100 μ L Hepes buffer) to yield a final 1:100 diluted serum or plasma sample to be used for protein determination. The 1:5 diluted, denatured serum or plasma samples are further diluted 1:5 in various buffers (1:25 total dilution) for binding onto different biochip surfaces under different binding conditions.

[0147] *Albumin and IgG Depletion of Serum.* Undiluted serum or plasma is used for albumin and IgG depletion using the Aurum Serum Protein Mini Kit (Bio-Rad). A spin column is filled with Affi-Gel Blue (to bind Albumin) and Affi-Gel Protein A (to bind IgG) resins, and placed in a 12x75mm test tube. The resins are allowed to settle for at least 5 min. The tip is broken off the bottom of the column, the cap removed, and the column is placed back in the test tube. Residual buffer is drained from the column by gravity flow. The column is washed twice with 1 ml of protein-binding buffer supplied with the kit. The column is drained completely each time. The column is placed in a 2 ml collection tube and centrifuged for 20 seconds at 10,000g to dry the resins. A yellow column tip is placed on the bottom of the column to stop flow, and the column is placed in a clean 2 ml collection tubes labeled "unbound". In a separate tube, 60 μ L of serum or plasma is mixed thoroughly with 180 μ L of protein-binding buffer, and 200 μ L of the diluted sample is added to the top of the resins bed. After allowing the samples to penetrate the resins, the column is gently vortexed. The vortexing step is repeated at 5 min and 10 min. The column is allowed to sit for another 5 min. The yellow tip is removed, and the column is centrifuged for 20 sec at 10,000g. The eluate was collected in the 2 ml tube labeled "unbound". The resins are washed with 200 μ L of binding buffer, vortexed gently and centrifuged for 20 sec at 10,000g. The eluate was collected in the same tube as above. This tube now contains 400 μ L of albumin- and IgG-depleted samples. The yield should be about 1.5 to 2 mg/ml protein. The bound albumin and IgG can be recovered from the column. For 1-D analysis, the column can be eluted with 500 μ L of Laemmli sample buffer (62.5mM Tris-Hcl pH 6.8; 10% glycerol; 2% SDS; 1mg/ml DTT; 0.05% Bromophenol Blue). For 2-D analysis, the column can be eluted with 500 μ L of ReadyPrep sequential extraction reagent 3 (Bio-Rad). The albumin- and IgG-depleted serum is denatured by diluting 2X in extraction buffer (9M Urea, 2% CHAPS, 2.3% DTT, 50mM Tris-HCl pH 9) (e.g., 50 μ L depleted serum + 50 μ L buffer) and incubating 30 min at RT with shaking.

[0148] *Protein Assay.* Protein concentrations in serum and plasma samples are measured using any protein assay procedure, *e.g.*, the Micro BCA Method (Pierce) or Coomassie Plus (Bradford) Method (Pierce), following the manufacturer's instructions.

[0149] *Serum Pre-fractionation.* If desired, serum is pre-fractionated on an anion exchange resin according to the protocol provided with the Ciphergen, Inc. EDM-Serum Fractionation Kit. Buffers needed for this protocol include: U9 Buffer (9M urea, 2% CHAPS, 50mM Tris-HCl pH 9); Rehydration Buffer, (50mM Tris-HCl, pH 9); Wash buffer 1 (50mM Tris-HCl with 0.1% OGP pH 9); Wash buffer 2 (50mM Hepes with 0.1% OGP pH 7); Wash buffer 3 (100mM NaAcetate with 0.1% OGP pH 5); Wash buffer 4 (100mM NaAcetate with 0.1% OGP pH 4); Wash buffer 5 (50mM NaCitrate with 0.1% OGP pH 3); and Wash buffer 6 (33.3% isopropanol/16.7% acetonitrile/0.1% trifluoroacetic acid). Wash buffer 6 should not be aliquoted for use until Wash buffer 5 has been applied to the resin to avoid evaporation of the volatile organic solvent. Materials needed for this protocol include: a 96-well filtration plate filled with dehydrated Q CERAMIC HYPERD F sorbent; microplate sealing strips; a v-bottom 96-well microplate labeled "samples"; v-bottom 96-well microplates labeled F1 through F6; 96-well microplate for collection of waste; adhesive sealing film for microplates (*e.g.*, E&K Scientific Cat. No. T396100); a 12 column, partitioned buffer reservoir (*e.g.*, Innovative Microplate Cat. No. S30019); pipette tips; a MicroMix 5 or equivalent mixer; a BIOMEK 2000 Laboratory Automation Workstation with PROTEINCHIP Biomarker Integration Package (optional); and a vacuum manifold (for manual use). When using a bioprocessor, make sure there are no air bubbles in the wells. To avoid introducing bubbles, the pipette tip is lowered very close to the spot surface while dispensing samples. The wells are completely emptied between washes. To ensure thorough mixing of sample with anion exchange resin, the 96 well plate was centrifuged at low speed for a few minutes.

[0150] The serum samples are thawed to ambient temperature, and then centrifuged at 20,000g for 10 min at 4°C. 20µL of serum sample is aliquoted to each well of a standard v-bottom 96-well microplate. 30µL of U9 Buffer is added to each well, the microplate is covered with adhesive sealing film and mixed on the MicroMix 5 (set at 20, 5, 20) or equivalent mixer for 20 min at 4°C.

[0151] The filtration plate is tapped on the bench several times to make sure that all of the dry Q HYPERD F beads settled to the bottom of the plate. The filtration plate is taken out of the pouch and the top seal on the filtration plate is carefully removed. With an 8-channel pipette, 200µL of Rehydration Buffer is added to each well. The filtration plate is mixed on

the MicroMix 5 (set at 20, 7, 60) or equivalent mixer for 60 min at RT. The waste collection plate is placed underneath the filtration plate and a vacuum is applied to remove the buffer from the filtration plate. 200 μ L of Rehydration Buffer is added to each well, and a vacuum is applied to remove the buffer in the filtration plate. This step is repeated three times, followed by washing the Q HYPERD F beads with U1 Solution (1:9 dilution of U9 Buffer in Rehydration Buffer). Q HYPERD F beads are stored in 50mM Tris-HCl pH 9 in a 50% suspension, equilibrated by adding 125 μ L Q HYPERD F beads to each well in the filter plate and then filtering the buffer, adding 150 μ L U1 Solution to each well and then filtering the buffer. The U1 Solution wash is repeated three times.

[0152] 50 μ L of sample from each well of the sample microplate is transferred to the corresponding well in the 96-well filtration plate. 50 μ L of U1 Solution is added to each well of the sample microplate, mixed 5 times, and then transferred to the corresponding well in the 96-well filtration plate. The filtration plate is covered adhesive sealing film and mixed on MicroMix 5 (set at 20, 7, 30) or equivalent mixer for 30 min at 4°C.

