APOLIPOPROTEIN FINGERPRINTING TECHNIQUE

BACKGROUND OF THE INVENTION

Be it known that WE, EMELITA DE GUZMAN BREYER, residing at 2840 Livesey Trail, Tucker, GA 30084, a citizen of the United States; and MARY K. ROBINSON, residing at 3649 Cherry Hill Place, Decatur, GA 30034, a citizen of the United States have invented certain new and useful improvements in a APOLIPOPROTEIN FINGERPRINTING TECHNIQUE of which the following is a specification.

CROSS-REFERENCE AND PRIORITY CLAIM

The present Patent Cooperation Treaty Application claims priority to and the benefit of U.S. Provisional patent application entitled "APOLIPOPROTEIN FINGERPRINTING TECHNIQUE filed March 23, 2006, on behalf of inventors Emelita De Guzman Breyer and Mary K. Robinson, having Serial No. 60/743,678.

BACKGROUND

I. Field of the Invention

The present invention relates to the field of compositions, analysis and quantification of apolipoproteins in biological and clinical samples. More particularly, this invention relates to methods, techniques, and protocols for the fingerprinting, profiling, determining, and/or quantifying of apolipoproteins present in samples of human plasma, serum and lipoprotein fractions.

II. Description of the Related Art.

Apolipoproteins, the protein components of lipoproteins, are able to solubilize hydrophobic lipids and facilitate cell targeting and transport. These components, synthesized in

the liver and intestine, are essential for maintaining the integrity of lipoprotein particles, serving as cofactors for enzymes that act on lipoproteins, and facilitating receptor-mediated interactions that remove lipids from circulation.

There are several groups of apolipoproteins: A (Apo A), B (Apo B), C (Apo C) and E (Apo E). Each of the three groups A, B and C consists of two or more distinct proteins. These are Apo Al, Apo All, Apo AIV, and AV for Apo A; Apo B100 and Apo B48 for Apo B; and Apo CI, Apo Cli, Apo CIII, and Apo CIV for Apo C. Apo CI, CIII, CIV and Apo E each consist of two or more isoforms. The apolipoproteins have various roles in disease and in health.

Apolipoprotein CIII is a 79 amino acid protein that exists in humans as three isoforms differing in the glycosylation at Threonine-74. CIII-0 isoform is the final product of sialidase enzyme reactions, has an absence of sialic acid, galactose and galactose amine residues, and accounts for 14% of the total isoforms. CIII-0 isoforms are inhibitors of very low density lipoproteins (VLDL) binding to the lipolysis stimulated receptor, which is an important route of clearance of high triglyceride lipoproteins in plasma. CIII-0 isoform has the lowest affinity to VLDL. CIII-1 isoform has 1 mole of sialic and counts for 51% of total isoforms. CIII-2 isoform is the initial form of CIII synthesized and secreted in the liver, has 2 mole of sialic acid, and accounts for 35% of total isoforms. CIII-2 isoform has a higher affinity to VLDL and is a poorer inhibitor of VLDL binding to the lipolysis stimulated receptor.

Current methods to identify and quantitate CIII isoforms involve isolation of lipoprotein fractions from plasma, delipidation of this fraction, and purification of the CIII from the water-soluble fraction of apolipoproteins. The different purified isoforms can be detected by isoelectric focusing gel electrophoresis, mass spectrometry, and fluorescence and absorbance spectroscopy. A couple of disadvantages of the current methods are that they are tedious in isolation of CIII from plasma, having only a 60-80% protein recovery, and that current methods for clinical diagnostics for CIII in plasma, such as ELISA and immunoturbidimetric assays, only can detect total CIII.

CIII isoforms are clinically significant for several reasons. The levels of the isoforms change with the level of glucose control in diabetic patients. For example, high HbA1C directly correlates to high CIII-0 levels. Hypertriglyceridemic subjects have an increased proportion of CIII as the CIII-2 isoform in VLDL. CIII-2 levels increase in females subjected to severe caloric restriction despite normal total CIII levels. The variation in CIII-2 positively correlates to changes in VLDL triglycerides while the variation of CIII-1 inversely correlates. With the limitations of the existing techniques in the accurate quantitation and detection of the CIII isoforms, their role in various metabolic processes of great importance in understanding lipid metabolism is still subject to controversy.

