NMR MEASUREMENTS OF GLYCA

Related Applications

[0001] This application claims the benefit of and priority to U.S. Provisional Application Serial No. 61/657,315, filed June 8, 2012, U.S. Provisional Application Serial No. 61/711,471, filed October 9, 2012, U.S. Provisional Application Serial No. 61/739,305, filed December 19, 2012, and U.S. Patent Application Serial No. 13/830,199, filed March 14, 2013, the contents of which are hereby incorporated by reference as if recited in full herein.

Field of the Invention

[0002] The present invention relates generally to analysis of *in vitro* biosamples. The invention may be particularly suitable for NMR analysis of human blood plasma and serum.

Background of the Invention

[0003] Conventionally, a patient's overall risk of coronary heart disease (CHD) and/or coronary artery disease (CAD) has been assessed based on measurements of cholesterol content of a patient's low density lipoproteins (LDL) and high density lipoproteins (HDL) (LDL-C, HDL-C) rather than the numbers of LDL and HDL particles. LDL-C and HDL-C are used to assess a patient's CHD risk and treatment decisions may be made to reduce the "bad" cholesterol (LDL-C) and/or increase the "good" cholesterol (HDL-C).

[0004] A"C-reactive protein" (CRP) test is a blood test that measures the amount of CRP protein in a blood sample. C-reactive protein is thought to measure general levels of inflammation in a patient's body. One type of CRP test, termed a high-sensitivity CRP test (hs-CRP), may be performed to find out if a person has an increased risk of having a heart attack.

[0005] NMR spectroscopy has been used to concurrently measure very low density lipoprotein (VLDL), LDL and HDL as VLDL, LDL and HDL particle subclasses from *in vitro* blood plasma or serum samples. *See*, U.S. Patent Nos. 4,933,844 and 6,617,167, the contents of which are hereby incorporated by reference as if recited in full herein. Generally stated, to evaluate the lipoproteins in a blood plasma and/or serum sample, the amplitudes of a plurality of NMR spectroscopy derived signals within a chemical shift region of NMR spectra are derived by deconvolution of the composite methyl signal envelope to yield

subclass concentrations. The subclasses are represented by many (typically over 60) discrete contributing subclass signals associated with NMR frequency and lipoprotein diameter. The NMR evaluations can interrogate the NMR signals to produce concentrations of different subpopulations, typically seventy-three discrete subpopulations, 27 for very low density lipoproteins (VLDL), 20 for LDL and 26 for HDL. These sub-populations can be further characterized as associated with a particular size range within the VLDL, LDL or HDL subclasses.

[0006] In the past, an "advanced" lipoprotein test panel, such as the LIPOPROFILE® lipoprotein test, available from LipoScience, Raleigh, N.C., has typically included a total high density lipoprotein particle (HDL-P) measurement (e.g., HDL-P number) that sums the concentration of all the HDL subclasses and a total low density lipoprotein particle (LDL-P) measurement that sums the concentration of all the LDL subclasses (e.g., LDL-P number). The LDL-P and HDL-P numbers represent the concentration of those respective particles in concentration units such as nmol/L.

[0007] Inflammation can be associated with many different disease states including, but not limited to, CHD. It is also believed that inflammation may modulate HDL functionality. *See, e.g.*, Fogelman, When Good Cholesterol Goes Bad, Nature Medicine, 2004. Carbohydrate components of glycoproteins can perform biological functions in protein sorting, immune and receptor recognition, inflammation and other cellular processes. There can also be variations in structure and differing degrees of glycosylation. *See*, Gates et al., Glycoprotein Analysis Manual, Overview, 1st edition, 2004, Sigma Aldrich, www.sigmaaldrich.com/img/assests/15402/Glyocprotein. The contents of the above referenced documents are hereby incorporated by reference as if recited in full herein.

