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

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

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USPC **435/7.21**; 435/7.1; 436/501; 436/518; 422/50; 422/430; 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-III 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.

21 Claims, 13 Drawing Sheets

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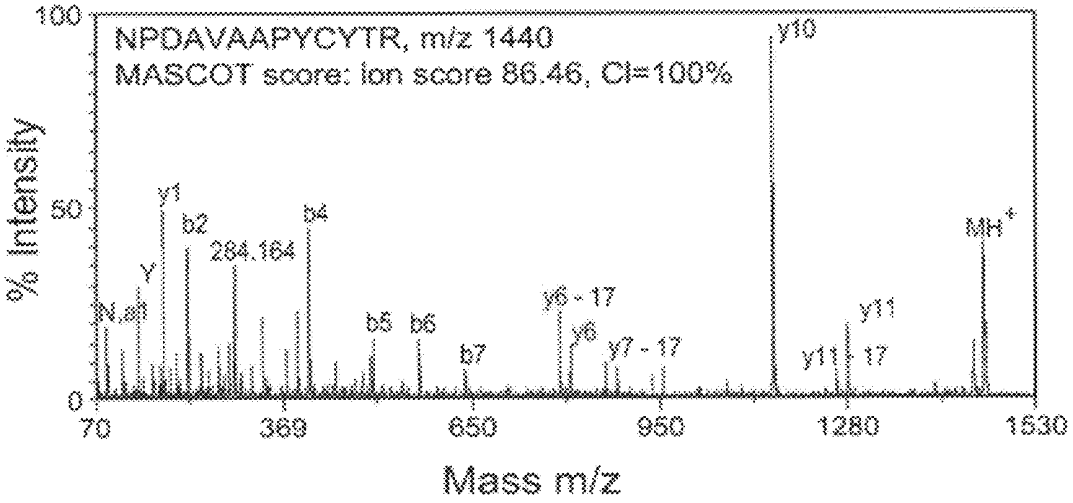
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100
50
0
% Intensity
N,a1
Y
y1
b2
284.164
b4
b5
b6
b7
y6 - 17
y6
y7 - 17
y10
y11
y11 - 17
MH+
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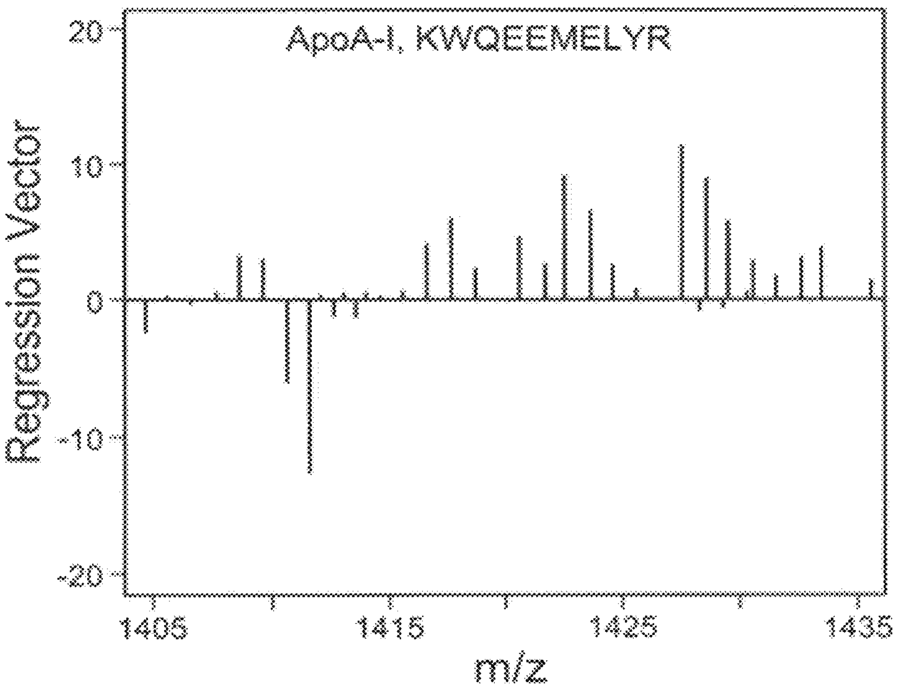
650
950
1280
1530
Mass m/z

Fig. 5C.


ApoA-I, KWQEEMELYR
Regression Vector
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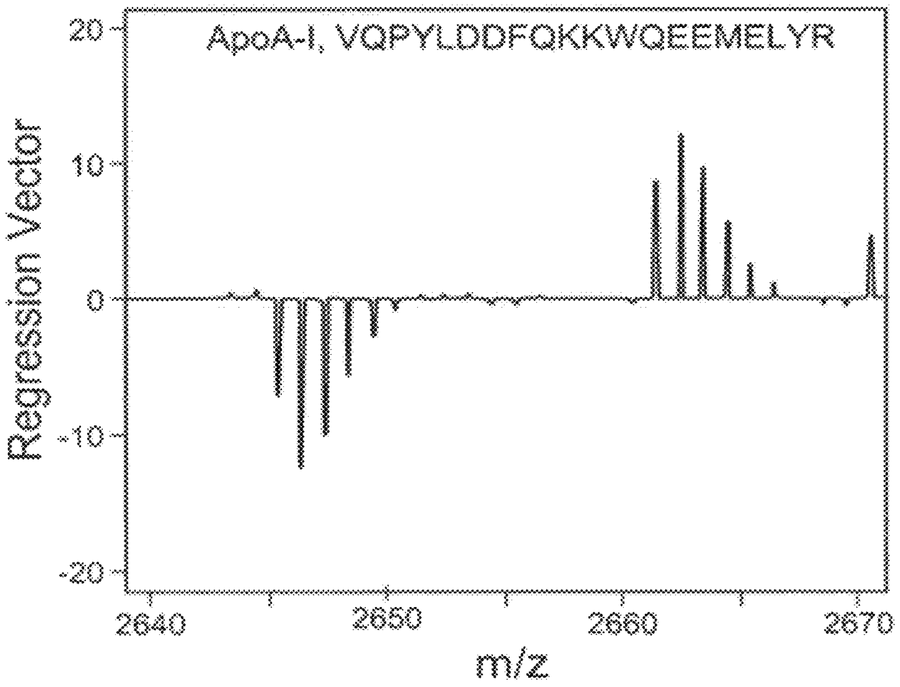
10
0
-10
-20
1405
1415
1425
1435
m/z

Fig.6A.


ApoA-I, VQPYLDDFQKKWQEEMELYR
Regression Vector
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10
0
-10
-20
2640
2650
2660
2670
m/z

Fig.6B.

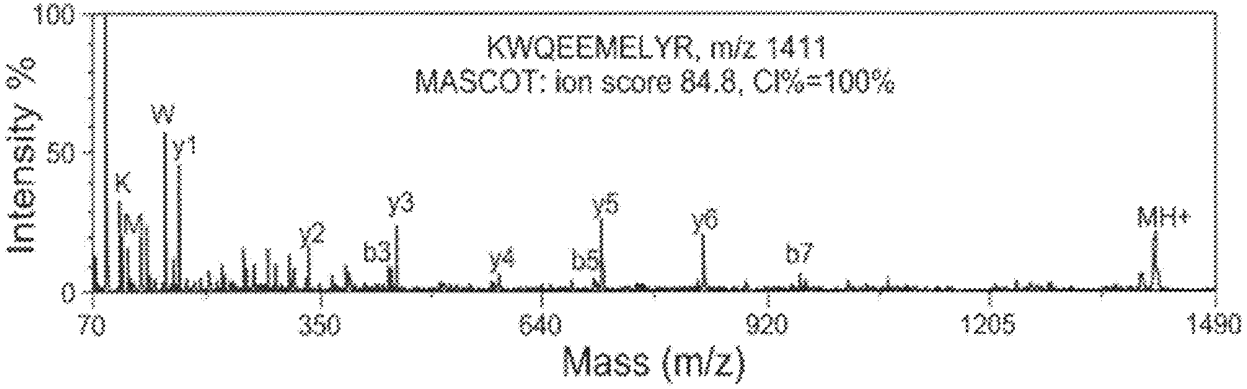
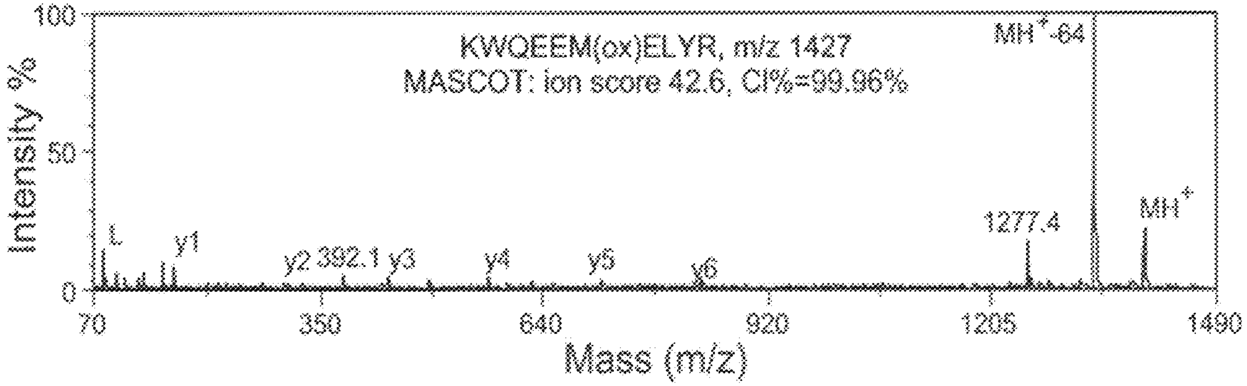

KWQEEMELYR, m/z 1411
MASCOT: ion score 84.8, CI%=100%
Intensity %
100
50
0
K
W
M
y1
y2
b3
y3
y4
b5
y5
y6
b7
MH+
70
350
640
920
1205
1490
Mass (m/z)

Fig. 6C.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MASCOT: ion score 42.6, CI%=99.96%
Intensity %
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50
0
L
y1
y2
392.1
y3
y4
y5
y6
1277.4
MH+-64
MH+
70
350
640
920
1205
1490
Mass (m/z)

Fig. 6D.