[0153] Fraction 1 is prepared by placing the 96-well microplate labeled F1 underneath the filtration plate, applying a vacuum and collecting the flow through into the F1 plate, adding 100 μ L of Wash Buffer 1 to each well of the filtration plate, mixing for 10 min on MicroMix 5 (set at 20, 7, 10) or equivalent mixer at RT, and applying a vacuum and collecting the eluant into the F1 plate. Fraction 1 contains the flow-through and the pH 9 eluant.

[0154] Fraction 2 is prepared by adding 100 μ L of Wash Buffer 2 to each well of the filtration plate, mixing for 10 min on MicroMix 5 (set at 20, 7, 10) or equivalent mixer at RT, placing the 96-well microplate labeled F2 underneath the filtration plate, and applying a vacuum and collecting the eluant into the F2 plate. This step is repeated once. Fraction 2 contains the pH 7 eluant.

[0155] Fraction 3 is prepared by adding 100 μ L of Wash Buffer 3 to each well of the filtration plate, mixing for 10 min on MicroMix 5 or equivalent mixer (set at 20, 7, 10) at RT, placing the 96-well microplate labeled F3 underneath the filtration plate, and applying a vacuum and collecting the eluant into the F3 plate. This step is repeated once. Fraction 3 contains the pH 5 eluant.

[0156] Fraction 4 is prepared by adding 100 μ L of Wash Buffer 4 to each well of the filtration plate, mixing for 10 min on MicroMix 5 (set at 20, 7, 10) or equivalent mixer at RT, placing the 96-well microplate labeled F4 underneath the filtration plate, and applying a

vacuum and collecting the eluant into the F4 plate. This step is repeated once. Fraction 4 contains the pH 4 eluant.

[0157] Fraction 5 is prepared by adding 100 μ L of Wash Buffer 5 to each well of the filtration plate, mixing for 10 min on MicroMix 5 (set at 20, 7, 10) or equivalent mixer at RT, placing the 96-well microplate labeled F5 underneath the filtration plate, and applying a vacuum and collecting the eluant into the F5 plate. This step is repeated once. Fraction 5 contains the pH 3 eluant.

[0158] Fraction 6 is prepared by adding 100 μ L of Wash Buffer 6 to each well of the filtration plate, mixing for 10 min on MicroMix 5 (set at 20, 7, 10) or equivalent mixer at RT, placing the 96-well microplate labeled F6 underneath the filtration plate, and applying a vacuum and collecting the eluant into the F6 plate. This step is repeated once. Fraction 6 contains the organic solvent eluant.

[0159] The six collection microplates are stored until proceeding with the PROTEINCHIP Array binding or equivalent protocol. If the samples are to be analyzed within 24 hours, store at 4°C, longer term storage should be at -20°C.

EXAMPLE 3

Preparation of Biochips

[0160] The following protocols are used to prepare the biochips used to identify and characterize the biomarkers of the invention.

[0161] *IMAC-Cu CHIP Spot Protocol.* If needed, each spot of the array is outlined with a PAP wax pen and allowed to air dry. 5 μ L of 100 mM copper sulfate is loaded onto each spot and incubated in a humidity chamber for 15 min. The solution is not allowed to dry. The loading process is repeated once. The loaded array is rinsed in running DW for about 10 sec. to remove excess copper. The spots are then rinsed (pipetting and aspirating) with an excess (5 to 10 μ L) of 50mM sodium acetate, pH 4 followed by aspiration. The array is rinsed in running DW for about 10 sec. 5 μ L of 0.5M NaCl in PBS (binding buffer) is added to each spot, incubated for 5 min, and then excess buffer is removed by aspiration without touching the active surface. Samples are diluted 5X with 0.5 MNaCl in PBS (binding buffer) and 2 to 3 μ L sample is applied per spot. The array is incubated in a humidity chamber for 30 min at RT, then the array is washed 5 times (pipetting & aspirating) with 5 μ L binding buffer, followed by washing twice (pipetting & aspirating) with 5 μ L DW. The array is tapped on the benchtop to remove excess water droplets, then wiped dry around the spots, taking care not to

smudge the wax circles. The EAM is applied while the spots are still moist following the procedure below.

[0162] *IMAC30 PROTEINCHIP Array.* The IMAC30 PROTEINCHIP Array is placed into the Bioprocessor (Ciphergen, Inc., Cat. No. C503-0008 - 8-well, C503-0006 - 96-well) and 50 μ L of 0.1M CuSO₄ is added to each well (volumes are adjusted depending on whether an 8 or 16 spot array is used), and incubated for 10 min at RT with vigorous shaking (*e.g.*, 250 rpm, or on a MicroMix, setting 20/7). Immediately after removing the copper solution from the wells, 150-250 μ L of de-ionized (DI) water is added to each well, and incubated for 2 min at RT with vigorous shaking. This step is repeated once. Immediately after removing the DI water from the wells, 150-250 μ L of 0.1M sodium acetate buffer pH 4 (neutralization buffer) is added to each well, and incubated for 5 min at RT with vigorous shaking. Immediately after removing the buffer solution from the wells, 150-250 μ L of DI water is added to each well, and incubated for 2 min at RT with vigorous shaking. Immediately after removing the DI water from the wells, 150-250 μ L of binding buffer is added to each well, and incubated for 5 min at RT with vigorous shaking. Immediately after removing the binding buffer from the wells, 50-150 μ L of sample (fractions diluted 1:5 with 0.5M NaCl in PBS binding buffer; the total protein concentration was 50–2000 μ g/mL in binding buffer) is added to each well, and incubated 30 min at RT with vigorous shaking. After removing the samples from the wells, the wells are washed with 150-250 μ L binding buffer for 5 min with agitation. This step is repeated twice. The wells are drained, and the array is removed from the Bioprocessor and allowed to air dry for 15-20 min. 1 μ L EAM solution is applied per spot (two applications of EAM solution can be used in order to increase the peak intensity), and allowed to air dry.

[0163] *CM10 PROTEINCHIP Array.* Buffers needed for this protocol include: Binding buffer (100mM sodium acetate, pH 6, 50mM Tris-base, pH 8.5, or 50mM Tris-HCl, pH 9.5); Sodium/ammonium acetate buffer (10–100mM), pH 4–6; Ammonium phosphate buffer (10–100mM), pH 6–8; HEPES Buffer, pH 7 (50 mM); Tris-HCl buffer (10–100mM), pH 7.5–9. The CM10 PROTEINCHIP Array is placed in the Bioprocessor (Ciphergen, Inc., Cat. No. C503-0008, 8-well; C503-0006, 96-well) and 150–250 μ L Binding buffer is added to each well, and incubated for 5 min at RT with vigorous shaking (*e.g.*, 250 rpm, or on a MicroMix1, setting 20/7). This washing step is repeated once. Immediately after removing the Binding buffer, 50–150 μ L sample (50–2000 μ g/mL total protein, diluted in Binding buffer) is added to each well, and incubated 30 min at RT with vigorous shaking. After

removing the samples from the wells, the wells were washed with 150–250 μ L Binding buffer for 5 min at RT, with agitation. This step is repeated twice. After removing the Binding buffer from the wells, the wells are washed with 150–250 μ L DI water for 5 min at RT, with agitation. The wells are drained, and the array is removed from the Bioprocessor and allowed to air dry for 15–20 min. 1 μ L SPA solution is applied per spot. After 5 min, a second 1 μ L of SPA is applied per spot, and allowed to air dry.