[0008] In the past, life insurance companies have considered various information regarding a prospective customer to identify whether to insure a person and at what price. One input used by some companies to predict a risk of all cause death for such analysis is a ratio of total cholesterol to HDL-C. However, it is believed that this is a relatively poor predictor of all-cause death.

Summary

[0009] Embodiments of the invention identify a novel NMR-derived biomarker termed "GlycA" based on deconvolution of a defined region of an NMR spectrum of a blood plasma or serum sample.

[0010] Embodiments of the invention identify a novel NMR-derived biomarker termed "GlycB" based on deconvolution of a defined region of an NMR spectrum of a blood plasma or serum sample.

[0011] Embodiments of the invention provide multiple-parameter risk assessments and/or screenings using GlycA and one or both of (i) Valine and (ii) at least one lipoprotein subclass input.

[0012] Embodiments of the invention provide multiple-parameter all-cause death risk assessments and/or screenings using GlycA and one or both of (i) Valine and (ii) at least one lipoprotein input, such as HDL-P.

[0013] Embodiments of the invention evaluate an *in vitro* biosample to calculate all-cause death risk using the equation: HDL-P*Valine/GlycA.

[0014] The computer program product may include computer readable program code that applies a conversion factor to the summed Lorentzian functions to generate the measurement of GlycA in μmol/L.

[0015] The computer program product can include computer readable program code that compares the GlycA measurement to a predefined range of measurements having associated degrees of CHD risk.

[0016] The computer program product can include computer readable program code that generates a patient report with the GlycA measurement and an associated with risk assessment.

[0017] The computer program code that provides the measurement can be configured to evaluate NMR spectra of an *in vitro* blood plasma or serum patient sample using NMR signal having a peak centered at about 2.00 ppm.

[0018] Still other embodiments are directed to systems that include: an NMR spectrometer for acquiring at least one NMR spectrum of an *in vitro* biosample; and at least one processor in communication with the NMR spectrometer, the at least one processor configured to obtain an NMR measurement of GlycA using the at least one NMR spectrum.

[0019] The at least one processor can be configured to: (i) obtain a composite NMR spectrum of a fitting region of an in vitro plasma biosample; (ii) deconvolve the composite NMR spectrum using a defined deconvolution model with high density lipoprotein (HDL) components, low density lipoprotein (LDL) components, VLDL(very low density lipoprotein)/chylomicron components, a defined protein signal component, and curve fit functions associated with at least a GlycA peak region; and (iii) electronically sum a defined number of the functions to generate the NMR measurement of GlycA.

[0020] The curve fit functions can comprise Lorentzian and/or other suitable functions.

[0021] The at least one processor can be configured to apply a conversion factor to the summed NMR measurement to generate a GlycA measurement in μmol/L.

[0022] The biosample can be a blood plasma or serum sample. The at least one processor is configured to obtain a concentration measurement of high density lipoprotein particles (HDL-P) and a concentration measurement of low density lipoprotein particles (LDL-P).

[0023] The at least one processor is configured to generate patient reports summarizing the respective LDL-P, HDL-P and GlycA measurements.

[0024] Yet other embodiments are directed to a patient report that includes a plurality of lipoprotein measurements including a quantitative measure of GlycA in μmol/L and/or arbitrary units and at least one of: (i) a low density lipoprotein particle number (LDL-P) in concentration units and (ii) a high density lipoprotein particle (HDL-P) number in concentration units.

[0025] The patient report can also includes at least one of NMR measures of magnesium and/or valine.

[0026] Yet other embodiments are directed to NMR analyzers. The NMR analyzers include: a NMR spectrometer; a flow probe in communication with the spectrometer; and a controller in communication with the spectrometer configured to obtain NMR signal of a defined single peak region of NMR spectra associated with GlycA of a fluid specimen in the flow probe and generate a patient report providing a GlycA level.

