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(54) **METHODS AND COMPOSITIONS FOR
DIAGNOSIS OR PROGNOSIS OF
CARDIOVASCULAR DISEASE**

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2008.

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G01N 31/00 (2006.01)
G01N 33/53 (2006.01)

(52) **U.S. Cl.** **435/7.21**; 435/7.1; 436/501; 436/518;
424/9.1; 424/520; 422/50; 530/300; 530/350

(58) **Field of Classification Search** None
See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods of screening a mammalian subject to determine if the subject is at risk to develop or is suffering from, cardiovascular disease. In one embodiment, the method comprises detecting a measurable feature of at least two biomarkers in an HDL subfraction, or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the subject, wherein the at least two biomarkers are selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof, and comparing the measurable features of the at least two biomarkers from the biological sample to a reference standard, wherein a difference in the measurable features of the at least two biomarkers from the biological sample and the reference standard is indicative of the presence or risk of cardiovascular disease in the subject.

18 Claims, 13 Drawing Sheets

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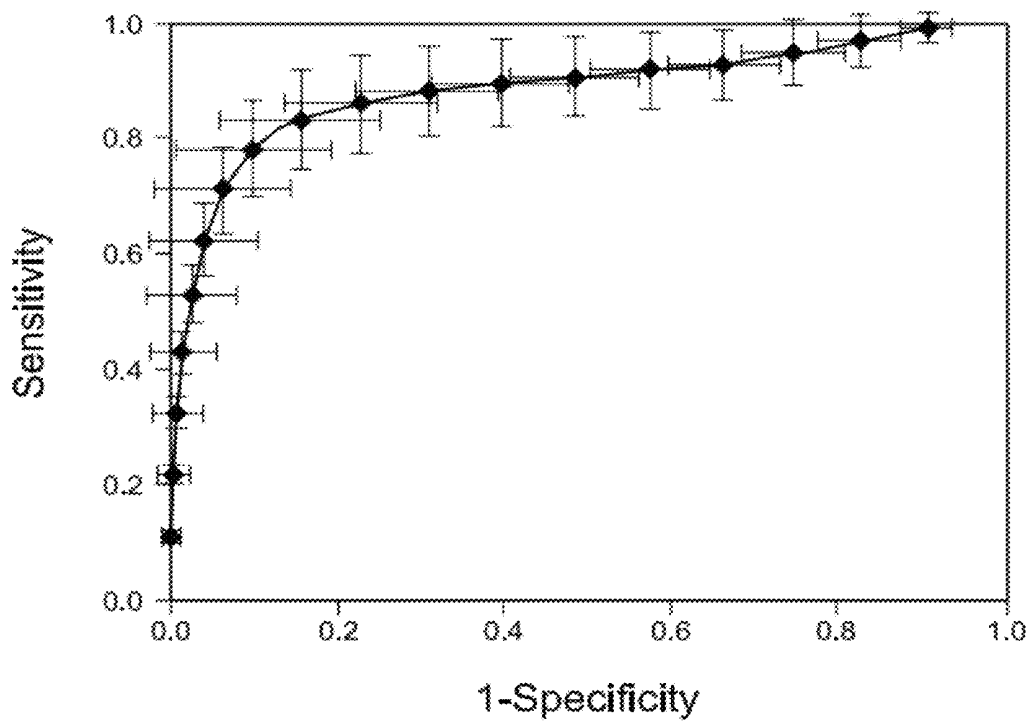


Fig. 1.

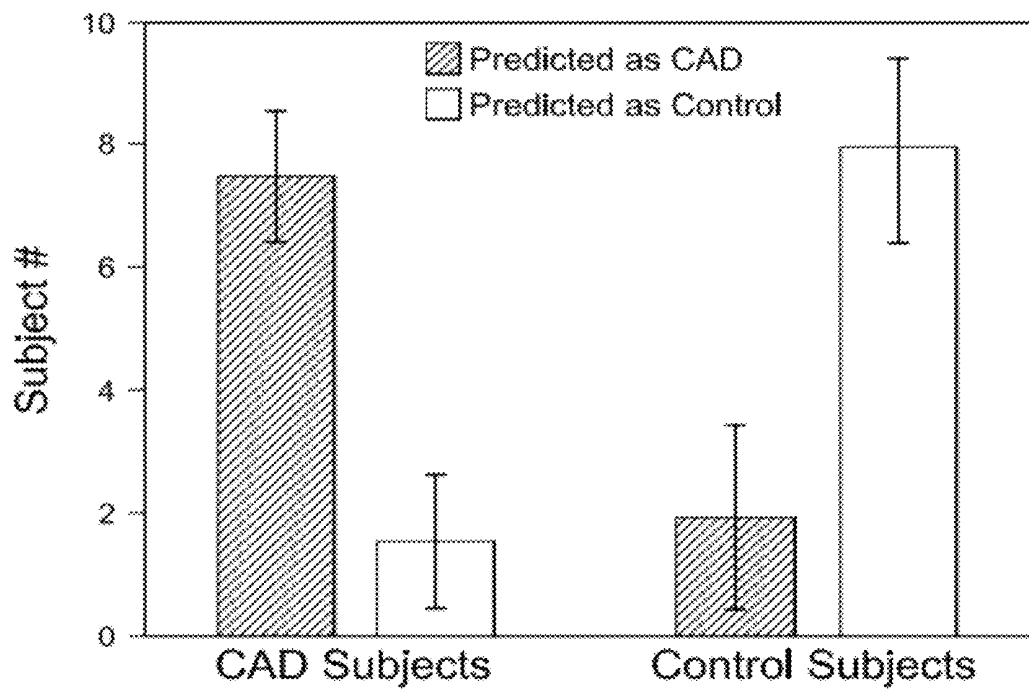


Fig. 2.

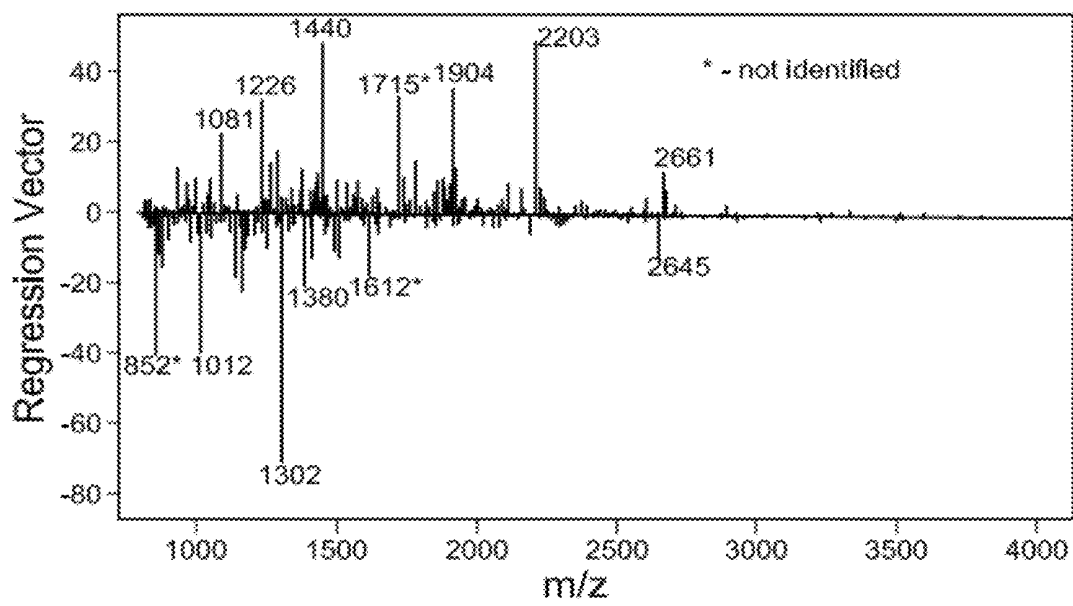


Fig. 4.

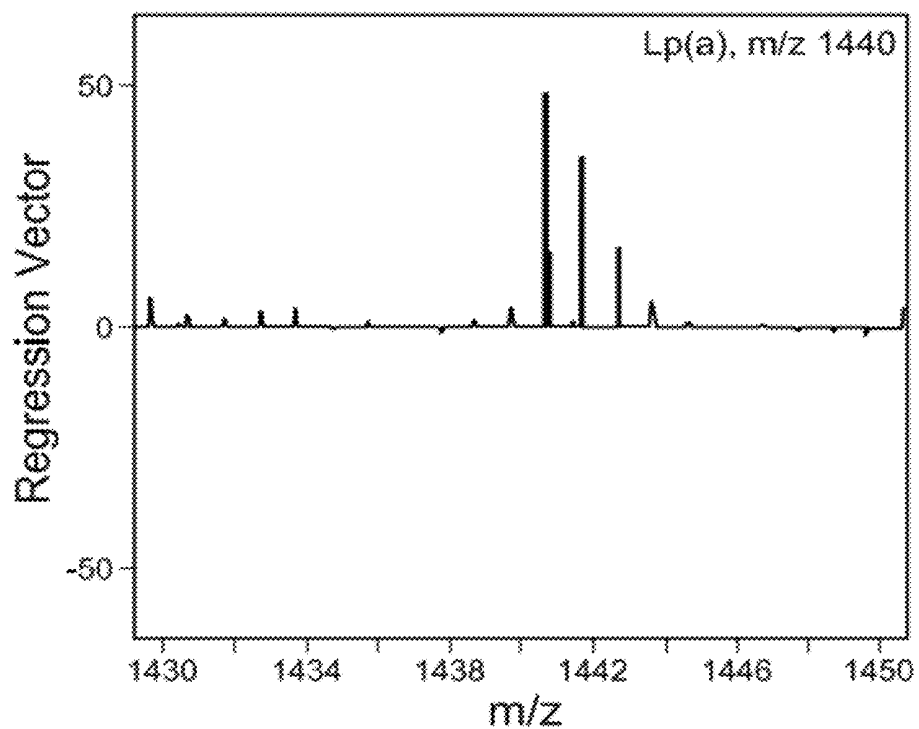


Fig. 5A.

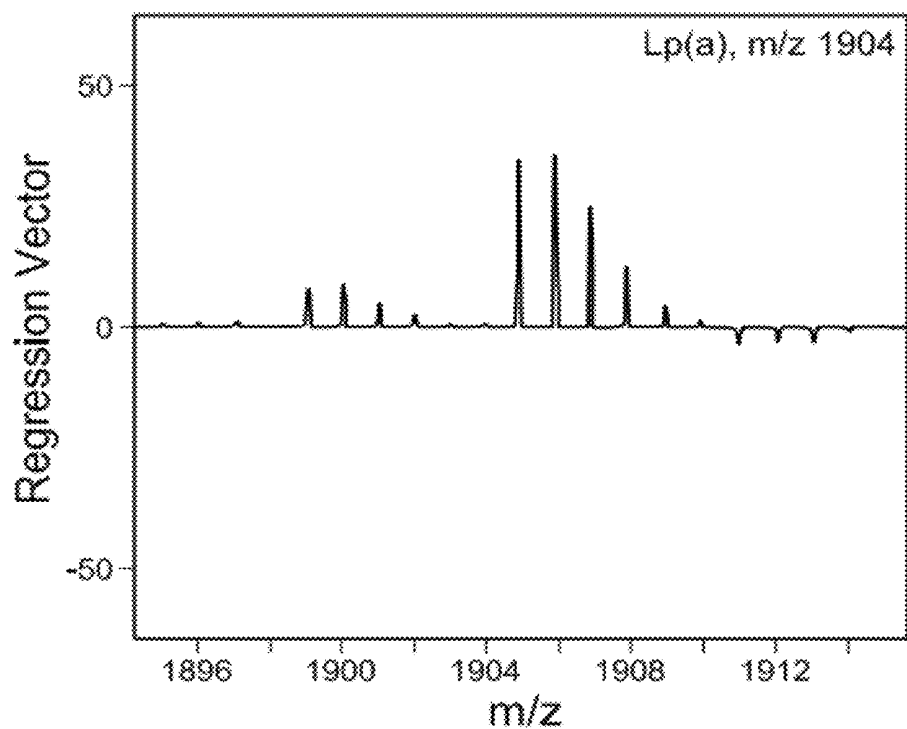


Fig. 5B.

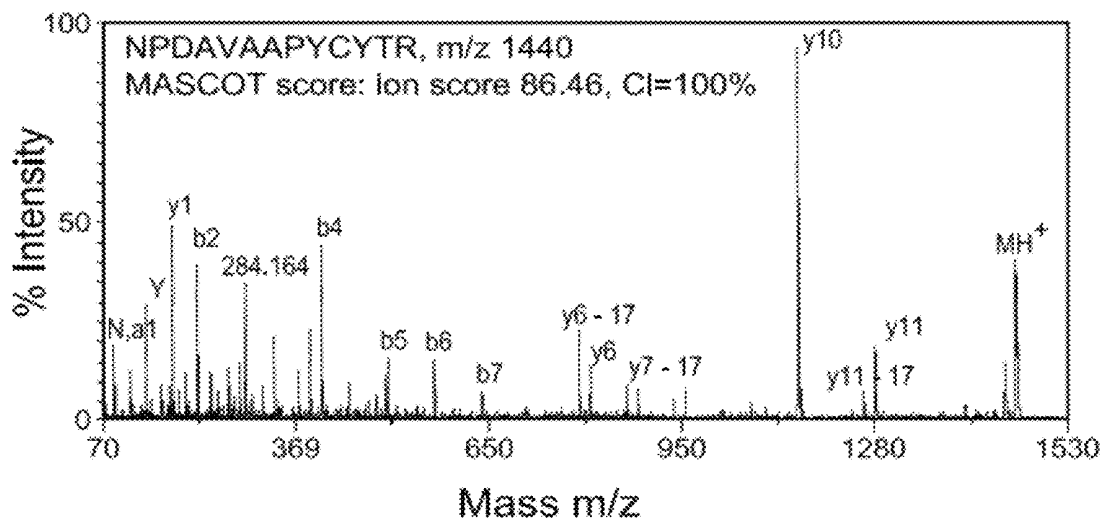


Fig. 5C.

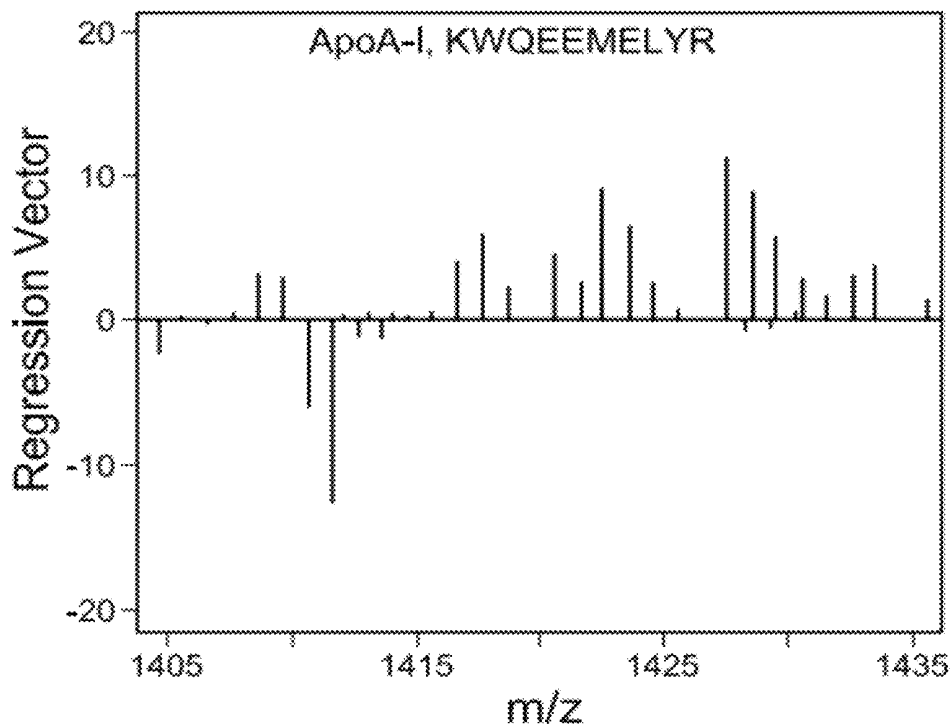


Fig. 6A.

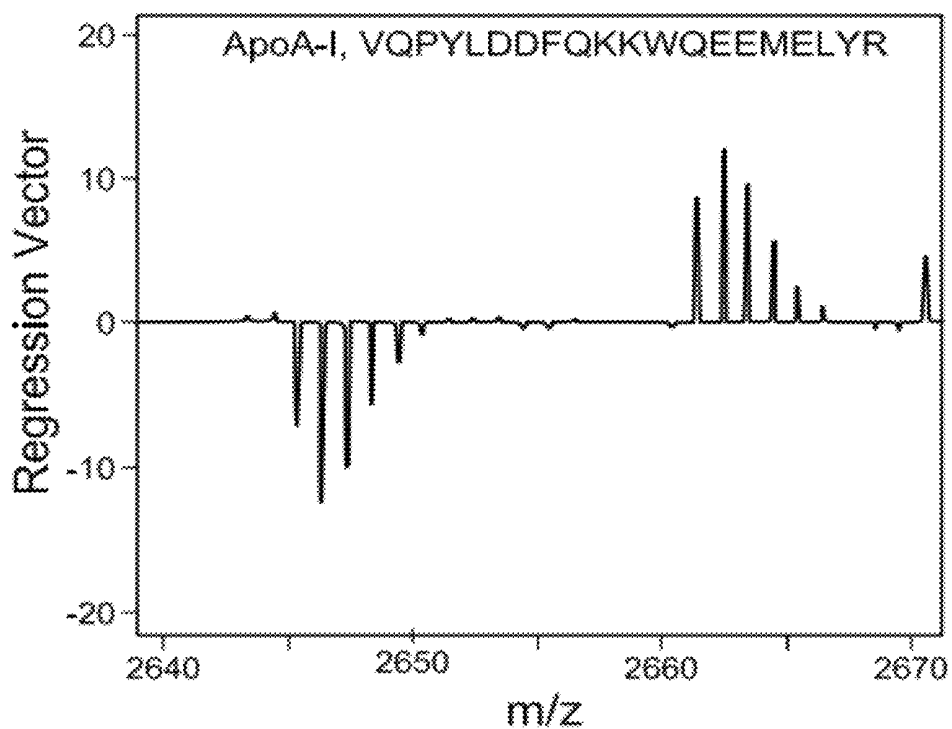


Fig. 6B.

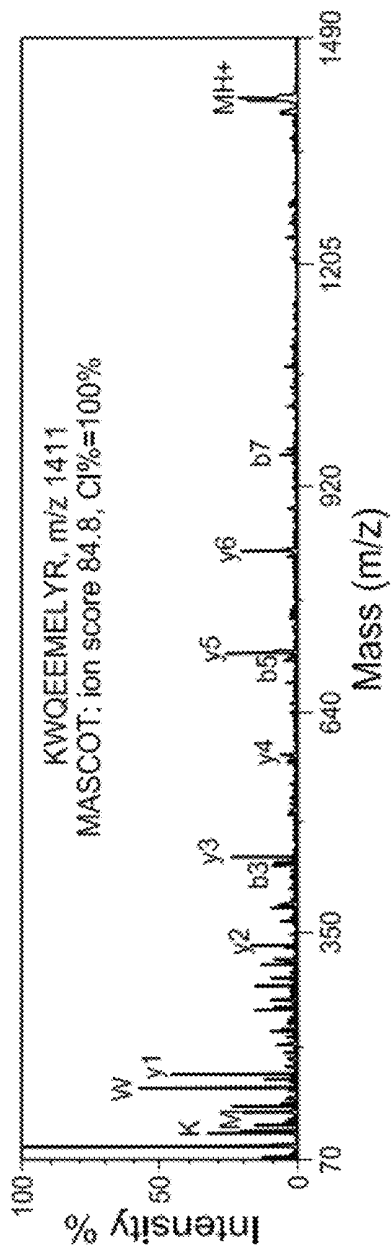


Fig. 6C.

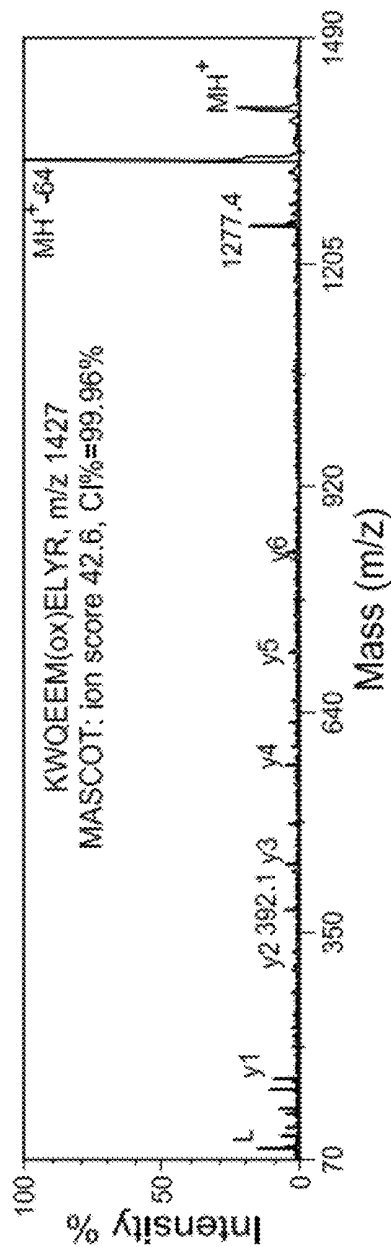


Fig. 6D.

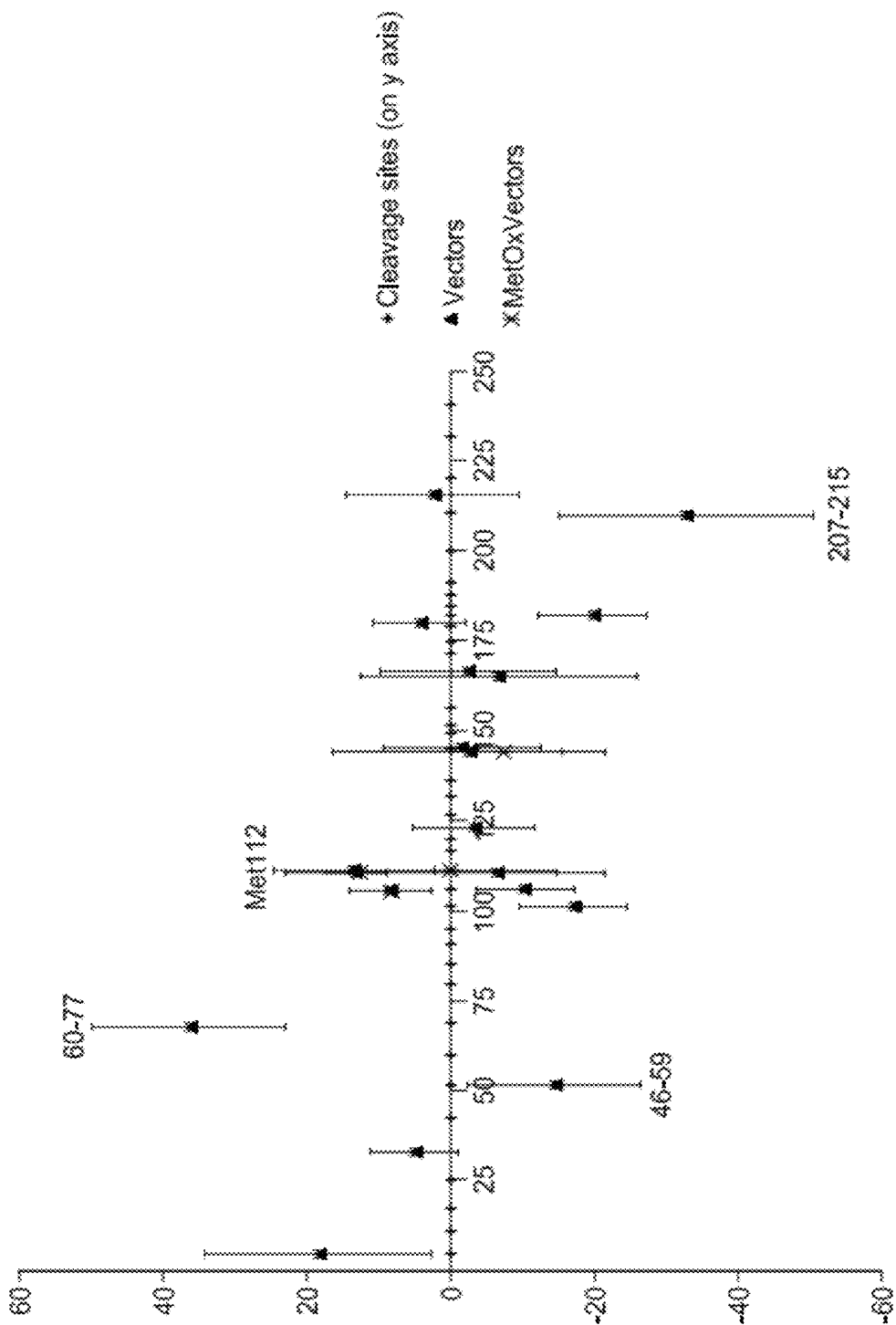


Fig. 7.