[0164] *EAM Preparation and Application.*

[0165] *Saturated CHCA.* A premixed solution of 100 μ L ACN + 100 μ L 1% TFA) is added to a pre-weighed CHCA tube, vortexed for 5 min, and centrifuged for 1 min at 10,000g. The supernatant is removed and diluted with an equal volume of ACN + 1% TFA. 1 μ L CHCA is added to each spot and allowed to air dry. This step can be repeated once. 1 μ L of 50%, 25% or 10% saturated CHCA solution can be used to detect biomarkers of lower masses.

[0166] *50% Saturated SPA.* A premixed solution of 200 μ L ACN + 200 μ L 1% TFA is added to a pre-weighed SPA tube, and vortexed for 5 min. 1 μ L 50% SPA is added to each spot, and allowed to air dry. This step is repeated once.

EXAMPLE 4

Data Collection and Analysis

[0167] The following protocols are used in preparation for collecting and analyzing the data leading to the identification and characterization of the biomarkers of the invention.

[0168] *Data Collection.*

[0169] 1. Check laser energy and use signal enhancer. Two laser energies are used to read the arrays. A low laser energy allows peaks in the low mass range (2–20 kDa) to be well visualized, while a high laser energy improves visualization of peaks in the high mass range (>20 kDa). The signal enhancer features of PROTEINCHIP Software 3.x can be turned on to further improve visualization of higher mass species in all acquired spectra.

[0170] 2. Check the appearance of spectra. The intensity and shape of the peaks should be noted. Peaks with flat tops or with non-normalized, baseline subtracted laser intensities greater than 60 generally are unreliable, since individual laser shots were probably off-scale.

[0171] 3. Perform a pre-qualification run. The PROTEINCHIP Reader parameters that require the most characterization are laser energy, detector sensitivity, and detector voltage. Spot protocols including specific energy settings should be determined by performing a pre-qualification run prior to the start of the study. A pre-qualification run consists of spotting a

standard sample (generally the same one used for monitoring of the project) onto a series of arrays and reading these at a range of laser energies and detector sensitivities and voltages.

[0172] *Data Analysis.*

[0173] 1. Choose five calibrants. Mass calibration should be performed by using five calibrants in the mass range of interest. Different calibrants should be used for the low mass range versus the high mass range.

[0174] 2. Normalize intensity values. Total ion current normalization should be used to normalize intensity values. Use a mass range appropriate to the analysis, but always omit the matrix region.

[0175] 3. Match time lag settings. Acquire data for calibration at the various time lag focusing settings that match the actual time lag focusing settings used to read the arrays containing the samples (and the Detector voltage settings should you end up changing this between spot protocols).

[0176] 4. Choose baseline subtraction setting. Use a baseline subtraction setting of eight times the fitting width.

[0177] 5. Find peaks. Data analysis requires a series of preprocessing steps, including baseline subtraction, mass calibration and total ion current normalization. Once these have been performed the true data analysis can be done, *i.e.*, finding peaks and determining their value in classifying samples. Spectra that do not show good binding of sample should be considered as unrepresentative and therefore not included in the analysis.

[0178] *Sequence of Steps During Preparation Phase.* Based on information collected so far, the preparation for experiments can be conducted per the following scheme.

[0179] 1. Detector. Voltage is optimized using IgG QC chips. Optimization is performed periodically (*e.g.*, weekly).

[0180] 2. Mass calibration. Spot one complete NP20 A-P array with protein MW standard, using SPA for > 20 KDa mass calibration, and verify spot-to- spot calibration. Spot one complete NP20 A-P array with peptide MW standard, using CHCA for < 10 kDa mass calibration, and verify spot-to-spot calibration. The mass calibration is performed each time chips are analyzed on the Bioprocessor.

[0181] 3. Test dilution effect. With a pooled serum sample, deplete albumin and IgG or pre-fractionate per procedures outlined above if desired, then assay protein content. Use “extraction buffer” to perform 1:1, 1:2, 1:5, 1:10 dilutions, spot samples onto various chip

arrays, use CHCA and SPA with low and high laser settings to analyze spots, and choose the best dilution for each chip surface and EAM.

[0182] 4. Test binding conditions I. With 2 control and 2 test serum samples, deplete albumin and IgG or pre-fractionate per procedures outlined above if desired, then assay protein content. Dilute the samples per results from step 3 above. Using selected chip arrays, choose different binding conditions, use CHCA and SPA, optimize low and high energy laser settings and sensitivity with previously determined detector voltage in step 1 above, and choose the best binding conditions for each chip surface and EAM.

[0183] 5. Test binding conditions II. With 4 control and 4 test serum samples; including those analyzed in step 4 above, spot in duplicate. Deplete albumin and IgG or pre-fractionate per procedures outlined above if desired, then assay protein content. Dilute the samples per results from step 3 above. Using binding conditions for each chip surface and EAM as determined in step 4 above, optimize low and high energy laser settings and sensitivity with previously determined detector voltage, check for reproducibility (including spectra from step 4), and choose and save optimized MS settings.

EXAMPLE 5

Serum Biomarkers for Age-Related Macular Degeneration (AMD)

[0184] The following protocol was used to generate mass spectra from the serum of 21 individuals, 13 of whom were diagnosed with AMD and 8 of whom were age-matched controls.

[0185] *Biochip Binding Protocol.* The IMAC30 PROTEINCHIP Array (Ciphergen, Inc.) was prepared as described in Example 3. Serum samples were bound to the IMAC30 Cu array basically as described in Example 3.

[0186] Energy absorbing molecules (EAM), frequently referred to as "matrix," were added to the IMAC30 Cu array as follows. The bioprocessor's top and gasket were removed and the array was allowed to air dry. For the cyano-hydroxy-cinnamic acid (CHCA) matrix, 1 μ L of 50% CHCA dissolved in 50% Acetonitrile + 0.25% TFA was added to each spot in the array and allowed to air dry. 1 μ L of 35% CHCA was added to each spot and allowed to air dry. For the sinapinic acid (SPA) matrix, 1 μ L 50% SPA in 50% Acetonitrile and 0.5% TFA was added to each spot in the array and allowed to air dry. 1 μ L 50% SPA was added to each spot and allowed to air dry.

[0187] *Data Acquisition Settings.* The conditions for data acquisition for the IMAC 30 Cu array were determined following the protocols described in Example 4.

[0188] *Identification of Biomarkers.* The spectra obtained were analyzed by standard mass spectroscopy analytic methods, *e.g.*, using the CIPHERGEN EXPRESS Data Manager Software with BIOMARKER WIZARD and Biomarker Pattern Software from Ciphergen Biosystems, Inc. The mass spectra for each group were subjected to scatter plot analysis. A Student's t test analysis was employed to compare AMD and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p < 0.05$ or $p < 0.1$ as indicated) between the two groups.