[0027] The controller can include or be in communication with at least one local or remote processor, wherein the at least one processor is configured to: (i) obtain a composite NMR spectrum of a fitting region of an in vitro plasma biosample; (ii) deconvolve the composite NMR spectrum using a defined deconvolution model with high density lipoprotein (HDL) components, low density lipoprotein (LDL) components, VLDL(very low density lipoprotein)/chylomicron components, a defined protein signal component, and curve fit functions (e.g., overlapping Lorentzian functions) applied to at least a GlycA peak region; and (iii) electronically sum a defined number of the applied Lorentzian functions to generate the GlycA level.

[0028] The at least one processor can be configured to apply a conversion factor to the summed NMR measurement to generate a GlycA measurement in μmol/L.

[0029] In some embodiments, NMR measures of NMR signal in a defined peak region of proton NMR spectrum associated with GlycA can be determined. The GlycA signal measurement can be used as an inflammation biomarker for assessing CHD risk.

[0030] The NMR signal for GlycA can include signal associated with N-acetylglucosylated proteins at between about 2.080 ppm and 1.845 ppm of the proton NMR spectra of blood plasma or serum (typically at 47 deg. C +/- 0.2)

[0031] The at least one processor can be configured to use the GlycA measurement as a denominator in a defined all-cause death risk ratio to generate a risk predictor associated with a risk of all-cause death.

[0032] It is believed that human plasma GlycA is more independent and a stronger inflammation biomarker for CHD/heart attack risk than hs-CRP, particularly when risk correlation is adjusted for other confounding factors (e.g., systolic blood pressure, age, gender, BMI, diabetes and smoking).

[0033] A patient's risk of having or developing CHD and/or a heart attack can be evaluated using a plurality of NMR derived measurements including LDL-P, HDL-P and GlycA.

[0034] Embodiments of the invention can generate a patient report that includes a calculated ratio of measurements of valine, HDL-P and GlycA using the ratio: (Valine*HDL-P)/GlycA.

[0035] The method can include electronically calculating LDL-P and HDL-P from NMR spectra of the biosample and the determining step can include identifying an increased risk of CHD when GlycA and LDL-P are above a respective population norm and HDL-P is below a population norm.

[0036] Some embodiments are directed to methods to evaluate or predict all-cause death risk of a person. The methods include: (a) electronically calculating a ratio of measurements of at least one of the following: Valine to GlycA, HDL-P to GlycA or (Valine*HDL-P)/GlycA; and (b) evaluating whether a person has an increased risk for all-cause death relative to a population, wherein a respective person has increased risk relative to a population when the calculated ratio is in a first tertile, quartile or quintile of the population. The calculating can be carried out using the ratio of (Valine*HDL-P)/GlycA.

[0037] Other embodiments are directed to methods of quantifying a level of metabolite A in a biosample. The methods include deconvolving a defined metabolite A peak region of a proton NMR spectrum having signal associated with lipoproteins and a single peak associated with metabolite A; applying a plurality of curve fitting functions to

the metabolite A peak region; and measuring a level of metabolite A using the applied curve fitting functions.

[0038] Yet other embodiments are directed to methods of quantifying valine in a biosample. The methods include: deconvolving an NMR spectrum using a deconvolution model of lipoproteins and protein components; identifying a multiplet signal in a region between 0.90-1.01 ppm; and calculating a level of valine in the biosample.

[0039] The method can include, before the deconvolving step, applying a quality control pre-analytical analysis using acquired NMR spectra.

[0040] Additional aspects of the invention are directed to methods of measuring GlycB. The methods include: (a) electronically obtaining a composite NMR spectrum of a fitting region of a biosample of a subject; (b) electronically deconvolving the composite NMR spectrum using a defined deconvolution model with high density lipoprotein (HDL) components, low density lipoprotein (LDL) components, VLDL (very low density lipoprotein)/chylomicron components, and a plurality of curve fit functions associated with at least a GlycB peak region; and (c) electronically generating a measure of GlycB using the curve fit functions.

[0041] The methods, computer products or systems can be configured to generate a ratio of GlycA/GlycB or GlycB/GlycA.