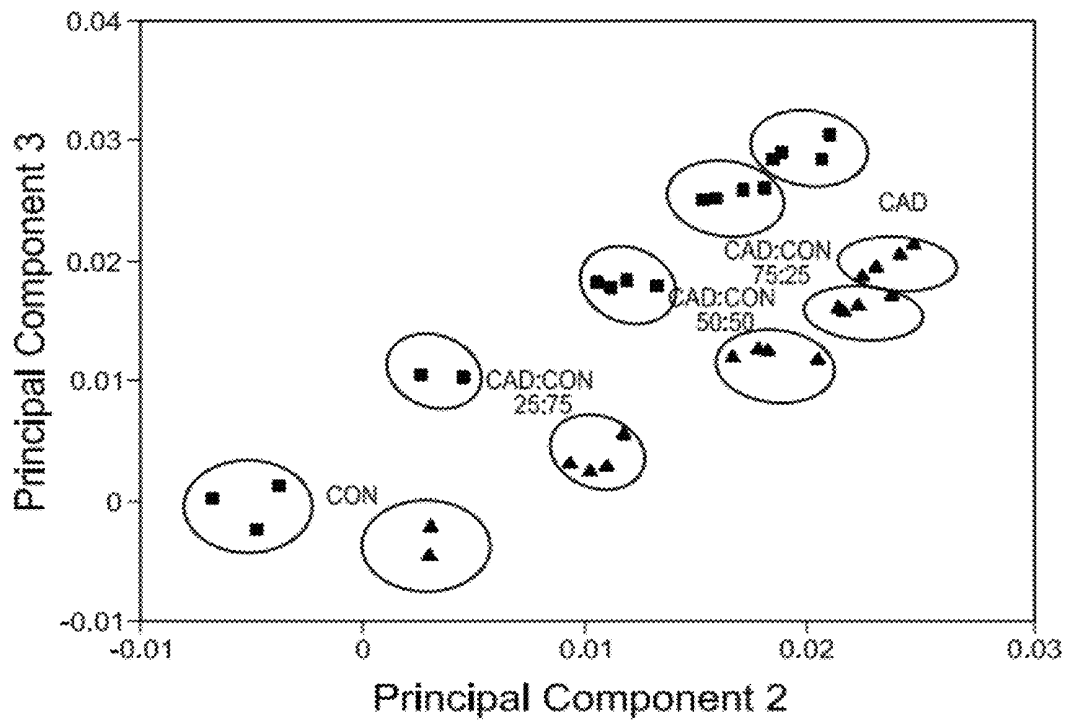


Fig. 8.

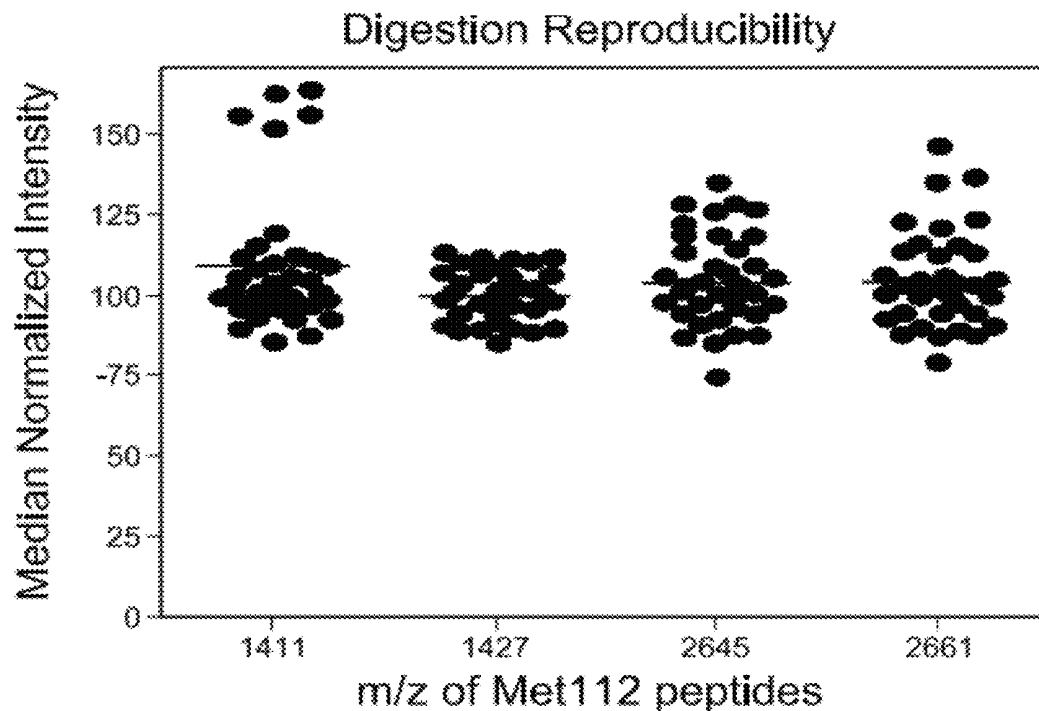


Fig. 9C.

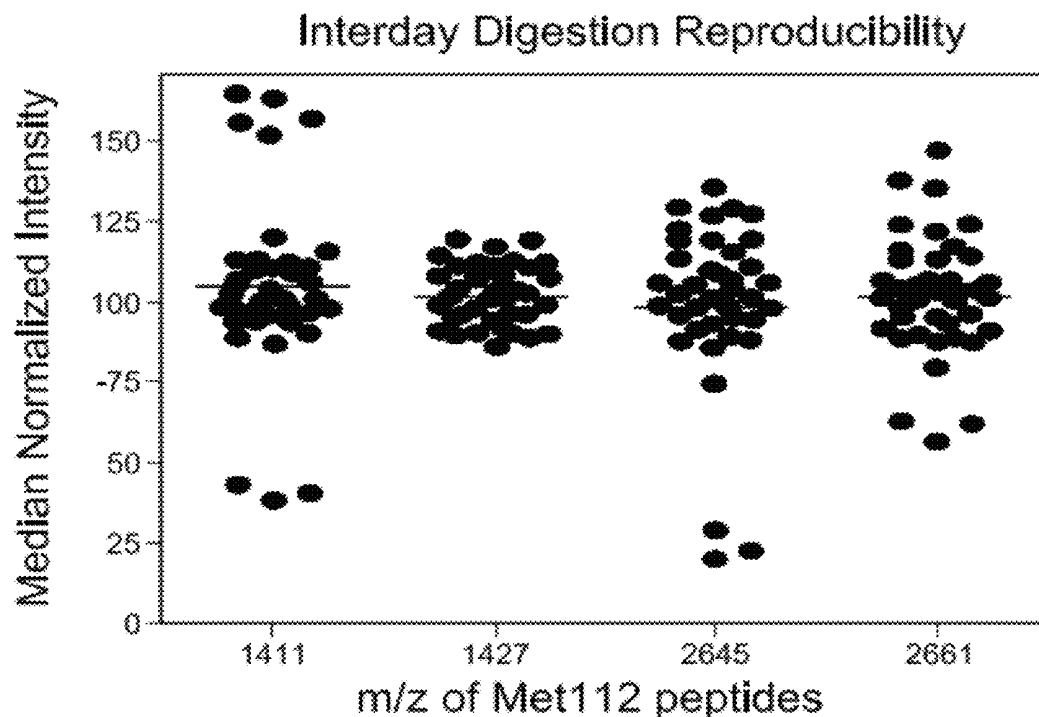


Fig. 9D.

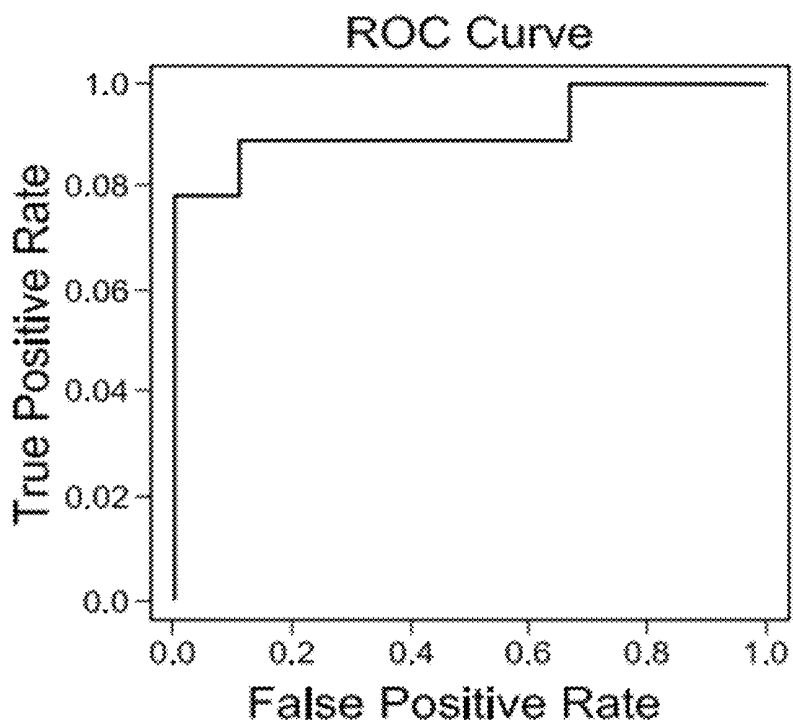


Fig.10A.

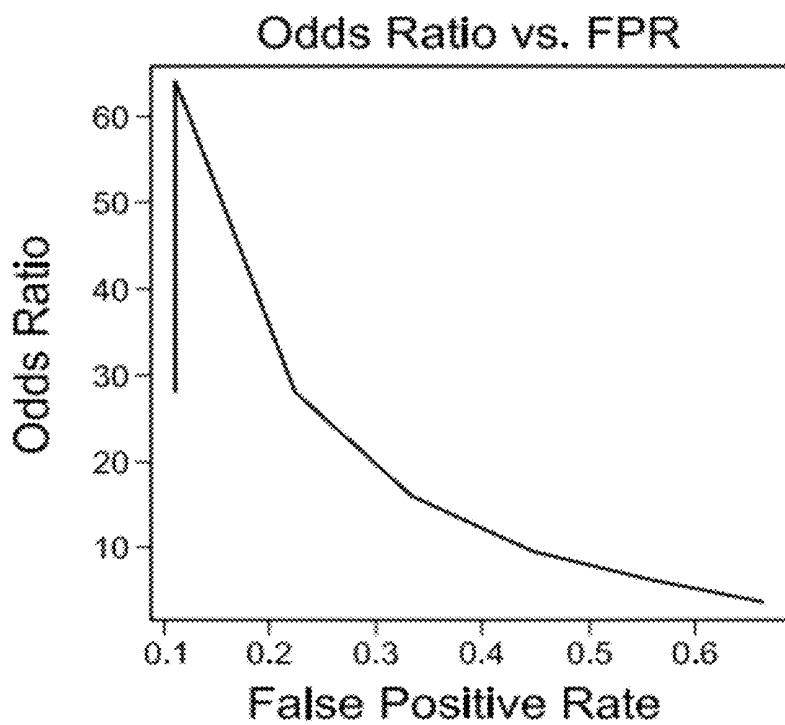


Fig.10B.

METHODS AND COMPOSITIONS FOR DIAGNOSIS OR PROGNOSIS OF CARDIOVASCULAR DISEASE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of Application No. 61/079,088, filed Jul. 8, 2008,

STATEMENT OF GOVERNMENT LICENSE RIGHTS

This invention was made with U.S. Government support under NIH grant number HL086798, awarded by the National Institutes of Health. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention generally relates to methods, reagents, and kits for diagnosing cardiovascular disease in a subject, and particularly relates to the use of lipoprotein-associated markers to diagnose cardiovascular disease in a subject.

BACKGROUND

Cardiovascular disease is a leading cause of morbidity and mortality, particularly in developed areas such as the United States and Western European countries. The incidence of mortality from cardiovascular disease has significantly decreased in the United States over the past 30 years (see Braunwald, E., N. *Engl. J. Med.* 337:1360-1369, 1997; Hoyert, D. L., et al., "Deaths: Preliminary Data for 2003" in *National Vital Statistics Reports*. Hyattsville: National Center for Health Statistics, 2005). Many factors have contributed to this improvement in patient outcome, including the identification of cardiovascular risk factors, the application of medical technologies to treat acute coronary syndrome, and the development of interventions that reduce cardiovascular risk factors. Despite these advances, however, cardiovascular disease remains a leading cause of morbidity and mortality in developed countries (see Hoyert D. L., et al., *National Vital Statistics Reports*, 2005).

Thus, there is a pressing need to identify markers that may be used for the rapid, accurate and non-invasive diagnosis and/or assessment of the risk of cardiovascular disease, and also to assess the efficacy of interventions designed to slow the initiation and progress of this disorder.

SUMMARY

In accordance with the foregoing, in one aspect, the present invention provides a method of screening a mammalian subject to determine if the subject is at risk to develop, or is suffering from, cardiovascular disease, the method comprising detecting a measurable feature of at least two biomarkers in an HDL subfraction, or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the subject, wherein the at least two biomarkers are selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof, and comparing the measurable features of the at least two biomarkers from the biological

sample and the reference standard is indicative of the presence or risk of cardiovascular disease in the subject.

In another aspect, the present invention provides a method for diagnosing and/or assessing the risk of CAD in a subject, comprising determining changes in a biomarker profile comprising the relative abundance of at least one, two, three, four, five, ten or more biomarkers in an HDL subfraction or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from a test subject as compared to the predetermined abundance of the at least one, two, three, four, five, ten or more biomarkers from a reference population of apparently healthy subjects, wherein the biomarkers are selected from the biomarkers set forth in TABLE 3, TABLE 4, and TABLE 5.

In another aspect, the present invention provides a method of screening a mammalian subject to determine if a test subject is at risk to develop, is suffering from, or recovering from, cardiovascular disease, the method comprising detecting an alteration in the conformational structure of apoA-I present in the HDL subfraction or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the test subject in comparison to a reference standard, wherein a difference in the conformation of the apoA-I between the biological sample from the test subject and the reference standard is indicative of the presence or risk of cardiovascular disease in the subject.

In another aspect, the present invention provides a method for determining the efficacy of a treatment regimen for treating and/or preventing cardiovascular disease in a subject by monitoring a measurable feature of at least two biomarkers selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof in an HDL subfraction or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the subject during treatment for cardiovascular disease.

In yet another aspect, the present invention provides a kit for determining susceptibility or presence of cardiovascular disease in a mammalian subject based on the detection of at least one measurable feature of at least one biomarker in a biological sample, an HDL subfraction thereof, or a complex containing apoA-I or apoA-II isolated from the biological sample, the kit comprising (i) one or more detection reagents for detecting the at least one measurable feature of the at least one biomarker selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, and (ii) written indicia indicating a positive correlation between the presence of the detected feature of the biomarker and the diagnosis or risk of developing cardiovascular disease.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIG. 1 presents graphical results demonstrating the receiver operating characteristic (ROC) curve of the prediction of cardiovascular disease (CAD) status based on random permutation analysis, as described in Example 2;

FIG. 2 graphically illustrates the prediction of CAD status by the proteomics CAD risk score "ProtCAD risk score" using a partial least squares discriminate analysis (PLS-DA) model built using a calibration group (as described in Example 2). Using a sensitivity of 80%, the ProtCAD risk

score of each subject in the validation group at each permutation was used to predict their CAD status, as described in Example 2;

FIG. 3 graphically illustrates the power of the ProtCAD risk score to discriminate between the CAD samples and healthy control samples based on leave-one-out analysis. The ProtCAD risk score was derived from PLS-DA analysis of MALDI-TOF-MS mass spectra of HDL tryptic digests, using a leave-one-out experiment for all 18 CAD and 20 control subjects, as described in Example 2;

FIG. 4 graphically illustrates the PLS-DA regression vectors (y-axis) of the leave-one-out PLS-DA model that distinguish CAD and control subjects. The x-axis (m/z) represents mass channels of the MALDI-TOF mass spectrum. Positive and negative features on the regression vector indicate an increase and decrease, respectively, of the signals from CAD samples relative to control samples, as described in Example 3;

FIG. 5A graphically illustrates the strong positive feature in the PLS-DA regression vector at m/z 1440.68 identified by LC-MALDI-TOF/TOF MS/MS as corresponding to peptides derived from Lp(a), as described in Example 3;

FIG. 5B graphically illustrates the strong positive feature in the PLS-DA regression vector at m/z 1904.91 identified by LC-MALDI-TOF/TOF MS/MS as corresponding to peptides derived from Lp(a), as described in Example 3;

FIG. 5C illustrates the results of the MASCOT database search of the MS/MS spectrum of the peptide of m/z 1440.68 that identified Lp(a) with a high confidence level (CI100%), as described in Example 3;

FIG. 6A to FIG. 6D graphically illustrate the PLS-DA regression vector features corresponding to apoA-I peptides containing Met112, with pairs of specific informative features at m/z 1411 and 1427 and m/z 2645 and 2661 corresponding to signals detected for M and M+16 respectively, wherein the positive features signify an increase of oxidized form of Met112 peptide, and negative features at m/z 1411 and m/z 2645 indicate a decreased level of the peptide containing unoxidized Met112 in the CAD samples, as described in Example 4;

FIG. 7 graphically illustrates the differential digestion efficiency in CAD HDL as compared to normal HDL, in which multiple features in the regression vector (y-axis) correspond to peptides derived from apoA-I (x-axis) with differential features at the N-terminal (residues 46-59, 60-77) and the C-terminal (residues 207-215) domains, indicating a conformational change in apoA-I in the HDL of CAD subjects, as described in Example 5;

FIG. 8 graphically illustrates the results of principle component analysis (PCA) of the average mass spectra from HDL₂ isolated from 3 control and 3 CAD subjects mixed in protein ratios (w/w) of 1:0, 1:3, 1:1, 3:1, and 0:1, digested with trypsin, and subjected to MALDI-TOF-MS (for simplicity only two pairs are shown in FIG. 8), as described in Example 1;

FIG. 9A graphically illustrates the reproducibility of the MALDI-TOF spectra with selected mass channels of Met112 peptides represented on the plot as median normalized intensities, as described in Example 1;

FIG. 9B graphically illustrates the reproducibility of the MALDI-TOF spectra of multiple spots of samples with selected mass channels of Met112 peptides represented on the plot as median normalized intensities, as described in Example 1;

FIG. 9C graphically illustrates the reproducibility of a series of trypsin digestions carried out on the same day followed by MALDI-TOF spectra with selected mass channels

of Met112 peptides represented on the plot as median normalized intensities, as described in Example 1;

FIG. 9D graphically illustrates the reproducibility of a series of trypsin digestions carried out on different days followed by MALDI-TOF spectra with selected mass channels of Met112 peptides represented on the plot as median normalized intensities, as described in Example 1;

FIG. 10A graphically illustrates a receiver operating characteristic (ROC) curve constructed using a ProtCAD score based on a PLS-DA model built from a leave-one-out approach, demonstrating high selectivity (true positive rate=y axis) and high specificity (false positive rate=x axis), as described in Example 2; and

FIG. 10B graphically illustrates the odds ratio of the ProtCAD score as a function of the false positive rate, demonstrating that at an 80% level of specificity (corresponding to a 90% sensitivity level as shown in FIG. 10A), the odds ratio was approximately 35, as described in Example 2.

DETAILED DESCRIPTION

As used herein, the term “cardiovascular disease” or “CAD,” generally refers to heart and blood vessel diseases, including atherosclerosis, coronary heart disease, cerebrovascular disease, and peripheral vascular disease. Cardiovascular disorders are acute manifestations of CAD and include myocardial infarction, stroke, angina pectoris, transient ischemic attacks, and congestive heart failure. Cardiovascular disease, including atherosclerosis, usually results from the build up of fatty material, inflammatory cells, extracellular matrix, and plaque. Clinical symptoms and signs indicating the presence of CAD include one or more of the following: chest pain and other forms of angina, shortness of breath, sweatiess, Q waves or inverted T waves on an EKG, a high calcium score by CT scan, at least one stenotic lesion on coronary angiography, or heart attack documented by changes in myocardial enzyme levels (e.g., troponin, CK levels).

As used herein, the term “biomarker” is a biological compound, such as a protein or a peptide fragment thereof, including a polypeptide or peptide that may be isolated from or measured in the biological sample, wherein the biomarker is differentially present or absent, or present in a different structure (i.e., post-translationally modified, or in an altered structural conformation) in a sample taken from a subject having established or potentially clinically significant CAD as compared to a comparable sample taken from an apparently normal subject that does not have CAD. A biomarker can be an intact molecule, or it can be a portion thereof or an altered structure thereof, that may be partially functional and recognized, for example, by a specific binding protein or other detection method. A biomarker is considered to be informative for CAD if a measurable feature of the biomarker is associated with the presence of CAD in a subject in comparison to a predetermined value or a reference profile from a control population. Such a measurable feature may include, for example, the presence, absence, or concentration of the biomarker, or a portion thereof, in the biological sample, an altered structure, such as, for example, the presence or amount of a post-translational modification, such as oxidation at one or more positions on the amino acid sequence of the biomarker or, for example, the presence of an altered conformation in comparison to the conformation of the biomarker in normal control subjects, and/or the presence, amount, or altered structure of the biomarker as a part of a profile of more than one biomarker. A measurable aspect of a biomarker is also referred to as a feature. A feature may be a ratio of two

or more measurable aspects of biomarkers. A biomarker profile comprises at least two measurable informative features, and may comprise at least three, four, five, 10, 20, 30 or more informative features. The biomarker profile may also comprise at least one measurable aspect of at least one feature relative to at least one internal standard.

As used herein, the term "predetermined value" refers to the amount and/or structure of one or more biomarkers in biological samples obtained from the general population or from a select population of subjects. For example, the select population may be comprised of apparently healthy subjects, such as individuals who have not previously had any sign or symptoms indicating the presence of CAD. In another example, the predetermined value may be comprised of subjects having established CAD. The predetermined value can be a cut-off value or a range. The predetermined value can be established based upon comparative measurements between apparently healthy subjects and subjects with established CAD, as described herein.

As used herein, the term "high density lipoprotein" or "HDL, or a subfraction thereof" includes protein or lipoprotein complexes with a density from about 1.06 to about 1.21 g/mL, or from about 1.06 to 1.10 g/mL, or from about 1.10 to about 1.21 g/mL, or a complex containing apoA-I or apoA-II. HDL may be prepared by density ultracentrifugation, as described in Mendez, A. J., et al., *J. Biol. Chem.* 266:10104-10111, 1991, from plasma, serum, bodily fluids, or tissue. The HDL₂ subfraction in the density range of about 1.110 to about 1.210 g/mL, and the HDL₂ subfraction in the density range of about 1.06 to about 1.125 g/mL may be isolated from plasma, serum, bodily fluids, tissue or total HDL by sequential density ultracentrifugation, as described in Mendez, supra. HDL is known to contain two major proteins, apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II); therefore, in some embodiments, the term "HDL, or a subfraction thereof" also includes an apoA-I and/or an apoA-II containing protein or lipoprotein complex which may be isolated, for example, by immunoaffinity with anti-apoA-I or anti-apoA-II antibodies.

As used herein, the term "HDL-associated" refers to any biological compounds that float in the density range of HDL (d=about 1.06 to about 1.21 g/mL) and/or molecules present in a complex containing apoA-I and/or apoA-II, including full-length proteins and fragments thereof, including peptides or lipid-protein complexes, such as microparticles, in HDL isolated from any sample, including lesions, blood, urine, cerebral spinal fluid, bronchoalveolar fluid, joint fluid, or tissue or fluid samples.

As used herein, the term "HDL₂-associated" refers to any biological compounds that float in the density range of HDL₂ (d=about 1.06 to about 1.125 g/mL) and/or molecules present in a complex containing apoA-I and/or apoA-II, including full-length proteins, and fragments thereof, including peptides, or lipid-protein complexes such as microparticles, in HDL isolated from any sample, including lesions, blood, urine, cerebral spinal fluid, bronchoalveolar fluid, joint fluid, or tissue or fluid samples.

As used herein, the term "mass spectrometer" refers to a device able to volatilize/ionize analytes to form gas-phase ions and determine their absolute or relative molecular masses. Suitable forms of volatilization/ionization are matrix-assisted laser desorption ionization (MALDI), electrospray, laser/light, thermal, electrical, atomized/sprayed and the like, or combinations thereof. Suitable forms of mass spectrometry include, but are not limited to, ion trap instruments, quadrupole instruments, electrostatic and magnetic sector instruments, time of flight instruments, time of flight

tandem mass spectrometer (TOF MS/MS), Fourier-transform mass spectrometers, and hybrid instruments composed of various combinations of these types of mass analyzers. These instruments may, in turn, be interfaced with a variety of sources that fractionate the samples (for example, liquid chromatography or solid-phase adsorption techniques based on chemical, or biological properties) and that ionize the samples for introduction into the mass spectrometer, including matrix-assisted laser desorption (MALDI), electrospray, or nanospray ionization (ESI) or combinations thereof.

As used herein, the term "affinity detection" or "affinity purified" refers to any method that selectively detects and/or enriches the protein or analyte of interest. This includes methods based on physical properties like charge, amino acid sequence, and hydrophobicity, and can involve many different compounds that have an affinity for the analyte of interest, including, but not limited to, antibodies, resins, RNA, DNA, proteins, hydrophobic materials, charged materials, and dyes.

As used herein, the term "antibody" encompasses antibodies and antibody fragments thereof derived from any antibody-producing mammal (e.g., mouse, rat, rabbit, and primate including human) that specifically bind to the biomarkers or portions thereof. Exemplary antibodies include polyclonal, monoclonal, and recombinant antibodies; multispecific antibodies (e.g., bispecific antibodies); humanized antibodies; murine antibodies; chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies; and anti-idiotypic antibodies, and may be any intact molecule or fragment thereof.

As used herein, the term "antibody fragment" refers to a portion derived from or related to a full length anti-biomarker antibody, generally including the antigen binding or variable region thereof. Illustrative examples of antibody fragments include Fab, Fab', F(ab)₂, F(ab')₂ and Fv fragments, scFv fragments, diabodies, linear antibodies, single-chain antibody molecules and multispecific antibodies formed from antibody fragments. Antibody and antibody fragments as used here may be incorporated into other proteins that can be produced by a variety of systems, including, but not limited to, bacteria, viruses, yeast, and mammalian cells.

As used herein, "a subject" includes all mammals, including without limitation humans, non-human primates, dogs, cats, horses, sheep, goats, cows, rabbits, pigs and rodents.

As used herein, the term "percent identity" or "percent identical," when used in connection with a biomarker used in the practice of the present invention, is defined as the percentage of amino acid residues in a biomarker sequence that are identical with the amino acid sequence of a specified biomarker after aligning the sequences to achieve the maximum percent identity. When making the comparison, no gaps are introduced into the biomarker sequences in order to achieve the best alignment.