The literature or prior art report various characterizations and correlations between various apolipoproteins and diseases. For example, Apo D is a multi-ligand, multifunctional transporter and is known to accumulate in a specific site of regenerating peripheral nerves in Alzheimer's disease. Further, Apo J so far has been reportedly implicated in several diverse physiological processes, such as sperm maturation, lipid transportation, complement inhibition, tissue remodeling, membrane recycling, cell-cell and cell-substratum interactions, stabilization of stressed proteins in a folding-competent state, and promotion or inhibition of apoptosis. Also, Apo H is known to bind tightly to negatively charged surfaces and to inhibit the activation of the intrinsic pathway of blood coagulation and the prothrombinase activity of activated platelets by covering the negatively charged surfaces necessary for both activities. Apo F associates with LDL and inhibits cholesterol ester transfer protein (CETP) activity, and appears to be an important regulator of cholesterol transport. Apo F associates to a lesser degree with VLDL, Apo Al and Apo All. Apo M was proposed to be involved in lipid transport. Apo CIV is a 14.5 kD size apolipoprotein in the same locus as CI and CII and no function appears to be reported in the literature.

Further, based on epidemiological correlations between cardiovascular disease and cholesterol levels, clinicians have long measured and standardized the measurement of

cholesterol levels to assess risks of heart disease. Lipoprotein particles (LDL and HDL) and the cholesterol associated with them also have been used in the assessment of cardiovascular risks. Many research studies have been conducted to relate health effects to lipoprotein particle sizes and densities, but the conclusions from these studies have not been consistent.

Over the last few years there has been considerable evidence that apolipoprotein levels are associated with a variety of conditions, and recently the National Heart, Lung, and Blood Institute (NHLBI), in a recent meeting with the Centers for Disease Control and Prevention, recommended that apolipoprotein B measurements be included for standardization in the near future. Apolipoproteins have been associated with cardiovascular disease, diabetes, stroke, obesity, Alzheimer's, HIV, and other diseases. Given the primary role of apolipoproteins in the transport and metabolism of lipids, these associations are not surprising. The difficulty of purifying, detecting, and quantifying apolipoproteins has not made it easy to conduct investigations between levels of these compounds and health effects.

Using prior art methodology, the quantification and measurement of apolipoprotein currently requires the step of separation of lipoprotein particles by analytical or sequential ultracentrifugation, column chromatography, electrophoresis, or precipitation. These techniques currently are too expensive and time consuming for routine clinical use. Another useful technique is high performance liquid chromatography, which is faster but much more complex and expensive. Other techniques used for measurement of Apo A and B content include enzyme immunoassay (ELISA), radioimmunoassay, fluorescence immunoassay, radial immunodiffusion, nephelometry, turbidimetry and electroimmunoassay. Recently, surface-enhanced laser desorption ionization mass spectrometry (SELDI or SELDITOF-MS, with TOF meaning time of flight and MS meaning mass spectrometry) has offered some new options for measuring apolipoproteins in plasma, serum, and lipoprotein fractions.

Accordingly, there is always a need for accurate, rapid, reproducible assays for the separation, identification, and quantification of apolipoproteins. There is a need for improved

techniques for the fingerprinting, profiling, determining, and/or quantifying of alipoproteins present in samples of human plasma, serum and lipoprotein fractions. It is to these needs among others that the present invention is directed.

BRIEF SUMMARY OF THIS INVENTION

Briefly, the technique of this invention combines immunological techniques with surface enhanced laser desorption/ionization, SELDI, to detect and measure multiple apolipoproteins directly in unfractionated plasma or serum, or in lipoprotein fractions. For quantification of internal standards, detection and measurement of known proteins very similar in antibody-binding properties to the proteins of interest, but with a visibly different molecular mass, would be required. The mass of the unknown polypeptide determined by mass spectrometry could be compared to the mass of a reference polypeptide of known identity and concentration.

Profiles of total plasma or serum require elaborate clustering and pattern recognition techniques to detect differences in samples of normal and healthy individuals. The use of specific antibodies in SELDI technology leads to a much simpler method of analysis. Another simplifying feature of the SELDI technology is retentate chromatography. Proteins of interest are retained by binding the proteins to a specific surface while other analytes are washed away. Absorption and desorption can be modified by adjustments in pH, salt concentration or organic solvents. Sinapinic acid or another appropriate matrix is mixed in a freshly prepared solution with tetrafluoroacetic acid and applied to the so-called chip and, upon drying, the matrix-embedded analyte molecules are desorbed by a laser, ionized from the solid phase, and accelerated as intact molecular ions.

One embodiment of the preferred technique of the present invention uses specific antibodies bound to surface-enhanced chips via Protein G to selectively absorb apolipoproteins directly from plasma samples. Protein G binds the fc portion of the antibodies, thereby enhancing the binding of the antibodies to specific antigens of interest in biological samples. The surface of

the surface-enhanced chips retains the specific apolipoproteins while other sample components are washed away. The EAM solution matrix facilitates laser desorption ionization of the apolipoproteins. The ionized apolipoproteins reach the detector at slightly different times based on their times of flight, which time differences can be converted to masses. The intensities of the peaks are related to the quantity of each protein.