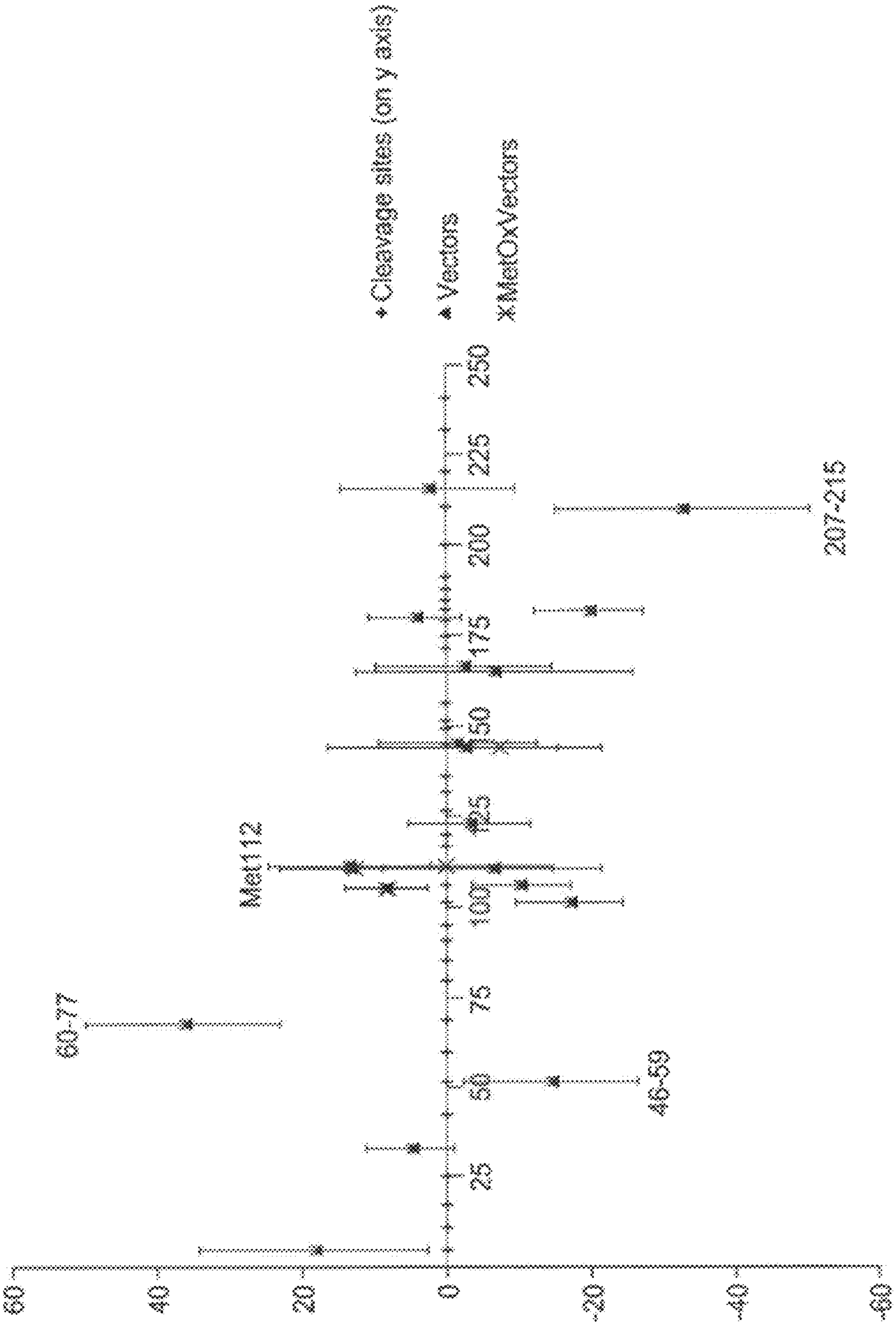
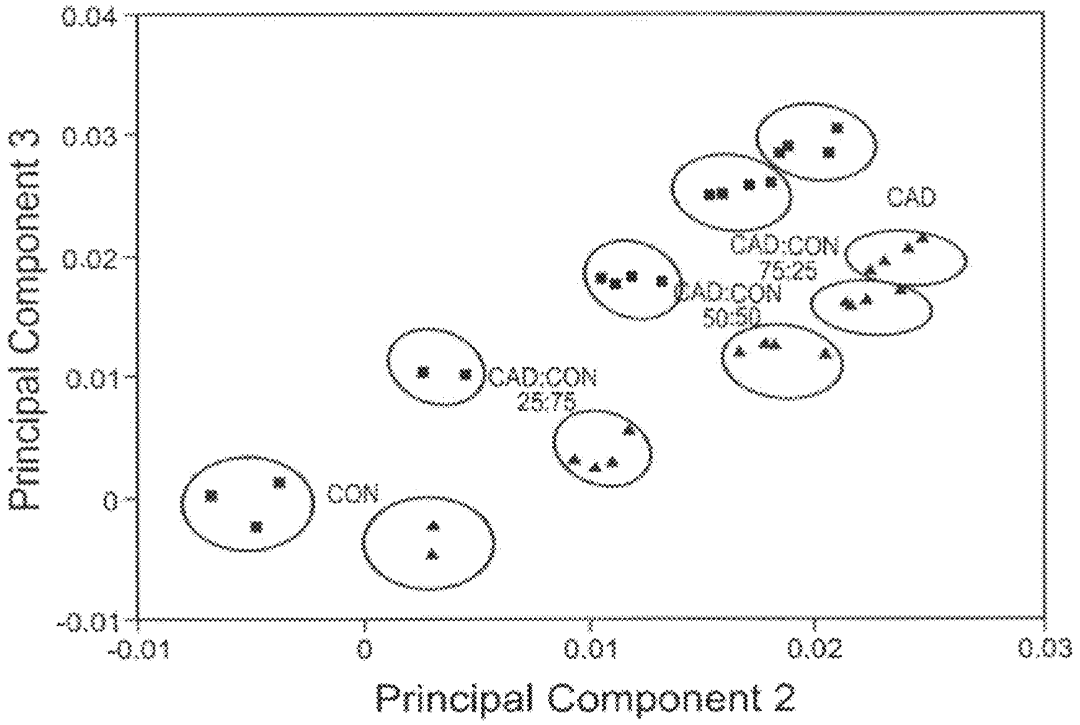

◆ Cleavage sites (on y axis)
▲ Vectors
X MetOxVectors
60
40
20
0
-20
-40
-60
25
50
75
100
125
150
175
200
225
250
60-77
Met112
46-59
207-215

Fig. 7.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0.03
0.02
0.01
0
-0.01
-0.01
0
0.01
0.02
0.03
Principal Component 3
Principal Component 2
CON
CAD:CON
25:75
CAD:CON
50:50
CAD:CON
75:25
CAD

Fig. 8.

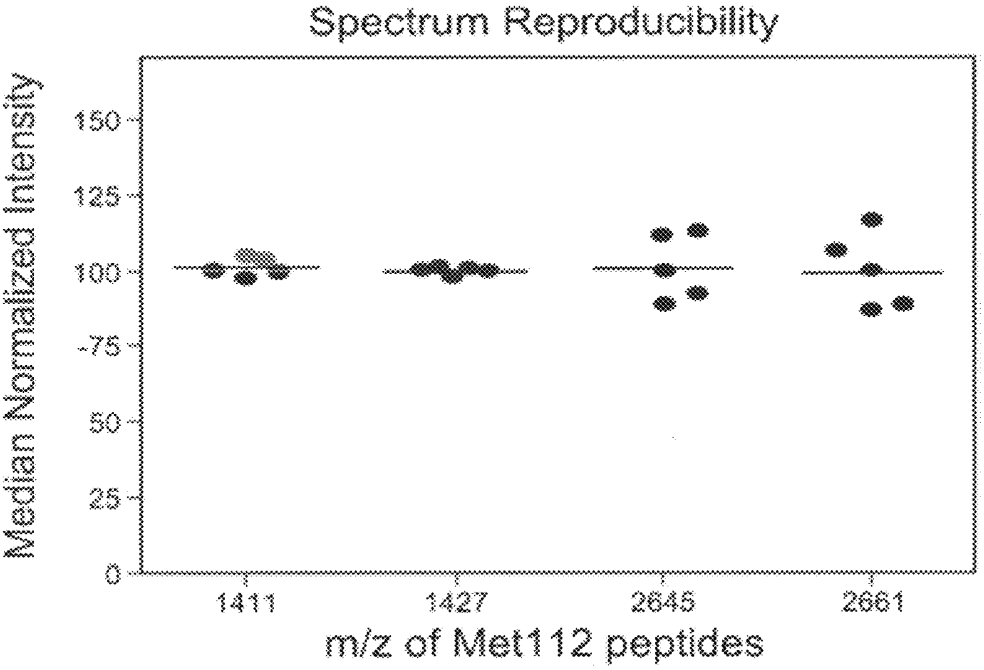
Spectrum Reproducibility
Median Normalized Intensity
150
125
100
75
50
25
0
1411
1427
2645
2661
m/z of Met112 peptides

Fig. 9A.