Amino acid sequence identity can be determined, for example, in the following manner. The amino acid sequence of a biomarker is used to search a protein sequence database, such as the GenBank database, using the BLASTP program. The program is used in the ungapped mode. Default filtering is used to remove sequence homologies due to regions of low complexity. The default parameters of BLASTP are utilized.

As used herein, the term "derivatives" of a biomarker, including proteins and peptide fragments thereof, include an insertion, deletion, or substitution mutant. Preferably, any substitution mutation is conservative in that it minimally disrupts the biochemical properties of the biomarker. Thus, where mutations are introduced to substitute amino acid residues, positively-charged residues (H, K, and R) preferably are substituted with positively-charged residues; negatively-

charged residues (D and E) are preferably substituted with negatively-charged residues; neutral polar residues (C, G, N, Q, S, T, and Y) are preferably substituted with neutral polar residues; and neutral non-polar residues (A, F, I, L, M, P, V, and W) are preferably substituted with neutral non-polar residues.

As used herein, the amino acid residues are abbreviated as follows: alanine (Ala;A), asparagine (Asn;N), aspartic acid (Asp;D), arginine (Arg;R), cysteine (Cys;C), glutamic acid (Glu;E), glutamine (Gln;Q), glycine (Gly;G), histidine (His;H), isoleucine (Ile;I), leucine (Leu;L), lysine (Lys;K), methionine (Met;M), phenylalanine (Phe;F), proline (Pro;P), serine (Ser;S), threonine (Thr;T), tryptophan (Trp;W), tyrosine (Tyr;Y), and valine (Val;V).

In the broadest sense, the naturally occurring amino acids can be divided into groups based upon the chemical characteristic of the side chain of the respective amino acids. By "hydrophobic" amino acid is meant either Ile, Leu, Met, Phe, Trp, Tyr, Val, Ala, Cys, or Pro. By "hydrophilic" amino acid is meant either Gly, Asn, Gln, Ser, Thr, Asp, Glu, Lys, Arg, or His. This grouping of amino acids can be further subclassed as follows. By "uncharged hydrophilic" amino acid is meant either Ser, Thr, Asn, or Gln. By "acidic" amino acid is meant either Glu or Asp. By "basic" amino acid is meant either Lys, Arg, or His.

In the past, studies have been done to identify proteins in the blood of a subject that could be used as markers for cardiovascular disease (see, e.g., Stanley et al., *Dis. Markers* 20:167-178, 2004). However, this approach has been hampered by the vast number of candidate proteins in blood plasma in concentrations that vary over six orders of magnitude, which complicate the discovery and validation processes (Qian, W. J., et al., *Proteomics* 5:572-584, 2005). Cholesterol is present in the blood as free and esterified cholesterol within lipoprotein particles, commonly known as chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs), and high density lipoproteins (HDLs). HDL particles vary in size and density due to the differences in the number of apolipoproteins on the surface of the particles and the amount of cholesterol esters in the core of HDL (see Asztaloe et al., *Am. J. Cardiol.* 91:12 E-17E, 2003). HDL is composed of two principal subfractions based on density: HDL₂ and the denser HDL₃.

Elevated LDL cholesterol and total cholesterol are directly related to an increased risk of cardiovascular disease. See Anderson et al., "Cholesterol and Mortality: 30 years of Follow Up from the Framingham Study," *JAMA* 257:2176-90, 1987. In contrast, it has been established that the risk of cardiovascular disease is inversely proportional to plasma levels of HDL and the major HDL apolipoprotein, apoA-I (Gordon, D. J., et al., *N. Engl. J. Med.* 321:1311-1316, 1989). Studies have shown that high HDL levels are associated with longevity (Barzilai, N., et al., *JAMA* 290:2030-2040, 2003). Consistent with these findings, an abnormally low HDL level is a well-accepted risk factor for the development of clinically significant atherosclerosis (particularly common in men with premature atherosclerosis (Gordon, D. J., et al., *N. Engl. J. Med.* 321:1311-1316, 1989; Wilson, P. W., et al., *Arteriosclerosis* 8:737-741, 1988)). The mechanism by which HDL renders its protective effect against atherosclerosis is the subject of continued debate. Some studies have implicated that HDL may directly protect against atherosclerosis by removing cholesterol from artery wall macrophages (see Tall, A. R., et al., *J. Clin. Invest.* 110:899-904, 2002; Oram, J. F., et al., *Arterioscler. Thromb. Vasc. Biol.* 23:720-727, 2003). Other studies have reported that HDL protects against LDL oxidative modification, which is believed to be central to the initiation

and progression of atherosclerosis (see, e.g., Parthasarathy, S., et al., *Biochim. Biophys. Acta* 1044:275-283, 1990; Barter, P. J., et al., *Circ Res* 95:764-772, 2004). However, while HDL/LDL ratios have been correlated with risk for cardiovascular disease on an overall population, HDL and/or LDL measurements have not been reliable indicators of risk at an individual level.

Animal studies indicate that one important mechanism by which HDL protects against development of atherosclerosis involves reverse cholesterol transport in which HDL accepts cholesterol from macrophage foam cells in the artery wall and transports it back to the liver for excretion. HDL's cardioprotective effects may also depend on its anti-inflammatory properties. Indeed, HDL contains multiple acute phase response proteins, protease inhibitors and complement regulatory proteins (Vaisar, T., et al., *J Clin Invest* 117(3):746-756 (2007)). Although HDL-cholesterol (HDL-C) levels are widely used to assess the risk for CAD, studies with genetically engineered animals convincingly demonstrate that changes in HDL metabolism can promote atherosclerosis by pathways that are independent of plasma levels of HDL-C. Also, the failure of recent clinical trials of a therapy that elevates HDL-C levels suggests that HDL can become dysfunctional in humans.

In accordance with the foregoing, in one aspect, a method of screening a mammalian test subject to determine if the subject is at risk to develop, or is suffering from, cardiovascular disease. The method comprises detecting a measurable feature of at least two biomarkers present in an HDL subfraction, or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the subject. The measurable features of the at least two biomarkers selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof, are then compared to a reference standard that is derived from measurements of the corresponding biomarkers present in comparable HDL subfractions or complexes isolated from biological samples obtained from a control population, such as a population of apparently healthy subjects. A difference in the measurable features of the at least two biomarkers between the test subject's sample and the reference standard, such as an average value from the control population, is indicative of the presence or risk of developing CAD in the subject. In some embodiments, the method further comprises determining whether the subject is exhibiting symptoms related to CAD.

The methods of this aspect of the invention are useful to screen any mammalian subject, including humans, non-human primates, canines, felines, murines, bovines, equines, and porcines. A human subject may be apparently healthy or may be diagnosed as having a low HDL:LDL ratio and/or as being at risk for CAD based on certain known risk factors such as high blood pressure, high cholesterol, obesity, or genetic predisposition for CAD. The methods described herein are especially useful to identify subjects that are at high risk of developing CAD in order to determine what type of therapy is most suitable and to avoid potential side effects due to the use of medications in low risk subjects. For example, prophylactic therapy is useful for subjects at some risk for CAD, including a low fat diet and exercise. For those at higher risk, a number of drugs may be prescribed by physicians, such as lipid-lowering medications as well as medications to lower blood pressure in hypertensive patients. For subjects at high risk, more aggressive therapy may be indicated, such as administration of multiple medications.

In order to conduct sample analysis, a biological sample containing HDL is provided to be screened, including, but not

limited to, whole blood or blood fractions (e.g., serum), bodily fluid, urine, cultured cells, tissue biopsies, or other tissue preparations. In some embodiments of the method of the invention, the biological samples include total HDL (density=about 1.06 to about 1.21 g/mL) or protein complexes that are isolated in this density range. In some embodiments of the method, a complex containing apoA-I and/or apoA-II is isolated from the biological sample. In other embodiments of the method of the invention, an HDL₂ subfraction (density=about 1.06 to about 1.125 g/mL) is isolated from the biological sample prior to analysis. The HDL₂ fraction may be isolated using any suitable method, such as, for example, through the use of ultracentrifugation, as described in Example 1.

In some embodiments, one or more of the biomarkers apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, including apoA-I oxidized at methionine residues and/or other HDL-associated peptides and/or proteins are isolated by liquid chromatography, affinity chromatography, or antibody-based methods from biological samples such as, but not limited to, blood, plasma, serum, urine, tissue, or atherosclerotic lesions.

As described in Examples 1-5, the present inventors have used matrix-assisted laser desorption mass spectrometry (MALDI-MS) to investigate the HDL proteome through the use of tryptic digestion. It was determined that the use of pattern recognition with two powerful linear algebraic techniques principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) could distinguish between tryptic digested HDL₂ subfractions generated from control and CAD subjects at a high level of specificity and selectivity, as described in Example 2. Tandem mass spectrometry of informative mass features used to distinguish between normal and CAD subjects revealed a set of biomarkers for CAD as shown in TABLE 2 which include apoA-I (SEQ ID NO:1), apoA-II (SEQ ID NO:2), apoB-100 (SEQ ID NO:3), Lp(a) (SEQ ID NO:4), apoC-I (SEQ ID NO:5), apoC-III (SEQ ID NO:6), SAA4 (SEQ ID NO:7) and ApoE (SEQ ID NO:8), and peptide fragments and measurable features thereof.

The informative features that were identified that are useful to distinguish between normal and CAD subjects fall into the following classes: (1) increased levels of particular peptides/proteins in CAD subjects as compared to normal controls, for example, peptides derived from Lp(a) and/or apoC-III as shown in TABLE 3 and TABLE 4; (2) decreased levels of particular peptides/proteins in CAD subjects as compared to normal controls, for example, peptides derived from apoC-I as shown in TABLE 3 and TABLE 5; (3) post-translational modifications of particular peptides/proteins in CAD subjects as compared to normal controls, for example oxidation of M112 in apoA-I as shown in TABLE 3 and TABLE 6; and (4) altered conformational structure of particular peptides/proteins in CAD subjects as compared to normal controls, for example, apoA-I as shown in FIG. 7 and described in Example 5.

These results demonstrate that HDL isolated from subjects with CAD is selectively enriched in oxidized amino acids and certain proteins, and that the distinct cargo carried by the lipoprotein in subjects with clinically significant CAD may be assessed in a mammalian subject to determine his or her risk for developing CAD, the presence of CAD, and/or the efficacy of treatment of the subject for CAD. Therefore, the identification of peptides/proteins that are present in HDL of subjects suffering from CAD in amounts or structures that differ from normal subjects provide new biomarkers which are useful in assays that are prognostic and/or diagnostic for

the presence of CAD and related disorders. The biomarkers may also be used in various assays to assess the effects of exogenous compounds for the treatment of CAD.

In one embodiment of this aspect of the invention, at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises an increased amount of at least one of the biomarkers in the HDL subfraction of the biological sample selected from the group consisting of apoA-I, apoB-100, apoC-III, and Lp(a), or portions and/or derivatives thereof, in comparison to the reference standard. For example, as demonstrated in Example 3, TABLE 3, and TABLE 4, tryptic peptides have been identified from apoA-I, apoB-100, apoC-III, and Lp(a) that were increased in HDL₂ of CAD subjects as compared to normal control subjects. As shown in Examples 1 and 2, these peptides with increased frequency in CAD subjects are informative features for the prognosis and/or diagnosis of CAD.

In another embodiment of this aspect of the invention, at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises a decreased amount of at least one of the biomarkers in the HDL subfraction of the biological sample selected from the group consisting of apoA-I and apoC-I, or portions and/or derivatives thereof, in comparison to the reference standard. For example, as demonstrated in Example 3, TABLE 3, and TABLE 5, tryptic peptides have been identified from apoA-I and apoC-I that were decreased in HDL₂ of CAD subjects as compared to normal control subjects. As shown in Examples 1 and 2, these peptides with decreased frequency in CAD subjects are informative features for the prognosis and/or diagnosis of CAD.

In another embodiment of the invention, at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises a post-translational modification of a peptide derived from apoA-I in the HDL subfraction of the biological sample, in comparison to the reference standard. For example, as demonstrated in Example 4 and TABLE 6, it has been determined that the oxidation state of apoA-I at M112 is indicative of the presence of CAD.

In the practice of the methods of the methods of this aspect of the invention, a measurable feature of at least two biomarkers (such as at least 3, at least 4, at least 5, or at least 6) selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III is detected. In accordance with this aspect of the invention, proteins having at least 90% identity (such as at least 95% identical, or at least 98% identical) with apoA-I (SEQ ID NO:1), apoA-II (SEQ ID NO:2), apoB-100 (SEQ ID NO:3), Lp(a) (SEQ ID NO:4), apoC-I (SEQ ID NO:5), and apoC-III (SEQ ID NO:6), and peptides derived therefrom, may be used as biomarkers for CAD, which are present at a differential level in CAD subjects as compared to normal control subjects. Peptide fragments derived from SEQ ID NOS: 1, 2, 3, 4, 5, and 6 may also be used as biomarkers, such as peptides from about 4 amino acids to at least about 20 amino acids or more. Representative peptide fragments that may be used as biomarkers in which an increased amount of the biomarker in HDL₂ is indicative of the presence or risk of CAD include the peptides with positive regression vector values shown in TABLE 3 and TABLE 4. Representative peptide fragments that may be used as biomarkers in which a decreased amount of the biomarker in HDL₂ is indicative of the presence or risk of CAD include the peptides with negative regression vector values shown in TABLE 3 and TABLE 5.

The presence and/or amount of the two or more HDL-associated biomarkers in a biological sample comprising total HDL, or a subfraction thereof, may be determined using any

suitable assay capable of detecting the amount of the one or more biomarkers. Such assay methods include, but are not limited to, mass spectrometry, liquid chromatography, thin layer chromatography, fluorometry, radioisotope detection, affinity detection, and antibody detection. Other detection paradigms may optionally be used, such as optical methods, electrochemical methods, atomic force microscopy, and radio frequency methods (e.g., multipolar resonance spectroscopy). Optical methods include, for example, microscopy, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, and transmittance.

In one embodiment, the presence and amount of one or more HDL-associated biomarkers is determined by mass spectrometry. In accordance with this embodiment, biological samples may be obtained and used directly, or may be separated into total HDL or an HDL₂ subfraction. The HDL-associated proteins are digested into peptides with any suitable enzyme such as trypsin, which cleaves adjacent to lysine (K) or arginine (R) residues in proteins. The peptides are then analyzed by a mass spectrometry method such as MALDI-TOF-MS or M/MS (solid phase), liquid chromatography (LC)-MS or MS/MS, μ LC-ESI-MS/MS, and iTRAQ™ ICAT, or other forms of isotope tagging. Any suitable method may be used for differential isotope labeling of proteins and/or peptide, such as the use of a compound or isotope-labeled compound that reacts with an amino acid functional group. Label-specific fragment ions allow one to quantify the differences in relative abundance between samples. For example, one useful approach to achieve quantitative results is the use of MALDI TOF/TOF or QTOF mass spectrometers and iTRAQ™, a commercially available stable isotope labeling system (Applied Biosystems, Foster City, Calif.). The iTRAQ™ labeling system allows selective labeling of up to four different samples which are distinguished from one another in the mixture by MS/MS analysis.

By way of representative example, the method of MALDI-TOF-MS/MS involves the following steps. The samples are prepared and separated with fluidic devices, such as microfluidic devices, and spotted on a MALDI plate for laser-desorption ionization. Mass spectra are taken every few seconds, followed by isolation of the most intense peptide ions, or the peptide ions of interest (e.g., one derived from specific peptides), fragmentation by collisions with an inert gas, and recording of a mass spectrum of the fragments. This fragment mass spectrum, known as MS/MS spectrum, tandem mass spectrum, or MS² spectrum, consists mainly of N- and C-terminal fragments of the peptide ions at the amide bonds, called b ions and y ions, respectively. The spectra are then matched to sequence databases, as further described in Example 3.

In a typical application of MS analysis, proteins in a biological sample are reduced, alkylated, digested into peptides with trypsin, and analyzed using multidimensional liquid chromatography and tandem mass spectrometry (MS/MS). Tryptic peptides are then subjected to multidimensional chromatography in concert with MS/MS analysis. In multidimensional chromatography, the first chromatographic dimension typically involves separation of digested peptides on a strong cation exchange column. The peptides are then typically separated through a reverse-phase column with increasing concentrations of acetonitrile and then introduced into the source of the mass spectrometer or fractionated directly onto a MALDI sample plate. Tandem mass spectra may be acquired in the data-dependent mode on an ion-trap, QTOF or MALDI-TOF/TOF instrument. The most abundant peaks from a survey scan are submitted to tandem MS analysis. In other applications, peaks that differ in intensity between samples of interest (e.g., a control population of apparently

healthy subjects and subjects with established CVD) are selected from the MS or MS/MS spectra by a suitable method such as pattern recognition, cluster analysis, or relative abundance (see Rocke, D. M., *Semin Cell Dev Biol* 15:703-13, 2004; Ghazalpour, A., et al., *Lipid Res* 45:1793-805, 2004). The collection of tandem mass spectra may be submitted for a database search against a database (e.g., the Human International Protein Index (IPI) database, using the SEQUEST search engine (see Kersey, P. J., et al., "The International Protein Index: An Integrated Database for Proteomics Experiments," *Proteomics* 4:1985-1988, 2004)), using software programs such as PeptideProphet, (Nesvizhskii, A. I., et al., *Anal. Chem.* 75:4646-4658, 2003) and ProteinProphet (Yan, W., et al., *Mol. Cell. Proteomics* 3:1039-1041, 2004) in order to refine peptide and protein identification.

To achieve semiquantitative results, protein abundance is estimated by the number of MS/MS spectra, the number of peptides detected, or by the percent of the protein sequence covered in the analysis. Quantitative results can be obtained with ICAT isotope tagging, iTRAQ™ isotope labeling, or other modifications or peptides involving stable isotopes. Label-specific ions or fragment ions allow quantification of differences between samples based on their relative abundance.

Mass spectrometry detection methods may include the use of isotope-labeled peptides or proteins. In accordance with one example of this detection method, as described by Zou, H., et al., *Cell* 107:715-726, 2001, a tryptic peptide is chosen from a protein of interest. The tryptic peptide is then synthesized to incorporate one or more stable isotope-labeled amino acids. The native peptide and the synthetic-labeled peptide share physical properties including size, charge, hydrophobicity, ionic character, and amenability to ionization. When mixed, they elute together chromatographically, migrate together electrophoretically, and ionize with the same intensity. However, they differ in molecular weight from as little as 1 to over 10 Daltons, depending on which stable isotope amino acid is chosen for incorporation. The native peptide and the synthetic peptide are easily distinguishable by mass spectrometry. The synthetic peptide is used in an assay by adding a known amount of the synthetic peptide to a biological sample. In another example of this detection method, an isotope-labeled protein is prepared by a suitable method, such as by using a bacterial expression system and growing the bacteria on medium enriched with ¹⁵N-Nitrate or other isotope-labeled nutrients. The isotope-labeled peptide or protein is added to the sample containing native proteins and the mixture is then digested and analyzed by mass spectrometry as described herein. Extracted ion chromatograms or selected ion chromatograms or peak ratios in a full scan mass spectrum are then generated for the native peptide and the synthetic peptide. The quantity of the native peptide is then calculated using ratios of ion current or peak ratios.

Another detection method that utilizes labeled peptide fragments is isotope-coded affinity tagging (ICAT). This technique, as described in Gygi, S. P., et al., *Nature Biotech.* 17:994-999, 1999, involves the use of isotope tags that covalently bind to specific amino acids (cysteines) within a protein of interest. For example, the tag may contain three functional elements including a biotin tag (used during affinity capture), an isotopically encoded linker chain (such as an ether linkage with either eight hydrogens or eight deuteriums), and the reactive group, which binds to and modifies the cysteine residues of the protein. The isotope tag is used in an assay by labeling a control sample with the light version of the tag and labeling a test sample with the heavy version of the tag. The two samples are then combined, enzymatically

digested, and the labeled cysteinyl residues may be captured using avidin affinity chromatography. The captured peptides are then analyzed by mass spectrometry, which can determine the relative abundance for each peptide-pair.

In another embodiment, antibodies are used in an immunoassay to detect one or more biomarkers in accordance with the method of this aspect of the invention. Such immunoassays may comprise an antibody to one or more of the biomarkers. The antibody is mixed with a sample suspected of containing the biomarker and monitored for biomarker-antibody binding. For example, the biomarker can be detected in an enzyme-linked immunosorbent assay (ELISA), in which a biomarker antibody is bound to a solid phase, such as a chip, and an enzyme-antibody conjugate is used to detect and/or quantify the biomarker(s) present in a sample.

In another aspect, the present invention provides a method of screening a mammalian subject to determine if the subject is at risk to develop, or is suffering from, or is recovering from a cardiovascular disease, the method comprising detecting an alteration in the conformational structure of apoA-I present in the HDL subfraction of a biological sample obtained from the test subject in comparison to a reference standard, wherein a difference in the conformation of the apoA-I between the biological sample from the subject and the reference standard is indicative of the presence or risk of cardiovascular disease in the subject.

In order to conduct sample analysis, a biological sample containing HDL is provided to be screened. Any HDL containing sample may be utilized with the methods described herein, including but not limited to whole blood or blood fractions (e.g., serum), bodily fluid, urine, cultured cells, biopsies or other tissue preparations. In some embodiments, the biological samples include total HDL (density=about 1.06 to about 1.21 g/mL) or protein complexes that are isolated in this density range. In some embodiments, an HDL₂ subfraction (density=about 1.06 to about 1.125 g/mL) is isolated from the biological sample prior to analysis. In some embodiments, the HDL subfraction may be isolated by affinity isolation with polyclonal antibodies against apoA-I, the major protein in HDL or with polyclonal antibodies raised against other HDL associated proteins.

As described in Example 5, and shown in FIG. 7, it was determined that two tryptic peptides originating from N-terminal regions of apoA-I were significantly increased in the HDL subfraction of CAD subjects as compared to normal controls, while one tryptic peptide originating from the C-terminal region of apoA-I was significantly decreased. Although these N-terminal and C-terminal peptides are distant in the apoA-I sequence, when mapped to the double-belt model of the lipid-associated HDL particle apoA-I (Davidson, W. S, et al., *J. Biol. Chem.* 282(31):22249-22253, 2007, or the spherical HDL particle apoA-I model, the peptides displaying significant changes in CAD subjects were found to be in close proximity, as discussed in Example 5.

The conformation of apoA-I may be determined using any suitable method, such as by digesting the HDL subfraction of the biological sample with trypsin, followed by mass spectrometry analysis to measure the presence and/or amount of the tryptic fragments of apoA-I as compared to a reference standard, such as apoA-I isolated from normal control subjects. For example, the reference standard could be an exogenous isotopically labeled apoA-I which serves as an internal reference to which the intensity of individual peptides derived from apoA-I from the HDL subfraction of the biological sample would be related by a first ratio (i.e., apoA-I peptide from biological test sample/apoA-I peptide from reference standard). This first ratio would then be compared to a second

ratio (i.e., apoA-I peptide from healthy control sample/apoA-I peptide from reference standard) to detect a difference in the amount of apoA-I peptides in the tested sample relative to the expected ratio in a healthy control sample, thereby indicating an altered apoA-I conformation.

In another example, the conformation of apoA-I may be determined by circular dichroism (CD), or with a monoclonal antibody that specifically detects the altered conformation of apoA-I. Methods of generating an antibody specific to an altered conformation of apoA-I are well known in the art, for example see Marcel, Y. L., et al., "Lipid Peroxidation Changes the Expression of Specific Epitopes of Apolipoprotein A-I," *J. Biol. Chem.* 264(33):19942-50, Nov. 25, 1989; Milthorpe P, et al., "Immunochemical characterization of apolipoprotein A-I from normal human plasma. In vitro modification of apo A-I antigens," *Arteriosclerosis* 6(3):285-96, May-June 1986; Marcel, Y. L., et al., "Monoclonal antibodies and the characterization of apolipoprotein structure and function," *Prog Lipid Res* 23(4):169-95, 1984; and Weech, P. K., et al., "Apolipoprotein A-I from normal human plasma: definition of three distinct antigenic determinants," *Biochim Biophys Acta* 835(2):390-401, Jul. 9, 1985., and Marcel Y. L. et al., "The epitopes of apolipoprotein A-I define distinct structural domains including a mobile middle region," *J. Biol. Chem.* 266(6):3644-53, 1991.

In another aspect, the invention provides a method for diagnosing and/or assessing the risk of CAD in a subject, comprising determining changes in a biomarker profile comprising the relative abundance of at least one, two, three, four, five, ten or more biomarkers present in the HDL fraction of a biological sample from a test subject as compared to the predetermined abundance of the at least one, two, three, four, five, ten or more biomarkers from a reference population of apparently healthy subjects. The biomarkers are selected from biomarkers set forth in TABLE 3, TABLE 4, and TABLE 5. The biomarker profile may optionally include an internal reference standard that is expected to be equally abundant in subjects with CAD and apparently healthy subjects.

In another aspect, the present invention provides a method for determining the efficacy of a treatment regimen for treating and/or preventing CAD by monitoring a measurable feature of at least two biomarkers selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof in a subject during treatment for CAD. The treatment for CAD varies depending on the symptoms and disease progression. The general treatments include lifestyle changes, medications, and may include surgery. Lifestyle changes include, for example, weight loss, a low saturated fat, low cholesterol diet, reduction of sodium, regular exercise, and a prohibition on smoking. Medications useful to treat CAD include, for example, cholesterol-lowering medications, antiplatelet agents (e.g., aspirin, ticlopidine, clopidogrel), glycoprotein IIb-IIIa inhibitors (such as abciximab, eptifibatid or tirofiban), or antithrombin drugs (blood-thinners such as heparin) to reduce the risk of blood clots. Beta-blockers may be used to decrease the heart rate and lower oxygen use by the heart. Nitrates, such as nitroglycerin, are used to dilate the coronary arteries and improve blood supply to the heart. Calcium-channel blockers are used to relax the coronary arteries and systemic arteries and thus reduce the workload for the heart. Medications suitable for reducing blood pressure are also useful to treat CAD, including ACE inhibitors, diuretics, and other medications.