The compositions, levels, and isoforms of apolipoproteins in plasma and in lipoprotein fractions are determinant of several disorders or conditions related to cardiovascular disease and other chronic disorders such as stroke, metabolic syndrome, diabetes, Alzheimer's, HIV and HIV patients under protease inhibitors (hypertriglyceridemic), and various types of lipoproteinemia. Thus, potential disorders that can benefit from a clinical diagnostic assay for CIII isoforms, such as the present invention, include those mentioned above. Clinical studies using lipid-lowering drugs would benefit from the apolipoprotein fingerprinting techniques of the present invention, and the techniques of the present invention would enable a look at the levels and distribution of apolipoproteins and their isoforms in plasma and in different lipoprotein fractions to show how these parameters change with different disorders and treatments. The techniques of the present invention also are ideal for assessing apolipoprotein status in newborns and for evaluating apolipoprotein modifications that occur with aging, nutrition, environmental exposures, and lifestyle changes.

Illustrative features of this invention include defining disease specific apolipoprotein biomarkers or profiles and developing quantitative assays that are rapid and useful for diagnosis or categorizing one or more diseases or conditions. These features, and other features and advantages of the present invention will become more apparent to those of ordinary skill in the relevant art when the following detailed description of the preferred embodiments is read in conjunction with the appended drawings.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be better understood by reading the Detailed Description of the Preferred and Alternate Embodiments with reference to the accompanying drawing figures, which illustrate representative isoform standards and profile reproducibility for the invention, in which like reference numerals denote similar structure and refer to like elements throughout, and in which:

FIGURE 1 is a representation of Reported Mass Spectra and Molecular Weights of the Different CIII Isoform Standards;

FIGURE 2 is a representation of Pooled Apolipoprotein CIII (CIII-0, CIII-1, and CIII-2 Isoforms) Profile CIPHERGEN Q10 chip;

FIGURE 3 is a representation of Pooled Apolipoprotein CIII (CIII-0, CIII-1, and CIII-2 Isoforms) Profile CIPHERGEN CM10 chip;

FIGURE 4 is a representation of CIII Isoforms Profile Reproducibility CIPHERGEN Anti-CIII PS 20 chip;

FIGURE 5 is a representation of CIII Isoforms Profile Reproducibility CIPHERGEN Anti-CIII PS 20 chip;

FIGURE 6 is a representation of Plasma CIII Isoform Profile Reproducibility;

FIGURE 7 is a representation of Plasma CIII Isoform Profile at Different Plasma Dilutions;

FIGURE 8 is a representation of Plasma CIII Isoform Profile at Different Plasma Dilutions; and

FIGURE 9 is a representation of Comparison of CIII Isoform Profile of Normal and Diabetic Subjects CIPHERGEN Anti-CIII PS 20 chip.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present Patent Cooperation Treaty Application claims priority to and the benefit of

U.S. Provisional patent application entitled "APOLIPOPROTEIN FINGERPRINTING TECHNIQUE filed March 23, 2006, on behalf of inventors Emelita De Guzman Breyer and Mary K. Robinson, having Serial No. 60/743,678.

This invention provides sensitive and rapid methods that can be useful in accessing cardiovascular diseases (e.g. lipid disorders, metabolic syndrome, and atherosclerosis), Alzheimer's disease, stress, stroke, diabetes that may be diagnosed using a profile comprising apolipoproteins. The profile correlates with a status of a disease, and therefore the status of a disease in a patient can be determined sensitively and rapidly using this invention. Thus, an ability of this invention is to define disease specific apolipoprotein biomarkers or profiles and to develop quantitative assays that are rapid and useful for diagnosis or categorizing one or more diseases or conditions.

More particularly, in one illustrative embodiment, the present invention provides a method of qualifying disease status in a subject comprising the steps of:

(1) measuring at least one biomarker in a sample from the subject, wherein the biomarker is selected from the group consisting of Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo All, Apo AIV, Apo AV, Apo B100, Apo B48, Apo E, Apo D, Apo H, Apo G, Apo F, Apo J, Apo L, Apo M, isoforms thereof, and combinations thereof;

(2) analyzing or quantifying or measuring the at least one biomarker in the 10 sample by spectrometry;

(3) preparing a profile of the at least one biomarker using the analysis, quantification, or measurement; and

(4) comparing the at least one biomarker to standard profiles that indicate disease,

whereby the presence or absence of the at least one biomarker in the sample indicates disease.