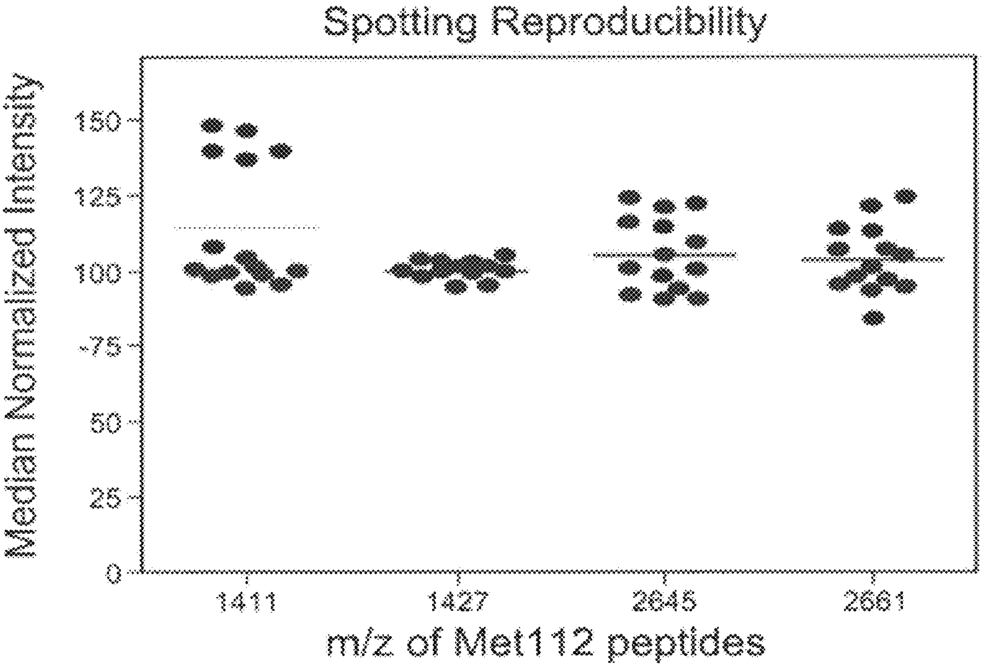
Spotting Reproducibility
Median Normalized Intensity
150
125
100
75
50
25
0
1411
1427
2645
2661
m/z of Met112 peptides

Fig. 9B.

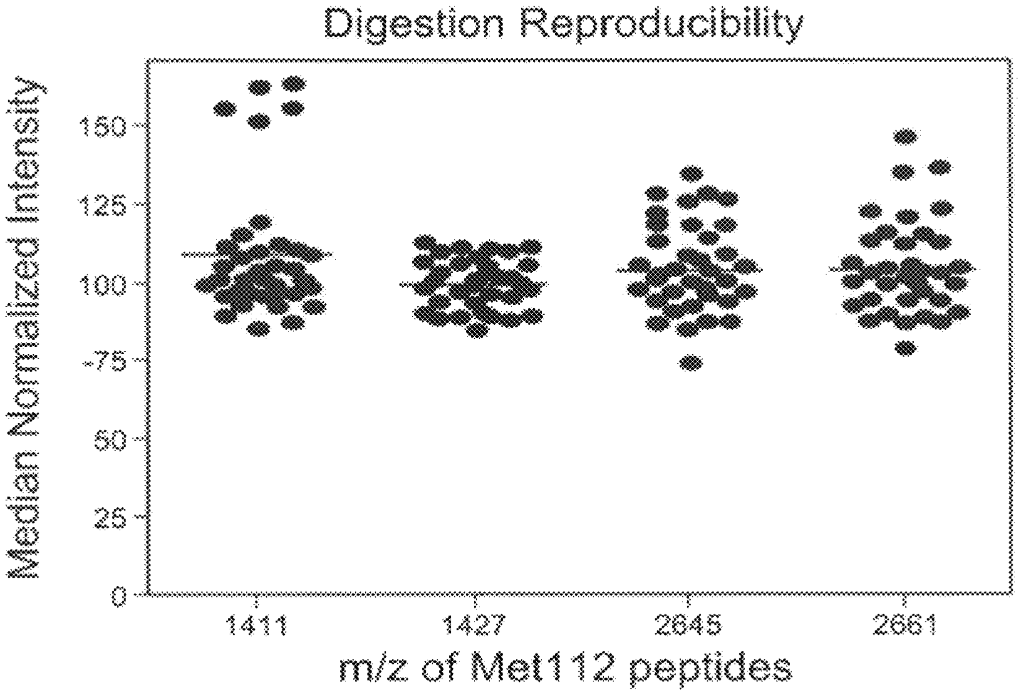
Digestion Reproducibility
Median Normalized Intensity
150
125
100
75
50
25
0
1411
1427
2645
2661
m/z of Met112 peptides

Fig.9C.

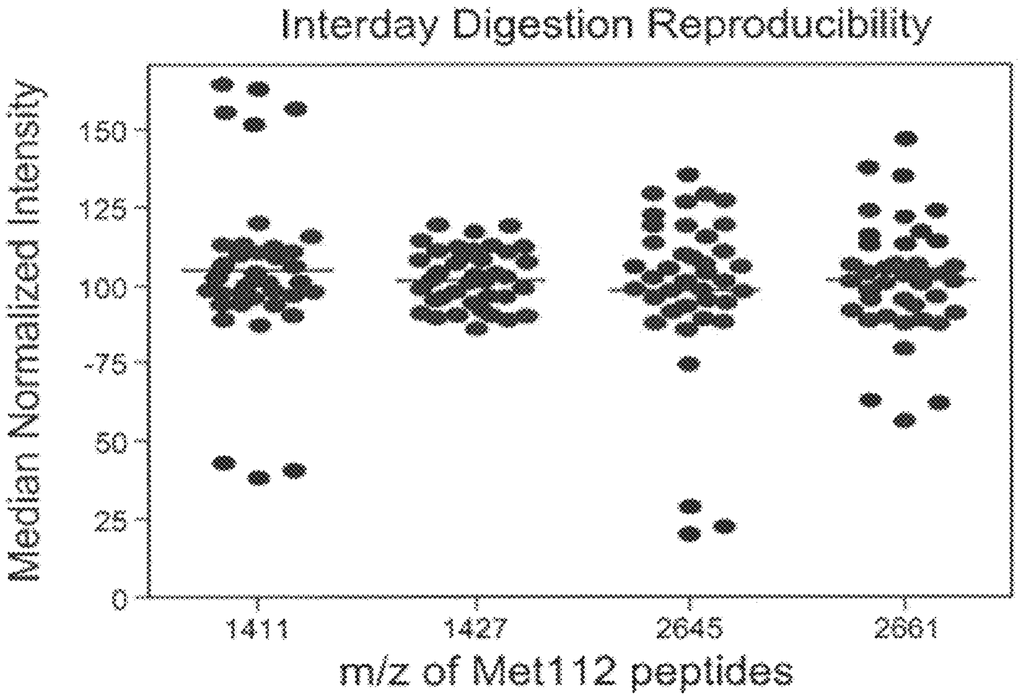
Interday Digestion Reproducibility
Median Normalized Intensity
150
125
100
75
50
25
0
1411
1427
2645
2661
m/z of Met112 peptides

Fig.9D.

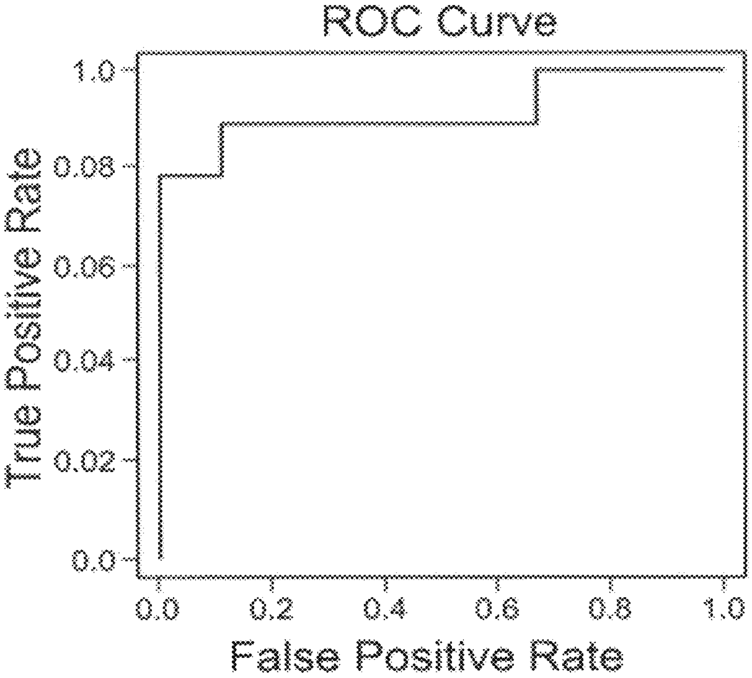

Fig.10A.

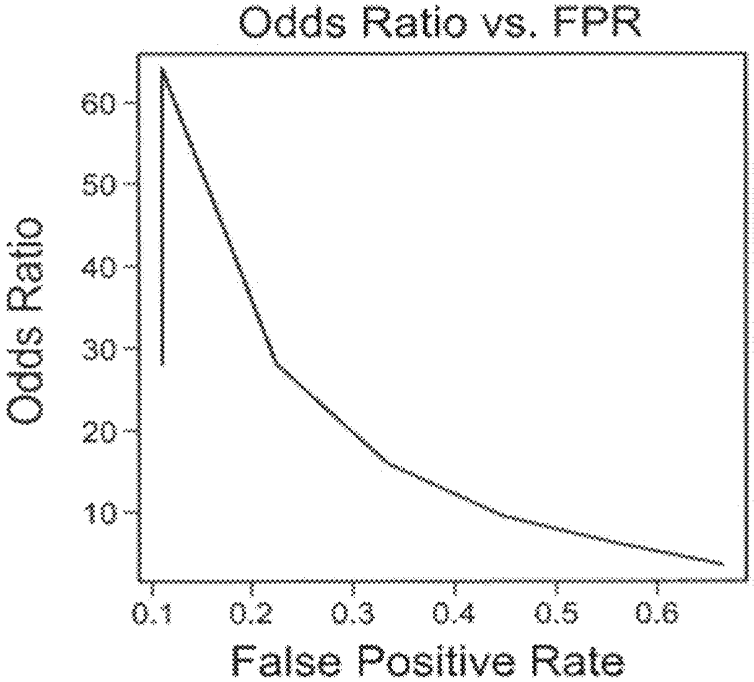

Fig.10B.

METHODS AND COMPOSITIONS FOR DIAGNOSIS OR PROGNOSIS OF CARDIOVASCULAR DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a division of U.S. application Ser. No. 12/499,711, filed idly 8, 2009 now U.S. Pat. No. 8,241,861, which claims the benefit of U.S. Provisional Application No. 61/079,088, filed Jul. 8, 2008, both which are expressly incorporated herein by reference in their entirety.

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.

STATEMENT REGARDING SEQUENCE LISTING

The sequence listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the sequence listing is 39406_Seq_Final 2012-07-05.txt. The text file is 75 KB; was created on Jul. 5, 2012; and is being submitted via EFS-Web with the fling of the specification.

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 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.