The treatment for cardiovascular disease may include surgical interventions such as coronary angioplasty, coronary

atherectomy, ablative laser-assisted angioplasty, catheter-based thrombolysis, mechanical thrombectomy, coronary stenting, coronary radiation implant, coronary brachytherapy (delivery of beta or gamma radiation into the coronary arteries), and coronary artery bypass surgery.

In another aspect, the present invention provides assays and kits comprising one or more detection reagents for determining susceptibility or presence of cardiovascular disease in a mammalian subject based on the detection of at least one measurable feature of at least one biomarker in a biological sample, an HDL subfraction thereof, or a complex containing apoA-I or apoA-II isolated from the biological sample. The biomarker is detected by mixing a detection reagent that detects at least one biomarker associated with CAD with a sample containing HDL-associated proteins (either an HDL subfraction or a complex containing apoA-I or apoA-II) and monitoring the mixture for detection of the biomarker with a suitable detection method such as spectrometry, immunoassay, or other method. In one embodiment, the assays are provided as a kit. In some embodiments, the kit comprises detection reagents for detecting at least two, three, four, five, ten or more HDL-associated biomarkers in biological samples from a test subject.

The kit also includes written indicia, such as instructions or other printed material for characterizing the risk of CAD based upon the outcome of the assay. The written indicia may include reference information or a link to information regarding the predetermined abundance of the at least one, two, three, four, five, ten or more HDL-associated biomarkers from a reference population of apparently healthy subjects and an indication of a correlation between the abundance of one or more HDL-associated biomarkers and the risk level and/or diagnosis of CAD.

The detection reagents may be any reagent for use in an assay or analytical method, such as mass spectrometry, capable of detecting at least one measurable feature of at least one biomarker selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III. In another embodiment, the detection reagents include proteins with peptides identical to those of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, such as peptides provided in TABLE 3. A variety of protocols for measuring the relative abundance of the biomarkers may be used, including mass spectrometry, ELISAs, RIAs, and FACs, which are well known in the art.

In one embodiment, the detection reagent comprises one or more antibodies which specifically bind one or more of the biomarkers provided in TABLE 3, TABLE 4, or TABLE 5 that may be used for the diagnosis and/or prognosis of CAD characterized by the relative abundance of the biomarker in the serum, or an HDL subfraction thereof. Standard values for protein levels of the biomarkers are established by combining biological samples taken from healthy subjects. Deviation in the amount of the biomarker between control subjects and CAD subjects establishes the parameters for diagnosing and/or assessing risk levels, or monitoring disease progression. The biomarkers and fragments thereof can be used as antigens to generate antibodies specific for the CAD biomarkers for use in immunodiagnostic assays. Purified samples of the biomarkers comprising the amino acid sequences shown in TABLE 3, TABLE 4, and TABLE 5 may be recovered and used to generate antibodies using techniques known to one of skill in the art.

In another embodiment, the detection reagent comprises isotope-labeled peptides, such as one or more of the peptides described in TABLE 3, TABLE 4, and TABLE 5 that correspond to the biomarker to be detected. In accordance with this

embodiment, the kit includes an enzyme, such as trypsin, and the amount of the biomarker in the tryptic digest of the sample is then quantified by isotope dilution mass spectrometry. The labeled peptides may be provided in association with a substrate, and the assay may be carried out in a multiplexed format. In one embodiment, a multiplexed format includes isotope-labeled peptides for at least two or more of the HDL-associated biomarkers described herein that are enriched in HDL of subjects with established CAD. The peptides are quantified of all the HDL-associated peptides in a biological sample obtained from a test subject using a technique such as isotope dilution mass spectrometry. The detection and quantification of multiple HDL-associated biomarker proteins may be used to increase the sensitivity and specificity of the assay to provide an accurate risk assessment and/or diagnosis of the presence of CAD in the test subject.

In one embodiment of the kit, the detection reagent is provided in association with or attached to a substrate. For example, a sample of blood, or HDL subfraction thereof, may be contacted with the substrate, having the detection reagent thereon, under conditions that allow binding between the biomarker and the detection reagent. The biomarker and/or the detection reagent are then detected with a suitable detection method. The substrate may be any suitable rigid or semi-rigid support including membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles, and capillaries. The substrate can have a variety of surface forms, such as wells, trenches, pins, channels, and pores to which the polypeptides are bound. For example, a chip, such as a biochip, may be a solid substrate having a generally planar surface to which a detection reagent is attached. For example, a variety of chips are available for the capture and detection of biomarkers, in accordance with the present invention, from commercial sources such as CIPHERGEN Biosystems (Fremont, Calif.), Packard BioScience Company (Meriden, Conn.), Zyomyx (Hayward, Calif.), and Phylos (Lexington, Mass.). An example of a method for producing such a biochip is described in U.S. Pat. No. 6,225,047. The biomarkers bound to the substrates may be detected in a gas phase ion spectrometer. The detector translates information regarding the detected ions into mass-to-charge ratios. Detection of a biomarker also provides signal intensity, thereby allowing the determination of quantity and mass of the biomarker.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

Example 1

This example demonstrates that subjects may be successfully classified as normal control or coronary artery disease (CAD) subjects by analyzing the proteomic profile of HDL₂ tryptic peptides using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) tandem mass spectrometry (MALDI-TOF-MS) and subjecting the results to principal component analysis (PCA), a well-established pattern recognition method.

Rationale:

The overall approach in this study was to isolate HDL₂ from control and CAD subjects, analyze a tryptic digest of HDL proteins by MALDI-TOF-MS, and use pattern recognition of the full scan mass spectra to classify subjects as either CAD subjects or control subjects.

Methods:

Sample isolation and preparation: All protocols involving human subjects were approved by the Human Studies Com-

mittees at the University of Washington. Blood samples were collected from 20 apparently healthy adult males and from 18 male patients with established CAD after an overnight fast. Blood samples were anticoagulated with EDTA. All subjects were male and matched for age and HDL cholesterol (HDL-C) levels. The CAD subjects had documented vascular disease, with symptoms consistent with angina and abnormal Q waves on their EKG or at least one stenotic lesion (>50% occlusion on coronary angiography). These CAD subjects were clinically stable, at least three months had elapsed since their acute coronary syndrome, and they had not taken lipid-lowering drugs for the six weeks prior to blood collection. The control subjects were apparently healthy and had no known history of CAD, were not hyperlipidemic, had no family history of premature CAD, and were not receiving any lipid-lowering therapy. None of the control subjects smoked cigarettes, had liver or renal disease, were diabetic, or had received lipid-lowering medications for at least six weeks before blood was collected.

The clinical characteristics of the two subject populations are summarized below in Table 1.

TABLE 1

CLINICAL CHARACTERISTICS OF STUDY SUBJECTS							
Number	Status	Age (yr)	% Male	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
20	Control	57 (6)	100	197 (13)	104 (29)	42 (8)	134 (14)
18	CAD	57 (6)	100	223 (27)	146 (67)	41 (8)	160 (25)

It is noted that although levels of plasma LDL and triglycerides were higher in the CAD subjects than in the control subjects, the two groups were otherwise well-matched for known risk factors for vascular disease.

HDL isolation: HDL₂ (d=1.063 to 1.125 g/mL) was isolated from plasma obtained from the blood samples by sequential density ultracentrifugation, according to the methods described in Mendez, A. J., et al., *J. Biol. Chem.* 266: 10104-10111, 1991. Protein concentration of HDL was determined using the Bradford assay (BioRad, Hercules, Calif.) with albumin as the standard.

Tryptic Digestion: HDL₂ was digested for 60 minutes with trypsin (1:50 w/w trypsin/HDL, sequencing grade trypsin, Promega WI) in 100 mM ammonium bicarbonate buffer in 80% aqueous acetonitrile (Strader, M. B., et al., *Anal Chem* 78(1):125-134, 2006). Digestion was terminated by addition of trifluoroacetic acid (TFA) to 1% final concentration.

The protein concentration of the HDL₂ digest was adjusted to 100 ng/μL with matrix solvent (70% acetonitrile, 0.1% TFA), and 0.5 μL of the digest was deposited on a MALDI target plate. Dried spots were overlaid with 0.5 μL of MALDI matrix (5 mg/mL alpha-cyano-4-hydroxy-cinnamic acid (CHCA) in matrix solvent).

Mass spectrometric analysis. Mass spectra were acquired on a matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) tandem mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer), operated in the reflection mode. Raw spectra were: (i) baseline-corrected and centroided using algorithms supplied by the manufacturer (ABI 4700 Explorer software, version 3.5); and (ii) internally mass calibrated using 5 tryptic fragments of apolipoprotein AI (apoA-I). The centroided spectra were then exported, using T2Extractor 5 (<http://www.proteomecommons.org/ar->

hive/1114637208624/) for further analysis. It was determined that internal calibration afforded mass accuracy better than 5 ppm across the acquisition mass range.

For pattern recognition analysis, a single mass spectrum was generated from at least 80 sub-spectra generated randomly from different sites across the sample spot, each sampled with 25 laser shots, for a total of 2,000 shots. To exclude low intensity and saturated sub-spectra, only those with an ion current ranging from 30 to 80×10⁴ cps were used to produce the mass spectrum.

The analytical precision of the different steps was evaluated by acquiring multiple spectra from: (1) the same MALDI spot; (2) multiple MALDI spots of the same tryptic digest; (3) multiple tryptic digests of the same sample; and (4) tryptic digestion of the same sample carried out on different days. As shown in FIGS. 9A-D, precision analysis of individual mass channels showed excellent reproducibility of the spectra from the same spot (FIG. 9A), multiple spots (FIG. 9B), parallel digestions (FIG. 9C), and interday digestion (FIG. 9D). The data indicated that precision was improved by averaging several spectra from the same spot. Thus, for the PLS-DA analysis, four spectra from the same spot were averaged to generate a master spectrum used for subsequent analyses.

Processing of MS Spectra: MATLAB (version 7.0, MathWorks Inc.) was used for pattern recognition analysis. The full scan mass spectrum of each sample was transformed into a vector format suitable for pattern recognition based on linear algebra by placing the signals in bins that ranged from m/z 800 to m/z 5,000. To ensure that every spectrum had the same mass channels, bin sizes were increased linearly over this range to yield 45,920 bins or channels per spectrum. After binning, data vectors were aligned to remove single bin shifts that occurred when signals were near the bin boarder. A threshold of 1/10,000 of the spectrum's total signal was used to remove baseline noise, and the spectra were aligned. For PLS-DA the data were separated into calibration and test sets prior to preprocessing to avoid overfitting. After alignment and filtering, 2,338 channels contained signals.

PCA Analysis: Processed MS spectra were then subjected to principal component analysis (PCA) and PLS-DA (Beebe, K. R., et al., *Chemometrics: A practical guide*. New York: Wiley—Interscience; 1998; and International Publication No. WO2006/083852 incorporated by reference herein in its entirety). PCA was used to assess the reproducibility of MALDI plate spotting and digestion and the sensitivity of MALDI-TOF-MS to changes in HDL protein composition. The latter was tested by mixing variable amounts of HDL₂ isolated from CAD and control subjects, as shown in FIG. 8.

Validation of the Pattern Recognition Model: In order to test the ability of pattern recognition to distinguish between CAD and control HDL, PCA analysis was performed after mixing variable proportions of HDL₂ isolated from control and CAD subjects. Mass spectra of 6 pairs of randomly chosen CAD and control samples were mixed in ratios of 1:0, 1:3, 1:1, 3:1, and 0:1. In separate experiments, blinded test samples from CAD or control subjects mixed at the same ratios were also included for the study.

Results:

PCA is a powerful linear algebraic technique for identifying factors that differentiate populations in a complex data set (Martens, H., et al., *Multivariate Calibration*. New York: John Wiley & Sons, 1989; Marengo, E., et al., *Proteomics* 5(3):654-666 (2005); Lee, K. R., et al., *Proteomics* 3(9): 1680-1686 (2003); Natale, C. D., et al., *Biosensors and Bioelectronics* 18(10):1209-1218 (2003)). Importantly, this unsupervised data reduction method creates pattern recognition models without a priori assumptions regarding relation-

ships between individual samples, (Beebe, K. R., et al., *Chemometrics: A practical Guide*. New York: Wiley-Interscience 1998).

PCA was initially used to test the ability of pattern recognition to distinguish between CAD and control HDL after mixing variable proportions of HDL₂ isolated from control and CAD subjects. The results of this analysis are shown in FIG. 8, where the square symbols and triangle symbols represent different pairs of CAD and control samples mixed at different ratios. As shown in FIG. 8, the bottom left corner of the graph shows control samples and the upper right corner of the graph shows CAD samples. Circles around the symbols represent a group of spectra from different mixed ratios. As shown in FIG. 8, the control and CAD subjects were well separated by PCA analysis. When the relative proportion of HDL₂ protein derived from control and CAD subjects in each sample was varied, there was a clear shift in the location of each sample on the PCA plot, as shown in FIG. 8, indicating that the method is sensitive to differences in the protein composition of HDL. Furthermore, the tight clustering of replicate spectra of the sample demonstrates the precision of this method.

These results demonstrate that subjects can be classified as CAD subjects based on the protein composition of their HDL₂ which differs substantially as compared to the protein composition of HDL₂ isolated from control subjects. These results further demonstrate that HDL₂ from subjects may be successfully classified into CAD or control subjects based on the MALDI-TOF-MS and PCA-based pattern profiling described. Use of tryptic peptides significantly enhances the precision and probability of identifying proteins and post-translational modifications and allows rapid analysis. Furthermore, as shown in FIG. 9, the tight clustering of replicate spectra demonstrates the precision of the analytical method. Thus, PCA provides a fast, simple, exploratory, and qualitative measure of differences in the protein cargo of HDL₂.

Example 2

This example demonstrates that subjects may be successfully classified into CAD or normal control subjects by analyzing tryptic digests of HDL₂-associated proteins by MALDI-TOF-MS using a highly precise pattern recognition linear algebraic algorithm, partial least squares determination analysis (PLS-DA).

Rationale:

Although PCA is a powerful technique for detecting and visualizing differences in patterns, it does not provide quantitative measures for predicting the disease status of individual samples. Therefore, another powerful linear algebraic technique, partial least squares discriminate analysis (PLS-DA) was used to develop a quantitative approach to classifying subjects with regard to CAD disease status. PLS-DA was selected rather than other pattern recognition techniques (such as K-nearest neighbor and Support Vector Machine) because it is well suited to analyzing the quantitative information in a mass spectrum which contains multiple independent signals as well as signals with significant redundancy and signals with incomplete selectivity.

Methods:

Sample isolation and preparation. HDL₂ fractions were isolated from the blood plasma of CAD and control subjects and digested with trypsin for 60 minutes, as described in Example 1. A sample from each subject was individually analyzed using MALDI-TOF MS as described in Example 1.

Processing of MS Spectra using Partial Least Squares Discriminate Analysis (PLS-DA) analysis. Matlab (version 7.0,

Mathworks Inc.) was used for pattern recognition analysis. The full scan mass spectrum of each sample was transformed to a vector format suitable for pattern recognition based on linear algebra by placing the signals in bins that ranged from m/z 800 to m/z 5,000. To ensure that every spectrum had the same mass channels, bin sizes were increased linearly over this range to yield 45,920 bins or channels per spectrum. After binning, data vectors were aligned to remove single bin shifts that occurred when signals were near the bin boarder. A threshold of 1/10,000 of the spectrum's total signal was used to remove baseline noise, and the spectra were aligned. For PLS-DA, the data were separated into calibration and test sets prior to preprocessing to avoid overfitting. After alignment and filtering, 2,338 channels contained signals.

Preprocessed MS spectra were then subjected to PLS-DA. PLS-DA is a supervised pattern recognition technique. It uses two sets of data, such as training sets with defined groups (such as cases vs. controls) to "supervise" the creation of a pattern recognition model (Barker, M., et al., *Journal of Chemometrics* 16:166-173 (2003)), which is subsequently applied to a second test set of samples of unknown status. Thus, PLS-DA can be used to determine if a new proteomics sample belongs to previously defined sample classes. Furthermore, it can reveal relationships among sample classes and identify features distinguishing the classes, and ultimately the corresponding proteins. Most importantly, PLS-DA yields a single discriminant score that quantifies similarity of the tested spectrum with the model and can be used to predict the disease status of individual samples (CAD or control).

PLS-DA models were built with a dummy response matrix containing discrete numerical values (1 or -1) for each class as described in International Publication No. WO2006/083853. In the present analysis, "1" represented the CAD class and "-1" represented the control class. For each sample being classified, the PLS-DA model then produced a discriminant value, which was termed the "proteomics CAD risk score" or "ProtCAD" score. The ProtCAD risk scores thus generated were used to predict disease status of the remaining control and CAD subjects (validation group) as described in International Publication No. WO2006/083853, hereby incorporated by reference. To provide a quantitative estimate of the performance of the PLS-DA model, two approaches were used to provide a quantitative estimate of the performance: (1) Random permutation analysis; and (2) Leave-one-out analysis.

Random Permutation Analysis: When data from only a small number of subjects are used to build a complex pattern recognition model, predictions are often affected by the selection of the calibration subjects. Therefore, a permutation analysis was used to test the ProtCAD score's ability to predict disease status. In each step of this analysis, the subjects were assigned to two groups: a calibration group and a validation group, each composed of ten randomly selected control subjects and nine CAD subjects. The calibration group was used to build a PLS-DA model, which was then used to predict the ProtCAD score for each subject in the validation group. This process was repeated 7,777 times to determine the precision of the PLS-DA predictions, as described in International Publication No. WO2006/083853.

Receiver operating characteristics (ROC) curves: Non-parametric empirical receiver operating characteristic (ROC) curves were constructed from the ProtCAD risk score (Pepe, M. S., *The Statistical Evaluation of Medical Tests for Classification and Prediction*, New York, Oxford University Press, 2003). Sensitivity and specificity were calculated from the known class identity of each subject in the validation

group. Area under the curve (AUC) calculations was determined using the trapezoidal rule (Fawcett, T., "An Introductory ROC Analysis," *Pattern Recognition Letters* 27:861-874, 2006). For each permutation, one ROC curve was generated, and by plotting the sensitivity (fraction of positive results) against specificity (the fraction of negative results), ROC quantitatively assessed the accuracy of the predictive test. A quantitative PLS-DA model based on full scan mass spectra of HDL₂ from a calibration group randomly selected subjects predicted CAD status in the validation group, with an average ROC_{AUC} of 0.9. ROC_{AUC} of 0.5 represents chance discrimination, whereas ROC_{AUC} 1.0 represents perfect discrimination. For a CAD diagnostic test, an ROC_{AUC} of 0.7-0.8 is generally considered acceptable, and values over 0.8 are considered excellent.

Leave-one-out ProtCAD prediction: In order to use the maximum number of available subjects, a leave-one-out approach was utilized to build a more powerful PLS-DA model, as described in International Publication No. WO2006/083853. The ProtCAD score for each subject was determined using a model built from the remaining subjects (e.g., 17 CAD and 19 controls). To predict the disease status of a subject, a ROC curve was first constructed and then the value of the ProtCAD score was compared to a threshold value corresponding to a selected sensitivity and selectivity on the ROC curve.

In preliminary experiments, it was determined that CAD predictions based on the entire mass spectrum outperformed predictions based on the ten most selective signals (as determined by PLS-DA, data not shown). Full spectrum PLS-DA can identify signals in regions normally associated with low selectivity and help identify outlier samples (e.g., problems with data acquisition from MS analysis, sample handling) or marked variations in sample protein composition (e.g., genetic variations in apoA-I, post-translational modifications). Such outliers would be overlooked by techniques that use only selected features of the spectrum. Therefore, all of the information in the full scan mass spectra was used to build models.

Results:

Two approaches were used to assess the ability of PLS-DA to distinguish the proteomic fingerprints of HDL isolated from control and CAD subjects. First, a PLS-DA model was built using data from randomly selected control and CAD subjects. Then the model was tested for its ability to predict disease status in a second set of subjects. The PLS-DA models were built with a dummy response matrix containing discrete numerical values for each class using ten control and nine CAD subjects (the calibration set) that were randomly selected from the 20 control and 18 CAD subjects. The PLS-DA model was then used to predict the disease status of the remaining ten control and nine CAD subjects (the validation set). For each sample in the validation set the approach produced a discriminant score, which was termed the "Proteomics CAD risk score" (ProtCAD risk score).

Random permutation analysis was used to provide a quantitative estimate of the performance of the PLS-DA model that was used to build the discriminant termed the Proteomics CAD risk score (ProtCAD risk score). The ProtCAD score was then used to predict the CAD status of the validation group (the remaining nine CAD and ten control subjects). A total of 7,777 random permutations were used to construct the ROC curve. FIG. 1 presents graphical results demonstrating the receiver operating characteristic (ROC) curve of the prediction of cardiovascular disease (CAD) status based on random permutation analysis. By plotting sensitivity (the fraction of true positive results, shown in the y-axis) against

specificity (the fraction of true negative results, shown in the x-axis), the ROC curve quantitatively assesses the accuracy of a predictive test. As shown in FIG. 1, the average ROC curve shows an area-under-the-curve of 0.880 ± 0.097 (mean, SD, N=7,777 iterations), indicating that PLS-DA analysis can predict disease status with high sensitivity and specificity.

FIG. 2 graphically illustrates the prediction of CAD status by the proteomics CAD risk score "ProtCAD risk score" using a partial least squares discriminate analysis (PLS-DA) model built using a calibration group. Using a sensitivity of 80%, the ProtCAD risk score of each subject in the validation group at each permutation was used to predict their CAD status. The results in FIG. 2 demonstrate that the ProtCAD score generated using the PLS-DA model is able to distinguish CAD and control subjects with high selectivity (p-value of 0.0001%). Using a clinically acceptable sensitivity of 80%, (see Pepe, M. S., *The Statistical Evaluation of Medical Tests for Classification and Prediction*, New York: Oxford University Press, 2003), the average PLS-DA model predicted CAD status with 76% specificity, as shown in FIG. 2.

The level of specificity shown in FIG. 2 corresponds to an odds ratio of 12.7, i.e., the odds ratio of the PLS-DA model for predicting CAD status here was 12.7 at 80% sensitivity and 76% specificity. These results demonstrate the power of this analytical approach for identifying subjects at risk for CAD.

In the second approach, disease status was predicted by using PLS-DA models built with the leave-one-out method as described in International Publication No. WO2006/083853. This strategy allowed the use of all available subjects for the analysis, which would be expected to yield the strongest predictive model. After systematically leaving out one subject at a time from the calibration set, the subject's ProtCAD score was predicted using a model built from the remaining samples.

FIG. 3 graphically illustrates the power of the ProtCAD risk score to discriminate between the CAD samples and healthy control samples based on leave-one-out analysis. The ProtCAD risk score was derived from PLS-DA analysis of MALDI-TOF-MS mass spectra of HDL tryptic digests, using a leave-one-out experiment for all 18 CAD and 20 control subjects. These ProtCAD scores distinguished the CAD and control subjects with high selectivity ($p < 0.0001$), as shown in FIG. 3. Furthermore, the larger number of subjects in the calibration set improved diagnostic power. As shown in FIG. 10A, the ROC curve constructed for ProtCAD scores from the leave-one-out approach showed an area-under-the-curve of 0.94 and a maximum odds ratio of 68. From the leave-one-out ProtCAD risk score ROC curve, we determined a threshold corresponding to 90% sensitivity (ProtCAD threshold = -0.06). Using this threshold, the model correctly classified 16 of 18 CAD subjects and 19 of 20 control subjects.

Therefore, these results demonstrate that pattern recognition analysis, when applied to MALDI-TOF-MS spectra of tryptic digests of HDL₂, clearly demonstrate differences in the HDL proteomic signature of apparently healthy subjects and CAD subjects. Moreover, a quantitative PLS-DA model based on full scan mass spectra of HDL₂ from a calibration group of randomly selected subjects predicted CAD status in the validation group, with an average ROC_{AUC} of 0.9 (ROC_{AUC} 0.5 represents chance discrimination, whereas ROC_{AUC} 1.0 represents perfect discrimination).

For a CAD diagnostic test, an ROCAUC of 0.7 to 0.8 is generally considered acceptable, and values over 0.8 are considered excellent (see Pepe, M. S., *The Statistical Evaluation of Medical Tests for Classification and Prediction*, New York, Oxford University Press, 2003). Furthermore, the odds ratio of the PLS-DA model for predicting CAD status was 12.7 at

80% sensitivity and 76% specificity. When the model was built with data from a larger number of subjects using the leave-one-out method, the ProtCAD risk score distinguished subjects with an even higher odds ratio of 68. These results compare favorably with other single lipoprotein-associated risk factors identified in previous studies (Yusuf, S., et al., *Lancet*, 364(9438):937-952, 2004; Walldius, G., and I. Jungner, *J. Intern. Med.* 259(5):493-519, 2006; Walldius, G., and I. Jungner, *Curr. Opin. Cardiol.* 22(4):359-367, 2007).

The standard method for predicting CAD, the Framingham risk score, combines seven demographic, biochemical and medical factors to predict CAD risk (Wilson, P. W., et al., *Circulation* 97(18):1837-1847 (1998)). The Framingham risk scores ROC_{AUC} ranges from 0.6-0.8 for predicting CAD risk over a ten year period. Its strongest predictors are age and sex, which are not modifiable risk factors. Moreover, LDL-C and HDL-C contribute little to the risk score in some models (Yusuf, S., et al., *Lancet* 364(9438):937-952, 2004; Walldius, G., et al., *Curr Opin Cardiol* 22(4):359-367, 2007; Walldius, G., et al., *J Intern Med* 259(5):493-519, 2006).

On the other hand, this example indicates that the HDL₂ isolated from CAD subjects with its characteristic proteome profile is faster and more accurate at predicting risk with a ROC_{AUC} of 0.9.

Conclusion:

As demonstrated herein, the protein composition of the HDL₂ in subjects with CAD as well as the protein composition of HDL₂ isolated from control subjects are different. Differences in the protein profiles can be accurately and quantitatively measured using the two different approaches together with the PLS-DA algorithm. These observations also show that PLS-DA analysis can correctly and with high sensitivity predict the status of a subject as a CAD subject or a control subject.