In a related illustrative embodiment the present invention provides a method of qualifying disease status in a subject comprising the steps of:

(1) measuring a plurality of biomarkers in a sample from the subject, wherein 20 the biomarkers are selected from the group consisting of Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo All, Apo AIV, Apo AV, Apo B100, Apo B48, Apo E, Apo D, Apo H, Apo G, Apo F, Apo J, Apo L, Apo M, isoforms thereof, and combinations thereof;

(2) analyzing or quantifying or measuring the biomarkers in the sample by spectrometry;

(3) preparing a profile of the biomarkers using the analysis, quantification, or measurement; and

(4) comparing the biomarkers to standard profiles that indicate disease,

whereby the presence or absence of the biomarkers in the sample indicates a disease or diseases.

In other illustrative embodiments, the measuring step comprises quantifying the amount of the at least one biomarker or biomarkers in the sample. In further illustrative embodiments, the invention involves resolution of the at least one biomarker or biomarkers including the use of SELDI mass spectrometry.

High-throughput protein profiling combined with effective use of bioinformatics tools provides a useful approach to screening for biomarkers. The system used in the present invention preferably utilizes chromatographic PROTEINCHIPO laboratory equipment, namely, an apparatus for screening samples, detecting the presence of analytes in samples, and identifying sample type, together with measuring patterns and equipment for making and screening molecular arrays to assay samples using SELDI. proteins bound to the arrays are read in a PROTEINCHIPO array reader, which is a time-of-flight mass spectrometer.

Additionally, the method of this invention takes advantages of the ability of the chip to detect modified proteins (glycosylated proteins etc) and degraded proteins. This method also can take advantage of the ability to detect apolipoproteins with pre- and/or post-translational modifications. Pre-translational modified forms include allelic variants, slice variants and RNA

editing forms. Post-translational modified forms include forms resulting from truncation, proteolytic cleavage (for example, fragments of a parent protein), glycosylation, lipidation, cysteinylation, glutationylation, phosphorylation, prenylation, acylation, acetylation, methylation, sulfation, sulfonation, hydroxylation, myristoylation, farnesylation, oxidation and ubiquitination. Modified forms of any biomarker of this invention also themselves may be used as biomarkers.

As may be seen, the profiles can comprise the compositions, levels, and isoforms of apolipoproteins extracted from plasma and in lipoprotein fractions, which are determinant of several diseases, disorders or conditions. As used herein, the term diseases includes disorders and conditions and is used specifically in relation to cardiovascular disease and other chronic disorders such as stroke, metabolic syndrome, diabetes, Alzheimer's, HIV, and various types of lipoproteinemia. For example, clinical studies conducted in conjunction with the diseases and using lipid-lowering drugs would benefit from the apolipoprotein fingerprinting technique of the present invention. For another example, use of the fingerprinting technique of the present invention would enable a look at the levels and distribution of apolipoproteins and their isoforms in plasma and in different lipoprotein fractions to show how these parameters change with different diseases and their treatments. For still another example, the technique of the present invention can be ideal for assessing apolipoprotein status in newborns and for evaluating apolipoprotein modifications that occur with aging, nutrition, environmental exposures, and lifestyle changes.

In yet another example, the present invention provides kits for qualifying disease status in which the kits can be used to measure the biomarkers of the present invention. For example, the kits can be used to measure any one or more of the biomarkers described herein, which biomarkers are differentially present in samples of diseased patient and normal subjects. For another example, the kits also can be used to monitor the patient's response to a course of treatment, enabling the physician to modify the treatment based upon the results of the test. For

still another example, the kits can be used to identify compounds that modulate expression of one or more of the biomarkers in in vitro or in vivo animal models for diseases.

One embodiment of the preferred technique of the present invention uses specific antibodies bound to surface-enhanced chips via Protein G to selectively absorb apolipoproteins directly from plasma samples. Protein G binds the fc portion of the antibodies, thereby enhancing the binding of the antibodies to specific antigens of interest in biological samples. The surface of the surface-enhanced chips retains the specific apolipoproteins while other sample components are washed away. The EAM solution matrix facilitates laser desorption ionization of the apolipoproteins. The ionized apolipoproteins reach the detector at slightly different times based on their times of flight, which time differences can be converted to masses. The intensities of the peaks are related to the quantity of each protein. As used herein, the terms antibody and antibodies include fragments of antibodies, immunoglobulins, affybodies, etcetera.