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

FIG. **5**C 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. **6**A to FIG. **6**D 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 2.645 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 ink 1411 and m/z 2645 indicate a decreased level of the peptide containing unoxidized Met1.12 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, 6077) 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 HDL_2 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. **9**A 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. **9**B 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. **9**C 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. **9**D 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. **10**A 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. **10**B graphically illustrates the odds ratio of the ProCAD 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. **10**A), 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, sweatiness, 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

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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 HDL_3 subfraction in the density range of about 1.110 to about 1.210 g/mL, and the HDL_2 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 "HDL_2-associated" refers to any biological compounds that float in the density range of HDL_2 (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-idiotype 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 Asztalos, B. F., et al., *Am. J. Cardiol.* 91(7):12E-17E, 2003). HDL is composed of two principal subfractions based on density: HDL_2 and the denser HDL_3.

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 HDL_2 subfraction (density=about 1.06 to about 1.125 g/mL) is isolated from the biological sample prior to analysis. The HDL_2 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 HDL_2 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-4 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-400, apoC-III, and Lp(a) that were increased in HDL_2 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 HDL_2 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-1, 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 HDL_2 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 HDL_2 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 HDL_2 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, μLC-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 MS^2 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.* 15:703-713, 2004; Ghazalpour, A., et al., *Lipid Res.* 45; 1793-1805, 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 HDL_2 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(30:22249-22253, 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):19942-19950, Nov. 25, 1989; Milthorp, 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, et al, "Monoclonal antibodies and the characterization of apolipoprotein structure and function," *Prog. Lipid Res.* 23(4):169-195, 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):3644-3653, 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, eptifibatide 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 HDL_2 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 Committees 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:

HDL_2 (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:

HDL_2 was digested for 60 minutes with trypsin (1:50 w/w trypsin/HDL, sequencing grade trypsin, Promega Wis.) 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 HDL_2 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/archive/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 80×10^4 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. **9**A-D, precision analysis of individual mass channels showed excellent reproducibility of the spectra from the same spot (FIG. **9**A), multiple spots (FIG. **9**B), parallel digestions (FIG. **9**C), and interday digestion (FIG. **9**D). 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 HDL_2 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 HDL_2 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 Bioelectron.* 18(10):1209-1218, 2003). Importantly, this unsupervised data reduction method creates pattern recognition models without a priori assumptions regarding relationships 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 HDL_2 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 HDL_2 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 HDL_2 which differs substantially as compared to the protein composition of HDL_2 isolated from control subjects. These results further demonstrate that HDL_2 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 HDL_2.

Example 2

This example demonstrates that subjects may be successfully classified into CAD or normal control subjects by analyzing tryptic digests of HDL_2-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.

HDL_2 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 $\frac{1}{10,000}$ 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., *J. Chemometr.* 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 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:

Nonparametric 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 HDL_2 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. **10**A, 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 HDL_2, 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 HDL_2 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 HDL_2 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 HDL_2 in subjects with CAD as well as the protein composition of HDL_2 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-TOE-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 HDL_3 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 peptides 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 HDL_2 subfractions isolated from normal control and CAD subjects by tryptic peptide analyses of HDL_2 fractions by tandem mass spectrometric (tandem MS) following PLS-DA based pattern recognition profiling.

Methods:

Sample Isolation and Preparation:

HDL_2 fractions were isolated from normal control and CAD subjects as described earlier in Example 1. Tryptic digests of HDL_2 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 HDL_2 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.

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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 (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) containing 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 HDL_2 apolipoproteins was altered in CAD subjects compared to the controls.

One group of informative features arose from proteins in the HDL_2 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	SVGFHLPSR	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

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD

m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
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	LTISEQNIQR	apoB-100	335-344	24
1202.645	-2.364	VKSPELQAEAK-III	apoA-II	52-62	23
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	WQEEMELYR	apoA-I	132-140	26
1284.6444	13.139	WQEEMELYR-I	apoA-I	132-140	26
1285.6211	4.945	WQEEMELYR-II	apoA-I	132-140	26
1286.5985	-1.058	WQEEMELYR-III	apoA-I	132-140	26
1299.5808	4.112	WQEEMoxELYR	apoA-I	132-140	26

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD

m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
1300.5688	4.532	WQEEMoxELYR-I	apoA-I	132-140	26
1301.6617	-16.664	THLAPYSDELR	apoA-I	185-195	27
1302.6514	-82.138	THLAPYSDELR-I	apoA-I	185-195	27
1302.6514	-82.138	LSPLGEEMRDR	apoA-I	165-175	28
1303.6417	-50.00	THLAPYSDELR-II	apoA-I	185-195	27
1303.6417	-50.00	LSPLGEEMRDR-I	apoA-I	165-175	28
1304.685	-25.542	KGNVATEISTER	apoB-100	196-207	29
1305.6769	-7.513	KGNVATEISTER-I	apoB-100	196-207	29
1306.6696	-0.949	KGNVATEISTER-II	apoB-100	196-207	29
1318.6407	2.632	LSPLGEEMoxRDR	apoA-I	165-175	28
1319.6432	1.355	LSPLGEEMoxRDR-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	FQFPGKPGIYTR	apoB-100	4202-4213	32
1411.7077	-12.606	KWQEEMELYR	apoA-I	131-140	33
1412.7244	-1.039	KWQEEMELYR-I	apoA-I	131-140	33
1412.7244	-1.039	DPDRFRPDGLPK	SAA4	117-128	34
1413.6854	-1.114	KWQEEMELYR-II	apoA-I	131-14-	33
1413.6854	-1.114	DPDRFRPDGLPK-I	SAA4	117-128	34
1414.7036	0.290	KWQEEMELYR-III	apoA-I	131-140	33
1414.7036	0.290	DPDRFRPDGLPK-II	SAA4	117-128	34
1415.7225	0.7011	KWQEEMELYR-IV	apoA-I	131-140	33
1415.7225	0.7011	DPDRFRPDGLPK-III	SAA4	117-128	34
1427.6644	11.458	KWQEEMoxELYR	apoA-I	131-140	33
1428.6927	9.069	KWQEEMoxELYR-I	apoA-I	131-140	33
1429.6645	5.938	KWQEEMoxELYR-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

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD

m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
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	THLAPYSDELRQR	apoA-I	185-197	38
1586.8608	0.9348	THLAPYSDELRQR-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	NLQNNAEWVYQGAIR	apoB-100	4107-4121	43
1776.9092	12.946	NLQNNAEWVYQGAIR-I	apoB-100	4107-4121	43
1777.9046	11.265	NLQNNAEWVYQGAIR-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
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	VQPYLDDEQKKWQEEMELYR-II	apoA-I	121-140	47
2648.3783	-5.448	VQPYLDDFQKKWQEEMELYR-III	apoA-I	121-140	47

TABLE 3-continued

PEPTIDES IDENTIFIED AS INFORMATIVE FOR CAD

m/z	Magnitude in Regression Vector	Peptide	Protein	start-stop	SEQ ID NO
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-III	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 to identified peptides	Protein Residues	Peptide Sequence	SEQ ID NO
1081.6043	+22.482	apo-B100	559-56	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)	117-192	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 (Met112ox)	121-140	VQPYLDDFQKKWQEEM(Ox)ELYR-I	47

TABLE 4-continued

INFORMATIVE FEATURES REPRESENTING INCREASED 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
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 HDL_2 of CAD subjects, as compared to normal subjects, FIG. **5**A graphically illustrates the strong positive informative feature in the PLS-DA regression vector at m/z 1440. As shown in FIG. **5**C, the positive informative feature at m/z 1440 was identified by LC-MALDI-TOF/TOF MS/MS as corresponding to the peptide NPDAVAAPYCYTR (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. **5**B, 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 HDL_2 of CAD subjects, as compared to normal subjects.

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	THLAPYSDELR	27
1302.6514	-82.138	apoA-I	185-195	THLAPYSDELR-I	27
1303.6417	-50.00	apoA-I	185-195	THLAPYSDELR-II	27
1302.6514	-82.138	apoA-I	165-175	LSPLGEEMRDR	28
1303.6417	-50.00	apoA-I	165-175	LSPLGEEMRDR-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	MoxREWFSEFQK	36
1505.7314	-12.066	apoC-I	64-74	MoxREWFSETFQK-I	36

TABLE 5-continued

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:
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	VQPYLDDFQKKWQEEMELYR	47
2646.3664	-12.328	apoA-I	121-140	VQPYLDDFQKKWQEEMELYR-I	47
2647.4251	-9.89	apoA-I	121-140	VQPYLDDFQKKWQREMELYR-II	47
2648.3783	-5.448	apoA-I	121-140	VQPYLDDFQKKWQEEMELYR-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 HDL_2 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).

Taken together, these results demonstrate that pattern recognition profiling performed on HDL_2 isolated from CAD and control subjects show altered patterns of apoproteins present in the HDL_2 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 HDL_2 in particular, has not been previously shown. Thus, these results demonstrate that co-isolation of Lp(a) with HDL_2 subfraction permits pattern recognition analysis of the 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 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. I., 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.

Example 4

This example demonstrates that subjects may be successfully classified as CAD or control subjects based on the oxidation status of their HDL_2 using PLS-DA based pattern recognition proteomic profiling.

Methods:

Sample Preparation and Analysis:

HDL_2 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 HDL_2 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 HDL_2.

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 ED NO:1), the major protein in HDL. MS/MS analysis confirmed the presence of these peptide sequences in the HDL_2 fraction and demonstrated that the methionine 112 residue had been converted to methionine sulfoxide (Met(O)).

As shown in FIGS. **6**A-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. **6**A 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. **6**C (MASCOT ion score of 84.8, CI=100%).

FIG. **6**B 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. **6**D (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 (MPG) 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 H_2O_2 is also capable of oxidizing methionines of apoA-I associated with HDL_3 (International Publication No. WO2006/014628). These HDL_3 subfractions are selectively enriched with oxidized amino acids.

Collectively, these observations support the conclusion that HDL_2 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 HDL_2 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.* 105(34):12224-12229, Aug. 26, 2008), oxidized apoA-I likely acts as a mediator of CAD, and serves as a useful biomarker for CAD. Thus, a subject may be evaluated fir 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 HDL_2 subfractions is altered in CAD subjects as compared to the conformation structure of apoA-I in HDL_2 subfractions of normal control subjects.