[0042] Further features, advantages and details of the present invention will be appreciated by those of ordinary skill in the art from a reading of the figures and the detailed description of the preferred embodiments that follow, such description being merely illustrative of the present invention. Features described with respect with one embodiment can be incorporated with other embodiments although not specifically discussed therewith. That is, it is noted that aspects of the invention described with respect to one embodiment, may be incorporated in a different embodiment although not specifically described relative thereto. That is, all embodiments and/or features of any embodiment can be combined in any way and/or combination. Applicant reserves the right to change any originally filed claim or file any new claim accordingly, including the right to be able to amend any originally filed claim to depend from and/or incorporate any feature of any other claim although not originally claimed in that manner. The foregoing and other aspects of the present invention are explained in detail in the specification set forth below.

[0043] As will be appreciated by those of skill in the art in light of the present disclosure, embodiments of the present invention may include methods, systems, apparatus and/or computer program products or combinations thereof.

Brief Description of the Figures

[0044] **Figure 1** is a an NMR spectrum showing the inflammation markers in the exemplary plasma NMR spectrum (N-acetyl methyl signals from glycosylated acute phase proteins, GlycA and GlycB, respectively) according to embodiments of the present invention.

[0045] **Figures 2A** and **2B** are schematic illustrations of the chemical structures of the carbohydrate portion of N-acetylglycosylated proteins showing the CH3 group that gives rise to the GlycA NMR signal.

[0046] **Figures 3A** and **3B** are schematic illustrations of the chemical structures of the carbohydrate portion of N-acetylneuraminic acid (also called sialic acid) modified glycoproteins showing the CH3 group that gives rise to the GlycB NMR signal.

[0047] **Figure 4A** is a an expansion of the plasma NMR spectrum containing the signal envelope from the plasma lipoproteins and the underlying GlycA and GlycB signals according to embodiments of the present invention.

[0048] **Figures 4B** and **4C** are graphs of the NMR spectral region shown in **Figure 4A** illustrating deconvolution models to yield NMR signal for measurement of GlycA and GlycB according to embodiments of the present invention.

[0049] **Figures 5A-5D** are GlycA/B NMR spectral regions illustrating spectral overlap from lipoprotein signals (from especially VLDL/Chylos) for samples with high TG (triglycerides).

[0050] **Figure 6A** is a table of different measures of GlycA concentration, depending on a protein component used in the deconvolution (e.g., "fitting") model.

[0051] **Figures 6B-6D** illustrate the GlycA and GlycB "fits" (deconvolution) of the same plasma sample using deconvolution models with different protein components (#1-#3 in the table in **Figure 6A**) according to embodiments of the present invention.

[0052] **Figure 7A** is a schematic screen shot of the deconvolution of a 10 mmol/L reference sample of N-acetylglucosamine, used to generate a conversion factor relating GlycA and GlycB signal areas to glycoprotein N-acetyl methyl group concentrations according to embodiments of the present invention.

[0053] **Figure 7B** is a table of components in a GlycA/B deconvolution model according to embodiments of the present invention.

[0054] **Figure 7C** is an NMR spectrum showing metabolite A present in a sample at typical normal (low) concentration according to embodiments of the present invention.

[0055] **Figure 7D** is an NMR spectrum showing metabolite A present in a sample at an elevated (high) concentration according to embodiments of the present invention.

[0056] **Figures 8A** and **8B** are graphs of distributions of NMR-measured GlycA levels (in methyl group concentration units) of two different populations. **Figure 8A** is from the MESA study of apparently healthy men and women. **Figure 8B** is from a study of women with Rheumatoid Arthritis.

[0057] **Figure 9** is a chart of prospective associations of hs-CRP and NMR-measured GlycA and NMR-measured valine levels with various disease outcomes in MESA (n=5680) according to embodiments of the present invention.

[0058] **Figure 10A** is a region of the plasma NMR spectrum containing methyl signals from lipoproteins and branched-chain amino acids.