Preferred methods of measuring the biomarkers include use of a biochip array. Biochip arrays useful in the invention include protein and nucleic acid arrays. One or more biomarkers are captured on the biochip array and subjected to laser ionization to detect the molecular weight of the biomarkers. Analysis of the biomarkers is, for example, by molecular weight of the one or more biomarkers against a threshold intensity that is normalized against total ion current. Preferably, logarithmic transformation is used for reducing peak intensity ranges to limit the number of biomarkers detected.

In preferred methods of the present invention, the step of preparing a profile of biomarkers and comparing the biomarkers with a disease status is performed by a software classification algorithm. Preferably, data is generated on immobilized subject samples on a biochip array by (1) subjecting the biochip array to laser ionization and detecting the intensity of a signal for mass/charge ratio; (2) transforming the data into computer readable form; and (3) executing an algorithm that classifies the data according to user input parameters for detecting signals that represent biomarkers that are present in diseased patients and that are lacking in non-

diseased subject controls. Step (1) preferably uses SELDI-TOF-MS, which generally speaking comprises (a) providing a probe adapted for use with a mass spectrometer comprising an adsorbent attached thereto; (b) contacting the subject sample with the adsorbent; (c) desorbing and ionizing the biomarker or biomarkers from the probe; and (d) detecting the ionized biomarkers with the mass spectrometer.

In one example, CIPHERGEN BIOMARKER PATTERNSTM software, which is a software package for supervised classification of SELDI mass spectral data sets derived from the CIPHERGEN PROTEINCHIP® array platform mass spectrometers and chemical synthesizers used to determine protein identification and structure, is used to detect a pattern in the spectra that are generated. The data is classified using a pattern recognition process that uses a classification model. In general, the spectra will represent samples from at least two different groups for which a classification algorithm is sought. For example, the groups can be pathological versus non-pathological (for example, cancer versus non-cancer), drug responder versus drug non-responder, toxic response versus non-toxic response, progressor to disease state versus non-progressor to disease state, or phenotypic condition present versus phenotypic condition absent.

The spectra that are generated in step (1) of embodiments of the invention can be classified using a pattern recognition process that uses a classification model. In some embodiments, data derived from the spectra (for example, mass spectra or time-of-flight spectra) that are generated using samples such as "known samples" can then be used to "train" a classification model. A "known sample" is a sample that is preclassified (for example, cancer or not cancer). Data derived from the spectra (for example, mass spectra or time-of-flight spectra) that are generated using samples such as "known samples" then can be used to "train" a classification model. A "known sample" is a sample that is pre-classified. The data that are derived from the spectra and are used to form the classification model can be referred to as a "training data set". Once trained, the classification model can recognize patterns in data derived

from spectra generated using unknown samples. The classification model can then be used to classify the unknown samples into classes. This can be useful, for example, in predicting whether or not a particular biological sample is associated with a certain biological condition (for example, diseased vs. non diseased).

The training data set that is used to form the classification model may comprise raw data or pre-processed data. In some embodiments, raw data can be obtained directly from time-of-flight spectra or mass spectra, and then may be optionally "preprocessed" in any suitable manner. For example, signals above a predetermined signal-to-noise ratio can be selected so that a subset of peaks in a spectrum is selected, rather than selecting all peaks in a spectrum. In another example, a predetermined number of peak "clusters" at a common value (for example, a particular time-of-flight value or mass-to-charge ratio value) can be used to select peaks. Illustratively, if a peak at a given mass-to-charge ratio is in less than 50% of the mass spectra in a group of mass spectra, then the peak at that mass-to-charge ratio can be omitted from the training data set. Pre-processing steps such as these can be used to reduce the amount of data that is used to train the classification model.

Classification models can be formed using any suitable statistical classification (or "learning") method that attempts to segregate bodies of data into classes based on objective parameters present in the data. Classification methods may be either supervised or unsupervised. Examples of supervised and unsupervised classification processes are described in Jain, "Statistical Pattern Recognition: A Review", IEEE Transactions on Pattern Analysis and Machine Intelligence, Vol. 22, No. 1, January 2000, which is herein incorporated by reference in its entirety.

In supervised classification, training data containing examples of known categories are presented to a learning mechanism, which learns one more sets of relationships that define each of the known classes. New data may then be applied to the learning mechanism, which then classifies the new data using the learned relationships. Examples of supervised classification

processes include linear regression processes (for example, multiple linear regression (MLR), partial least squares (PLS) regression and principal components regression (PCR)), binary decision trees (for example, recursive partitioning processes such as CART--classification and regression trees), artificial neural networks such as back propagation networks, discriminant analyses (for example, Bayesian classifier or Fischer analysis), logistic classifiers, and support vector classifiers (support vector machines).