Rationale:

The structural conformation of apoA-I has been suggested to influence its ability to transfer cholesterol ester from HDL_2 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. 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:

HDL_2 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 HDL_2 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 HDL_2. 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 HDL_2 subfraction of CAD subjects in comparison to the structural conformation of apoA-I present in the HDL_2 subjection 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 (Gangani, R. A., et al., *Proc. Natl. Acad. Sci.* 105(34):12176-12181, Aug. 26, 2008), 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 HDL_2 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 HDL_2 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 HDL_2 subfractions of CAD subjects compared to control subjects. Thus, dysfunctionality of HDL_2 of CAD subjects likely results from changes in the proteome profile and conformation of the associated HDL_2 proteins. These results demonstrate that pattern recognition profiling using tryptic peptides of HDL_2 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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Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp Val Leu Lys Asp
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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

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            20                  25                  30

Leu Val Ser Gln Tyr Phe Gln Thr Val Thr Asp Tyr Gly Lys Asp Leu
        35                  40                  45

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
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<210> SEQ ID NO 3
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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
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Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys Leu Thr
                325                 330                 335

Ile Ser Glu Gln Asn Ile Gln Arg Ala Asn Leu Phe Asn Lys Leu Val
            340                 345                 350

Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu Leu Pro
        355                 360                 365

Gln Leu Ile Glu Val Ser Ser Pro Ile Thr Leu Gln Ala Leu Val Gln
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Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn Met Gly
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Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala Ala Ile
        515                 520                 525

Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu Val Leu
    530                 535                 540

Leu Gln Thr Phe Leu Asp Asp Ala Ser Pro Gly Asp Lys Arg Leu Ala
545                 550                 555                 560

Ala Tyr Leu Met Leu Met Arg Ser Pro Ser Gln Ala Asp Ile Asn Lys
                565                 570                 575

Ile Val Gln Ile Leu Pro Trp Glu Gln Asn Glu Gln Val Lys Asn Phe
            580                 585                 590

Val Ala Ser His Ile Ala Asn Ile Leu Asn Ser Glu Glu Leu Asp Ile
        595                 600                 605

Gln Asp Leu Lys Lys Leu Val Lys Glu Ala Leu Lys Glu Ser Gln Leu
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Pro Thr Val Met Asp Phe Arg Lys Phe Ser Arg Asn Tyr Gln Leu Tyr
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785                 790                 795                 800

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Ala His Val Ala Leu Lys Ala Gly Lys Leu Lys Phe Ile Ile Pro Ser
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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

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    1115                 1120                 1125

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    1130                 1135                 1140

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    1145                 1150                 1155

Ser Thr  Val Ser Lys Arg Val  Ala Trp His Tyr Asp  Glu Glu Lys
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Ile Glu  Phe Glu Trp Asn Thr  Gly Thr Asn Val Asp  Thr Lys Lys
    1175                 1180                 1185

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
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    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

Glu Asn  Tyr Glu Leu Thr Leu  Lys Ser Asp Thr Asn  Gly Lys Tyr
    1565                 1570                 1575

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
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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
    1745                 1750                 1755

Ile Ala  Gly Leu Ser Leu Asp  Phe Ser Ser Lys Leu  Asp Asn Ile
    1760                 1765                 1770

Tyr Ser  Ser Asp Lys Phe Tyr  Lys Gln Thr Val Asn  Leu Gln Leu
    1775                 1780                 1785

Gln Pro  Tyr Ser Leu Val Thr  Thr Leu Asn Ser Asp  Leu Lys Tyr
    1790                 1795                 1800

Asn Ala  Leu Asp Leu Thr Asn  Asn Gly Lys Leu Arg  Leu Glu Pro
    1805                 1810                 1815

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
    1910                 1915                 1920

Thr Gly  Gln Leu Tyr Ser Lys  Phe Leu Leu Lys Ala  Glu Pro Leu
    1925                 1930                 1935

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

Ser Ala  Leu Leu Thr Pro Ala  Glu Gln Thr Gly Thr  Trp Lys Leu
    1970                 1975                 1980

Lys Thr  Gln Phe Asn Asn Asn  Glu Tyr Ser Gln Asp  Leu Asp Ala
    1985                 1990                 1995

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

Leu Leu  Ser Glu Pro Ile Asn  Ile Ile Asp Ala Leu  Glu Met Arg
    2030                 2035                 2040
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Asp Ala  Val Glu Lys Pro Gln  Glu Phe Thr Ile Val  Ala Phe Val
    2045                 2050                 2055

Lys Tyr  Asp Lys Asn Gln Asp  Val His Ser Ile Asn  Leu Pro Phe
    2060                 2065                 2070

Phe Glu  Thr Leu Gln Glu Tyr  Phe Glu Arg Asn Arg  Gln Thr Ile
    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
    2105                 2110                 2115

Pro Gln  Gln Ala Asn Asp Tyr  Leu Asn Ser Phe Asn  Trp Glu Arg
    2120                 2125                 2130

Gln Val  Ser His Ala Lys Glu  Lys Leu Thr Ala Leu  Thr Lys Lys
    2135                 2140                 2145

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
    2180                 2185                 2190

Leu Lys  Ile Ala Ile Ala Asn  Ile Ile Asp Glu Ile  Ile Glu Lys
    2195                 2200                 2205

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
    2225                 2230                 2235

Asn Lys  Ser Gly Ser Ser Thr  Ala Ser Trp Ile Gln  Asn Val Asp
    2240                 2245                 2250

Thr Lys  Tyr Gln Ile Arg Ile  Gln Ile Gln Glu Lys  Leu Gln Gln
    2255                 2260                 2265

Leu Lys  Arg His Ile Gln Asn  Ile Asp Ile Gln His  Leu Ala Gly
    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
    2360                 2365                 2370

Ile Gln  Lys Leu Ser Asn Val  Leu Gln Gln Val Lys  Ile Lys Asp
    2375                 2380                 2385

Tyr Phe  Glu Lys Leu Val Gly  Phe Ile Asp Asp Ala  Val Lys Lys
    2390                 2395                 2400

Leu Asn  Glu Leu Ser Phe Lys  Thr Phe Ile Glu Asp  Val Asn Lys
    2405                 2410                 2415

Phe Leu  Asp Met Leu Ile Lys  Lys Leu Lys Ser Phe  Asp Tyr His
    2420                 2425                 2430

Gln Phe  Val Asp Glu Thr Asn  Asp Lys Ile Arg Glu  Val Thr Gln
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    2435                 2440                 2445

Arg Leu  Asn Gly Glu Ile Gln  Ala Leu Glu Leu Pro  Gln Lys Ala
    2450                 2455                 2460

Glu Ala  Leu Lys Leu Phe Leu  Glu Glu Thr Lys Ala  Thr Val Ala
    2465                 2470                 2475

Val Tyr  Leu Glu Ser Leu Gln  Asp Thr Lys Ile Thr  Leu Ile Ile
    2480                 2485                 2490

Asn Trp  Leu Gln Glu Ala Leu  Ser Ser Ala Ser Leu  Ala His Met
    2495                 2500                 2505

Lys Ala  Lys Phe Arg Glu Thr  Leu Glu Asp Thr Arg  Asp Arg Met
    2510                 2515                 2520

Tyr Gln  Met Asp Ile Gln Gln  Glu Leu Gln Arg Tyr  Leu Ser Leu
    2525                 2530                 2535

Val Gly  Gln Val Tyr Ser Thr  Leu Val Thr Tyr Ile  Ser Asp Trp
    2540                 2545                 2550

Trp Thr  Leu Ala Ala Lys Asn  Leu Thr Asp Phe Ala  Glu Gln Tyr
    2555                 2560                 2565

Ser Ile  Gln Asp Trp Ala Lys  Arg Met Lys Ala Leu  Val Glu Gln
    2570                 2575                 2580

Gly Phe  Thr Val Pro Glu Ile  Lys Thr Ile Leu Gly  Thr Met Pro
    2585                 2590                 2595

Ala Phe  Glu Val Ser Leu Gln  Ala Leu Gln Lys Ala  Thr Phe Gln
    2600                 2605                 2610

Thr Pro  Asp Phe Ile Val Pro  Leu Thr Asp Leu Arg  Ile Pro Ser
    2615                 2620                 2625

Val Gln  Ile Asn Phe Lys Asp  Leu Lys Asn Ile Lys  Ile Pro Ser
    2630                 2635                 2640

Arg Phe  Ser Thr Pro Glu Phe  Thr Ile Leu Asn Thr  Phe His Ile
    2645                 2650                 2655

Pro Ser  Phe Thr Ile Asp Phe  Val Glu Met Lys Val  Lys Ile Ile
    2660                 2665                 2670

Arg Thr  Ile Asp Gln Met Leu  Asn Ser Glu Leu Gln  Trp Pro Val
    2675                 2680                 2685

Pro Asp  Ile Tyr Leu Arg Asp  Leu Lys Val Glu Asp  Ile Pro Leu
    2690                 2695                 2700

Ala Arg  Ile Thr Leu Pro Asp  Phe Arg Leu Pro Glu  Ile Ala Ile
    2705                 2710                 2715

Pro Glu  Phe Ile Ile Pro Thr  Leu Asn Leu Asn Asp  Phe Gln Val
    2720                 2725                 2730

Pro Asp  Leu His Ile Pro Glu  Phe Gln Leu Pro His  Ile Ser His
    2735                 2740                 2745

Thr Ile  Glu Val Pro Thr Phe  Gly Lys Leu Tyr Ser  Ile Leu Lys
    2750                 2755                 2760

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
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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

Phe Asp  Leu Ser Val Lys Ala  Gln Tyr Lys Lys Asn  Lys His Arg
    3170                3175                3180

His Ser  Ile Thr Asn Pro Leu  Ala Val Leu Cys Glu  Phe Ile Ser
    3185                3190                3195

Gln Ser  Ile Lys Ser Phe Asp  Arg His Phe Glu Lys  Asn Arg Asn
    3200                3205                3210

Asn Ala  Leu Asp Phe Val Thr  Lys Ser Tyr Asn Glu  Thr Lys Ile
    3215                3220                3225