[0059] **Figure 10B** is an example of a fitting function/deconvolution model that uses four valine signals (two doublets) to calculate NMR measures of valine according to embodiments of the present invention.

[0060] **Figure 11** is a chart of characteristics of MESA subjects by NMR measured GlycA quartile (in "NMR signal area units") according to embodiments of the present invention.

[0061] **Figure 12** is a chart showing the independent associations of hsCRP, fibrinogen, and GlycA with future CHD events in MESA according to embodiments of the present invention.

[0062] **Figure 13** is a table of various statistical evaluations of the associations with all-cause death in MESA (345 deaths, N=5607) of various parameters including lipids, lipoproteins, inflammation markers, and GlycA when added to a base prediction model including 8 covariates according to embodiments of the present invention.

[0063] **Figure 14** is a graph of NMR measured GlycA (NMR signal area units versus CRP (mg/dL) showing linear regressions for hsCRP values <5 and ≥5 mg/dL based on MESA data according to embodiments of the present invention.

[0064] **Figure 15A** is a graph of mean carotid IMT (microns) by quintile of LDL-P, HDL-P and GlycA according to embodiments of the present invention.

[0065] **Figure 15B** is a graph of mean carotid IMT by quintile of a risk index comprised of LDL-P/HDL-P*GlycA according to embodiments of the present invention.

[0066] **Figure 16** is a schematic illustration of different lipoprotein parameters according to embodiments of the present invention.

[0067] **Figure 17** is a schematic illustration of a system for analyzing clinical disease states and/or risk including a GlycA evaluation module and/or circuit using according to embodiments of the present invention.

[0068] **Figure 18** is a schematic illustration of a NMR spectroscopy apparatus according to embodiments of the present invention.

[0069] **Figure 19** is a schematic diagram of a data processing system according to embodiments of the present invention.

[0070] **Figure 20** is a flow chart of exemplary operations that can be used to calculate NMR measures of GlycA according to embodiments of the present invention.

[0071] **Figure 21A** is a flow chart of exemplary operations that can be used to asses a clinical disease state including, by way of example only, CHD risk, according to embodiments of the present invention.

[0072] **Figure 21B** is a flow chart of exemplary operations that can be used to asses a risk of all-cause death according to embodiments of the present invention.

[0073] **Figure 22A** is an example of a patient report that includes a GlycA measurement according to embodiments of the present invention.

[0074] **Figure 22B** is an example of a patient report with the GlycA measurement and a closely spaced corresponding risk summary according to embodiments of the present invention.

[0075] **Figures 22C–22E** are further examples of patient reports according to embodiments of the present invention.

[0076] **Figure 23A** is a prophetic example of a graph of GlycA versus time that can monitor change to evaluate a patient's risk status and/or one or more clinical disease states, change in status, and/or clinical efficacy of a therapy or even used for clinical trials or to contradict planned therapies and the like according to embodiments of the present invention.

[0077] **Figure 23B** illustrates a prophetic example of a graph of a calculated ACD risk ratio over time that can be monitored to assess health status or a change of health status (or a change in risk) according to embodiments of the present invention.

[0078] **Figure 24** is a table from logistic regression analyses adjusted for age, gender, race, smoking, systolic blood pressure, hypertension medications, body mass index, diabetes and LDL-P illustrating all cause death prediction according to embodiments of the present invention.

[0079] **Figure 25A** is a graph of Hazard Ratio versus quintile of (HDL-P*Valine/GlycA) according to some embodiments of the present invention.

[0080] **Figure 25B** is a three dimensional graph of adjusted death rate for GlycA and (HDL-P*Valine) in quartiles for each parameter according to embodiments of the present invention.

[0081] **Figure 26A** is a flow diagram of an NMR valine test protocol according to embodiments of the present invention.

[0082] **Figure 26B** is a flow chart of exemplary pre-analytical processing that can be used prior to obtaining NMR signal of biosamples according to embodiments of the present invention.