Once captured on a substrate, for example a biochip or antibody, any suitable method can be used to measure a biomarker or biomarkers in a sample. For example, biomarkers can be detected and/or measured by a variety of detection methods including for example, gas phase ion spectrometry methods, optical methods, electrochemical methods, atomic force microscopy and radio frequency methods. Using these methods, one or more biomarkers can be detected in the sample.

In preferred methods of the present invention, multiple biomarkers are measured. The use of multiple biomarkers increases the predictive value of the test and provides greater utility in diagnosis, toxicology, patient stratification and patient monitoring. The process called "pattern recognition" detects the patterns formed by multiple biomarkers greatly improves the sensitivity and specificity of clinical proteomics for predictive medicine. Subtle variations in data from clinical samples, for example obtained using SELDI, indicate that certain patterns of protein expression can predict phenotypes such as the presence or absence of a certain disease, a particular stage of disease progression, or a positive or adverse response to drug treatments.

Data generation in mass spectrometry begins with the detection of ions by an ion detector as described above. Ions that strike the detector generate an electric potential that is digitized by a high speed time-array recording device that digitally captures the analog signal. The CIPHERGEN PROTEINCHIP® array system employs an analog-to-digital converter (ADC) to accomplish this. The ADC integrates detector output at regularly spaced time intervals into time-dependent bins. The time intervals typically are one to four nanoseconds long. Furthermore, the

time-of-flight spectrum ultimately analyzed typically does not represent the signal from a single pulse of ionizing energy against a sample, but rather the sum of signals from a number of pulses. This reduces noise and increases dynamic range. This time-of-flight data is then subject to data processing. In the CIPHERGEN PROTEINCHIPO array software, data processing typically includes TOF-to-M/Z transformation, baseline subtraction, and high frequency noise filtering. In this step, the signals are converted from the time domain to the mass domain.

Baseline subtraction improves data quantification by eliminating artificial, reproducible instrument offsets that perturb the spectrum. It involves calculating a spectrum baseline using an algorithm that incorporates parameters such as peak width, and then subtracting the baseline from the mass spectrum. Peak data from one or more spectra can be subject to further analysis by, for example, creating a spreadsheet in which each row represents a particular mass spectrum, each column represents a peak in the spectra defined by mass, and each cell includes the intensity of the peak in that particular spectrum. Various statistical or pattern recognition approaches can applied to the data. As disclosed above, the CIPHERGEN BIOMARKER PATTERNSTM software is used to detect a pattern in the spectra that are generated. The data is classified using a pattern recognition process that uses a classification model. In general, the spectra will represent samples from at least two different groups for which a classification algorithm is sought.

Samples can be collected from patients or other subjects who want to establish various disease statuses. The subjects may be particular humans who believe that they are or may be at higher risk for certain types of diseases, from for example their medical history. Other subjects may be people who have had certain diseases and wish to monitor the effectiveness of their treatment. Further, it is possible to obtain the samples from subjects as part of routine examinations.

Any biomarker, individually, is useful in aiding in the determination of disease status. First, the selected biomarker is measured in a subject sample using the methods described herein,

for example capture on a SELDI biochip followed by detection by mass spectrometry. Then, the measurement is compared with a diagnostic amount or control that distinguishes that disease status from a non-disease status. The diagnostic amount will reflect that a particular biomarker is up-regulated or downregulated in a disease status compared with a non-disease status. As is well understood in the art, the particular diagnostic amount used can be adjusted to increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician. The test amount as compared with the diagnostic amount thus indicates disease status.

EXAMPLE METHODOLOGIES

The example methodologies disclosed below are representative methods for using the preferred embodiments of the invention. In these methodologies, a serum sample can be collected from a patient and then fractionated using an anion exchange resin. The biomarkers in the sample are captured using an IMAC copper PROTEINCHIP® array. The biomarkers then are detected using SELDI. The results then are entered into a computer system, which contains an algorithm that is designed using the same parameters that were used in the learning algorithm and classification algorithm to originally determine the biomarkers. The algorithm produces a diagnosis based upon the data received relating to each biomarker. See FIGS. 1-9 for experimental data regarding correlations between CIII isoforms and cardiovascular disease. Exemplary correlations between various apolipoproteins and diseases, as previously discussed, were confirmed in the literature.

The diagnosis can be determined by examining the data produced from the SELDI tests with the classification algorithm that is developed using the biomarkers. The classification algorithm depends on the particulars of the test protocol used to detect the biomarkers. These particulars include, for example, sample preparation, chip type and mass spectrometer parameters. If the test parameters change, the algorithm must change. Similarly, if the algorithm changes, the test protocol can change.