[0189] Examples of the biomarkers thus discovered are presented in Table 1 below. The "Assay" column refers to the type of biochip to which the biomarkers bound, the type of EAM used with the biochip, and the laser energy used to ionize the biomarkers.

Table 1. Serum Biomarkers Associated with AMD

No.	Mass	Assay	P-value	Up/Down
1	1287	IMAC-Cu 35% CHCA Low Laser	< 0.05	↑
2	2926	"	< 0.1	↓
3	3307	"	< 0.1	↓
4	3329	"	< 0.05	↓
5	3350	"	< 0.05	↓
6	3452	"	< 0.05	↓
7	4094	"	< 0.1	↓
8	4627	"	< 0.05	↓
9	6233	IMAC-Cu SPA Low Laser	< 0.05	↑
10	6516	"	< 0.1	↓
11	9250	"	< 0.1	↓
12	9452	"	< 0.05	↓
13	9593	"	< 0.05	↓
14	9664	"	< 0.05	↓
15	11445	"	< 0.1	↑
16	13933	"	< 0.1	↓
17	39577	"	< 0.1	↓
18	55985	"	< 0.1	↓
19	60308	"	< 0.1	↓
20	12811	IMAC-Cu SPA High Laser	< 0.1	↑
21	27921	"	< 0.1	↓
22	59581	"	< 0.1	↓
23	157301	"	< 0.1	↓

EXAMPLE 6

Serum and Plasma Biomarkers for Age-Related Macular Degeneration (AMD)

[0190] The following protocol was used to generate mass spectra from the serum and plasma of 21 individuals, 14 of whom were diagnosed with AMD and 7 of whom were age-matched controls.

[0191] *Depletion of Albumin and IgG.* Pre-fractionation to deplete albumin and IgG from the serum and plasma was performed as described in Example 2.

[0192] *Biochip Binding Protocol.* The IMAC30 and CM10 PROTEINCHIP Arrays (Ciphergen, Inc.) were prepared as described in Example 3. Serum and plasma samples were bound to IMAC30 and CM10 arrays basically as described in Example 3. The pH4 fraction for the CM10 array was prepared as described in Example 2.

[0193] EAM or matrix (SPA) were added to the IMAC30 Cu array as described in Example 5. EAM or matrix were added to the CM10 array as follows. The bioprocessor's top and gasket were removed and the array was allowed to air dry. For the SPA matrix, 400 μ L of 50% acetonitrile, 0.5% TFA were added to a SPA tube and mixed for 5' at RT. 1 μ L of the mixture was added to each spot in the array and allowed to air dry. This step was repeated once.

[0194] *Data Acquisition Settings.* The conditions for data acquisition for the IMAC30 Cu array or the CM10 array were determined following the protocols described in Example 4.

[0195] *Identification of Biomarkers.* The spectra obtained were analyzed as described in Example 5. A Student's t-test analysis was employed to compare AMD and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p < 0.05$ or $p < 0.1$, as indicated) between the two groups.

[0196] Examples of albumin and IgG depleted biomarkers thus discovered are presented in Tables 2 (serum) and 3 (plasma) below. The "Assay" column refers to the type of biochip to which the biomarkers bound, the type of EAM used with the biochip, and the laser energy used to ionize the biomarkers.

Table 2. Albumin and IgG Depleted Serum Biomarkers Associated with AMD

No.	Mass	Assay	P-value	Up/Down
1	3039	IMAC-Cu SPA Low Laser	< 0.1	↑
2	3070	"	< 0.05	↑
3	3216	"	< 0.05	↑
4	3402	"	< 0.05	↑
5	4303	"	< 0.1	↓
6	4951	"	< 0.05	↑
7	4976	"	< 0.05	↑
8	8858	"	< 0.05	↑
9	9268	"	< 0.1	↑
10	11834	"	< 0.05	↑
11	72818	"	< 0.1	↑
12	12577	IMAC-Cu SPA High Laser	< 0.05	↓
13	14003	"	< 0.1	↓
14	36008	"	< 0.05	↑
15	56020	"	< 0.1	↓
16	148144	"	< 0.1	↑
17	163398	"	< 0.1	↓
18	199667	"	< 0.1	↓
19	3003	CM10 pH4 SPA Low Laser	< 0.05	↑
20	3061	"	< 0.05	↑
21	3169	"	< 0.1	↓
22	3176	"	< 0.1	↓
23	3346	"	< 0.1	↑
24	3691	"	< 0.1	↓
25	4088	"	< 0.1	↓
26	4144	"	< 0.05	↑
27	4300	"	< 0.1	↑
28	4490	"	< 0.05	↑
29	4603	"	< 0.05	↓
30	4775	"	< 0.05	↑
31	5816	"	< 0.05	↓
32	5873	"	< 0.1	↓
33	6823	"	< 0.05	↓
34	6992	"	< 0.1	↑
35	7698	"	< 0.1	↓
36	9211	"	< 0.1	↓
37	10218	CM10 pH4 SPA High Laser	< 0.1	↓
38	14628	"	< 0.1	↓

Table 3. Albumin and IgG Depleted Plasma Biomarkers Associated with AMD

No.	Mass	Assay	P-value	Up/Down
39	3029	IMAC-Cu SPA Low Laser	< 0.1	↓
40	3123	"	< 0.05	↑
41	3211	"	< 0.1	↓
42	3247	"	< 0.1	↓
43	3498	"	< 0.05	↑
44	3740	"	< 0.1	↑
45	3925	"	< 0.1	↑
46	3955	"	< 0.1	↑
47	3990	"	< 0.05	↑
48	4006	"	< 0.1	↑
49	4200	"	< 0.1	↑
50	4632	"	< 0.05	↓
51	5691	"	< 0.05	↑
52	5850	"	< 0.05	↓
53	6405	"	< 0.05	↓
54	7698	"	< 0.1	↓
55	7768	"	< 0.1	↓
56	8880	"	< 0.1	↓
57	11468	"	< 0.05	↑
58	14579	"	< 0.1	↑
59	18963	IMAC-Cu SPA High Laser	< 0.1	↓
60	3052	CM10 pH4 SPA Low Laser	< 0.1	↓
61	3062	"	< 0.1	↑
62	3092	"	< 0.1	↑
63	3105	"	< 0.1	↑
64	3160	"	< 0.05	↑
65	3257	"	< 0.1	↑
66	3284	"	< 0.1	↓
67	3396	"	< 0.05	↑
68	3458	"	< 0.1	↓
69	3509	"	< 0.1	↑
70	3532	"	< 0.1	↑
71	3537	"	< 0.1	↑
72	3600	"	< 0.05	↑
73	3681	"	< 0.05	↑
74	3708	"	< 0.05	↑
75	3867	"	< 0.05	↑
76	3943	"	< 0.05	↓
77	3997	"	< 0.05	↑
78	4152	"	< 0.1	↑
79	4529	"	< 0.1	↑
80	4550	"	< 0.1	↓
81	6987	"	< 0.05	↑

No.	Mass	Assay	P-value	Up/Down
82	33117	"	< 0.05	↑
83	39724	"	< 0.1	↑
84	145931	"	< 0.05	↓
85	23489	CM10 pH4 SPA High Laser	< 0.1	↓
86	58655	"	< 0.05	↑
87	60449	"	< 0.05	↑

EXAMPLE 7

Urine Biomarkers for Age-Related Macular Degeneration (AMD)

[0197] The following protocol was used to generate mass spectra from the urine of 21 individuals, 14 of whom were diagnosed with AMD and 7 of whom were age-matched controls.