The methods of proteomic fingerprinting of HDL by MALDI-TOF-MS offer a number of important advantages for building classification models. First, it has been demonstrated that HDL is causally linked to CAD pathogenesis. Second, the HDL proteome is much simpler than the plasma proteome (which has been estimated to contain $>10^4$ different proteins and peptides with relative concentrations ranging over 12 orders of magnitude), which greatly facilitates MS analysis. Third, the interrogation of tryptic digests significantly enhances the precision of the mass spectrometric measurements, and thereby increases the probability of identifying proteins and post-translational modifications. In contrast to the methods described herein, surface-enhanced laser desorption ionization (SELDI) MS analysis, which has been widely used for pattern recognition, typically samples intact proteins, which makes it difficult to identify the proteins responsible for informative signals in quantitative models. SELDI MS also has a limited mass range and low mass resolution, which bias detection of informative features toward degraded and low MW proteins. Finally, the high mass accuracy of MALDI-TOF-MS facilitates the subsequent identification of proteins and posttranslational modifications by tandem MS. MALDI-TOF-MS of tryptic digests also greatly improves the precision of signals, which is important for pattern recognition analysis.

Previous studies using shotgun proteomics to investigate the protein composition of HDL₃ using liquid chromatography in concert with electrospray ionization (ESI) to introduce peptides into the mass spectrometer (Vaisar, T., et al., *J. Clin. Invest.* 117(3):746-756 (2007)). In contrast, in the present study the peptides were ionized with MALDI. It is well established that ESI and MALDI ionize different classes of pep-

tides with different efficiencies. For example, hydrophobic peptides are much more readily introduced into the gas phase by MALDI.

Example 3

This example describes the identification of proteins differentially present in HDL₂ subfractions isolated from normal control and CAD subjects by tryptic peptide analyses of HDL₂ fractions by tandem mass spectrometric (tandem MS) following PLS-DA based pattern recognition profiling.

Methods:

Sample isolation and preparation: HDL₂ fractions were isolated from normal control and CAD subjects as described earlier in Example 1. Tryptic digests of HDL₂ fractions were subjected to liquid chromatographic separation with direct application of the sample effluent from the liquid chromatograph onto a MALDI sample plate and subjected to MALDI-TOF/TOF tandem mass spectrometric analysis (LC-MALDI-TOF/TOF). Subjects were confirmed as either CAD or normal subjects by pattern recognition proteomic profiling of HDL₂ proteins using PLS-DA, as described in Example 2.

The PLS-DA models are characterized by regression vectors which indicate channels on the m/z axis of a mass spectrum that differentiate the two sample classes.

FIG. 4 graphically illustrates the PLS-DA regression vectors (y-axis) of the leave-one-out PLS-DA model that distinguish CAD and control subjects. The x-axis (m/z) represents mass channels of the MALDI-TOF mass spectrum. Positive and negative features on the regression vector indicate an increase and decrease of the signals from CAD samples (and therefore relative amount of peptide present) relative to control samples. Each mass channel in the regression vector that had significant differences between CAD and normal subjects was called an informative feature.

Channels in the regression vectors with positive values correspond to the peptides (and indirectly to the proteins) with increased relative abundance in CAD samples. Channels with negative values in the regression vector have decreased abundance in CAD samples. As shown in FIG. 4, a subset of 13 informative features were identified with the most significant increase or decrease in CAD subjects as compared to normal control subjects in a full scan mass spectrum that contributed to the ability to differentiate CAD subjects from normal subjects. The peptides associated with these informative features were identified by tandem MS using the MALDI-TOF/TOF analyzer capable of MS and MS/MS analysis interfaced with an off-line capillary liquid chromatograph and coupled with a MALDI plate spotter. As described in International Publication No. WO2006/083853, chromatographic information may be used to more strongly validate that the peptide identified is actually producing the observed peak in the regression vector.

Identification of significant features by Liquid-Chromatography (LC) matrix-assisted laser desorption ionization (MALDI): To identify features that were enriched or depleted in the mass spectra of HDL isolated from CAD subjects, CAD HDL tryptic digests were fractionated by liquid chromatography and the peptide digest was subjected to MS/MS analysis by MALDI-TOF/TOF. A tryptic digest of HDL was injected onto a trap column (NanoTrap C18, LC Packings), washed, and eluted onto an analytical capillary HPLC column (PepMap C18, LC Packings) using an Ultimate 300 HPLC (LC Packings Inc.). Separation was achieved by linear gradient 5-50% B over 40 minutes (A-5% aqueous can, 0.1% TFA, B-80% aqueous can, 0.1% TFA). The eluent from the column was mixed with matrix (CHCA, 5 mg/ml in 70% AcN) con-

taining internal standard peptides and spotted on-line (Shimadzu Accuspot MALDI plate spotter) on a MALDI target plate. Targeted MS/MS analysis of selected peptide ions was based on informative mass features of HDL proteomics fingerprints that were identified in the PLS-DA analysis. Peptides were identified by MASCOT database search (v2.0, Matrix Science) against the human SwissProt protein database with the following parameters; trypsin cleavage with up to two missed cleavages, methionine oxidation variable modification, precursor tolerance 15 ppm, and fragment ion tolerance 0.2 Da. Peptide matches were only accepted if the MASCOT probability based Mowse score identified the peptide with a very high score indicating a match to the database with >99% confidence.

Results:

It was determined that the relative abundance of HDL₂ apolipoproteins was altered in CAD subjects compared to the controls.

One group of informative features arose from proteins in the HDL₂ fraction that were differentially abundant in CAD and control subjects. As shown in FIG. 4, informative features with positive regression vector values were observed at channels 1081, 1226, 1440, 1715*, 1904, 2203, and 2661 m/z in the CAD subjects relative to the control subjects, indicating that the peptides (and therefore proteins) represented by these regression vectors at these channels were more abundant in CAD subjects.

As further shown in FIG. 4, informative features with negative regression vector values were observed at channels 852*,

1012, 1302, 1380, 1612, and 2645 m/z in the CAD subjects relative to the control subjects, indicating that the peptides (and therefore proteins) represented by these regression vectors at these channels were reduced in CAD subjects as compared to normal control subjects.

TABLE 2 provides a set of informative biomarkers corresponding to features from FIG. 4 that were identified using the targeted LC-MALDI-TOF/TOF approach.

TABLE 2

BIOMARKERS IDENTIFIED AS PROGNOSTIC AND/OR DIAGNOSTIC INDICATORS OF CAD		
Protein	Refseq ID Numbers	SEQ ID NO:
apoA-I	NP_000030.1	SEQ ID NO: 1
apoA-II	NP_001634	SEQ ID NO: 2
apoB-100	NP_000375	SEQ ID NO: 3
Lp(a)	NP_005568.1	SEQ ID NO: 4
apoC-I	NP_001636.1	SEQ ID NO: 5
apoC-III	NP_000031.1	SEQ ID NO: 6
SAA4 (serum amyloid A4-confirm)	NP_006503	SEQ ID NO: 7
apoE	NP_000032	SEQ ID NO: 8

Targeted tandem MS analysis was carried out to identify the peptides corresponding to the informative features shown in FIG. 4. The results are shown in TABLE 3.

TABLE 3

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD					
m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
861.5088	-11.679	ITLPDFR	apoB-100	2706-2712	9
999.5271	-5.9951	SVGFHLPSPR	apoB-100	1325-1333	10
1012.6055	-39.9366	AKPALEDLR	apoA-I	231-239	11
1013.5781	-21.0822	AKPALEDLR-I	apoA-I	231-239	11
1014.5921	-5.15	AKPALEDLR-II	apoA-I	231-239	11
1031.5333	-5.700	LSPLGEEMR	apoA-I	165-172	12
1032.524	-3.785	LSPLGEEMR-I	apoA-I	165-173	12
1033.5571	5.470	LSPLGEEMR-II	apoA-I	165-173	14
1033.5571	5.470	LQAEAFQAR	apoE	270-278	13
1047.4997	-6.987	LSPLGEEMoxR	apoA-I	165-173	12
1048.5057	-3.295	LSPLGEEMoxR-I	apoA-I	165-173	12
1049.5128	-1.862	LSPLGEEMoxR-II	apoA-I	165-173	12
1081.6043	22.482	LAAYLMLMR	apoB-100	559-567	15
1141.6155	2.912	HINIDQFVR	apoB-100	2101-2109	16
1156.6456	-3.813	SKEQLTPLIK	apoA-II	68-77	17
1156.6456	-3.813	SPAFTDLHLR	apoB-100	3980-3989	18
1157.6638	-22.261	LEALKENGGAR	apoA-I	202-212	19
1157.6638	-22.261	SKEQLTPLIK-I	apoA-II	68-77	17
1158.6367	-15.025	LEALKENGGAR-I	apoA-I	202-212	19
1158.6367	-15.025	SKEQLTPLIK-II	apoA-II	68-77	17
1159.6567	-5.85	LEALKENGGAR-II	apoA-I	202-212	19
1159.6567	-5.85	SKEQLTPLIK-III	apoA-II	68-77	17
1160.6312	-0.846	LEALKENGGAR-III	apoA-I	202-212	19
1160.6312	-0.846	SKEQLTPLIK-IV	apoA-II	68-77	17
1166.5888	-8.57	FRETLEDTR	apoB-100	2512-2520	20
1167.5691	-5.47	FRETLEDTR-I	apoB-100	2512-2520	20
1168.597	-1.685	FRETLEDTR-II	apoB-100	2512-2520	20
1169.579	-10.437	SLDEHYHIR	apoB-100	2211-2219	21
1170.6086	-7.161	SLDEHYHIR	apoB-100	2211-2219	21
1171.5924	-1.733	SLDEHYHIR	apoB-100	2211-2219	21
1178.6429	-6.301	VLVDHFGYTK	apoB-100	733-742	22
1179.6334	-5.424	VLVDHFGYTK-I	apoB-100	733-742	22
1199.6662	-6.224	VKSPELQAEAK	apoA-II	52-62	23
1200.6743	-4.688	VKSPELQAEAK-I	apoA-II	52-62	23
1201.6352	-4.73	VKSPELQAEAK-II	apoA-II	52-62	23
1201.6352	-4.73	LTISQNIQR	apoB-100	335-344	24
1202.645	-2.364	VKSPELQAEAK-III	apoA-II	52-62	23

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD					
m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
1202.645	-2.364	LTISEQNIQR-I	apoB-100	335-344	24
1226.547	31.827	DEPPQSPWDR	apoA-I	25-34	25
1227.5777	22.165	DEPPQSPWDR-I	apoA-I	25-34	25
1283.6171	17.33	WQEEMELYSR	apoA-I	132-140	26
1284.6444	13.139	WQEEMELYSR-I	apoA-I	132-140	26
1285.6211	4.945	WQEEMELYSR-II	apoA-I	132-140	26
1286.5985	-1.058	WQEEMELYSR-III	apoA-I	132-140	26
1299.5808	4.112	WQEEMoxELYSR	apoA-I	132-140	26
1300.5688	4.532	WQEEMoxELYSR-I	apoA-I	132-140	26
1301.6617	-16.664	THLAPYSDELRSR	apoA-I	185-195	27
1302.6514	-82.138	THLAPYSDELRSR-I	apoA-I	185-195	27
1302.6514	-82.138	LSPLGEMRDR	apoA-I	165-175	28
1303.6417	-50.00	THLAPYSDELRSR-II	apoA-I	185-195	27
1303.6417	-50.00	LSPLGEMRDR-I	apoA-I	165-175	28
1304.685	-25.542	KGNVATELSTER	apoB-100	196-207	29
1305.6769	-7.513	KGNVATELSTER-I	apoB-100	196-207	29
1306.6696	-0.949	KGNVATELSTER-II	apoB-100	196-207	29
1318.6407	2.632	LSPLGEMoxRDR	apoA-I	165-175	28
1319.6432	1.355	LSPLGEMoxRDR-I	apoA-I	165-175	28
1380.7137	-20.692	VQPYLDDFQKK	apoA-I	121-131	30
1381.7081	-15.84	VQPYLDDFQKK	apoA-I	121-131	30
1400.6834	6.728	DYVSQFEGSALGK	apoA-I	52-64	31
1401.6922	4.3536	DYVSQFEGSALGK-I	apoA-I	52-64	31
1402.7018	-2.083	DYVSQFEGSALGK-II	apoA-I	52-64	31
1403.7121	-4.059	DYVSQFEGSALGK-III	apoA-I	52-64	31
1404.7231	-2.216	DYVSQFEGSALGK-III	apoA-I	52-64	31
1410.748	-6.002	FQFPKPGIYTR	apoB-100	4202-4213	32
1411.7077	-12.606	KWQEEMELYSR	apoA-I	131-140	33
1412.7244	-1.039	KWQEEMELYSR-I	apoA-I	131-140	33
1412.7244	-1.039	DPDRFRPDGLPK	SAA4	117-128	34
1413.6854	-1.114	KWQEEMELYSR-II	apoA-I	131-140	33
1413.6854	-1.114	DPDRFRPDGLPK-I	SAA4	117-128	34
1414.7036	0.290	KWQEEMELYSR-III	apoA-I	131-140	33
1414.7036	0.290	DPDRFRPDGLPK-II	SAA4	117-128	34
1415.7225	0.7011	KWQEEMELYSR-IV	apoA-I	131-140	33
1415.7225	0.7011	DPDRFRPDGLPK-III	SAA4	117-128	34
1427.6644	11.458	KWQEEMoxELYSR	apoA-I	131-140	33
1428.6927	9.069	KWQEEMoxELYSR-I	apoA-I	131-140	33
1429.6645	5.938	KWQEEMoxELYSR-II	apoA-I	131-140	33
1440.6864	48.538	NPDAVAAPYCYTR	Lp (a)	79-91	35
1441.6664	35.366	NPDAVAAPYCYTR-I	Lp (a)	79-91	35
1442.7047	16.693	NPDAVAAPYCYTR-II	Lp (a)	79-91	35
1488.7235	-10.607	MREWFSETFQK	apoC-I	64-74	36
1489.7361	-8.862	MREWFSETFQK-I	apoC-I	64-74	36
1490.6898	-3.125	MREWFSETFQK-II	apoC-I	64-74	36
1504.7079	-12.493	MoXREWFSETFQK	apoC-I	64-74	36
1505.7314	-12.066	MoXREWFSETFQK-I	apoC-I	64-74	36
1506.6954	-5.215	MoXREWFSETFQK-II	apoC-I	64-74	36
1568.8737	9.352	LAARLEALKENGGAR	apoA-I	198-212	37
1569.8781	6.904	LAARLEALKENGGAR-I	apoA-I	198-212	37
1585.8456	4.483	THLAPYSDELRSR	apoA-I	185-197	38
1586.8608	0.9348	THLAPYSDELRSR-I	apoA-I	185-197	38
1612.7768	-17.535	LLDNWDSVTSTFSK	apoA-I	70-83	39
1716.8928	6.669	DALSSVQESQVAQQAR	apoC-III	45-60	40
1717.8545	3.025	DALSSVQESQVAQQAR-I	apoC-III	45-60	40
1718.8855	-0.99	DALSSVQESQVAQQAR-II	apoC-III	45-60	40
1723.9809	2.583	QKVEPLRAELQEGAR	apoA-I	141-155	41
1723.9809	2.583	IVQILPWEQNEQVK	apoB-100	577-590	42
1724.9466	1.7167	QKVEPLRAELQEGAR-I	apoA-I	141-155	41
1724.9466	1.7167	IVQILPWEQNEQVK-I	apoB-100	577-590	42
1725.9818	1.707	QKVEPLRAELQEGAR-II	apoA-I	141-155	41
1725.9818	1.707	IVQILPWEQNEQVK-II	apoB-100	577-590	42
1775.9145	15.536	NLQNNAEWVYQGAI	apoB-100	4107-4121	43
1776.9092	12.946	NLQNNAEWVYQGAI-I	apoB-100	4107-4121	43
1777.9046	11.265	NLQNNAEWVYQGAI-II	apoB-100	4107-4121	43
1904.9087	34.681	TPEYYPNAGLIMNYCR	Lp (a)	177-192	44
1905.8995	35.544	TPEYYPNAGLIMNYCR-I	Lp (a)	177-192	44
1906.8908	24.997	SEAEDASLLSFMQGYMK	apoC-III	21-37	45
1906.8908	24.997	TPEYYPNAGLIMNYCR-II	Lp (a)	177-192	44
1907.8826	12.840	SEAEDASLLSFMQGYMK-I	apoC-III	21-37	45
1907.8826	12.840	TPEYYPNAGLIMNYCR-III	Lp (a)	177-192	44
1922.8989	7.340	SEAEDASLLSFMoxQGYMK	apoC-III	21-37	45
2202.1435	41.389	LREQLGPVTQEFWDNLEK	apoA-I	84-101	46
2203.2007	49.396	LREQLGPVTQEFWDNLEK-I	apoA-I	84-101	46

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD						
m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO	
2204.1703	35.316	LREQLGPVTQEFWDNLEK-II	apoA-I	84-101	46	
2205.1404	16.202	LREQLGPVTQEFWDNLEK-III	apoA-I	84-101	46	
2206.1991	6.574	LREQLGPVTQEFWDNLEK-IIII	apoA-I	84-101	46	
2645.4139	-7.042	VQPYLDDFQKKWQEEMELYR	apoA-I	121-140	47	
2646.3664	-12.328	VQPYLDDFQKKWQEEMELYR-I	apoA-I	121-140	47	
2647.4251	-9.890	VQPYLDDFQKKWQEEMELYR-II	apoA-I	121-140	47	
2648.3783	-5.448	VQPYLDDFQKKWQEEMELYR-IIII	apoA-I	121-140	47	
2661.3337	8.767	VQPYLDDFQKKWQEEMoxELYR	apoA-I	121-140	47	
2662.3985	12.259	VQPYLDDFQKKWQEEMoxELYR-I	apoA-I	121-140	47	
2663.3571	9.880	VQPYLDDFQKKWQEEMoxELYR-II	apoA-I	121-140	47	
2664.4226	5.8323	VQPYLDDFQKKWQEEMoxELYR-IIII	apoA-I	121-140	47	

The m/z values are peaks that were obtained for the markers using mass spectrometry system using the methods described herein.

As shown in TABLE 3, a marker may be represented at multiple m/z points in a spectrum. This can be due to the fact that multiple isotopes (represented in TABLE 3 as "I, II, III, IIII") were observed, and/or that multiple charge states of the marker were observed, or that multiple isoforms of the marker were observed, for example, a post-translational modification

such as oxidation. These multiple representations of a particular marker can be analyzed individually or grouped together. An example of how multiple representations of a marker may be grouped is that the intensities for the multiple peaks can be summed.

As shown below in TABLE 4 and TABLE 5, targeted tandem MS analysis identified the peptides corresponding to ten of the 13 informative features shown in FIG. 4 (i.e., most significant features that contributed to the PLS-DA model).

TABLE 4

INFORMATIVE FEATURES REPRESENTING INCREASED PROTEIN/PEPTIDE LEVELS IN CAD SUBJECTS AS COMPARED TO NORMAL SUBJECTS						
Channel m/z	Magnitude in Regression Vector	Protein corresponding peptides	Protein Residues	Peptide Sequence	SEQ ID NO	
1081.6043	+22.482	apo-B100	559-567	LAAYLMLMR	15	
1226.547	+31.83	apo-A1	25-34	DEPPQSPWDR	25	
1227.5777	+22.165	apo-AI	25-34	DEPPQSPWDR-I	25	
1440.6864	+48.538	Lp (a)	79-91	NPDAVAAPYCYTR	35	
1441.6664	+35.366	Lp (a)	79-91	NPDAVAAPYCYTR-I	35	
1442.7047	+16.693	Lp (a)	79-91	NPDAVAAPYCYTR-II	35	
1904.9087	+34.681	Lp (a)	177-192	TPEYYPNAGLIMNYCR	44	
1905.8995	+35.544	Lp (a)	177-192	TPEYYPNAGLIMNYCR-I	44	
1906.8908	+24.997	Lp (a)	177-192	TPEYYPNAGLIMNYCR-II	44	
1907.8826	+12.840	Lp (a)	177-192	TPEYYPNAGLIMNYCR-III	44	
1906.8908	+24.997	apoC-III	21-37	SEAEDASLLSFMQGYMK	45	
1907.8826	+12.840	apoC-III	21-37	SEAEDASLLSFMQGYMK-I	45	
1922.8989	+7.340	apoC-III	21-37	SEAEDASLLSFMoxQGYMK	45	
2202.1435	+41.39	apoA-I	84-101	LREQLGPVTQEFWDNLEK	46	
2203.2007	+49.39	apoA-I	84-101	LREQLGPVTQEFWDNLEK-I	46	
2204.1703	+35.32	apoA-I	84-101	LREQLGPVTQEFWDNLEK-II	46	
2205.1404	+16.202	apoA-I	84-101	LREQLGPVTQEFWDNLEK-III	46	
2206.1991	+6.574	apoA-I	84-101	LREQLGPVTQEFWDNLEK-IIII	46	
2661.3337	+8.767	apoA-I (Met112ox)	121-140	VQPYLDDFQKKWQEEM (Ox) ELYR	47	
2662.3985	+12.259	apoA-I (Met 112ox)	121-140	VQPYLDDFQKKWQEEM (Ox) ELYR-I	47	
2663.3571	+9.880	apoA-I (Met112ox)	121-140	VQPYLDDFQKKWQEEM (Ox) ELYR-II	47	
2664.4226	+5.8323	apoA-I (met112ox)	121-140	VQPYLDDFQKKWQEEM (Ox) ELYR-III	47	

As shown above in TABLE 4, identification of the tryptic peptides associated with the positive regression vector values shown in FIG. 4 revealed that, surprisingly, two peptides identified at m/z 1440 to 1442 (SEQ ID NO: 35) and m/z 1904 to 1906 (SEQ ID 44) derived from apolipoprotein(a) (Lp(a)) were increased in HDL₂ of CAD subjects, as compared to normal subjects. FIG. 5A graphically illustrates the strong positive informative feature in the PLS-DA regression vector at m/z 1440. As shown in FIG. 5C, the positive informative feature at m/z 1440 was identified by LC-MALDI-TOF/TOF MS/MS as corresponding to the peptide NPDVAAPY-CYTR (SEQ ID NO:35) which corresponds to amino acids 79-91 of Lp(a) (SEQ ID NO:4), with a MASCOT ion score of 86.46 (CI=100%). As shown in FIG. 5B, another strong positive informative feature in the PLS-DA regression vector at m/z 1904 was identified as corresponding to the peptide TPEYYPNAGLIMNYCR (SEQ ID NO:44), which corresponds to amino acids 177-192 of Lp(a) (SEQ ID NO:4).

As further shown in TABLE 4, tryptic peptides identified at m/z 1906-1922 (SEQ ID NO: 45) derived from apoC-III (SEQ ID NO:6) were increased in HDL₂ of CAD subjects, as compared to normal subjects.

Taken together, these results demonstrate that pattern recognition profiling performed on HDL₂ isolated from CAD and control subjects show altered patterns of apoproteins present in the HDL₂ fractions which fall into two classes: (1) increased levels of peptides/proteins in CAD subjects as compared to normal controls; or (2) decreased levels of peptides/proteins in CAD subjects as compared to normal controls.

The observation that levels of Lp(a) were found to be increased in CAD subjects in comparison to normal controls was a surprising result because Lp(a) has been shown to be associated with small dense low density lipoproteins (LDLs), and its association with HDLs in general, and HDL₂ in particular, has not been previously shown. Thus, these results demonstrate that co-isolation of Lp(a) with HDL₂ subfraction permits pattern recognition analysis of the HDL₂ subfraction in the prediction, diagnosis, and prognosis of CAD subjects.

It was also observed that levels of apoC-III peptides were found to be elevated in CAD subjects, whereas those of apoC-I were decreased. In this regard, although not wishing to be bound by theory, it is noted that apoC-III inhibits lipoprotein lipase and the hepatic uptake of triglyceride-rich lipoproteins, which might promote an increase in atherogenic

TABLE 5

INFORMATIVE FEATURES REPRESENTING DECREASED PROTEIN/PEPTIDE LEVELS IN CAD SUBJECTS AS COMPARED TO NORMAL SUBJECTS					
Channel m/z	Magnitude in Regression Vector	Protein corresponding to Identified Peptides	Protein Residues	Peptide sequence	SEQ ID NO:
1012.6055	-39.93	apoA-I	231-239	AKPALEDLR	11
1013.5781	-21.082	apoA-I	231-239	AKPALEDLR-I	11
1014.5921	-5.15	apoA-I	231-239	AKPALEDLR-II	11
1157.6638	-22.261	apoA-I	202-212	LEALKENGGAR	19
1158.6367	-15.025	apoA-I	202-212	LEALKENGGAR-I	19
1159.6567	-5.85	apoA-I	202-212	LEALKENGGAR-II	19
1160.6312	-0.846	apoA-I	202-212	LEALKENGGAR-III	19
1301.6617	-16.664	apoA-I	185-195	THLAPYSDELRL	27
1302.6514	-82.138	apoA-I	185-195	THLAPYSDELRL-I	27
1303.6417	-50.00	apoA-I	185-195	THLAPYSDELRL-II	27
1302.6514	-82.138	apoA-I	165-175	LSPPLGEEMRDR	28
1303.6417	-50.00	apoA-I	165-175	LSPPLGEEMRDR-I	28
1380.7137	-20.69	apoA-I	121-131	VQPYLDDFQKK	30
1381.7081	-15.84	apoA-I	121-131	VQPYLDDFQKK-I	30
1488.7235	-10.607	apoC-I	64-74	MREWFSETFQK	36
1489.7361	-8.862	apoC-I	64-74	MREWFSETFQK-I	36
1490.6898	-3.125	apoC-I	64-74	MREWFSETFQK-II	36
1504.7079	-12.493	apoC-I	64-74	MoxREWFSETFQK	36
1505.7314	-12.066	apoC-I	64-74	MoxREWFSETFQK-I	36
1506.6954	-5.215	apoC-I	64-74	MoxREWFSETFQK-II	36
1612.7768	-17.53	apoA-I	70-83	LLDNWDSVTSTFSK	39
2645.4139	-7.042	apoA-I	121-140	VQPYLDDFQKKWQEMELYR	47
2646.3664	-12.328	apoA-I	121-140	VQPYLDDFQKKWQEMELYR-I	47
2647.4251	-9.89	apoA-I	121-140	VQPYLDDFQKKWQEMELYR-II	47
2648.3783	-5.448	apoA-I	121-140	VQPYLDDFQKKWQEMELYR-III	47

As shown above in TABLE 5, identification of the tryptic peptides associated with the negative regression vector values shown in FIG. 4 revealed several peptides from apoA-I and a peptide from apoC-I were decreased in HDL₂ of CAD subjects compared to that of control subjects. The peptides derived from apoA-I (SEQ ID NO:1) that were identified as decreased in CAD subjects included SEQ ID NO:11, SEQ ID NO:19, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:39 and SEQ ID NO:47, as shown in TABLE 5.