For the present invention, purified apolipoproteins, plasma samples from normal subjects, and plasma samples from diabetic subjects were analyzed using the PG-20 protein chip surface. The specific apolipoproteins that were analyzed according to the method of the invention are present in plasma, serum and lipoprotein fraction in various levels. The presence of each apolipoprotein is determined using antibodies specific for each of the apolipoproteins. The below disclosed protocol is limited to the molecular weight range that can be analyze biy current MS technology. Initially, B100 (500 kD) and B48 (approx 250 kD) are either first isolated by binding to the antibody-surface,released and then chemically or enzymatically fragmented prior to MS analysis or by direct digest while bound to the PG20 chip using the below protocol.

1. Sample Preparation

Before the SELDI-TOF-MS procedure, plasma samples were diluted with buffer 5 and the antibody was coupled to the PROTEINCHIP® as follows:

Preparation of plasma sample:

(1) Prepare U9Buffer (9M urea, 50 mM HEPES, 0.5% CHAPS, pH 7); and

(2) Dilute plasma sample with U9 Buffer (1:2) and vortex in cold for 30 minutes.

Coupling of Antibody to PROTEINCHIP® Array

(1) Dilute antibody to 0.2 mg/ml and add 2 µl to spots on array;

(2) Transfer array to a humidity chamber and incubate for 1 hour at room temperature;

(3) Aspirate antibody without touching surface of spot;

(4) Wash chip in 15 mL conical tube for 10 minutes with 8 mL of wash buffer;

(5) Empty and wash with 8 mL of phosphate-buffered saline for 5 minutes, and repeat; and

(6) Blot excess PBS from the surface of the chip.

2. SELDI-TOF-MS Procedure

(1) Add 2 µl of plasma sample to each spot;

(2) Transfer array to a humidity chamber and incubate for 1 hour at room temperature;

(3) Wash chip in 15 mL conical tube with 8 mL of wash buffer for 10 minutes;

(4) Empty and wash with 8 mL of Phosphate-buffered Saline for 5 minutes, and repeat;

(5) Empty and wash with 8 mL of 1 mM HEPES 1 minute, and repeat;

(6) Remove chip from tube, flick off liquid, and air-dry for 10 minutes;

(7) Prepare EAM solution by adding 100 µl of 99.8% acetonitrile and 100µl of 1.0% trifluoroacetic acid to 1 vial (10 mg) of sinapinic acid powder, and vortex for 5 minutes to dissolve the powder; and

(8) Add 5 µl of EAM solution to each spot and air dry for 5 minutes, repeat, and air dry for 10 minutes.

The procedure may be modified for use with a bioprocessor that allows larger volumes and automated addition of samples and reagents. Both positive and negative controls are usually included in experiments. TNF-a antibody and antigen are included in the kit for this purpose.

3. Generation of Fingerprints

The procedure for generating apolipoprotein fingerprints for plasma samples and purified apolipoproteins is described below:

(1) The PROTEINCHIP® array reader and software (Ciphergen Biosystems, Inc.) are used to generate the profiles.

(2) Spot protocols are generated and used to mad the chips.

(3) Data collection parameters must be adjusted for each antigen and are based on molecular mass. Instructions are included in the PROTEINCHIP® array Antibody Capture Kit supplied by Ciphergen Biosystems, Inc.

(4) The data was analyzed and the apolipoprotein fingerprints were generated by, for example, comparing the data intrinsically with itself and comparing the data extrinsically with

norms such as, for example, control subjects.

Alternatively, this invention can be carried out by coupling antibodies to beads, capturing the apolipoproteins on the beads, eluting purified proteins from the beads, and then detecting by any version of mass spectrometry, including matrix assisted laser desorptionronization (MALDI) and electrospray methods. Methods for coupling antibodies to beads are well known in the art and such methods are suitable for the present invention. MALDI is a laser-based soft ionization method in which the sample is embedded in a chemical matrix that greatly facilitates the production of intact gas-phase ions from large, nonvolatile, and thermally labile compounds such as proteins, oligonucleotides, synthetic polymers, and large inorganic compounds. The matrix plays a key role in this technique by absorbing the laser light energy and causing a small part of the target substrate to vaporize.

The foregoing detailed description of the preferred embodiments and the appended figures have been presented only for illustrative and descriptive purposes and are not intended to be exhaustive or to limit the scope and spirit of the invention. The embodiments were selected and described to best explain the principles of the invention and its practical applications. One of ordinary skill in the art will recognize that many variations can be made to the invention disclosed in this specification without departing from the scope and spirit of the invention.