[0198] *Biochip Binding Protocol.* The IMAC30 and CM10 PROTEINCHIP Arrays (Ciphergen, Inc.) were prepared as described in Example 3. Urine samples were bound to IMAC30 Cu and CM10 arrays basically as described in Example 3. The pH 4 fraction for the CM10 array was prepared as described in Example 2.

[0199] EAM or matrix (CHCA) were added to the IMAC30 Cu array as described in Example 5, except the concentrations of CHCA were 35% and 15%. EAM or matrix (SPA) were added to the CM10 array as described in Example 6.

[0200] *Data Acquisition Settings.* The conditions for data acquisition for the IMAC30 Cu array and the CM10 array were determined following the protocols described in Example 4.

[0201] *Identification of Biomarkers.* The spectra obtained were analyzed as described in Example 5. A Student's t-test analysis was employed to compare AMD and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p < 0.05$ or $p < 0.1$, as indicated) between the two groups.

[0202] Examples of the biomarkers thus discovered are presented in Table 4 below. The "Assay" column refers to the type of biochip to which the biomarkers bound, the type of EAM used with the biochip, and the laser energy used to ionize the biomarkers.

Table 4. Urine Biomarkers Associated with AMD

No.	Mass	Assay	P-value	Up/Down
1	3856	CM10 pH4 SPA	< 0.1	↑
2	5619	"	< 0.1	↑
3	5704	"	< 0.1	↑
4	19089	"	< 0.1	↓

No.	Mass	Assay	P-value	Up/Down
5	1914	IMAC-Cu 35% CHCA	< 0.1	↓
6	2564	"	< 0.05	↓
7	2663	"	< 0.1	↓
8	3027	"	< 0.05	↓
9	4602	"	< 0.1	↑
10	4700	"	< 0.1	↓
11	5916	"	< 0.05	↓
12	6189	"	< 0.05	↓
13	6316	"	< 0.05	↓
14	6864	"	< 0.05	↓
15	9625	"	< 0.1	↑
16	9866	"	< 0.1	↓
17	11724	"	< 0.05	↓
18	11783	"	< 0.05	↓
19	164	IMAC-Cu 15% CHCA	< 0.1	↓
20	203	"	< 0.1	↑
21	643	"	< 0.1	↑

EXAMPLE 8

Biomarkers Associated with Age-Related Macular Degeneration (AMD) and Abdominal Aortic Aneurysm (AAA)

[0203] An abdominal aortic aneurysm (AAA) is a vascular disorder involving swelling or stretching of the abdominal aorta that supplies blood to the abdomen, pelvis and legs. The serious medical risk is rupture of the aorta, which causes severe pain, internal bleeding and, absent prompt treatment, death. Aneurysms are also a source of blood clots, which can cause many complications, including a heart attack or stroke. AAA develops slowly over time and is most common in older individuals, with the average age at diagnosis being 65-70 years. Risk factors for AAA include high blood pressure, smoking, cholesterol and obesity. AAA is currently diagnosed by abdominal ultrasound, abdominal CT scanning and aortic angiography.

[0204] Some individuals that have been diagnosed with AMD also have been diagnosed with AAA. As described below, it has been found that several biomarkers are present at different levels in the serum, plasma or urine of individuals diagnosed with both AMD and AAA, compared to age-matched controls. Such biomarkers may be useful to diagnose individuals as having both AMD and AAA. This information may be useful in designing treatment strategies for AAA/AMD patients.

[0205] The following protocol was used to generate mass spectra from: (A) the serum of 15 individuals, 7 of whom were diagnosed with both AAA and AMD and 8 of whom were age-

matched controls (Table 5, Nos. 1-32); (B) the urine of 14 individuals, 7 of whom were diagnosed with both AAA and AMD and 7 of whom were age-matched controls (Table 5, Nos. 33-58); and (C) the serum and plasma of 14 individuals, 7 of whom were diagnosed with both AAA and AMD and 7 of whom were age-matched controls (Table 5, Nos. 59-192).

[0206] *Depletion of Albumin and IgG.* Pre-fractionation to deplete albumin and IgG from the serum and plasma in (C) was performed as described in Example 6.

[0207] *Biochip Binding Protocol.* The IMAC30 and CM10 PROTEINCHIP Arrays (Ciphergen, Inc.) were prepared as described in Example 3. Serum and plasma samples were bound to the IMAC30 Cu and CM10 arrays basically as described in Example 3. The pH 4 fraction for the CM10 array was prepared as described in Example 2.

[0208] EAM or matrix (CHCA or SPA) were added to the IMAC30 Cu array and to the CM10 array as described in Example 5. The final CHCA concentration was 35% or 15%, as indicated in Table 5.

[0209] *Data Acquisition Settings.* The conditions for data acquisition for the IMAC30 Cu array and the CM10 array were determined following the protocols described in Example 4.

[0210] *Identification of Biomarkers.* The spectra obtained were analyzed as described in Example 5. A Student's t-test analysis was employed to compare AMD/AAA and control groups for each protein cluster in the scatter plot, and proteins were selected that differed significantly ($p < 0.05$ or $p < 0.1$, as indicated) between the two groups.

[0211] Examples of the biomarkers thus discovered are presented in Table 5 below. The "Source" column refers to source of the biomarkers, *i.e.*, serum, plasma or urine. The "Assay" column refers to the type of biochip to which the biomarkers bound, the type of EAM used with the biochip, and the laser energy used to ionize the biomarkers.