The negative regression vector at m/z 1504-1506 was identified as corresponding to the peptide MREWFSETFQK (SEQ ID NO: 36) which corresponds to amino acids 64-74 of ApoC-I (SEQ ID NO: 5).

triglyceride-rich lipoproteins (see Ooi, E. M., et al., *Clin Sci (Lond)* 114:611-624, 2008. It is further noted that ApoC-I inhibits cholesterol ester transfer protein (CETP) (see Shachter, N. S., et al., *Curr Opin Lipidol* 12:297-304 (2001); Sparks, D. L., et al., *J. Lipid Res* 30:1491-1498, 1989. Thus, it is believed that alterations in apoC-I and apoC-III levels are likely contribute to lipid remodeling and the formation of pro-atherogenic HDL particles.

Therefore it is demonstrated that simultaneous profiling of these biomarkers in subjects using pattern recognition analysis may be used to aid in the diagnosis and prognosis of cardiovascular diseases in mammalian subjects.

This example demonstrates that subjects may be successfully classified as CAD or control subjects based on the oxidation status of their HDL₂ using PLS-DA based pattern recognition proteomic profiling.

Methods:

Sample preparation and analysis: HDL₂ fractions were isolated from subjects, and samples from each individual subject were subjected to MALDI-TOF/TOF MS and PLS-DA analyses, as described in Example 2. Subjects were classified as either CAD or normal control subjects by pattern recognition proteomic profiling of HDL₂ proteins using PLS-DA. The PLS-DA models were characterized by regression vectors as described in Example 3. The PLS-DA model regression vector analysis is centered on post-translationally modified peptides derived from apoA-I, the major protein in HDL₂.

Results:

In addition to the first two groups of informative features (increased or decreased peptide levels in CAD subjects as compared to normal subjects) as described in Example 2, a third group of informative features in the PLS-DA model was identified that centered on post-translationally modified peptides derived from apoA-I (SEQ ID NO:1), the major protein in HDL. MS/MS analysis confirmed the presence of these peptide sequences in the HDL₂ fraction and demonstrated that the methionine 112 residue had been converted to methionine sulfoxide (Met(O)).

As shown in FIGS. 6A-D and summarized below in TABLE 6, this third group of informative features included both native peptides KWQEEMELYR (SEQ ID NO:33) and VQPYLDDFQKKWQEEMELYR (SEQ ID NO: 47) and the corresponding oxidized peptides that contained methionine 112 (Met112). FIG. 6A graphically illustrates the negative regression vector at m/z 1411.7077 and the positive regression vector at m/z 1427.6644 which were identified as corresponding to the native form of the apoA-I peptide KWQEEMELYR (SEQ ID NO: 33), and the Met112 oxidized form KWQEEM(O)ELYR of SEQ ID NO:33, respectively, as shown in FIG. 6C (MASCOT ion score of 84.8, CI=100%).

FIG. 6B graphically illustrates the negative regression vector at m/z 2646.3664 and the positive regression vector at m/z 2662.3985 which were identified as corresponding to the native form of the apoA-I peptide VQPYLDDFQKKWQEEMELYR (SEQ ID NO:47), and the Met112 oxidized form VQPYLDDFQKKWQEEM(O)ELYR of SEQ ID NO:47, respectively, as shown in FIG. 6D (MASCOT ion score of 42.6, CI=99.96%).

TABLE 6

INFORMATIVE FEATURES REPRESENTING POSTTRANSLATIONALLY MODIFIED PEPTIDES IN CAD SUBJECTS AS COMPARED TO NORMAL SUBJECTS					
Channel m/z	Magnitude in Regression Vector	Protein/peptide location	Modification	Peptide Sequence	SEQ ID
1411.7077	-12.606	apoA-I (131-140)	native	KWQEEMELYR	33
1427.6644	+11.458	apoA-I (131-140)	oxidized M112 (MetOx)	KWQEEM(O)ELYR	33
2646.3664	-12.328	apoA-I (121-140)	native	VQPYLDDFQKKWQEEMELYR-I	47
2662.3985	+12.259	apoA-I (121-140)	oxidized M112 (MetOx)	VQPYLDDFQKKWQEEM(O)ELYR-I	47

Strikingly, as shown in FIG. 6 and summarized above in TABLE 6, the signals for the Met 112 oxidized (Met112(O)) apoA-I peptides (SEQ ID NO:33 and SEQ ID NO:47) were found to be increased in CAD subjects as compared to normal control subjects, while the levels of the corresponding native Met112 peptides (SEQ ID NO:33 and SEQ ID NO:47) were found to be decreased in CAD subjects as compared to normal control subjects.

It is noted however, that no difference in relative levels of other methionine containing native and oxidized peptides, such as those derived from apoC-I, were observed between normal controls and CAD subjects was observed in this analysis (data not shown), suggesting that the difference in levels of oxygenated Met112 did not result from ex vivo oxidation.

While not wishing to be bound by theory, oxidation has been proposed as one mechanism for generating dysfunctional HDL resulting in decreased reverse cholesterol transport, thereby disrupting normal cholesterol homeostasis. Lipid hydroperoxides and reactive intermediates produced by Myeloperoxidase (MPO) oxidize apoA-I. It has been shown that oxidation of methionine residues impairs apoA-I's ability to promote cholesterol efflux by the ABCA1 pathway (Shao, B., et al., *J Biol Chem* 281(14):9001-9004 (2006) and to activate LCAT, two key steps in removing cholesterol from lipid-laden macrophages. apoA-I co-localizes with HOCl oxidation adducts in human atherosclerotic tissues. MPO-produced HOCl is known to modify HDL in vivo. Antibodies specific for apoA-I and HOCl-modified proteins immunostained coronary arteries obtained from patients undergoing cardiac transplantation (O'Brien et al., *Circulation* 98:519-527, 1998). apoA-I co-localized with epitopes recognized by HOP-I antibody, which is specific for proteins oxidized by HOCl (Hazell et al., *J. Clin. Invest.* 97:1535-1544, 1996) in the intima of atherosclerotic lesions. The co-localization of HOCl-modified proteins with apoA-I suggests that HOCl oxidizes specific proteins in the human artery wall.

Oxidized HDLs are also present in the circulation of CVD patients, (International Publication No. WO2006/014628). Circulating HDL from cardiovascular patients has 8-times higher 3-chlorotyrosine than normal subjects. Levels of chlorinated HDL are elevated in the blood of humans suffering from clinically significant atherosclerosis. In addition, MPO-produced H₂O₂ is also capable of oxidizing methionines of apoA-I associated with HDL₃ (International Publication No. WO2006/014628). These HDL₃ subfractions are selectively enriched with oxidized amino acids.

Collectively, these observations support the conclusion that HDL₂ from control and CAD subjects differ in their protein cargoes and levels of oxidized methionine residues.

Because pattern recognition analysis makes no assumptions about the origins of the differential signals seen in the regression vectors for each sample, it provides a powerful tool for identifying post-translationally modified peptides that would be very difficult to identify using classic proteomic approaches. The results demonstrated in this example indicate that oxidized methionines (Met(O)) in apoA-I are detectable by pattern recognition profiling of an HDL₂ subfraction. Since oxidation of methionine residues impairs apoA-I's ability to promote cholesterol efflux by the ABCA1 pathway (Shao, B., et al., "Myeloperoxidase impairs ABCA1-dependent cholesterol efflux through methionine oxidation and site-specific tyrosine chlorination of apolipoprotein A-I," *J. Biol. Chem.* 281:9001-4, 2006) and to activate Lecithin:Cholesterol Acyltransferase (LCAT) (Shao, B., et al., "Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I," *Proc. Natl. Acad. Sci.* 2008, In press), oxidized apoA-I likely acts as a mediator of CAD, and serves as a useful biomarker for CAD. Thus, a subject may be evaluated for the presence of oxidized apoA-I (SEQ ID NO:1) to determine the risk, diagnosis, prognosis of CAD in the subject and/or to measure the efficacy of treatment of a subject suffering from CAD.

Example 5

This example demonstrates that the conformational structure of apoA-I in HDL₂ subfractions is altered in CAD subjects as compared to the conformation structure of apoA-I in HDL₂ subfractions of normal control subjects.

Rationale:

The structural conformation of apoA-I has been suggested to influence its ability to transfer cholesterol ester from HDL₂ particles to scavenger receptor BI as part of reverse cholesterol transport and cholesterol ester clearance in the liver (de Beer, M. C., et al., *J. of Lipid Res* 42:309-313, February 2001). Contact between the N-terminal fold and the C-terminal domain of apoA-I has been suggested to stabilize the lipid-bound conformation of the protein. Since methionines in apoA-I are oxidized in CAD subjects, as demonstrated in Example 4, an experiment was carried out to determine if such post-translational modifications lead to local changes in the structural conformation of apoA-I. Alterations in a protein's local structure is said to affect susceptibility of the protein to proteolytic digestion, which in turn can affect the apparent abundance of peptides assessed by MS.

Methods:

Sample preparation and analysis: HDL₂ fractions were isolated from subjects and treated with trypsin as disclosed in Example 1. The samples from each individual subject were subjected to MALDI-TOF/TOF MS, as described in Example 2. Subjects were classified as either CAD or normal subjects by pattern recognition proteomic profiling of HDL₂ proteins using PLS-DA. The PLS-DA models were characterized by regression vectors as described in Example 3. The PLS-DA model regression vector analysis is centered on apoA-I peptides of HDL₂. The differential signals reflecting the relative abundance of the trypsinized peptides was measured.

Results:

In addition to the three groups of informative features (increased or decreased peptide levels in CAD subjects as compared to normal subjects) as described in Example 2, and post-translationally modified peptides derived from apoA-I as described in Example 3, a fourth group of informative features in the PLS-DA model was identified based on the altered structural conformation of apoA-I present in the HDL₂ subfraction of CAD subjects in comparison to the structural conformation of apoA-I present in the HDL₂ subfraction of normal subjects.

Informative features corresponding to tryptic peptides derived from the N-terminal and C-terminal regions of

apoA-I (SEQ ID NO:1) were identified. FIG. 7 graphically illustrates the regression vector values (y-axis) for the amino acid sequence of apoA-I (x-axis).

It was determined that two tryptic apoA-I peptides originating from N-terminal regions of the mature protein (residues 1-10: DEPPQSPWDR (SEQ ID NO:48) and residues 60-77: LREQLGPVTQEFWDNLEK (SEQ ID NO:49) were significantly increased in CAD subjects as compared to normal controls, while one C-terminal region peptide (residues 207-215, AKPALEDLR (SEQ ID NO:50) was significantly decreased as compared to normal controls, as shown in FIG. 7. Also, a tryptic peptide (peptide 46-59: LLDNWDSVTSTFSK (SEQ ID NO:52) was apparently decreased in abundance. These observations suggest that tryptic digests of apoA-I in HDL isolated from control and CAD subjects give different patterns of peptides, perhaps because of conformational differences of the apoA-I in the two different classes of subjects. Indeed, although the above-referenced N-terminal peptides (SEQ ID NO:48 and SEQ ID NO:49) and C-terminal peptides (SEQ ID NO:49) are distant in apoA-I sequence, when mapped to the double-belt model of the lipid-associated apoA-I (Davidson, W. S., et al., *J. Biol. Chem.* 282(31): 22249-22253 (2007)) or spherical HDL particle apoA-I model (Davidson W. S. PNAS 2008, in press), the peptides displaying significant changes in CAD subjects were found to be in close proximity (data not shown).

Additionally, it was determined that the peptides (residues 97-107, VQPYLDDFQKK SEQ ID NO: 51) proximal to Met112 was significantly decreased in the CAD samples (FIG. 7), as is the peptide containing Met112, SEQ ID NO:33.

It was recently proposed that contact between the globular N-terminal fold and the C-terminal fold of apoA-I stabilizes the lipid-bound conformation of the protein. It is important to note that alterations in a proteins' local structure can effect susceptibility to proteolytic digestion, which in turn can affect the apparent abundance of peptides in a MS analysis. As demonstrated in this example, the differential levels of N-terminal and C-terminal apoA-I peptides indicates that the secondary and/or tertiary conformations at the N and C-termini of apoA-I differ in the HDL₂ of CAD subjects as compared to normal control subjects. Further in this regard, as described in Example 4 and summarized in TABLE 6, it was also determined that levels of apoA-I peptides containing Met(O)112 were elevated in the HDL₂ of CAD subjects concomitantly with a decrease in Met112 peptides in the CAD subjects. The peptides directly adjacent to the peptides containing Met112 also displayed significant changes in CAD subjects as compared to normal controls. While not wishing to be bound by theory, these observations suggest that oxidation of methionine residues in apoA-I is increased in CAD subjects and such oxidation may lead to local changes in the conformation of the apoA-I protein which can be detected by tryptic digestion followed by analysis by mass spectrometry.

The results described in this example demonstrate that the altered conformation of apoA-I at its N- and C-termini is detectable using PLS-DA-based pattern recognition profiling. Changes in relative abundance of certain tryptic peptides demonstrate that apoA-I exists in altered secondary and/or tertiary conformation in HDL₂ subfractions of CAD subjects compared to control subjects. Thus, dysfunctionality of HDL₂ of CAD subjects likely results from changes in the proteome profile and conformation of the associated HDL₂ proteins. These results demonstrate that pattern recognition profiling using tryptic peptides of HDL₂ subfractions from subjects can be used to determine the conformation status of apoA-I in order to classify subjects as normal or CAD patients.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