Lys Phe  Asp Lys Tyr Lys Ala  Glu Lys Ser His Asp  Glu Leu Pro
    3230                3235                3240
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Arg Thr  Phe Gln Ile Pro Gly  Tyr Thr Val Pro Val  Val Asn Val
    3245                 3250                 3255

Glu Val  Ser Pro Phe Thr Ile  Glu Met Ser Ala Phe  Gly Tyr Val
    3260                 3265                 3270

Phe Pro  Lys Ala Val Ser Met  Pro Ser Phe Ser Ile  Leu Gly Ser
    3275                 3280                 3285

Asp Val  Arg Val Pro Ser Tyr  Thr Leu Ile Leu Pro  Ser Leu Glu
    3290                 3295                 3300

Leu Pro  Val Leu His Val Pro  Arg Asn Leu Lys Leu  Ser Leu Pro
    3305                 3310                 3315

Asp Phe  Lys Glu Leu Cys Thr  Ile Ser His Ile Phe  Ile Pro Ala
    3320                 3325                 3330

Met Gly  Asn Ile Thr Tyr Asp  Phe Ser Phe Lys Ser  Ser Val Ile
    3335                 3340                 3345

Thr Leu  Asn Thr Asn Ala Glu  Leu Phe Asn Gln Ser  Asp Ile Val
    3350                 3355                 3360

Ala His  Leu Leu Ser Ser Ser  Ser Ser Val Ile Asp  Ala Leu Gln
    3365                 3370                 3375

Tyr Lys  Leu Glu Gly Thr Thr  Arg Leu Thr Arg Lys  Arg Gly Leu
    3380                 3385                 3390

Lys Leu  Ala Thr Ala Leu Ser  Leu Ser Asn Lys Phe  Val Glu Gly
    3395                 3400                 3405

Ser His  Asn Ser Thr Val Ser  Leu Thr Thr Lys Asn  Met Glu Val
    3410                 3415                 3420

Ser Val  Ala Thr Thr Thr Lys  Ala Gln Ile Pro Ile  Leu Arg Met
    3425                 3430                 3435

Asn Phe  Lys Gln Glu Leu Asn  Gly Asn Thr Lys Ser  Lys Pro Thr
    3440                 3445                 3450

Val Ser  Ser Ser Met Glu Phe  Lys Tyr Asp Phe Asn  Ser Ser Met
    3455                 3460                 3465

Leu Tyr  Ser Thr Ala Lys Gly  Ala Val Asp His Lys  Leu Ser Leu
    3470                 3475                 3480

Glu Ser  Leu Thr Ser Tyr Phe  Ser Ile Glu Ser Ser  Thr Lys Gly
    3485                 3490                 3495

Asp Val  Lys Gly Ser Val Leu  Ser Arg Glu Tyr Ser  Gly Thr Ile
    3500                 3505                 3510

Ala Ser  Glu Ala Asn Thr Tyr  Leu Asn Ser Lys Ser  Thr Arg Ser
    3515                 3520                 3525

Ser Val  Lys Leu Gln Gly Thr  Ser Lys Ile Asp Asp  Ile Trp Asn
    3530                 3535                 3540

Leu Glu  Val Lys Glu Asn Phe  Ala Gly Glu Ala Thr  Leu Gln Arg
    3545                 3550                 3555

Ile Tyr  Ser Leu Trp Glu His  Ser Thr Lys Asn His  Leu Gln Leu
    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
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    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

Tyr Glu  Gly Leu Gln Glu Trp  Glu Gly Lys Ala His  Leu Asn Ile
    3965                3970                3975

Lys Ser  Pro Ala Phe Thr Asp  Leu His Leu Arg Tyr  Gln Lys Asp
    3980                3985                3990

Lys Lys  Gly Ile Ser Thr Ser  Ala Ala Ser Pro Ala  Val Gly Thr
    3995                4000                4005

Val Gly  Met Asp Met Asp Glu  Asp Asp Asp Phe Ser  Lys Trp Asn
    4010                4015                4020

Phe Tyr  Tyr Ser Pro Gln Ser  Ser Pro Asp Lys Lys  Leu Thr Ile
    4025                4030                4035
```

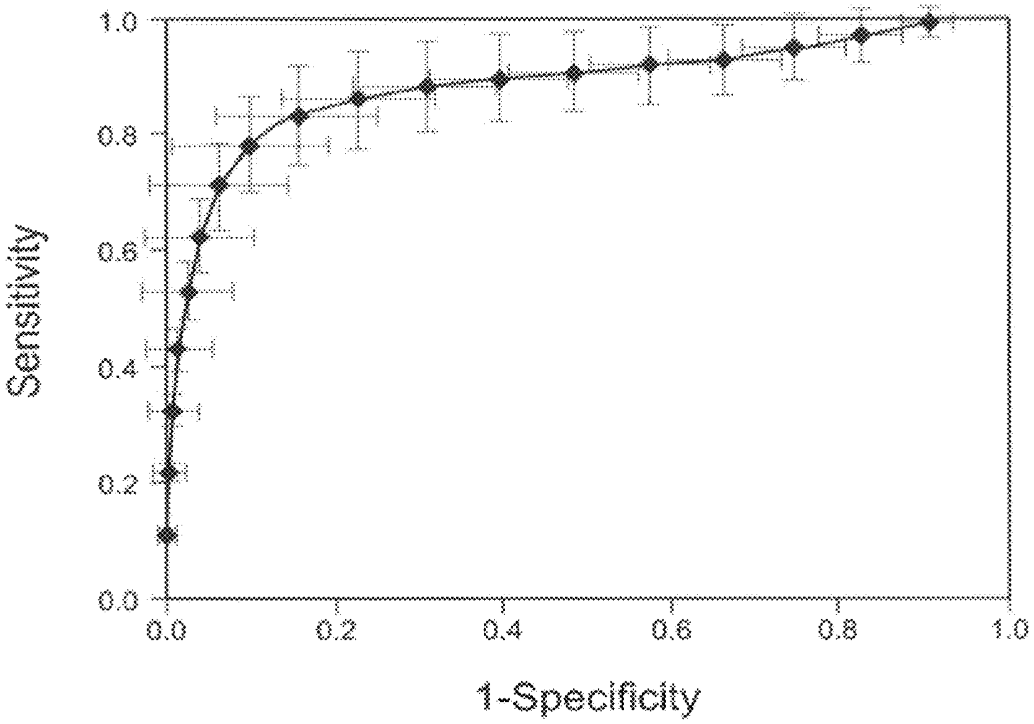

Fig.1.

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Phe Lys  Thr Glu Leu Arg Val  Arg Glu Ser Asp Glu  Glu Thr Gln
    4040                 4045                 4050

Ile Lys  Val Asn Trp Glu Glu  Glu Ala Ala Ser Gly  Leu Leu Thr
    4055                 4060                 4065

Ser Leu  Lys Asp Asn Val Pro  Lys Ala Thr Gly Val  Leu Tyr Asp
    4070                 4075                 4080

Tyr Val  Asn Lys Tyr His Trp  Glu His Thr Gly Leu  Thr Leu Arg
    4085                 4090                 4095

Glu Val  Ser Ser Lys Leu Arg  Arg Asn Leu Gln Asn  Asn Ala Glu
    4100                 4105                 4110

Trp Val  Tyr Gln Gly Ala Ile  Arg Gln Ile Asp Asp  Ile Asp Val
    4115                 4120                 4125

Arg Phe  Gln Lys Ala Ala Ser  Gly Thr Thr Gly Thr  Tyr Gln Glu
    4130                 4135                 4140

Trp Lys  Asp Lys Ala Gln Asn  Leu Tyr Gln Glu Leu  Leu Thr Gln
    4145                 4150                 4155

Glu Gly  Gln Ala Ser Phe Gln  Gly Leu Lys Asp Asn  Val Phe Asp
    4160                 4165                 4170

Gly Leu  Val Arg Val Thr Gln  Glu Phe His Met Lys  Val Lys His
    4175                 4180                 4185

Leu Ile  Asp Ser Leu Ile Asp  Phe Leu Asn Phe Pro  Arg Phe Gln
    4190                 4195                 4200

Phe Pro  Gly Lys Pro Gly Ile  Tyr Thr Arg Glu Glu  Leu Cys Thr
    4205                 4210                 4215

Met Phe  Ile Arg Glu Val Gly  Thr Val Leu Ser Gln  Val Tyr Ser
    4220                 4225                 4230

Lys Val  His Asn Gly Ser Glu  Ile Leu Phe Ser Tyr  Phe Gln Asp
    4235                 4240                 4245

Leu Val  Ile Thr Leu Pro Phe  Glu Leu Arg Lys His  Lys Leu Ile
    4250                 4255                 4260

Asp Val  Ile Ser Met Tyr Arg  Glu Leu Leu Lys Asp  Leu Ser Lys
    4265                 4270                 4275

Glu Ala  Gln Glu Val Phe Lys  Ala Ile Gln Ser Leu  Lys Thr Thr
    4280                 4285                 4290

Glu Val  Leu Arg Asn Leu Gln  Asp Leu Leu Gln Phe  Ile Phe Gln
    4295                 4300                 4305

Leu Ile  Glu Asp Asn Ile Lys  Gln Leu Lys Glu Met  Lys Phe Thr
    4310                 4315                 4320

Tyr Leu  Ile Asn Tyr Ile Gln  Asp Glu Ile Asn Thr  Ile Phe Ser
    4325                 4330                 4335

Asp Tyr  Ile Pro Tyr Val Phe  Lys Leu Leu Lys Glu  Asn Leu Cys
    4340                 4345                 4350

Leu Asn  Leu His Lys Phe Asn  Glu Phe Ile Gln Asn  Glu Leu Gln
    4355                 4360                 4365

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
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```
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 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

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
```