[0083] **Figure 26C** is a flow diagram of operations that can be used to evaluate valine using NMR according to embodiments of the present invention. The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

Detailed Description of Embodiments of the Invention

[0084] The present invention now is described more fully hereinafter with reference to the accompanying drawings, in which embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0085] Like numbers refer to like elements throughout. In the figures, the thickness of certain lines, layers, components, elements or features may be exaggerated for clarity. Broken lines illustrate optional features or operations unless specified otherwise.

[0086] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. As used herein, phrases such as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used

herein, phrases such as "between about X and Y" mean "between about X and about Y." As used herein, phrases such as "from about X to Y" mean "from about X to about Y."

[0087] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. Well-known functions or constructions may not be described in detail for brevity and/or clarity.

[0088] It will be understood that, although the terms first, second, etc. may be used herein to describe various elements, components, regions, layers and/or sections, these elements, components, regions, layers and/or sections should not be limited by these terms. These terms are only used to distinguish one element, component, region, layer or section from another region, layer or section. Thus, a first element, component, region, layer or section discussed below could be termed a second element, component, region, layer or section without departing from the teachings of the present invention. The sequence of operations (or steps) is not limited to the order presented in the claims or figures unless specifically indicated otherwise.

[0089] The term "programmatically" means carried out using computer program and/or software, processor or ASIC directed operations. The term "electronic" and derivatives thereof refer to automated or semi-automated operations carried out using devices with electrical circuits and/or modules rather than via mental steps and typically refers to operations that are carried out programmatically. The terms "automated" and "automatic" means that the operations can be carried out with minimal or no manual labor or input. The term "semi- automated" refers to allowing operators some input or activation, but the calculations and signal acquisition as well as the calculation of the concentrations of the ionized constituent(s) is done electronically, typically programmatically, without requiring manual input. The term "about" refers to +/- 10% (mean or average) of a specified value or number.

[0090] The terms" CAD" and "CHD" are used interchangeably to correspond to a patient or subject's risk of developing or having coronary artery and/or coronary heart disease, respectively. The term cardio vascular disease (CVD) refers to a combined outcome that is typically CHD plus stroke.

[0091] The term "all-cause death" refers to dying for any reason, either as a result of a disease such as CHD or cancer, or accident, or natural causes such as old age.

[0092] The term "GlycA" refers to a new biomarker that is derived from a measure of composite NMR signal from carbohydrate portions of acute phase reactant glycoproteins containing N-acetylglucosamine and/or N-acetylgalactosamine moieties, more particularly from the protons of the 2-NAcGlc and 2-NAcGal methyl groups. The GlycA signal is centered at about 2.00 ppm in a plasma NMR spectrum at about 47 deg C. The peak location is independent of spectrometer field but may vary depending on analysis temperature of the biosample. Thus, the GlycA peak region may vary if the temperature of the test sample varies. The GlycA NMR signal may include a subset of NMR signal at the defined peak region so as to include only clinically relevant signal contributions and may exclude a protein contribution to the signal in this region as will be discussed further below.

[0093] The term "GlycB" refers to a new biomarker that is derived from a measure of composite NMR signal from the carbohydrate portions of acute phase reactant glycoproteins containing N-acetylneuraminic acid (sialic acid) moieties, more particularly from the protons of the 5-N-acetyl methyl groups. The GlycB signal is centered at about 2.04 ppm in the plasma NMR spectrum at about 47 deg C. The peak location is independent of spectrometer field but may vary depending on analysis temperature of the biosample. Thus, the GlycB peak region may vary if the temperature of the test sample varies.

[0094] As used herein, the chemical shift locations (ppm) refer to NMR spectra referenced internally to CaEDTA signal at 2.519 ppm. Thus, the noted peak locations discussed and/or claimed herein may vary depending on how the chemical shift is generated or referenced as is well known to those of skill in the art. Thus, to be clear, certain of the described and/or claimed peak locations have equivalent different peak locations in other corresponding chemical shifts as is well known to those of skill in the art.