Table 5. Biomarkers Associated with Both AAA and AMD

No.	Mass	Source	Assay	P-value	Up/Down
1	2935	Serum	IMAC-Cu 35% CHCA Low Laser	< 0.05	↓
2	3318		"	< 0.1	↓
3	3329		"	< 0.05	↓
4	3350		"	< 0.05	↓
5	3949		"	< 0.1	↓
6	3958		"	< 0.05	↓
7	4094		"	< 0.1	↓
8	4284		"	< 0.05	↓
9	4347		"	< 0.05	↓
10	4646		"	< 0.05	↓

No.	Mass	Source	Assay	P-value	Up/Down
11	6190		"	< 0.05	↑
12	6652		"	< 0.1	↑
13	11753		"	< 0.1	↑
14	6233	Serum	IMAC-Cu SPA Low Laser	< 0.05	↑
15	6470		"	< 0.1	↓
16	8739		"	< 0.05	↓
17	9593		"	< 0.05	↓
18	11010		"	< 0.1	↓
19	11643		"	< 0.05	↑
20	11841		"	< 0.05	↑
21	42832		"	< 0.1	↓
22	118128		"	< 0.1	↓
23	11487	Serum	IMAC-Cu SPA High Laser	< 0.1	↑
24	12545		"	< 0.05	↓
25	13681		"	< 0.05	↓
26	13812		"	< 0.05	↓
27	14013		"	< 0.05	↓
28	18252		"	< 0.05	↑
29	22682		"	< 0.1	↓
30	35905		"	< 0.05	↑
31	39572		"	< 0.1	↑
32	146602		"	< 0.1	↑
33	2672	Urine	CM10 pH4 SPA Low Laser	< 0.1	↑
34	4062		"	< 0.05	↑
35	4311		"	< 0.1	↓
36	4458		"	< 0.1	↓
37	5704		"	< 0.1	↑
38	5742		"	< 0.05	↑
39	6253		"	< 0.05	↓
40	45878		"	< 0.1	↑
41	60948		"	< 0.1	↓
42	91571		"	< 0.1	↑
43	1914	Urine	IMAC-Cu 35% CHCA Low Laser	< 0.1	↓
44	3027		"	< 0.1	↓
45	4602		"	< 0.1	↑
46	5916		"	< 0.1	↓
47	6130		"	< 0.05	↓
48	6189		"	< 0.05	↓
49	9625		"	< 0.1	↑
50	137	Urine	IMAC-Cu 15% CHCA Low Laser	< 0.1	↓
51	179		"	< 0.05	↓
52	423		"	< 0.1	↓
53	430		"	< 0.1	↑
54	445		"	< 0.1	↓
55	461		"	< 0.05	↑
56	637		"	< 0.1	↑
57	643		"	< 0.1	↑

No.	Mass	Source	Assay	P-value	Up/Down
58	671		"	< 0.05	↓
59	3035	Serum*	IMAC-Cu SPA Low Laser	< 0.1	↓
60	3314		"	< 0.1	↑
61	3941		"	< 0.1	↓
62	4100		"	< 0.05	↑
63	4346		"	< 0.1	↓
64	4450		"	< 0.05	↓
65	5290		"	< 0.1	↓
66	5814		"	< 0.05	↓
67	5836		"	< 0.05	↑
68	6378		"	< 0.1	↓
69	6391		"	< 0.1	↓
70	6557		"	< 0.1	↓
71	7501		"	< 0.05	↓
72	7857		"	< 0.1	↓
73	7905		"	< 0.1	↓
74	8858		"	< 0.1	↑
75	9211		"	< 0.05	↓
76	9414		"	< 0.1	↓
77	11638		"	< 0.05	↑
78	11834		"	< 0.05	↑
79	125870		"	< 0.1	↑
80	12557	Serum*	IMAC-Cu SPA High Laser	< 0.05	↓
81	13837		"	< 0.1	↓
82	15480		"	< 0.05	↓
83	23562		"	< 0.1	↑
84	34270		"	< 0.1	↑
85	36008		"	< 0.1	↑
86	37788		"	< 0.1	↑
87	44593		"	< 0.1	↑
88	3061	Serum*	CM10 pH4 SPA Low Laser	< 0.1	↑
89	3189		"	< 0.1	↓
90	3507		"	< 0.1	↓
91	3685		"	< 0.1	↑
92	3849		"	< 0.05	↓
93	4132		"	< 0.1	↓
94	4144		"	< 0.05	↑
95	4490		"	< 0.1	↑
96	4603		"	< 0.05	↓
97	4775		"	< 0.1	↑
98	5873		"	< 0.1	↓
99	6377		"	< 0.1	↓
100	6778		"	< 0.05	↓
101	6823		"	< 0.05	↓
102	7498		"	< 0.1	↓
103	7698		"	< 0.05	↓
104	8076		"	< 0.1	↓

No.	Mass	Source	Assay	P-value	Up/Down
105	8693		"	< 0.05	↓
106	9211		"	< 0.05	↓
107	9414		"	< 0.1	↓
108	13771		"	< 0.05	↓
109	25454		"	< 0.1	↑
110	10508	Serum*	CM10 pH4 SPA High Laser	< 0.1	↓
111	13713		"	< 0.1	↓
112	18291		"	< 0.1	↑
113	39702		"	< 0.05	↑
114	40545		"	< 0.1	↑
115	73047		"	< 0.1	↓
116	3123	Plasma*	IMAC-Cu SPA Low Laser	< 0.05	↑
117	3188		"	< 0.05	↓
118	3247		"	0.1	↓
119	3285		"	< 0.05	↓
120	3307		"	0.1	↓
121	3387		"	< 0.05	↓
122	3474		"	< 0.05	↑
123	3658		"	< 0.05	↓
124	3748		"	< 0.05	↑
125	3822		"	0.1	↓
126	3850		"	0.1	↓
127	3935		"	0.1	↓
128	4133		"	0.1	↑
129	4632		"	0.1	↓
130	5447		"	< 0.05	↑
131	5789		"	0.1	↑
132	5856		"	0.1	↑
133	5916		"	< 0.05	↑
134	7501		"	0.1	↓
135	7698		"	< 0.05	↓
136	7768		"	< 0.05	↓
137	7903		"	< 0.05	↓
138	8074		"	< 0.05	↓
139	9209		"	< 0.05	↓
140	9419		"	< 0.05	↓
141	11635		"	< 0.05	↑
142	11834		"	< 0.05	↑
143	14579		"	< 0.05	↑
144	13834	Plasma*	IMAC-Cu SPA High Laser	< 0.1	↓
145	15274		"	< 0.05	↓
146	18295		"	< 0.05	↑
147	74846		"	0.1	↓
148	118670		"	< 0.05	↑
149	3052	Plasma*	CM10 pH4 SPA Low Laser	< 0.05	↓
150	3081		"	< 0.05	↓
151	3088		"	0.1	↑

No.	Mass	Source	Assay	P-value	Up/Down
152	3105		"	0.1	↑
153	3113		"	< 0.05	↑
154	3257		"	< 0.05	↑
155	3284		"	< 0.05	↓
156	3303		"	< 0.05	↓
157	3317		"	< 0.05	↓
158	3325		"	< 0.05	↓
159	3412		"	< 0.05	↓
160	3435		"	< 0.05	↓
161	3475		"	0.1	↓
162	3514		"	0.1	↑
163	3614		"	< 0.05	↓
164	3681		"	< 0.05	↑
165	3709		"	< 0.05	↓
166	3860		"	0.1	↑
167	4100		"	< 0.05	↑
168	4170		"	0.1	↑
169	4187		"	0.1	↓
170	4307		"	< 0.05	↑
171	4438		"	< 0.05	↓
172	4493		"	< 0.05	↑
173	5059		"	0.1	↑
174	5105		"	< 0.05	↑
175	5211		"	< 0.05	↑
176	6072		"	0.1	↑
177	6559		"	< 0.05	↑
178	8693		"	< 0.05	↓
179	9632		"	< 0.05	↑
180	11619		"	< 0.05	↑
181	22087		"	0.1	↑
182	33117		"	< 0.05	↑
183	44269		"	< 0.05	↑
184	145931		"	0.1	↓
185	10791	Plasma*	CM10 pH4 SPA High Laser	< 0.05	↑
186	11476		"	< 0.05	↑
187	11631		"	0.1	↑
188	11677		"	< 0.05	↑
189	11876		"	< 0.05	↑
190	14626		"	0.1	↑
191	24295		"	0.1	↑
192	28851		"	0.1	↑

*Albumin and IgG depleted serum or plasma.