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```
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
        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
```

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```
        675                 680                 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 Tyr 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

Ala Phe  Val Pro Pro Asn Val  Ile Leu Ala Pro Ser  Leu Glu Ala
    1025                 1030                 1035

Phe Phe  Glu Gln Ala Leu Thr  Glu Glu Thr Pro Gly  Val Gln Asp
    1040                 1045                 1050

Cys Tyr  Tyr His Tyr Gly Gln  Ser Tyr Arg Gly Thr  Tyr Ser Thr
    1055                 1060                 1065

Thr Val  Thr Gly Arg Thr Cys  Gln Ala Trp Ser Ser  Met Thr Pro
    1070                 1075                 1080

His Gln  His Ser Arg Thr Pro  Glu Asn Tyr Pro Asn  Ala Gly Leu
    1085                 1090                 1095
```

```
Thr Arg  Asn Tyr Cys Arg Asn  Pro Asp Ala Glu Ile  Arg Pro Trp
    1100                 1105                 1110

Cys Tyr  Thr Met Asp Pro Ser  Val Arg Trp Glu Tyr  Cys Asn Leu
    1115                 1120                 1125

Thr Gln  Cys Leu Val Thr Glu  Ser Ser Val Leu Ala  Thr Leu Thr
    1130                 1135                 1140

Val Val  Pro Asp Pro Ser Thr  Glu Ala Ser Ser Glu  Glu Ala Pro
    1145                 1150                 1155

Thr Glu  Gln Ser Pro Gly Val  Gln Asp Cys Tyr His  Gly Asp Gly
    1160                 1165                 1170

Gln Ser  Tyr Arg Gly Ser Phe  Ser Thr Thr Val Thr  Gly Arg Thr
    1175                 1180                 1185

Cys Gln  Ser Trp Ser Ser Met  Thr Pro His Trp His  Gln Arg Thr
    1190                 1195                 1200

Thr Glu  Tyr Tyr Pro Asn Gly  Gly Leu Thr Arg Asn  Tyr Cys Arg
    1205                 1210                 1215

Asn Pro  Asp Ala Glu Ile Ser  Pro Trp Cys Tyr Thr  Met Asp Pro
    1220                 1225                 1230

Asn Val  Arg Trp Glu Tyr Cys  Asn Leu Thr Gln Cys  Pro Val Thr
    1235                 1240                 1245

Glu Ser  Ser Val Leu Ala Thr  Ser Thr Ala Val Ser  Glu Gln Ala
    1250                 1255                 1260

Pro Thr  Glu Gln Ser Pro Thr  Val Gln Asp Cys Tyr  His Gly Asp
    1265                 1270                 1275

Gly Gln  Ser Tyr Arg Gly Ser  Phe Ser Thr Thr Val  Thr Gly Arg
    1280                 1285                 1290

Thr Cys  Gln Ser Trp Ser Ser  Met Thr Pro His Trp  His Gln Arg
    1295                 1300                 1305

Thr Thr  Glu Tyr Tyr Pro Asn  Gly Gly Leu Thr Arg  Asn Tyr Cys
    1310                 1315                 1320

Arg Asn  Pro Asp Ala Glu Ile  Arg Pro Trp Cys Tyr  Thr Met Asp
    1325                 1330                 1335

Pro Ser  Val Arg Trp Glu Tyr  Cys Asn Leu Thr Gln  Cys Pro Val
    1340                 1345                 1350

Met Glu  Ser Thr Leu Leu Thr  Thr Pro Thr Val Val  Pro Val Pro
    1355                 1360                 1365

Ser Thr  Glu Leu Pro Ser Glu  Glu Ala Pro Thr Glu  Asn Ser Thr
    1370                 1375                 1380

Gly Val  Gln Asp Cys Tyr Arg  Gly Asp Gly Gln Ser  Tyr Arg Gly
    1385                 1390                 1395

Thr Leu  Ser Thr Thr Ile Thr  Gly Arg Thr Cys Gln  Ser Trp Ser
    1400                 1405                 1410

Ser Met  Thr Pro His Trp His  Arg Arg Ile Pro Leu  Tyr Tyr Pro
    1415                 1420                 1425

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
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```
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
    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
```

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```
    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

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

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
<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
```

-continued

```
<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

<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
```

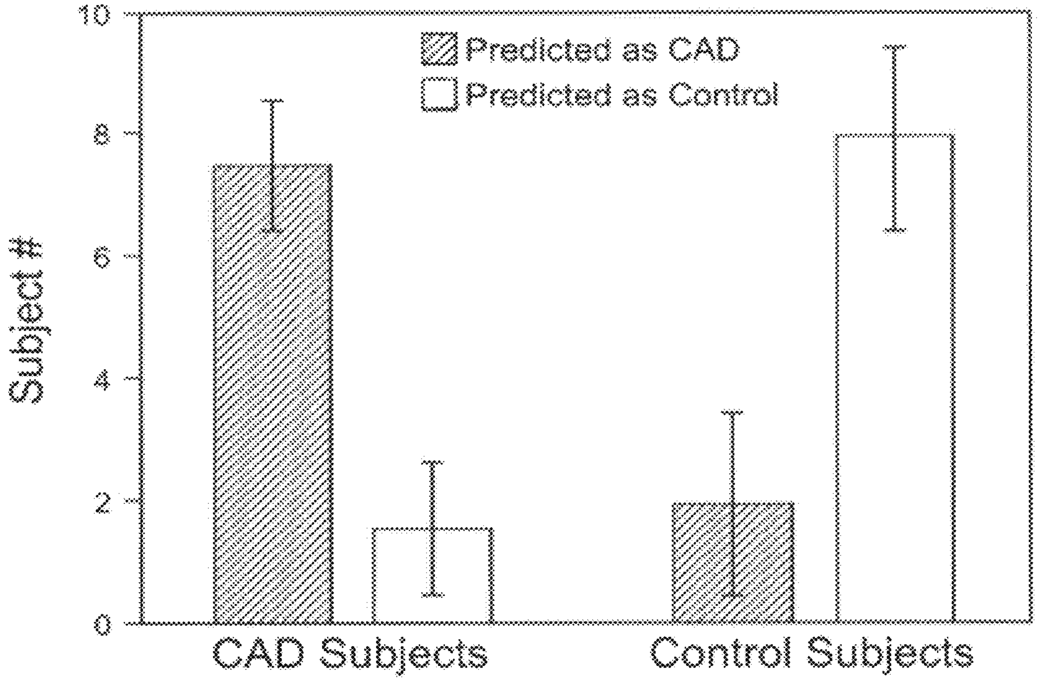
▨ Predicted as CAD
☐ Predicted as Control
Subject #
10
8
6
4
2
0
CAD Subjects
Control Subjects

Fig.2.

```
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)
<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

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


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<400> SEQUENCE: 36

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


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


<210> SEQ ID NO 39
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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
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<400> SEQUENCE: 40

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


<210> SEQ ID NO 41
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<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
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<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
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1               5                   10                  15


<210> SEQ ID NO 44
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Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg
1               5                   10                  15


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Lys


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1               5                   10                  15

Glu Lys


<210> SEQ ID NO 47
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Glu Leu Tyr Arg
            20


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1               5                   10


<210> SEQ ID NO 49
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1               5                   10                  15

Glu Lys


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<400> SEQUENCE: 52

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```

The invention claimed is:

1. A method for determining the efficacy of a treatment regimen for treating and/or preventing cardiovascular disease in a subject, the method comprising 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.

2. The method of claim **1**, wherein the monitoring comprises detecting the measurable feature of the at least two biomarkers in biological samples obtained at a one or more time points during the treatment for cardiovascular disease.

3. The method of claim **2**, further comprising comparing the measurable features of the at least two biomarkers as detected in biological samples obtained at two or more time points during the treatment for cardiovascular disease.

4. The method of claim **3**, wherein a difference in the measurable features of the at least two biomarkers from biological samples obtained from the subject at the two or more time points during treatment is indicative of the efficacy of the treatment regimen for treating and/or preventing cardiovascular disease in the subject.

5. The method of claim **4**, wherein at least one of the measurable features indicative of the efficacy of the treatment regimen for treating and/or preventing 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 amount of the at least one of the biomarkers in the HDL subfraction or in the complex containing apoA-I or apoA-II determined in a biological sample obtained at a later time point.

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

7. The method of claim **5**, wherein the biomarker is apoC-III or a portion or derivative thereof.

8. The method of claim **5**, wherein the biomarker is Lp(a) or a portion or derivative thereof.

9. The method of claim **4**, wherein at least one of the measurable features indicative of the efficacy of the treatment regimen for treating and/or preventing cardiovascular disease comprises a decreased 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 and apoC-I, or portions and/or derivatives thereof, in comparison to the amount of the at least one of the biomarkers in the HDL subfraction or in the complex containing apoA-I or apoA-II determined in a biological sample obtained at a later time point.

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

11. The method of claim **9**, wherein the biomarker is apoC-I, or a portion or derivative thereof.

12. The method of claim **4**, wherein at least one of the measurable features indicative of the efficacy of the treatment regimen for treating and/or preventing cardiovascular disease comprises an increased or decreased presence or amount of 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 presence or amount of the post-translational modification of the at least one of the biomarkers in the HDL subfraction or in the complex determined in a biological sample obtained at a later time point.

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

14. The method of claim **4**, wherein at least one of the measurable features indicative of the efficacy of the treatment regimen for treating and/or preventing cardiovascular disease comprises an altered structural conformation of apoA-I in the HDL subfraction of the biological sample, in comparison to the structural conformation of apoA-I in the HDL subfraction or in the complex determined in a biological sample obtained at a later time point.

15. The method of claim **4**, wherein the measurable features of the at least two biomarkers from the biological samples are determined using mass spectrometry analysis.

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

17. The method of claim **15**, wherein the mass spectrometry analysis is carried out with a matrix-assisted laser desorption ionization (MALDI) mass spectrometer or LCMS.