SEQUENCE LISTING

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<211> LENGTH: 267

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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Gln Ala Arg His Phe Trp Gln Gln Asp Glu Pro Pro Gln Ser Pro Trp
20     25     30
Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
35     40     45
Ser Gly Arg Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
50     55     60
Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr
65     70     75     80
Phe Ser Lys Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp
85     90     95
Asp Asn Leu Glu Lys Glu Thr Glu Gly Leu Arg Gln Glu Met Ser Lys
100    105    110
Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr Leu Asp Asp Phe
115    120    125
Gln Lys Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg Gln Lys Val Glu
130    135    140
Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu
145    150    155    160
Leu Gln Glu Lys Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg Ala
165    170    175
Arg Ala His Val Asp Ala Leu Arg Thr His Leu Ala Pro Tyr Ser Asp
180    185    190
Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn
195    200    205
Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu
210    215    220
Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln
225    230    235    240
Gly Leu Leu Pro Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala
245    250    255
Leu Glu Glu Tyr Thr Lys Lys Leu Asn Thr Gln
260    265

```

<210> SEQ ID NO 2

<211> LENGTH: 100

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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Glu Gly Ala Leu Val Arg Arg Gln Ala Lys Glu Pro Cys Val Glu Ser
20     25     30
Leu Val Ser Gln Tyr Phe Gln Thr Val Thr Asp Tyr Gly Lys Asp Leu
35     40     45

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-continued

Met Glu Lys Val Lys Ser Pro Glu Leu Gln Ala Glu Ala Lys Ser Tyr
 50 55 60

Phe Glu Lys Ser Lys Glu Gln Leu Thr Pro Leu Ile Lys Lys Ala Gly
 65 70 75 80

Thr Glu Leu Val Asn Phe Leu Ser Tyr Phe Val Glu Leu Gly Thr Gln
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Pro Ala Thr Gln
 100

<210> SEQ ID NO 3
 <211> LENGTH: 4563
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

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 20 25 30

Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe Lys His
 35 40 45

Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
 50 55 60

Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
 65 70 75 80

Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
 85 90 95

Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
 100 105 110

Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
 115 120 125

Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
 130 135 140

Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
 145 150 155 160

Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys Gln Val
 165 170 175

Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe Thr Val
 180 185 190

Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg Asp
 195 200 205

Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile Ser Pro
 210 215 220

Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu Ile Ser
 225 230 235 240

Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys His Val
 245 250 255

Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe Ser Tyr
 260 265 270

Lys Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Thr Leu Lys Leu
 275 280 285

Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly Thr Lys
 290 295 300

Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro Pro Lys
 305 310 315 320

-continued

Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
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 Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Glu Glu Leu
 770 775 780
 Gly Phe Ala Ser Leu His Asp Leu Gln Leu Leu Gly Lys Leu Leu Leu
 785 790 795 800
 Met Gly Ala Arg Thr Leu Gln Gly Ile Pro Gln Met Ile Gly Glu Val
 805 810 815
 Ile Arg Lys Gly Ser Lys Asn Asp Phe Phe Leu His Tyr Ile Phe Met
 820 825 830
 Glu Asn Ala Phe Glu Leu Pro Thr Gly Ala Gly Leu Gln Leu Gln Ile
 835 840 845
 Ser Ser Ser Gly Val Ile Ala Pro Gly Ala Lys Ala Gly Val Lys Leu
 850 855 860
 Glu Val Ala Asn Met Gln Ala Glu Leu Val Ala Lys Pro Ser Val Ser
 865 870 875 880
 Val Glu Phe Val Thr Asn Met Gly Ile Ile Ile Pro Asp Phe Ala Arg
 885 890 895
 Ser Gly Val Gln Met Asn Thr Asn Phe Phe His Glu Ser Gly Leu Glu
 900 905 910
 Ala His Val Ala Leu Lys Ala Gly Lys Leu Lys Phe Ile Ile Pro Ser
 915 920 925
 Pro Lys Arg Pro Val Lys Leu Leu Ser Gly Gly Asn Thr Leu His Leu
 930 935 940
 Val Ser Thr Thr Lys Thr Glu Val Ile Pro Pro Leu Ile Glu Asn Arg
 945 950 955 960
 Gln Ser Trp Ser Val Cys Lys Gln Val Phe Pro Gly Leu Asn Tyr Cys
 965 970 975
 Thr Ser Gly Ala Tyr Ser Asn Ala Ser Ser Thr Asp Ser Ala Ser Tyr
 980 985 990
 Tyr Pro Leu Thr Gly Asp Thr Arg Leu Glu Leu Glu Leu Arg Pro Thr
 995 1000 1005
 Gly Glu Ile Glu Gln Tyr Ser Val Ser Ala Thr Tyr Glu Leu Gln
 1010 1015 1020
 Arg Glu Asp Arg Ala Leu Val Asp Thr Leu Lys Phe Val Thr Gln
 1025 1030 1035
 Ala Glu Gly Ala Lys Gln Thr Glu Ala Thr Met Thr Phe Lys Tyr
 1040 1045 1050
 Asn Arg Gln Ser Met Thr Leu Ser Ser Glu Val Gln Ile Pro Asp
 1055 1060 1065
 Phe Asp Val Asp Leu Gly Thr Ile Leu Arg Val Asn Asp Glu Ser
 1070 1075 1080
 Thr Glu Gly Lys Thr Ser Tyr Arg Leu Thr Leu Asp Ile Gln Asn
 1085 1090 1095
 Lys Lys Ile Thr Glu Val Ala Leu Met Gly His Leu Ser Cys Asp
 1100 1105 1110
 Thr Lys Glu Glu Arg Lys Ile Lys Gly Val Ile Ser Ile Pro Arg
 1115 1120 1125
 Leu Gln Ala Glu Ala Arg Ser Glu Ile Leu Ala His Trp Ser Pro
 1130 1135 1140
 Ala Lys Leu Leu Leu Gln Met Asp Ser Ser Ala Thr Ala Tyr Gly
 1145 1150 1155
 Ser Thr Val Ser Lys Arg Val Ala Trp His Tyr Asp Glu Glu Lys

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1160	1165	1170
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Met Thr Ser Asn Phe Pro Val Asp Leu Ser Asp Tyr Pro Lys Ser 1190 1195 1200		
Leu His Met Tyr Ala Asn Arg Leu Leu Asp His Arg Val Pro Gln 1205 1210 1215		
Thr Asp Met Thr Phe Arg His Val Gly Ser Lys Leu Ile Val Ala 1220 1225 1230		
Met Ser Ser Trp Leu Gln Lys Ala Ser Gly Ser Leu Pro Tyr Thr 1235 1240 1245		
Gln Thr Leu Gln Asp His Leu Asn Ser Leu Lys Glu Phe Asn Leu 1250 1255 1260		
Gln Asn Met Gly Leu Pro Asp Phe His Ile Pro Glu Asn Leu Phe 1265 1270 1275		
Leu Lys Ser Asp Gly Arg Val Lys Tyr Thr Leu Asn Lys Asn Ser 1280 1285 1290		
Leu Lys Ile Glu Ile Pro Leu Pro Phe Gly Gly Lys Ser Ser Arg 1295 1300 1305		
Asp Leu Lys Met Leu Glu Thr Val Arg Thr Pro Ala Leu His Phe 1310 1315 1320		
Lys Ser Val Gly Phe His Leu Pro Ser Arg Glu Phe Gln Val Pro 1325 1330 1335		
Thr Phe Thr Ile Pro Lys Leu Tyr Gln Leu Gln Val Pro Leu Leu 1340 1345 1350		
Gly Val Leu Asp Leu Ser Thr Asn Val Tyr Ser Asn Leu Tyr Asn 1355 1360 1365		
Trp Ser Ala Ser Tyr Ser Gly Gly Asn Thr Ser Thr Asp His Phe 1370 1375 1380		
Ser Leu Arg Ala Arg Tyr His Met Lys Ala Asp Ser Val Val Asp 1385 1390 1395		
Leu Leu Ser Tyr Asn Val Gln Gly Ser Gly Glu Thr Thr Tyr Asp 1400 1405 1410		
His Lys Asn Thr Phe Thr Leu Ser Cys Asp Gly Ser Leu Arg His 1415 1420 1425		
Lys Phe Leu Asp Ser Asn Ile Lys Phe Ser His Val Glu Lys Leu 1430 1435 1440		
Gly Asn Asn Pro Val Ser Lys Gly Leu Leu Ile Phe Asp Ala Ser 1445 1450 1455		
Ser Ser Trp Gly Pro Gln Met Ser Ala Ser Val His Leu Asp Ser 1460 1465 1470		
Lys Lys Lys Gln His Leu Phe Val Lys Glu Val Lys Ile Asp Gly 1475 1480 1485		
Gln Phe Arg Val Ser Ser Phe Tyr Ala Lys Gly Thr Tyr Gly Leu 1490 1495 1500		
Ser Cys Gln Arg Asp Pro Asn Thr Gly Arg Leu Asn Gly Glu Ser 1505 1510 1515		
Asn Leu Arg Phe Asn Ser Ser Tyr Leu Gln Gly Thr Asn Gln Ile 1520 1525 1530		
Thr Gly Arg Tyr Glu Asp Gly Thr Leu Ser Leu Thr Ser Thr Ser 1535 1540 1545		
Asp Leu Gln Ser Gly Ile Ile Lys Asn Thr Ala Ser Leu Lys Tyr 1550 1555 1560		

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Lys	Asn	Phe	Ala	Thr	Ser	Asn	Lys	Met	Asp	Met	Thr	Phe	Ser	Lys
1580						1585					1590			
Gln	Asn	Ala	Leu	Leu	Arg	Ser	Glu	Tyr	Gln	Ala	Asp	Tyr	Glu	Ser
1595						1600					1605			
Leu	Arg	Phe	Phe	Ser	Leu	Leu	Ser	Gly	Ser	Leu	Asn	Ser	His	Gly
1610						1615					1620			
Leu	Glu	Leu	Asn	Ala	Asp	Ile	Leu	Gly	Thr	Asp	Lys	Ile	Asn	Ser
1625						1630					1635			
Gly	Ala	His	Lys	Ala	Thr	Leu	Arg	Ile	Gly	Gln	Asp	Gly	Ile	Ser
1640						1645					1650			
Thr	Ser	Ala	Thr	Thr	Asn	Leu	Lys	Cys	Ser	Leu	Leu	Val	Leu	Glu
1655						1660					1665			
Asn	Glu	Leu	Asn	Ala	Glu	Leu	Gly	Leu	Ser	Gly	Ala	Ser	Met	Lys
1670						1675					1680			
Leu	Thr	Thr	Asn	Gly	Arg	Phe	Arg	Glu	His	Asn	Ala	Lys	Phe	Ser
1685						1690					1695			
Leu	Asp	Gly	Lys	Ala	Ala	Leu	Thr	Glu	Leu	Ser	Leu	Gly	Ser	Ala
1700						1705					1710			
Tyr	Gln	Ala	Met	Ile	Leu	Gly	Val	Asp	Ser	Lys	Asn	Ile	Phe	Asn
1715						1720					1725			
Phe	Lys	Val	Ser	Gln	Glu	Gly	Leu	Lys	Leu	Ser	Asn	Asp	Met	Met
1730						1735					1740			
Gly	Ser	Tyr	Ala	Glu	Met	Lys	Phe	Asp	His	Thr	Asn	Ser	Leu	Asn
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Ile	Ala	Gly	Leu	Ser	Leu	Asp	Phe	Ser	Ser	Lys	Leu	Asp	Asn	Ile
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Tyr	Ser	Ser	Asp	Lys	Phe	Tyr	Lys	Gln	Thr	Val	Asn	Leu	Gln	Leu
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Gln	Pro	Tyr	Ser	Leu	Val	Thr	Thr	Leu	Asn	Ser	Asp	Leu	Lys	Tyr
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Leu	Lys	Leu	His	Val	Ala	Gly	Asn	Leu	Lys	Gly	Ala	Tyr	Gln	Asn
1820						1825					1830			
Asn	Glu	Ile	Lys	His	Ile	Tyr	Ala	Ile	Ser	Ser	Ala	Ala	Leu	Ser
1835						1840					1845			
Ala	Ser	Tyr	Lys	Ala	Asp	Thr	Val	Ala	Lys	Val	Gln	Gly	Val	Glu
1850						1855					1860			
Phe	Ser	His	Arg	Leu	Asn	Thr	Asp	Ile	Ala	Gly	Leu	Ala	Ser	Ala
1865						1870					1875			
Ile	Asp	Met	Ser	Thr	Asn	Tyr	Asn	Ser	Asp	Ser	Leu	His	Phe	Ser
1880						1885					1890			
Asn	Val	Phe	Arg	Ser	Val	Met	Ala	Pro	Phe	Thr	Met	Thr	Ile	Asp
1895						1900					1905			
Ala	His	Thr	Asn	Gly	Asn	Gly	Lys	Leu	Ala	Leu	Trp	Gly	Glu	His
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Thr	Gly	Gln	Leu	Tyr	Ser	Lys	Phe	Leu	Leu	Lys	Ala	Glu	Pro	Leu
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Ala	Phe	Thr	Phe	Ser	His	Asp	Tyr	Lys	Gly	Ser	Thr	Ser	His	His
1940						1945					1950			
Leu	Val	Ser	Arg	Lys	Ser	Ile	Ser	Ala	Ala	Leu	Glu	His	Lys	Val
1955						1960					1965			

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Ser	Ala	Leu	Leu	Thr	Pro	Ala	Glu	Gln	Thr	Gly	Thr	Trp	Lys	Leu
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Tyr	Asn	Thr	Lys	Asp	Lys	Ile	Gly	Val	Glu	Leu	Thr	Gly	Arg	Thr
2000						2005					2010			
Leu	Ala	Asp	Leu	Thr	Leu	Leu	Asp	Ser	Pro	Ile	Lys	Val	Pro	Leu
2015						2020					2025			
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Lys	Tyr	Asp	Lys	Asn	Gln	Asp	Val	His	Ser	Ile	Asn	Leu	Pro	Phe
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2075						2080					2085			
Ile	Val	Val	Leu	Glu	Asn	Val	Gln	Arg	Asn	Leu	Lys	His	Ile	Asn
2090						2095					2100			
Ile	Asp	Gln	Phe	Val	Arg	Lys	Tyr	Arg	Ala	Ala	Leu	Gly	Lys	Leu
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Pro	Gln	Gln	Ala	Asn	Asp	Tyr	Leu	Asn	Ser	Phe	Asn	Trp	Glu	Arg
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Gln	Val	Ser	His	Ala	Lys	Glu	Lys	Leu	Thr	Ala	Leu	Thr	Lys	Lys
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Tyr	Arg	Ile	Thr	Glu	Asn	Asp	Ile	Gln	Ile	Ala	Leu	Asp	Asp	Ala
2150						2155					2160			
Lys	Ile	Asn	Phe	Asn	Glu	Lys	Leu	Ser	Gln	Leu	Gln	Thr	Tyr	Met
2165						2170					2175			
Ile	Gln	Phe	Asp	Gln	Tyr	Ile	Lys	Asp	Ser	Tyr	Asp	Leu	His	Asp
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Leu	Lys	Ile	Ala	Ile	Ala	Asn	Ile	Ile	Asp	Glu	Ile	Ile	Glu	Lys
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Leu	Lys	Ser	Leu	Asp	Glu	His	Tyr	His	Ile	Arg	Val	Asn	Leu	Val
2210						2215					2220			
Lys	Thr	Ile	His	Asp	Leu	His	Leu	Phe	Ile	Glu	Asn	Ile	Asp	Phe
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2255						2260					2265			
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2270						2275					2280			
Lys	Leu	Lys	Gln	His	Ile	Glu	Ala	Ile	Asp	Val	Arg	Val	Leu	Leu
2285						2290					2295			
Asp	Gln	Leu	Gly	Thr	Thr	Ile	Ser	Phe	Glu	Arg	Ile	Asn	Asp	Val
2300						2305					2310			
Leu	Glu	His	Val	Lys	His	Phe	Val	Ile	Asn	Leu	Ile	Gly	Asp	Phe
2315						2320					2325			
Glu	Val	Ala	Glu	Lys	Ile	Asn	Ala	Phe	Arg	Ala	Lys	Val	His	Glu
2330						2335					2340			
Leu	Ile	Glu	Arg	Tyr	Glu	Val	Asp	Gln	Gln	Ile	Gln	Val	Leu	Met
2345						2350					2355			
Asp	Lys	Leu	Val	Glu	Leu	Ala	His	Gln	Tyr	Lys	Leu	Lys	Glu	Thr

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2360	2365	2370
Ile Gln Lys Leu Ser Asn Val 2375	Leu Gln Gln Val Lys 2380	Ile Lys Asp 2385
Tyr Phe Glu Lys Leu Val 2390	Gly Phe Ile Asp Asp 2395	Ala Val Lys Lys 2400
Leu Asn Glu Leu Ser Phe 2405	Lys Thr Phe Ile Glu 2410	Asp Val Asn Lys 2415
Phe Leu Asp Met Leu Ile 2420	Lys Lys Leu Lys Ser 2425	Phe Asp Tyr His 2430
Gln Phe Val Asp Glu Thr 2435	Asn Asp Lys Ile Arg 2440	Glu Val Thr Gln 2445
Arg Leu Asn Gly Glu Ile 2450	Gln Ala Leu Glu Leu 2455	Pro Gln Lys Ala 2460
Glu Ala Leu Lys Leu Phe 2465	Leu Glu Glu Thr Lys 2470	Ala Thr Val Ala 2475
Val Tyr Leu Glu Ser Leu 2480	Gln Asp Thr Lys Ile 2485	Thr Leu Ile Ile 2490
Asn Trp Leu Gln Glu Ala 2495	Leu Ser Ser Ala Ser 2500	Leu Ala His Met 2505
Lys Ala Lys Phe Arg Glu 2510	Thr Leu Glu Asp Thr 2515	Arg Asp Arg Met 2520
Tyr Gln Met Asp Ile Gln 2525	Gln Glu Leu Gln Arg 2530	Tyr Leu Ser Leu 2535
Val Gly Gln Val Tyr Ser 2540	Thr Leu Val Thr Tyr 2545	Ile Ser Asp Trp 2550
Trp Thr Leu Ala Ala Lys 2555	Asn Leu Thr Asp Phe 2560	Ala Glu Gln Tyr 2565
Ser Ile Gln Asp Trp Ala 2570	Lys Arg Met Lys Ala 2575	Leu Val Glu Gln 2580
Gly Phe Thr Val Pro Glu 2585	Ile Lys Thr Ile Leu 2590	Gly Thr Met Pro 2595
Ala Phe Glu Val Ser Leu 2600	Gln Ala Leu Gln Lys 2605	Ala Thr Phe Gln 2610
Thr Pro Asp Phe Ile Val 2615	Pro Leu Thr Asp Leu 2620	Arg Ile Pro Ser 2625
Val Gln Ile Asn Phe Lys 2630	Asp Leu Lys Asn Ile 2635	Lys Ile Pro Ser 2640
Arg Phe Ser Thr Pro Glu 2645	Phe Thr Ile Leu Asn 2650	Thr Phe His Ile 2655
Pro Ser Phe Thr Ile Asp 2660	Phe Val Glu Met Lys 2665	Val Lys Ile Ile 2670
Arg Thr Ile Asp Gln Met 2675	Leu Asn Ser Glu Leu 2680	Gln Trp Pro Val 2685
Pro Asp Ile Tyr Leu Arg 2690	Asp Leu Lys Val Glu 2695	Asp Ile Pro Leu 2700
Ala Arg Ile Thr Leu Pro 2705	Asp Phe Arg Leu Pro 2710	Glu Ile Ala Ile 2715
Pro Glu Phe Ile Ile Pro 2720	Thr Leu Asn Leu Asn 2725	Asp Phe Gln Val 2730
Pro Asp Leu His Ile Pro 2735	Glu Phe Gln Leu Pro 2740	His Ile Ser His 2745
Thr Ile Glu Val Pro Thr 2750	Phe Gly Lys Leu Tyr 2755	Ser Ile Leu Lys 2760

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Ile	Gln	Ser	Pro	Leu	Phe	Thr	Leu	Asp	Ala	Asn	Ala	Asp	Ile	Gly
2765						2770					2775			
Asn	Gly	Thr	Thr	Ser	Ala	Asn	Glu	Ala	Gly	Ile	Ala	Ala	Ser	Ile
2780						2785					2790			
Thr	Ala	Lys	Gly	Glu	Ser	Lys	Leu	Glu	Val	Leu	Asn	Phe	Asp	Phe
2795						2800					2805			
Gln	Ala	Asn	Ala	Gln	Leu	Ser	Asn	Pro	Lys	Ile	Asn	Pro	Leu	Ala
2810						2815					2820			
Leu	Lys	Glu	Ser	Val	Lys	Phe	Ser	Ser	Lys	Tyr	Leu	Arg	Thr	Glu
2825						2830					2835			
His	Gly	Ser	Glu	Met	Leu	Phe	Phe	Gly	Asn	Ala	Ile	Glu	Gly	Lys
2840						2845					2850			
Ser	Asn	Thr	Val	Ala	Ser	Leu	His	Thr	Glu	Lys	Asn	Thr	Leu	Glu
2855						2860					2865			
Leu	Ser	Asn	Gly	Val	Ile	Val	Lys	Ile	Asn	Asn	Gln	Leu	Thr	Leu
2870						2875					2880			
Asp	Ser	Asn	Thr	Lys	Tyr	Phe	His	Lys	Leu	Asn	Ile	Pro	Lys	Leu
2885						2890					2895			
Asp	Phe	Ser	Ser	Gln	Ala	Asp	Leu	Arg	Asn	Glu	Ile	Lys	Thr	Leu
2900						2905					2910			
Leu	Lys	Ala	Gly	His	Ile	Ala	Trp	Thr	Ser	Ser	Gly	Lys	Gly	Ser
2915						2920					2925			
Trp	Lys	Trp	Ala	Cys	Pro	Arg	Phe	Ser	Asp	Glu	Gly	Thr	His	Glu
2930						2935					2940			
Ser	Gln	Ile	Ser	Phe	Thr	Ile	Glu	Gly	Pro	Leu	Thr	Ser	Phe	Gly
2945						2950					2955			
Leu	Ser	Asn	Lys	Ile	Asn	Ser	Lys	His	Leu	Arg	Val	Asn	Gln	Asn
2960						2965					2970			
Leu	Val	Tyr	Glu	Ser	Gly	Ser	Leu	Asn	Phe	Ser	Lys	Leu	Glu	Ile
2975						2980					2985			
Gln	Ser	Gln	Val	Asp	Ser	Gln	His	Val	Gly	His	Ser	Val	Leu	Thr
2990						2995					3000			
Ala	Lys	Gly	Met	Ala	Leu	Phe	Gly	Glu	Gly	Lys	Ala	Glu	Phe	Thr
3005						3010					3015			
Gly	Arg	His	Asp	Ala	His	Leu	Asn	Gly	Lys	Val	Ile	Gly	Thr	Leu
3020						3025					3030			
Lys	Asn	Ser	Leu	Phe	Phe	Ser	Ala	Gln	Pro	Phe	Glu	Ile	Thr	Ala
3035						3040					3045			
Ser	Thr	Asn	Asn	Glu	Gly	Asn	Leu	Lys	Val	Arg	Phe	Pro	Leu	Arg
3050						3055					3060			
Leu	Thr	Gly	Lys	Ile	Asp	Phe	Leu	Asn	Asn	Tyr	Ala	Leu	Phe	Leu
3065						3070					3075			
Ser	Pro	Ser	Ala	Gln	Gln	Ala	Ser	Trp	Gln	Val	Ser	Ala	Arg	Phe
3080						3085					3090			
Asn	Gln	Tyr	Lys	Tyr	Asn	Gln	Asn	Phe	Ser	Ala	Gly	Asn	Asn	Glu
3095						3100					3105			
Asn	Ile	Met	Glu	Ala	His	Val	Gly	Ile	Asn	Gly	Glu	Ala	Asn	Leu
3110						3115					3120			
Asp	Phe	Leu	Asn	Ile	Pro	Leu	Thr	Ile	Pro	Glu	Met	Arg	Leu	Pro
3125						3130					3135			
Tyr	Thr	Ile	Ile	Thr	Thr	Pro	Pro	Leu	Lys	Asp	Phe	Ser	Leu	Trp
3140						3145					3150			
Glu	Lys	Thr	Gly	Leu	Lys	Glu	Phe	Leu	Lys	Thr	Thr	Lys	Gln	Ser
3155						3160					3165			

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Phe Asp 3170	Leu Ser Val Lys 3175	Ala Gln Tyr Lys Lys 3175	Asn Lys His Arg 3180
His Ser 3185	Ile Thr Asn Pro 3190	Leu Ala Val Leu Cys 3190	Glu Phe Ile Ser 3195
Gln Ser 3200	Ile Lys Ser Phe 3205	Asp Arg His Phe Glu Lys 3205	Asn Arg Asn 3210
Asn Ala 3215	Leu Asp Phe Val Thr 3220	Lys Ser Tyr Asn Glu Thr Lys Ile 3220	
Lys Phe 3230	Asp Lys Tyr Lys 3235	Ala Glu Lys Ser His Asp 3235	Glu Leu Pro 3240
Arg Thr 3245	Phe Gln Ile Pro Gly 3250	Tyr Thr Val Pro Val Val Asn Val 3250	
Glu Val 3260	Ser Pro Phe Thr Ile 3265	Glu Met Ser Ala Phe Gly Tyr Val 3265	
Phe Pro 3275	Lys Ala Val Ser Met 3280	Pro Ser Phe Ser Ile Leu Gly Ser 3280	
Asp Val 3290	Arg Val Pro Ser Tyr 3295	Thr Leu Ile Leu Pro Ser Leu Glu 3295	
Leu Pro 3305	Val Leu His Val Pro 3310	Arg Asn Leu Lys Leu Ser Leu Pro 3310	
Asp Phe 3320	Lys Glu Leu Cys Thr 3325	Ile Ser His Ile Phe Ile Pro Ala 3325	
Met Gly 3335	Asn Ile Thr Tyr Asp 3340	Phe Ser Phe Lys Ser Ser Val Ile 3340	
Thr Leu 3350	Asn Thr Asn Ala Glu 3355	Leu Phe Asn Gln Ser Asp Ile Val 3355	
Ala His 3365	Leu Leu Ser Ser Ser 3370	Ser Ser Val Ile Asp Ala Leu Gln 3370	
Tyr Lys 3380	Leu Glu Gly Thr Thr 3385	Arg Leu Thr Arg Lys Arg Gly Leu 3385	
Lys Leu 3395	Ala Thr Ala Leu Ser 3400	Leu Ser Asn Lys Phe Val Glu Gly 3400	
Ser His 3410	Asn Ser Thr Val Ser 3415	Leu Thr Thr Lys Asn Met Glu Val 3415	
Ser Val 3425	Ala Thr Thr Thr Lys 3430	Ala Gln Ile Pro Ile Leu Arg Met 3430	
Asn Phe 3440	Lys Gln Glu Leu Asn 3445	Gly Asn Thr Lys Ser Lys Pro Thr 3445	
Val Ser 3455	Ser Ser Met Glu Phe 3460	Lys Tyr Asp Phe Asn Ser Ser Met 3460	
Leu Tyr 3470	Ser Thr Ala Lys Gly 3475	Ala Val Asp His Lys Leu Ser Leu 3475	
Glu Ser 3485	Leu Thr Ser Tyr Phe 3490	Ser Ile Glu Ser Ser Thr Lys Gly 3490	
Asp Val 3500	Lys Gly Ser Val Leu 3505	Ser Arg Glu Tyr Ser Gly Thr Ile 3505	
Ala Ser 3515	Glu Ala Asn Thr Tyr 3520	Leu Asn Ser Lys Ser Thr Arg Ser 3520	
Ser Val 3530	Lys Leu Gln Gly Thr 3535	Ser Lys Ile Asp Asp Ile Trp Asn 3535	
Leu Glu 3545	Val Lys Glu Asn Phe 3550	Ala Gly Glu Ala Thr Leu Gln Arg 3550	
Ile Tyr 3555	Ser Leu Trp Glu His 3555	Ser Thr Lys Asn His Leu Gln Leu 3555	

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3560	3565	3570
Glu Gly Leu Phe Phe Thr	Asn Gly Glu His Thr Ser	Lys Ala Thr
3575	3580	3585
Leu Glu Leu Ser Pro Trp	Gln Met Ser Ala Leu Val	Gln Val His
3590	3595	3600
Ala Ser Gln Pro Ser Ser	Phe His Asp Phe Pro Asp	Leu Gly Gln
3605	3610	3615
Glu Val Ala Leu Asn Ala	Asn Thr Lys Asn Gln Lys	Ile Arg Trp
3620	3625	3630
Lys Asn Glu Val Arg Ile	His Ser Gly Ser Phe Gln	Ser Gln Val
3635	3640	3645
Glu Leu Ser Asn Asp Gln	Glu Lys Ala His Leu Asp	Ile Ala Gly
3650	3655	3660
Ser Leu Glu Gly His Leu	Arg Phe Leu Lys Asn Ile	Ile Leu Pro
3665	3670	3675
Val Tyr Asp Lys Ser Leu	Trp Asp Phe Leu Lys Leu	Asp Val Thr
3680	3685	3690
Thr Ser Ile Gly Arg Arg	Gln His Leu Arg Val Ser	Thr Ala Phe
3695	3700	3705
Val Tyr Thr Lys Asn Pro	Asn Gly Tyr Ser Phe Ser	Ile Pro Val
3710	3715	3720
Lys Val Leu Ala Asp Lys	Phe Ile Ile Pro Gly Leu	Lys Leu Asn
3725	3730	3735
Asp Leu Asn Ser Val Leu	Val Met Pro Thr Phe His	Val Pro Phe
3740	3745	3750
Thr Asp Leu Gln Val Pro	Ser Cys Lys Leu Asp Phe	Arg Glu Ile
3755	3760	3765
Gln Ile Tyr Lys Lys Leu	Arg Thr Ser Ser Phe Ala	Leu Asn Leu
3770	3775	3780
Pro Thr Leu Pro Glu Val	Lys Phe Pro Glu Val Asp	Val Leu Thr
3785	3790	3795
Lys Tyr Ser Gln Pro Glu	Asp Ser Leu Ile Pro Phe	Phe Glu Ile
3800	3805	3810
Thr Val Pro Glu Ser Gln	Leu Thr Val Ser Gln Phe	Thr Leu Pro
3815	3820	3825
Lys Ser Val Ser Asp Gly	Ile Ala Ala Leu Asp Leu	Asn Ala Val
3830	3835	3840
Ala Asn Lys Ile Ala Asp	Phe Glu Leu Pro Thr Ile	Ile Val Pro
3845	3850	3855
Glu Gln Thr Ile Glu Ile	Pro Ser Ile Lys Phe Ser	Val Pro Ala
3860	3865	3870
Gly Ile Val Ile Pro Ser	Phe Gln Ala Leu Thr Ala	Arg Phe Glu
3875	3880	3885
Val Asp Ser Pro Val Tyr	Asn Ala Thr Trp Ser Ala	Ser Leu Lys
3890	3895	3900
Asn Lys Ala Asp Tyr Val	Glu Thr Val Leu Asp Ser	Thr Cys Ser
3905	3910	3915
Ser Thr Val Gln Phe Leu	Glu Tyr Glu Leu Asn Val	Leu Gly Thr
3920	3925	3930
His Lys Ile Glu Asp Gly	Thr Leu Ala Ser Lys Thr	Lys Gly Thr
3935	3940	3945
Phe Ala His Arg Asp Phe	Ser Ala Glu Tyr Glu Glu	Asp Gly Lys
3950	3955	3960

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Tyr 3965	Glu	Gly	Leu	Gln	Glu	Trp 3970	Glu	Gly	Lys	Ala	His 3975	Leu	Asn	Ile
Lys 3980	Ser	Pro	Ala	Phe	Thr	Asp 3985	Leu	His	Leu	Arg	Tyr 3990	Gln	Lys	Asp
Lys 3995	Lys	Gly	Ile	Ser	Thr	Ser 4000	Ala	Ala	Ser	Pro	Ala 4005	Val	Gly	Thr
Val 4010	Gly	Met	Asp	Met	Asp	Glu 4015	Asp	Asp	Asp	Phe	Ser 4020	Lys	Trp	Asn
Phe 4025	Tyr	Tyr	Ser	Pro	Gln	Ser 4030	Ser	Pro	Asp	Lys	Lys 4035	Leu	Thr	Ile
Phe 4040	Lys	Thr	Glu	Leu	Arg	Val 4045	Arg	Glu	Ser	Asp	Glu 4050	Glu	Thr	Gln
Ile 4055	Lys	Val	Asn	Trp	Glu	Glu 4060	Glu	Ala	Ala	Ser	Gly 4065	Leu	Leu	Thr
Ser 4070	Leu	Lys	Asp	Asn	Val	Pro 4075	Lys	Ala	Thr	Gly	Val 4080	Leu	Tyr	Asp
Tyr 4085	Val	Asn	Lys	Tyr	His	Trp 4090	Glu	His	Thr	Gly	Leu 4095	Thr	Leu	Arg
Glu 4100	Val	Ser	Ser	Lys	Leu	Arg 4105	Arg	Asn	Leu	Gln	Asn 4110	Asn	Ala	Glu
Trp 4115	Val	Tyr	Gln	Gly	Ala	Ile 4120	Arg	Gln	Ile	Asp	Asp 4125	Ile	Asp	Val
Arg 4130	Phe	Gln	Lys	Ala	Ala	Ser 4135	Gly	Thr	Thr	Gly	Thr 4140	Tyr	Gln	Glu
Trp 4145	Lys	Asp	Lys	Ala	Gln	Asn 4150	Leu	Tyr	Gln	Glu	Leu 4155	Leu	Thr	Gln
Glu 4160	Gly	Gln	Ala	Ser	Phe	Gln 4165	Gly	Leu	Lys	Asp	Asn 4170	Val	Phe	Asp
Gly 4175	Leu	Val	Arg	Val	Thr	Gln 4180	Glu	Phe	His	Met	Lys 4185	Val	Lys	His
Leu 4190	Ile	Asp	Ser	Leu	Ile	Asp 4195	Phe	Leu	Asn	Phe	Pro 4200	Arg	Phe	Gln
Phe 4205	Pro	Gly	Lys	Pro	Gly	Ile 4210	Tyr	Thr	Arg	Glu	Glu 4215	Leu	Cys	Thr
Met 4220	Phe	Ile	Arg	Glu	Val	Gly 4225	Thr	Val	Leu	Ser	Gln 4230	Val	Tyr	Ser
Lys 4235	Val	His	Asn	Gly	Ser	Glu 4240	Ile	Leu	Phe	Ser	Tyr 4245	Phe	Gln	Asp
Leu 4250	Val	Ile	Thr	Leu	Pro	Phe 4255	Glu	Leu	Arg	Lys	His 4260	Lys	Leu	Ile
Asp 4265	Val	Ile	Ser	Met	Tyr	Arg 4270	Glu	Leu	Leu	Lys	Asp 4275	Leu	Ser	Lys
Glu 4280	Ala	Gln	Glu	Val	Phe	Lys 4285	Ala	Ile	Gln	Ser	Leu 4290	Lys	Thr	Thr
Glu 4295	Val	Leu	Arg	Asn	Leu	Gln 4300	Asp	Leu	Leu	Gln	Phe 4305	Ile	Phe	Gln
Leu 4310	Ile	Glu	Asp	Asn	Ile	Lys 4315	Gln	Leu	Lys	Glu	Met 4320	Lys	Phe	Thr
Tyr 4325	Leu	Ile	Asn	Tyr	Ile	Gln 4330	Asp	Glu	Ile	Asn	Thr 4335	Ile	Phe	Ser
Asp 4340	Tyr	Ile	Pro	Tyr	Val	Phe 4345	Lys	Leu	Leu	Lys	Glu 4350	Asn	Leu	Cys
Leu 4355	Asn	Leu	His	Lys	Phe	Asn 4360	Glu	Phe	Ile	Gln	Asn 4365	Glu	Leu	Gln

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Glu Ala Ser Gln Glu Leu Gln Gln Ile His Gln Tyr Ile Met Ala
4370 4375 4380

Leu Arg Glu Glu Tyr Phe Asp Pro Ser Ile Val Gly Trp Thr Val
4385 4390 4395

Lys Tyr Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn
4400 4405 4410

Leu Leu Val Ala Leu Lys Asp Phe His Ser Glu Tyr Ile Val Ser
4415 4420 4425

Ala Ser Asn Phe Thr Ser Gln Leu Ser Ser Gln Val Glu Gln Phe
4430 4435 4440

Leu His Arg Asn Ile Gln Glu Tyr Leu Ser Ile Leu Thr Asp Pro
4445 4450 4455

Asp Gly Lys Gly Lys Glu Lys Ile Ala Glu Leu Ser Ala Thr Ala
4460 4465 4470

Gln Glu Ile Ile Lys Ser Gln Ala Ile Ala Thr Lys Lys Ile Ile
4475 4480 4485

Ser Asp Tyr His Gln Gln Phe Arg Tyr Lys Leu Gln Asp Phe Ser
4490 4495 4500

Asp Gln Leu Ser Asp Tyr Tyr Glu Lys Phe Ile Ala Glu Ser Lys
4505 4510 4515

Arg Leu Ile Asp Leu Ser Ile Gln Asn Tyr His Thr Phe Leu Ile
4520 4525 4530

Tyr Ile Thr Glu Leu Leu Lys Lys Leu Gln Ser Thr Thr Val Met
4535 4540 4545

Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu Leu Thr Ile Ile Leu
4550 4555 4560

<210> SEQ ID NO 4
 <211> LENGTH: 2040
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 4

Met Glu His Lys Glu Val Val Leu Leu Leu Leu Phe Leu Lys Ser
1 5 10 15

Ala Ala Pro Glu Gln Ser His Val Val Gln Asp Cys Tyr His Gly Asp