[0212] Although the present invention has been described in detail with reference to specific embodiments, those of skill in the art will recognize that modifications and

improvements are within the scope and spirit of the invention, as set forth in the claims which follow. All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents (patents, published patent applications, and unpublished patent applications) is not intended as an admission that any such document is pertinent prior art, nor does it constitute any admission as to the contents or date of the same. The invention having now been described by way of written description, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description is for purposes of illustration and not limitation of the following claims.

WHAT IS CLAIMED IS:

1. A method for diagnosing age-related macular degeneration (AMD) in an individual, the method comprising:
 - a) determining levels of at least two AMD-associated protein markers (biomarkers) in a biological sample from the individual; and
 - b) comparing the levels of the at least two biomarkers to reference levels of the at least two biomarkers characteristic of a control population of individuals without AMD,wherein a difference in the levels of the at least two biomarkers between the biological sample from the individual and the control population indicates that the individual has an increased likelihood of having AMD, and
wherein the at least two biomarkers are biomarkers listed in Table 1, 2, 3 or 4.
2. The method of claim 1, wherein the levels of the at least two biomarkers are measured by surface enhanced laser desorption ionization (SELDI).
3. The method of claim 1, wherein the biological sample is blood, serum, plasma, or urine from the individual.
4. The method of claim 3, wherein the biological sample is serum.
5. The method of claim 4, wherein the at least two biomarkers are selected from the group listed in Table 1 consisting of 1287, 3329, 3350, 3452, 4627, 6233, 9452, 9593, and 9664.
6. The method of claim 4, wherein the at least two biomarkers listed in Table 1 are 1287 and 6233.
7. The method of claim 4, wherein the at least two biomarkers are selected from the group listed in Table 1 consisting of 3329, 3350, 3452, 4627, 9452, 9593, and 9664.

8. The method of claim 4, wherein the biological sample is serum depleted of albumin and IgG.
9. The method of claim 8, wherein the at least two biomarkers are selected from the group listed in Table 2 consisting of 3070, 3216, 3402, 4951, 4976, 8858, 11834, 12577, 36008, 3003, 3061, 4144, 4490, 4603, 4775, 5816, and 6823.
10. The method of claim 8, wherein the at least two biomarkers are selected from the group listed in Table 2 consisting of 3070, 3216, 3402, 4951, 4976, 8858, 11834, 36008, 3003, 3061, 4144, 4490, and 4775.
11. The method of claim 8, wherein the at least two biomarkers are selected from the group listed in Table 2 consisting of 12577, 4603, 5816, and 6823.
12. The method of claim 3, wherein the biological sample is plasma.
13. The method of claim 12, wherein the biological sample is plasma depleted of albumin and IgG.
14. The method of claim 13, wherein the at least two biomarkers are selected from the group listed in Table 3 consisting of 3123, 3498, 3990, 4632, 5691, 5850, 6405, 11468, 3160, 3396, 3600, 3681, 3708, 3867, 3943, 3997, 6987, 33117, 145931, 58655, and 60449.
15. The method of claim 13, wherein the at least two biomarkers are selected from the group listed in Table 3 consisting of 3123, 3498, 3990, 5691, 11468, 3160, 3396, 3600, 3681, 3708, 3867, 3997, 6987, 33117, 58655, and 60449.
16. The method of claim 13, wherein the at least two biomarkers are selected from the group listed in Table 3 consisting of 4632, 5850, 6405, 3943, and 145931.
17. The method of claim 3, wherein the biological sample is urine.

18. The method of claim 17, wherein the at least two biomarkers are selected from the group listed in Table 4 consisting of 2564, 3027, 5916, 6189, 6316, 6864, 11724, and 11783.

19. The method of claim 1, wherein the at least two biomarkers are a set of biomarkers comprising at least 2, at least 3, at least 4, or at least 5 of the biomarkers listed in Table 1, 2, 3 or 4.

20. The method of claim 19, wherein the set of biomarkers comprises at least 2, at least 3, at least 4, or at least 5 of the biomarkers present at elevated levels in individuals diagnosed with AMD as compared to a control population in Table 1, 2, 3 or 4.

21. The method of claim 19, wherein the set of biomarkers comprises at least 2, at least 3, at least 4, or at least 5 of the biomarkers present at reduced levels in individuals diagnosed with AMD as compared to a control population in Table 1, 2, 3 or 4.