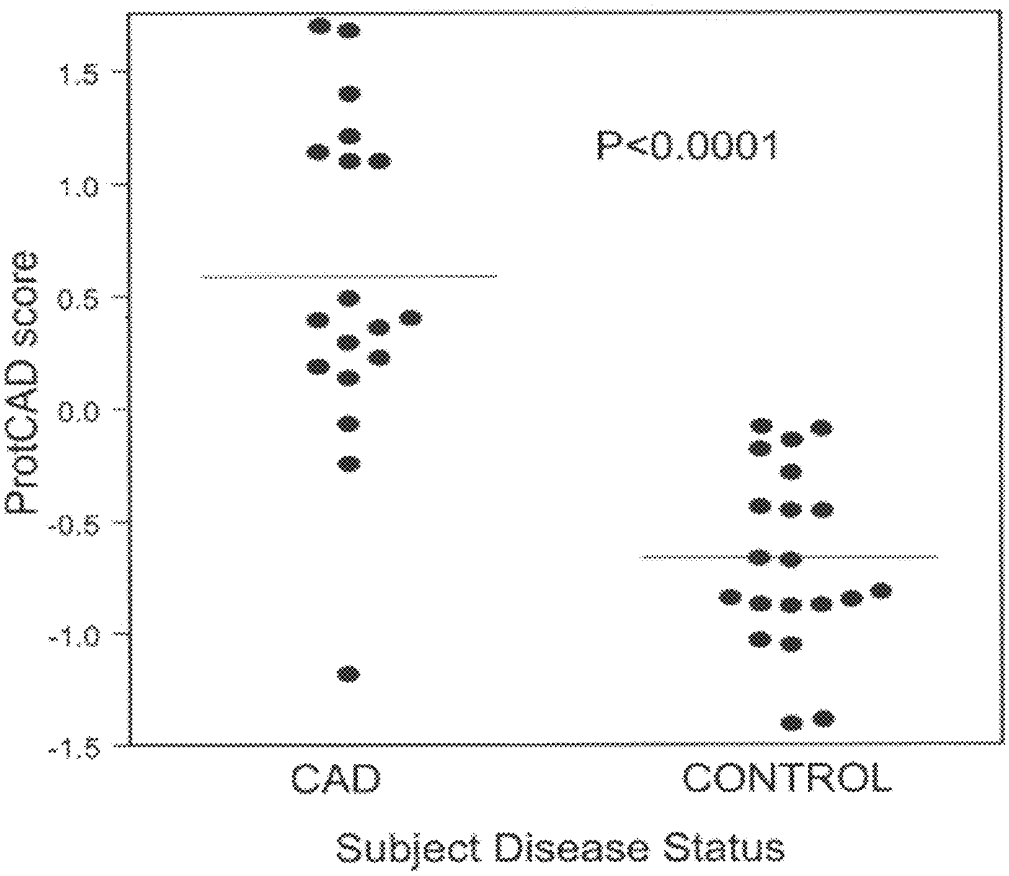
18. The method of claim **1**, wherein the HDL subfraction of the biological sample is the HDL2 subfraction.

19. 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.

20. 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.

21. 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.

* * * * *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CAD
CONTROL
Subject Disease Status

Fig.3.

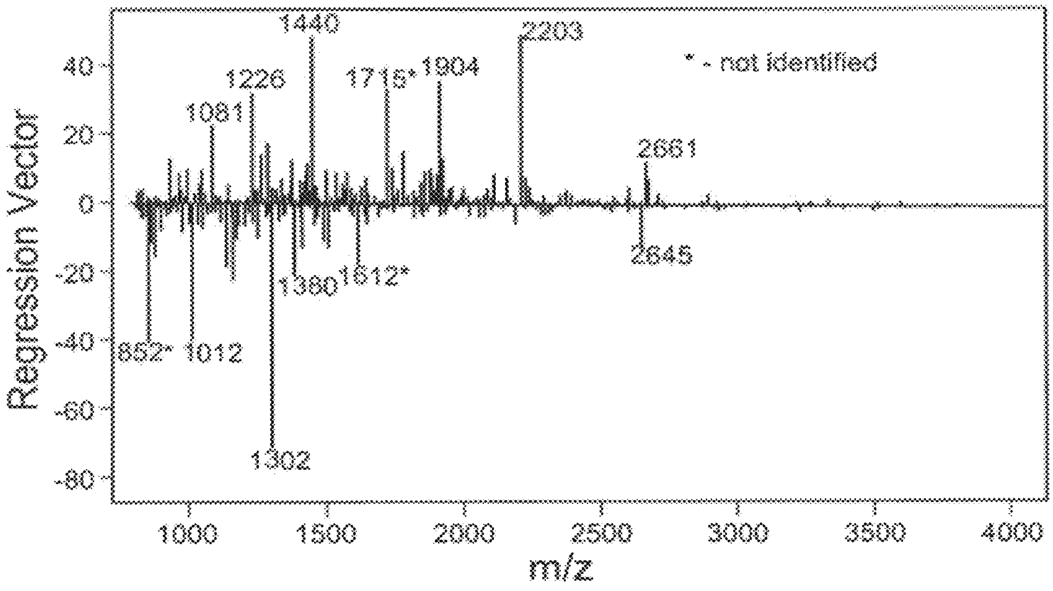
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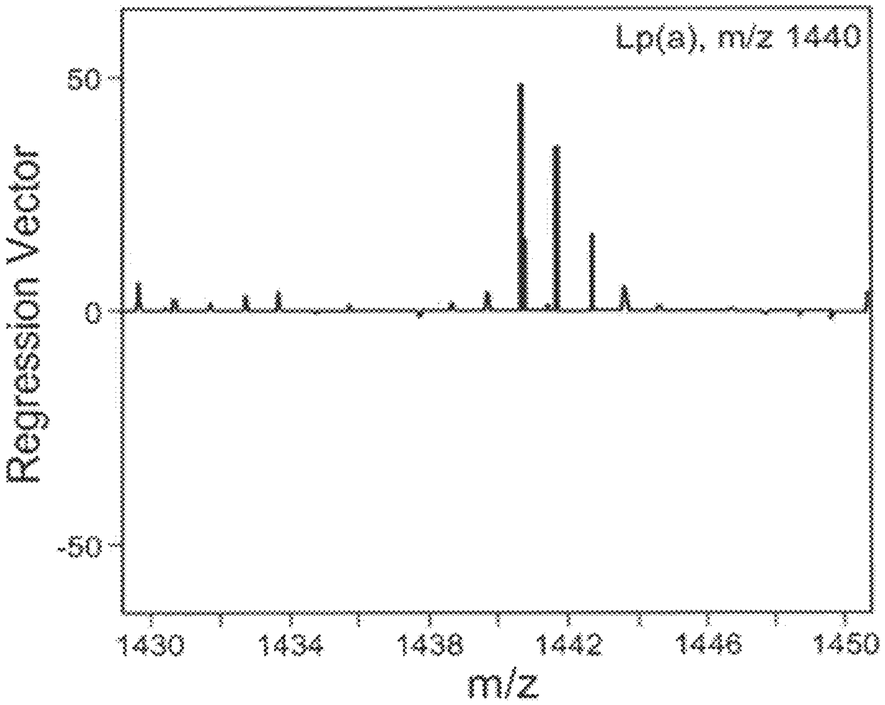
Fig. 4.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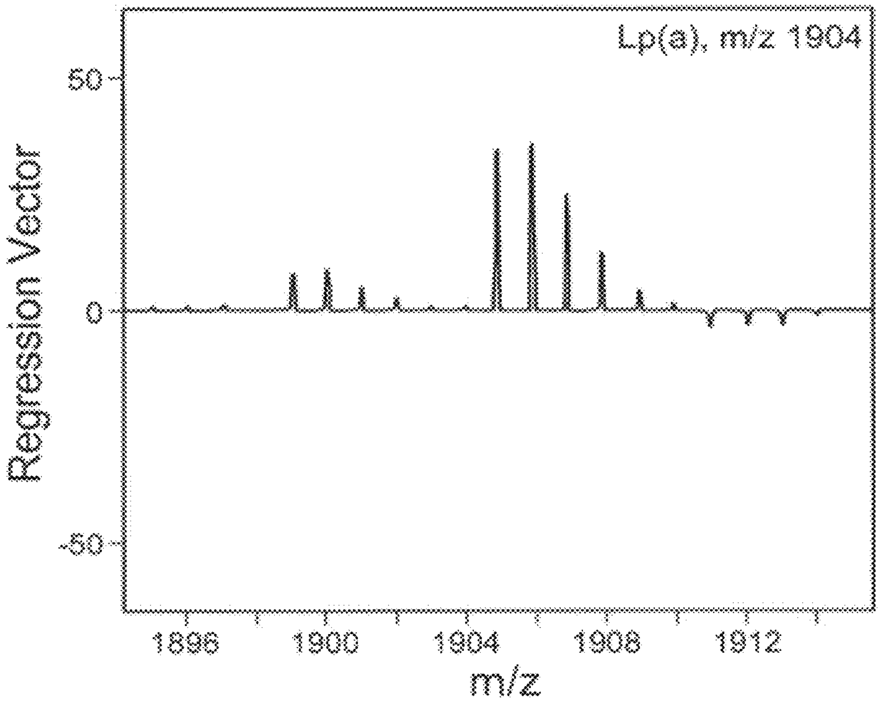
m/z

Fig. 5A.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m/z

Fig. 5B.

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		
公开(公告)号	US8460889	公开(公告)日	2013-06-11
申请号	US13/543745	申请日	2012-07-06
[标]申请(专利权)人(译)	INSILICOS 华盛顿大学		
申请(专利权)人(译)	INSILICOS，LLC 华盛顿大学学报		
当前申请(专利权)人(译)	华盛顿大学 INSILICOS，LLC		
[标]发明人	HEINECKE JAY W VAISAR TOMAS PRAZEN BRYAN NILSSON ERIK		
发明人	HEINECKE, JAY W. VAISAR, TOMAS PRAZEN, BRYAN NILSSON, ERIK		
IPC分类号	G01N33/53 G01N31/00		
CPC分类号	G01N33/92 G01N2333/775 G01N2800/32		
审查员(译)	COOK，LISA		
优先权	61/079088 2008-07-08 US		
其他公开文献	US20120288880A1		
外部链接	Espacenet USPTO		

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-III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。

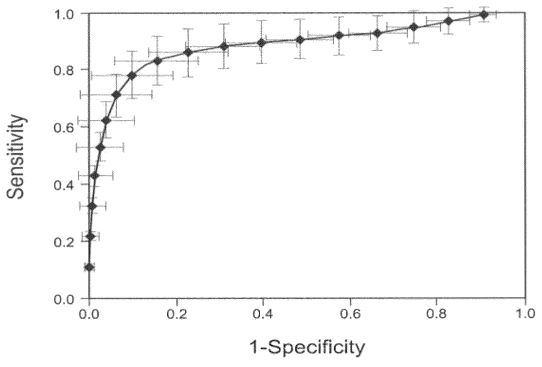

Fig. 1.