20 25 30

Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr
35 40 45

Cys Gln Ala Trp Ser Ser Met Thr Pro His Gln His Asn Arg Thr Thr
50 55 60

Glu Asn Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro
65 70 75 80

Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg
85 90 95

Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala
100 105 110

Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser
115 120 125

Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His
130 135 140

Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly
145 150 155 160

Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg
165 170 175

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Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg
 180 185 190
 Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly
 195 200 205
 Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly
 210 215 220
 Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala
 225 230 235 240
 Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys
 245 250 255
 Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val
 260 265 270
 Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His
 275 280 285
 Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr
 290 295 300
 Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp
 305 310 315 320
 Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala
 325 330 335
 Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu
 340 345 350
 Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln
 355 360 365
 Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr
 370 375 380
 Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His
 385 390 395 400
 Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met
 405 410 415
 Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr
 420 425 430
 Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser
 435 440 445
 Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro
 450 455 460
 Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly
 465 470 475 480
 Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr
 485 490 495
 Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr
 500 505 510
 Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu
 515 520 525
 Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys
 530 535 540
 Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln
 545 550 555 560
 Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro
 565 570 575
 Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg
 580 585 590
 Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly

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595				600				605							
Thr	Tyr	Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser
610							615					620			
Met	Thr	Pro	His	Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala
625					630					635					640
Gly	Leu	Ile	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro
				645					650					655	
Tyr	Cys	Tyr	Thr	Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu
			660						665					670	
Thr	Gln	Cys	Ser	Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val
			675											685	
Thr	Pro	Val	Pro	Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu
	690					695					700				
Gln	Arg	Pro	Gly	Val	Gln	Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr
705					710					715					720
Arg	Gly	Thr	Tyr	Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp
			725						730					735	
Ser	Ser	Met	Thr	Pro	His	Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Pro	Pro
			740						745					750	
Asn	Ala	Gly	Leu	Ile	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala
		755					760					765			
Ala	Pro	Tyr	Cys	Tyr	Thr	Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys
		770				775					780				
Asn	Leu	Thr	Gln	Cys	Ser	Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro
785					790					795					800
Thr	Val	Thr	Pro	Val	Pro	Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro
			805							810				815	
Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln	Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln
			820							825				830	
Ser	Tyr	Arg	Gly	Thr	Tyr	Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln
		835					840							845	
Ala	Trp	Ser	Ser	Met	Thr	Pro	His	Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr
		850					855				860				
Tyr	Pro	Asn	Ala	Gly	Leu	Ile	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Pro
865					870					875					880
Val	Ala	Ala	Pro	Tyr	Cys	Tyr	Thr	Arg	Asp	Pro	Ser	Val	Arg	Trp	Glu
			885						890					895	
Tyr	Cys	Asn	Leu	Thr	Gln	Cys	Ser	Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala
			900						905					910	
Pro	Pro	Thr	Ile	Thr	Pro	Ile	Pro	Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln
		915					920							925	
Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln	Glu	Cys	Tyr	His	Gly	Asn
	930					935					940				
Gly	Gln	Ser	Tyr	Gln	Gly	Thr	Tyr	Phe	Ile	Thr	Val	Thr	Gly	Arg	Thr
945					950					955					960
Cys	Gln	Ala	Trp	Ser	Ser	Met	Thr	Pro	His	Ser	His	Ser	Arg	Thr	Pro
			965						970					975	
Ala	Tyr	Tyr	Pro	Asn	Ala	Gly	Leu	Ile	Lys	Asn	Tyr	Cys	Arg	Asn	Pro
			980						985					990	
Asp	Pro	Val	Ala	Ala	Pro	Trp	Cys	Tyr	Thr	Thr	Asp	Pro	Ser	Val	Arg
		995					1000							1005	
Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Arg	Cys	Ser	Asp	Ala	Glu	Trp	Thr	
	1010					1015								1020	

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Asn	Ala	Gly	Leu	Thr	Arg	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Glu
1430						1435					1440			
Ile	Arg	Pro	Trp	Cys	Tyr	Thr	Met	Asp	Pro	Ser	Val	Arg	Trp	Glu
1445						1450					1455			
Tyr	Cys	Asn	Leu	Thr	Arg	Cys	Pro	Val	Thr	Glu	Ser	Ser	Val	Leu
1460						1465					1470			
Thr	Thr	Pro	Thr	Val	Ala	Pro	Val	Pro	Ser	Thr	Glu	Ala	Pro	Ser
1475						1480					1485			
Glu	Gln	Ala	Pro	Pro	Glu	Lys	Ser	Pro	Val	Val	Gln	Asp	Cys	Tyr
1490						1495					1500			
His	Gly	Asp	Gly	Arg	Ser	Tyr	Arg	Gly	Ile	Ser	Ser	Thr	Thr	Val
1505						1510					1515			
Thr	Gly	Arg	Thr	Cys	Gln	Ser	Trp	Ser	Ser	Met	Ile	Pro	His	Trp
1520						1525					1530			
His	Gln	Arg	Thr	Pro	Glu	Asn	Tyr	Pro	Asn	Ala	Gly	Leu	Thr	Glu
1535						1540					1545			
Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ser	Gly	Lys	Gln	Pro	Trp	Cys	Tyr
1550						1555					1560			
Thr	Thr	Asp	Pro	Cys	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Gln
1565						1570					1575			
Cys	Ser	Glu	Thr	Glu	Ser	Gly	Val	Leu	Glu	Thr	Pro	Thr	Val	Val
1580						1585					1590			
Pro	Val	Pro	Ser	Met	Glu	Ala	His	Ser	Glu	Ala	Ala	Pro	Thr	Glu
1595						1600					1605			
Gln	Thr	Pro	Val	Val	Arg	Gln	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser
1610						1615					1620			
Tyr	Arg	Gly	Thr	Phe	Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln
1625						1630					1635			
Ser	Trp	Ser	Ser	Met	Thr	Pro	His	Arg	His	Gln	Arg	Thr	Pro	Glu
1640						1645					1650			
Asn	Tyr	Pro	Asn	Asp	Gly	Leu	Thr	Met	Asn	Tyr	Cys	Arg	Asn	Pro
1655						1660					1665			
Asp	Ala	Asp	Thr	Gly	Pro	Trp	Cys	Phe	Thr	Met	Asp	Pro	Ser	Ile
1670						1675					1680			
Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Arg	Cys	Ser	Asp	Thr	Glu	Gly
1685						1690					1695			
Thr	Val	Val	Ala	Pro	Pro	Thr	Val	Ile	Gln	Val	Pro	Ser	Leu	Gly
1700						1705					1710			
Pro	Pro	Ser	Glu	Gln	Asp	Cys	Met	Phe	Gly	Asn	Gly	Lys	Gly	Tyr
1715						1720					1725			
Arg	Gly	Lys	Lys	Ala	Thr	Thr	Val	Thr	Gly	Thr	Pro	Cys	Gln	Glu
1730						1735					1740			
Trp	Ala	Ala	Gln	Glu	Pro	His	Arg	His	Ser	Thr	Phe	Ile	Pro	Gly
1745						1750					1755			
Thr	Asn	Lys	Trp	Ala	Gly	Leu	Glu	Lys	Asn	Tyr	Cys	Arg	Asn	Pro
1760						1765					1770			
Asp	Gly	Asp	Ile	Asn	Gly	Pro	Trp	Cys	Tyr	Thr	Met	Asn	Pro	Arg
1775						1780					1785			
Lys	Leu	Phe	Asp	Tyr	Cys	Asp	Ile	Pro	Leu	Cys	Ala	Ser	Ser	Ser
1790						1795					1800			
Phe	Asp	Cys	Gly	Lys	Pro	Gln	Val	Glu	Pro	Lys	Lys	Cys	Pro	Gly
1805						1810					1815			
Ser	Ile	Val	Gly	Gly	Cys	Val	Ala	His	Pro	His	Ser	Trp	Pro	Trp

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1820	1825	1830
Gln Val Ser Leu Arg Thr	Arg Phe Gly Lys His Phe	Cys Gly Gly
1835	1840	1845
Thr Leu Ile Ser Pro Glu Trp	Val Leu Thr Ala Ala	His Cys Leu
1850	1855	1860
Lys Lys Ser Ser Arg Pro Ser	Ser Tyr Lys Val Ile	Leu Gly Ala
1865	1870	1875
His Gln Glu Val Asn Leu Glu	Ser His Val Gln Glu	Ile Glu Val
1880	1885	1890
Ser Arg Leu Phe Leu Glu Pro	Thr Gln Ala Asp Ile	Ala Leu Leu
1895	1900	1905
Lys Leu Ser Arg Pro Ala Val	Ile Thr Asp Lys Val	Met Pro Ala
1910	1915	1920
Cys Leu Pro Ser Pro Asp Tyr	Met Val Thr Ala Arg	Thr Glu Cys
1925	1930	1935
Tyr Ile Thr Gly Trp Gly Glu	Thr Gln Gly Thr Phe	Gly Thr Gly
1940	1945	1950
Leu Leu Lys Glu Ala Gln Leu	Leu Val Ile Glu Asn	Glu Val Cys
1955	1960	1965
Asn His Tyr Lys Tyr Ile Cys	Ala Glu His Leu Ala	Arg Gly Thr
1970	1975	1980
Asp Ser Cys Gln Gly Asp Ser	Gly Gly Pro Leu Val	Cys Phe Glu
1985	1990	1995
Lys Asp Lys Tyr Ile Leu Gln	Gly Val Thr Ser Trp	Gly Leu Gly
2000	2005	2010
Cys Ala Arg Pro Asn Lys Pro	Gly Val Tyr Ala Arg	Val Ser Arg
2015	2020	2025
Phe Val Thr Trp Ile Glu Gly	Met Met Arg Asn Asn	
2030	2035	2040

<210> SEQ ID NO 5
 <211> LENGTH: 83
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Arg Leu Phe Leu Ser Leu Pro Val Leu Val Val Val Leu Ser Ile
 1 5 10 15
 Val Leu Glu Gly Pro Ala Pro Ala Gln Gly Thr Pro Asp Val Ser Ser
 20 25 30
 Ala Leu Asp Lys Leu Lys Glu Phe Gly Asn Thr Leu Glu Asp Lys Ala
 35 40 45
 Arg Glu Leu Ile Ser Arg Ile Lys Gln Ser Glu Leu Ser Ala Lys Met
 50 55 60
 Arg Glu Trp Phe Ser Glu Thr Phe Gln Lys Val Lys Glu Lys Leu Lys
 65 70 75 80
 Ile Asp Ser

<210> SEQ ID NO 6
 <211> LENGTH: 99
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Gln Pro Arg Val Leu Leu Val Val Ala Leu Leu Ala Leu Leu Ala
 1 5 10 15

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Ser Ala Arg Ala Ser Glu Ala Glu Asp Ala Ser Leu Leu Ser Phe Met
 20 25 30

Gln Gly Tyr Met Lys His Ala Thr Lys Thr Ala Lys Asp Ala Leu Ser
 35 40 45

Ser Val Gln Glu Ser Gln Val Ala Gln Gln Ala Arg Gly Trp Val Thr
 50 55 60

Asp Gly Phe Ser Ser Leu Lys Asp Tyr Trp Ser Thr Val Lys Asp Lys
 65 70 75 80

Phe Ser Glu Phe Trp Asp Leu Asp Pro Glu Val Arg Pro Thr Ser Ala
 85 90 95

Val Ala Ala

<210> SEQ ID NO 7
 <211> LENGTH: 130
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 7

Met Arg Leu Phe Thr Gly Ile Val Phe Cys Ser Leu Val Met Gly Val
 1 5 10 15

Thr Ser Glu Ser Trp Arg Ser Phe Phe Lys Glu Ala Leu Gln Gly Val
 20 25 30

Gly Asp Met Gly Arg Ala Tyr Trp Asp Ile Met Ile Ser Asn His Gln
 35 40 45

Asn Ser Asn Arg Tyr Leu Tyr Ala Arg Gly Asn Tyr Asp Ala Ala Gln
 50 55 60

Arg Gly Pro Gly Gly Val Trp Ala Ala Lys Leu Ile Ser Arg Ser Arg
 65 70 75 80

Val Tyr Leu Gln Gly Leu Ile Asp Tyr Tyr Leu Phe Gly Asn Ser Ser
 85 90 95

Thr Val Leu Glu Asp Ser Lys Ser Asn Glu Lys Ala Glu Glu Trp Gly
 100 105 110

Arg Ser Gly Lys Asp Pro Asp Arg Phe Arg Pro Asp Gly Leu Pro Lys
 115 120 125

Lys Tyr
 130

<210> SEQ ID NO 8
 <211> LENGTH: 317
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 8

Met Lys Val Leu Trp Ala Ala Leu Leu Val Thr Phe Leu Ala Gly Cys
 1 5 10 15

Gln Ala Lys Val Glu Gln Ala Val Glu Thr Glu Pro Glu Pro Glu Leu
 20 25 30

Arg Gln Gln Thr Glu Trp Gln Ser Gly Gln Arg Trp Glu Leu Ala Leu
 35 40 45

Gly Arg Phe Trp Asp Tyr Leu Arg Trp Val Gln Thr Leu Ser Glu Gln
 50 55 60

Val Gln Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala
 65 70 75 80

Leu Met Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu
 85 90 95

Glu Glu Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser
 100 105 110

-continued

Lys Glu Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp
 115 120 125
 Val Cys Gly Arg Leu Val Gln Tyr Arg Gly Glu Val Gln Ala Met Leu
 130 135 140
 Gly Gln Ser Thr Glu Glu Leu Arg Val Arg Leu Ala Ser His Leu Arg
 145 150 155 160
 Lys Leu Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg
 165 170 175
 Leu Ala Val Tyr Gln Ala Gly Ala Arg Glu Gly Ala Glu Arg Gly Leu
 180 185 190
 Ser Ala Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val
 195 200 205
 Arg Ala Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu Arg
 210 215 220
 Ala Gln Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met Gly
 225 230 235 240
 Ser Arg Thr Arg Asp Arg Leu Asp Glu Val Lys Glu Gln Val Ala Glu
 245 250 255
 Val Arg Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala
 260 265 270
 Glu Ala Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu
 275 280 285
 Asp Met Gln Arg Gln Trp Ala Gly Leu Val Glu Lys Val Gln Ala Ala
 290 295 300
 Val Gly Thr Ser Ala Ala Pro Val Pro Ser Asp Asn His
 305 310 315

<210> SEQ ID NO 9
 <211> LENGTH: 7
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Ile Thr Leu Pro Asp Phe Arg
 1 5

<210> SEQ ID NO 10
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Ser Val Gly Phe His Leu Pro Ser Arg
 1 5

<210> SEQ ID NO 11
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Ala Lys Pro Ala Leu Glu Asp Leu Arg
 1 5

<210> SEQ ID NO 12
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE

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<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Wherein Met at position 8 is optionally oxidized

<400> SEQUENCE: 12

Leu Ser Pro Leu Gly Glu Glu Met Arg
1 5

<210> SEQ ID NO 13

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Leu Gln Ala Glu Ala Phe Gln Ala Arg
1 5

<210> SEQ ID NO 14

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Leu Ser Pro Leu Gly Glu Glu Met Arg
1 5

<210> SEQ ID NO 15

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Leu Ala Ala Tyr Leu Met Leu Met Arg
1 5

<210> SEQ ID NO 16

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

His Ile Asn Ile Asp Gln Phe Val Arg
1 5

<210> SEQ ID NO 17

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Ser Lys Glu Gln Leu Thr Pro Leu Ile Lys
1 5 10

<210> SEQ ID NO 18

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

Ser Pro Ala Phe Thr Asp Leu His Leu Arg
1 5 10

<210> SEQ ID NO 19

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 19

Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg
1 5 10

<210> SEQ ID NO 20

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 20

Phe Arg Glu Thr Leu Glu Asp Thr Arg
1 5

<210> SEQ ID NO 21

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

Ser Leu Asp Glu His Tyr His Ile Arg
1 5

<210> SEQ ID NO 22

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

Val Leu Val Asp His Phe Gly Tyr Thr Lys
1 5 10

<210> SEQ ID NO 23

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

Val Lys Ser Pro Glu Leu Gln Ala Glu Ala Lys
1 5 10

<210> SEQ ID NO 24

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

Leu Thr Ile Ser Glu Gln Asn Ile Gln Arg
1 5 10

<210> SEQ ID NO 25

<211> LENGTH: 10

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg
1 5 10

<210> SEQ ID NO 26

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<220> FEATURE:

<221> NAME/KEY: MISC_FEATURE

<222> LOCATION: (5)..(5)

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<223> OTHER INFORMATION: Wherein Met at position 5 is optionally oxidized

<400> SEQUENCE: 26

Trp Gln Glu Glu Met Glu Leu Tyr Arg
1 5

<210> SEQ ID NO 27

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg
1 5 10

<210> SEQ ID NO 28

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<220> FEATURE:

<221> NAME/KEY: MISC_FEATURE

<222> LOCATION: (8)..(8)

<223> OTHER INFORMATION: Wherein Met at position 8 is optionally oxidized

<400> SEQUENCE: 28

Leu Ser Pro Leu Gly Glu Glu Met Arg Asp Arg
1 5 10

<210> SEQ ID NO 29

<211> LENGTH: 12

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg
1 5 10

<210> SEQ ID NO 30

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys
1 5 10

<210> SEQ ID NO 31

<211> LENGTH: 13

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

Asp Tyr Val Ser Gln Phe Glu Gly Ser Ala Leu Gly Lys
1 5 10

<210> SEQ ID NO 32

<211> LENGTH: 12

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32

Phe Gln Phe Pro Gly Lys Pro Gly Ile Tyr Thr Arg
1 5 10

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<210> SEQ ID NO 33
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (6)..(6)
 <223> OTHER INFORMATION: Wherein Met at position 6 is optionally oxidized

<400> SEQUENCE: 33

Lys Trp Gln Glu Glu Met Glu Leu Tyr Arg
 1 5 10

<210> SEQ ID NO 34
 <211> LENGTH: 12
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

Asp Pro Asp Arg Phe Arg Pro Asp Gly Leu Pro Lys
 1 5 10

<210> SEQ ID NO 35
 <211> LENGTH: 13
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg
 1 5 10

<210> SEQ ID NO 36
 <211> LENGTH: 11
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (1)..(1)
 <223> OTHER INFORMATION: Wherein Met at position 1 is optionally oxidized

<400> SEQUENCE: 36

Met Arg Glu Trp Phe Ser Glu Thr Phe Gln Lys
 1 5 10

<210> SEQ ID NO 37
 <211> LENGTH: 15
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

Leu Ala Ala Arg Leu Glu Ala Leu Lys Glu Asn Gly Gly Ala Arg
 1 5 10 15

<210> SEQ ID NO 38
 <211> LENGTH: 13
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 38

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg
 1 5 10

<210> SEQ ID NO 39
 <211> LENGTH: 14
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 39

Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys
 1 5 10

<210> SEQ ID NO 40

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 40

Asp Ala Leu Ser Ser Val Gln Glu Ser Gln Val Ala Gln Gln Ala Arg
 1 5 10 15

<210> SEQ ID NO 41

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 41

Gln Lys Val Glu Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg
 1 5 10 15

<210> SEQ ID NO 42

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 42

Ile Val Gln Ile Leu Pro Trp Glu Gln Asn Glu Gln Val Lys
 1 5 10

<210> SEQ ID NO 43

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 43

Asn Leu Gln Asn Asn Ala Glu Trp Val Tyr Gln Gly Ala Ile Arg
 1 5 10 15

<210> SEQ ID NO 44

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg
 1 5 10 15

<210> SEQ ID NO 45

<211> LENGTH: 17

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<220> FEATURE:

<221> NAME/KEY: MISC_FEATURE

<222> LOCATION: (12)..(12)

<223> OTHER INFORMATION: Wherein Met at position 12 is optionally oxidized

<400> SEQUENCE: 45

Ser Glu Ala Glu Asp Ala Ser Leu Leu Ser Phe Met Gln Gly Tyr Met
 1 5 10 15

Lys

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<210> SEQ ID NO 46
 <211> LENGTH: 18
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 46

 Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu
 1 5 10 15

 Glu Lys

 <210> SEQ ID NO 47
 <211> LENGTH: 20
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (16)..(16)
 <223> OTHER INFORMATION: Wherein Met at position 16 is optionally
 oxidized

 <400> SEQUENCE: 47

 Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys Trp Gln Glu Glu Met
 1 5 10 15

 Glu Leu Tyr Arg
 20

 <210> SEQ ID NO 48
 <211> LENGTH: 10
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 48

 Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg
 1 5 10

 <210> SEQ ID NO 49
 <211> LENGTH: 18
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 49

 Leu Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu
 1 5 10 15

 Glu Lys

 <210> SEQ ID NO 50
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 50

 Ala Lys Pro Ala Leu Glu Asp Leu Arg
 1 5

 <210> SEQ ID NO 51
 <211> LENGTH: 11
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

 <400> SEQUENCE: 51

 Val Gln Pro Tyr Leu Asp Asp Phe Gln Lys Lys
 1 5 10

 <210> SEQ ID NO 52
 <211> LENGTH: 14

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<212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 52

Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys
 1 5 10

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of screening a mammalian subject to determine if the subject is at risk to develop, or is suffering from, cardiovascular disease, the method comprising detecting a measurable feature of at least two biomarkers in an HDL subfraction, or in a complex containing apoA-I or apoA-II isolated from a biological sample obtained from the subject, wherein the at least two biomarkers are selected from the group consisting of apoA-I, apoA-II, apoB-100, Lp(a), apoC-I, and apoC-III, combinations or portions and/or derivatives thereof, and comparing the measurable features of the at least two biomarkers from the biological sample to a reference standard, wherein a difference in the measurable features of the at least two biomarkers from the biological sample and the reference standard is indicative of the presence or risk of cardiovascular disease in the subject.

2. The method of claim 1, wherein the HDL subfraction of the biological sample is the HDL2 subfraction.

3. The method of claim 1, wherein the biological sample is selected from the group consisting of a blood sample, a serum sample, a plasma sample, a tissue sample, a bodily fluid sample, and a urine sample.

4. The method of claim 1, wherein at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises an increased amount of at least one of the biomarkers in the HDL subfraction or in the complex containing apoA-I or apoA-II isolated from the biological sample selected from the group consisting of apoA-I, apoB-100, apoC-III and Lp(a), or portions and/or derivatives thereof, in comparison to the reference standard.

5. The method of claim 4, wherein the biomarker is apoA-I, or a portion or derivative thereof.

6. The method of claim 4, wherein the biomarker is apoC-III or a portion or derivative thereof.

7. The method of claim 4, wherein the biomarker is Lp(a) or a portion or derivative thereof.

8. The method of claim 1, wherein at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises a decreased amount of at least one of the biomarkers in the HDL subfraction or in the com-

plex containing apoA-I or apoA-II isolated from the biological sample selected from the group consisting of apoA-I and apoC-I, or portions and/or derivatives thereof, in comparison to the reference standard.

9. The method of claim 8, wherein the biomarker is apoA-I, or a portion or derivative thereof.

10. The method of claim 8, wherein the biomarker is apoC-I, or a portion or derivative thereof.

11. The method of claim 1, wherein at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises a post-translational modification of a peptide derived from apoA-I in the HDL subfraction or complex isolated from the biological sample, in comparison to the reference standard.

12. The method of claim 11, wherein the post-translational modification of apoA-I is oxidation of at least one Methionine residue.

13. The method of claim 1, wherein at least one of the measurable features indicative of the presence or risk of cardiovascular disease comprises an altered structural conformation of apoA-I in the HDL subfraction of the biological sample, in comparison to a reference standard.

14. The method of claim 1, wherein the measurable features of the at least two biomarkers from the biological sample are determined using mass spectrometry analysis.

15. The method of claim 14, wherein the mass spectrometry analysis is performed on a tryptic digestion of the HDL subfraction or complex isolated from the biological sample.

16. The method of claim 14, wherein the mass spectrometry analysis is carried out with a matrix-assisted laser desorption ionization (MALDI) mass spectrometer or LCMS.

17. The method of claim 1, wherein the cardiovascular disease is the predisposition to myocardial infarction, atherosclerosis, coronary artery disease, peripheral artery disease, heart failure, or stroke.

18. The method of claim 1, wherein the measurable features of the at least two biomarkers in the HDL subfraction or complex isolated from the biological sample are detected using at least one antibody specific to each of the at least one of the two biomarkers.

* * * * *

专利名称(译)	用于心血管疾病的诊断或预后的方法和组合物		
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申请号	US12/499711	申请日	2009-07-08
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申请(专利权)人(译)	INSILICOS , LLC 华盛顿大学		
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摘要(译)

本发明提供了筛选哺乳动物受试者的方法，以确定受试者是否有发展或患有心血管疾病的风险。在一个实施方案中，该方法包括检测HDL亚组分中的至少两种生物标志物的可测量特征，或检测从获自受试者的生物样品中分离的含有apoA-I或apoA-II的复合物中的至少两种生物标志物的可测量特征，其中所述至少两种生物标志物是选自apoA-I， apoA-II， apoB-100， Lp (a)， apoC-I和apoC-III，其组合或部分和/或衍生物，并比较至少可测量的特征从生物样品到参考标准的两种生物标志物，其中来自生物样品和参照标准的至少两种生物标志物的可测量特征的差异指示受试者中心血管疾病的存在或风险。

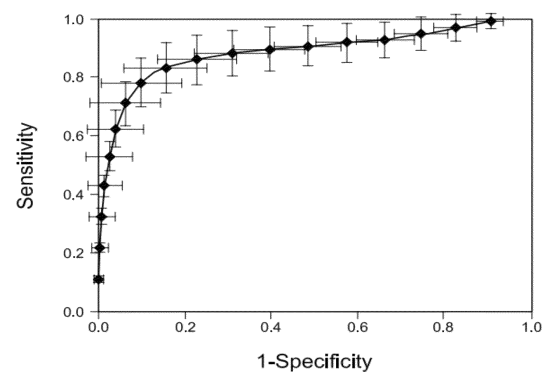


Fig. 1.