22. A method for assessing the efficacy of a treatment for AMD in an individual, comprising:

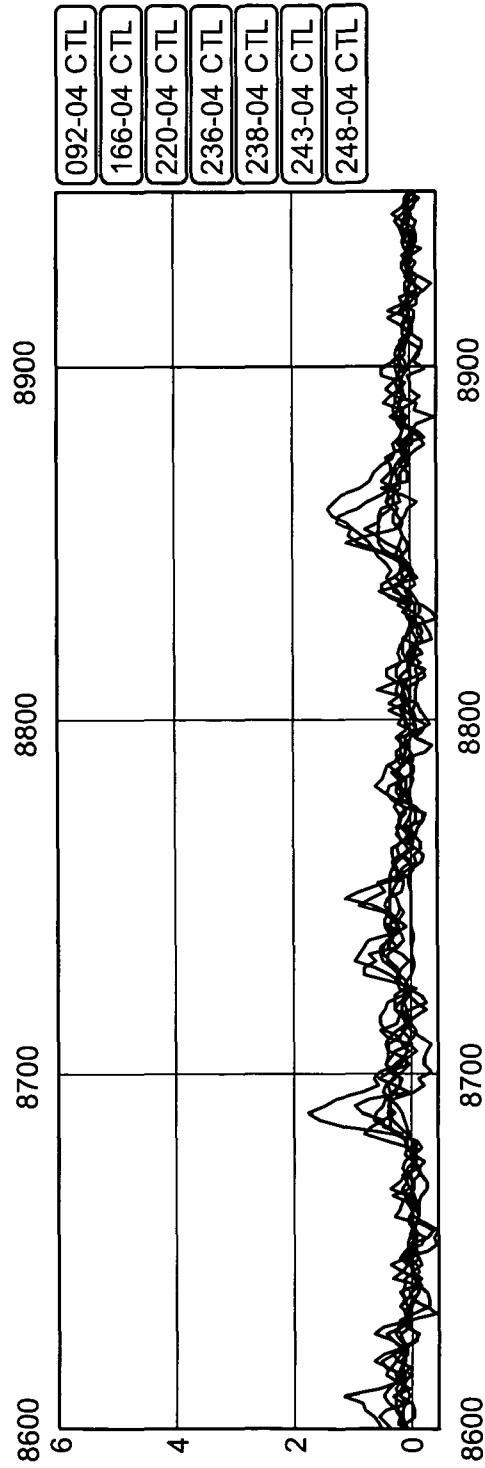
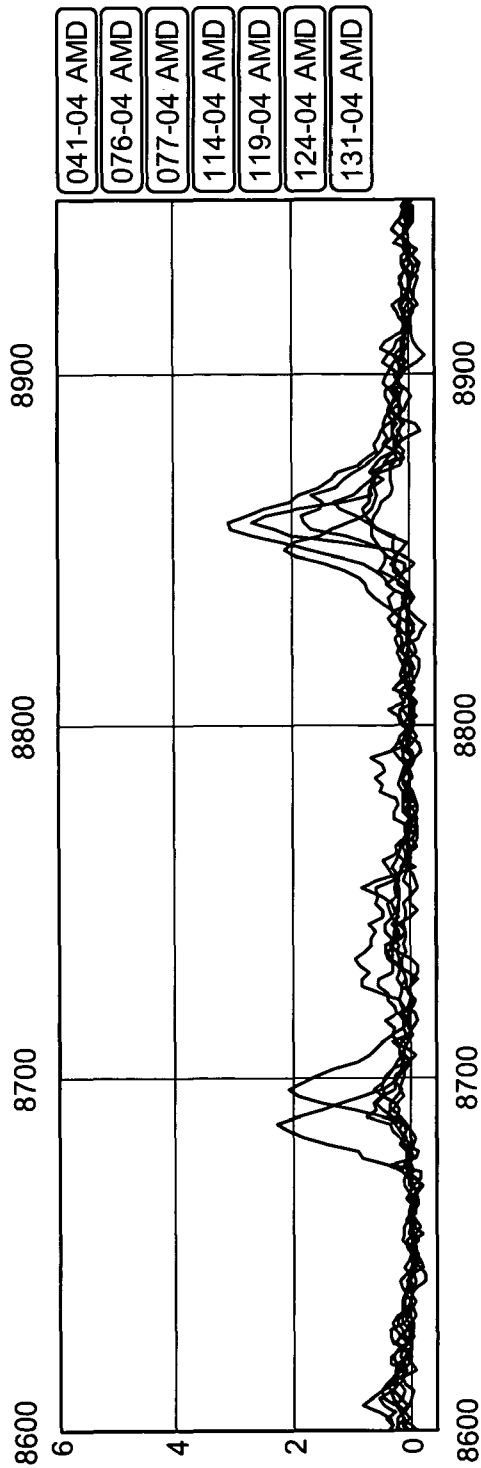
a) determining levels of at least two biomarkers listed in Tables 1-4 in a biological sample from the individual either before treatment or at a first time point after treatment with an agent;

b) comparing the levels of the at least two biomarkers in a) to reference levels of the at least two biomarkers characteristic of a control population of individuals without AMD;

c) determining levels of the at least two biomarkers at a later time point during treatment or after treatment with the agent; and

d) comparing the levels of the at least two biomarkers in c) to the reference levels of the at least two biomarkers characteristic of a control population of individuals without AMD,

wherein a decreased difference in the levels of the at least two biomarkers between the individual and the control population measured in d) compared to b) indicates that the treatment is effective.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/79104

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/53; G01N 24/00 (2009.01)

USPC - 435/7.1; 436/173

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - G01N 33/53; G01N 24/00 (2009.01)

USPC - 435/7.1; 436/173

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 436/174; 424/9.2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(USPT,PGPB,EPAB,JPAB); Google Scholar; TagIdent

Search terms: age-related macular degeneration or AMD, biomarker, marker, protein, peptide, polypeptide, mass spectrometry, time-of-flight, surface enhanced laser desorption ionization or SELDI, mass-to-charge ratio

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0072241 A1 (HOLLYFIELD et al.) 29 March 2007 (29.03.2007) para [0005], [0007], [0034]	1-6, 19-20 and 22
Y	US 2006/0204951 A1 (FOLKMAN et al.) 14 September 2006 (14.09.2006) para [0026], [0079]-[0080], [0169], [0174]	1-6, 19-20 and 22
Y	DRESIOS et al., Eukaryotic ribosomal proteins lacking a eubacterial counterpart: important players in ribosomal function, Mol Microbiol, March 2006, vol 59, No 6, pp 1651-63. p 1659, col 1, para 1.	1-6, 19-20 and 22
Y	US 2007/0014723 A1 (SAWYER et al.) 18 January 2007 (18.01.2007) para [0024] and [0057]	1-6, 19-20 and 22

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

9 February 2009 (09.02.2009)

Date of mailing of the international search report

27 FEB 2009

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 08/79104

Continuation of : Box No. III Observations where unity of invention is lacking

Group IV: Claims 1-3, 12-16, and 19-22 are directed to a method of diagnosing age-related macular degeneration (AMD) in an individual or of assessing the efficacy of a treatment for AMD, limited to albumin- and IgG- depleted plasma biomarkers in table 3.

Groups V+: Claims 1-3, 17-19, 21, and 22 are directed to a method of diagnosing age-related macular degeneration (AMD) in an individual or of assessing the efficacy of a treatment for AMD, limited to urine biomarkers in table 4 which are present at reduced levels, where biomarkers 2564 and 3027 will be searched. Applicant may have additional biomarker(s) searched upon paying additional search fee(s).

Groups VI+: Claims 1-3, 17, 19, 20, and 22 are directed to a method of diagnosing age-related macular degeneration (AMD) in an individual or of assessing the efficacy of a treatment for AMD, limited to urine biomarkers in table 4 which are present at elevated levels, where biomarkers 3856 and 5619 will be searched. Applicant may have additional biomarker(s) searched upon paying additional search fee(s).

The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The common technical feature of the listed as Groups I-VI is a method of diagnosing age-related macular degeneration (AMD) in an individual or of assessing the efficacy of a treatment for AMD comprising determining elevated or reduced levels of biomarkers in serum, plasma, or urine. However, these do not represent an improvement over the prior art of US 2005/0250745 A1 (Seddon) which teaches a method of diagnosing age-related macular degeneration (AMD) in an individual or of assessing the efficacy of a treatment for AMD comprising determining elevated or reduced levels of biomarkers (para [0012]) in serum (para [0099], [0101], plasma (para [0130]; [0178]), or urine. (para [0047]). Accordingly, unity of invention is lacking under PCT Rule 13.1.