CLAIMS

What is claimed is:

1. A method for obtaining a profile of at least one selected apolipoprotein biomarker in biological samples, comprising:

a) providing the sample;

b) measuring the at least one selected apolipoprotein biomarker by applying the sample to a surface and removing unbound sample components;

c) analyzing the sample by spectrometry; and

d) preparing the profile using the analysis, whereby at least one disease may be diagnosed.

2. The method as claimed in Claim 1, wherein the at least one selected apolipoprotein biomarker is selected from the group consisting of Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo All, Apo AIV, Apo AV, Apo B48, Apo B100, Apo D, Apo E, Apo F, Apo G, Apo H, Apo J, Apo L, Apo M, isoforms thereof, and combinations thereof.

3. The method as claimed in Claim 2 wherein the sample is applied to and allowed to bind with the surface-enhanced, antibody capture protein coated, antibody-bound surface, and the unbound sample components are washed away.

4. The method as claimed in Claim 2 wherein the at least one selected apolipoprotein biomarker is detected by laser desorption ionization time-of-flight mass spectrometry or other appropriate mass spectrometry method.

5. The method as claimed in Claim 1 wherein the sample is selected from the group consisting of human plasma, serum, and human lipoprotein fractions.

6. The method as claimed in Claim 1 the at least one disease is selected from the group consisting of diabetes, stroke, stress, Alzheimer's, cardiovascular diseases, lipid disorders, metabolic syndrome, obesity, and atherosclerosis.

7. A method for determining the concentration and modifications of specific apolipoproteins in a biological sample, the sample being obtained from a patient and the sample including plasma, serum, and lipoprotein fractions, the method comprising the steps of;

a) adding a specific volume of an internal standard to the sample;

b) applying the sample to a surface-enhanced, Protein G-coated, antibody-bound chip and removing unbound sample components;

c) analyzing the sample by mass spectrometry; and

d) determining the concentration of the apolipoproteins using values of internal standards,

wherein the application of this method is to evaluate the concentration of isoforms of the apolipoproteins for use as a diagnostic tool for detecting diabetes, stroke, stress, Alzheimer's, cardiovascular diseases, lipid disorders, metabolic syndrome, obesity, and atherosclerosis.

8. The method as claimed in Claim 7, wherein the specific apolipoproteins are bound to specific antibodies.

9. The method as claimed in Claim 8, wherein the specific antibodies are immobilized on the antibody capture protein coated surface.

10. The method as claimed in Claimed in Claim 9 wherein the sample is applied to and allowed to bind with the antibody capture protein coated surface, and the unbound sample components are washed away.

11. The method as claimed in Claim 9, wherein the apolipoproteins are detected by laser desorption ionization time-of-flight mass spectrometry or other appropriate mass spectrometry method.

12. The method as claimed in Claim 9 wherein apolipoproteins are selected from the group consisting of Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo All, Apo AIV, Apo AV, Apo B48, Apo B100, Apo D, Apo E, Apo F, Apo G, Apo H, Apo J, Apo L and Apo M and the specific antibodies are selected from the group consisting of antibodies to Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo Cll, Apo AIV, Apo AV, Apo B48, Apo B100, Apo D, Apo E, Apo F, Apo G, Apo H, Apo J, Apo L and Apo M.

13. The method as claimed in Claim 12, wherein the samples are selected from human plasma, serum, and human lipoprotein fractions.

14. The method as claimed in Claim 7, wherein concentration values for specific apolipoproteins in the sample are based on internal standards developed by modifying selected purified proteins and setting reference values using high resolution mass spectrometry.

15. The method as claimed in Claim 7, wherein the modifications are selected from the group consisting of glycation, sialylation, fragmentation, and amino acid solutions.

16. A method for use in assessing risk factors, disease state, drug treatment and response in the field of pharmacogenetics.

17. A method for obtaining a profile of at least one selected apolipoprotein biomarker

in a biological sample from a subject, comprising the steps of:

a) providing the biological sample from a subject;

b) applying the biological sample to the surface of a capture reagent that specifically captures the at least one apolipoprotein biomarker, allowing the at least one apolipoprotein biomarker to bind to the capture reagent, and removing unbound sample components;

c) analyzing the captured at least one apolipoprotein by laser desorption/ionization mass spectrometry; and

d) preparing the profile using the analysis,

wherein the at least one selected apolipoprotein biomarker is selected from the group consisting of Apo CI, Apo CII, Apo CIII, Apo CIV, Apo Al, Apo AII, Apo AIV, Apo AV, Apo B48, Apo B100, Apo D, Apo E, Apo F, Apo G, Apo H, Apo J, Apo L, Apo M, isoforms thereof, and combinations thereof.

18. The method as claimed in Claim 17, further comprising the step of:

a) using the profile to diagnose a disease in the subject.