[0095] The term "biosample" refers to *in vitro* blood, plasma, serum, CSF, saliva, lavage, sputum, or tissue samples of humans or animals. Embodiments of the invention may be particularly suitable for evaluating human blood plasma or serum biosamples. The blood plasma or serum samples may be fasting or non-fasting. GlycA and GlycB are not found in urine and are most suitable for evaluation in blood plasma or serum samples.

[0096] The terms "population norm" and "standard" refer to values defined by a large study or studies such as the Framingham Offspring Study or the Multi-Ethnic Study of Atherosclerosis (MESA) or other study having a large enough sample to be representative of the general population. However, the instant invention is not limited to the population values

in MESA as the presently defined normal and at-risk population values or levels may change over time. Thus, a reference range associated with values from a defined population can be provided and used to assess elevated or reduced levels and/or risk of having a clinical disease state.

[0097] The term "patient" is used broadly and refers to an individual that provides a biosample for testing or analysis.

[0098] The term "clinical disease state" means an at-risk medical condition that may indicate medical intervention, therapy, therapy adjustment or exclusion of a certain therapy (e.g., pharmaceutical drug) and/or monitoring is appropriate. Identification of a likelihood of a clinical disease state can allow a clinician to treat, delay or inhibit onset of the condition accordingly. Examples of clinical disease states include, but are not limited to, CHD, CVD, stroke, type 2 diabetes, prediabetes, dementia, Alzheimers, cancer, arthritis, rheumatoid arthritis (RA), kidney disease, pulmonary disease, COPD (chronic obstructive pulmonary disease), peripheral vascular disease, congestive heart failure, organ transplant response, and/or medical conditions associated with immune deficiency, abnormalities in biological functions in protein sorting, immune and receptor recognition, inflammation, pathogenicity, metastasis and other cellular processes.

[0099] The term "HDL-P" refers to a high density lipoprotein particle (HDL-P) measurement (e.g., HDL-P number) that sums the concentration of defined HDL subclasses. HDL-P can include a total high density lipoprotein particle (HDL-P) measurement that sums the concentration (μmol/L) of all the HDL subclasses (large, medium and small) in the size range between about 7 nm to about 14 nm. In some embodiments, the HDL-P measurement may employ selected combinations of the HDL subclasses in the noted size range.

[00100] As is known to those of skill in the art, valine is an α-amino acid with the chemical formula $HO_2CCH(NH_2)CH(CH_3)_2$. When measured by NMR, the value can be unitless. The valine measurement may be multiplied by a defined conversion factor to convert the value into μmol/L concentration units. The current Valine embodiment has a conversion factor of 2271 to report Valine in μM units; however, this value can vary by ± 10% without unduly affecting the reported value significantly.

[00101] Embodiments of the invention use NMR quantified measures of GlycA of a biosample with a measure of valine and/or at least one lipoprotein subclass to screen for and/or identify a likelihood of a subject's health risk such as, but not limited to, a predictor of a subject's risk of all-cause death.

[00102] GlycA can be represented by a single peak region centered at about 2.00 ppm (at 47 degrees C +/-0.2) in plasma NMR spectra. The peak region location may change with temperature.

[00103] It is believed that GlycA and/or GlycB are responsive to global protein glycosylation level and can reflect global inflammation status. NMR measures of GlycA, alone or with measures of other defined analytes, or ratios of GlycA/GlycB (or vice versa) can be performed as a standard screening test to identify at-risk individuals or subjects.

[00104] GlycA, on its own, may indicate a global measure of inflammation that may be non-specific with respect to a clinical disease state, but such value, particularly when combined with other defined patient-specific parameters may still be a better predictor of all-cause death than conventional total-cholesterol (TC)/ HDL-C ratios. *See*, e.g., the discussion below with respect to **Figures 24** and **25A/25B**, for example.

[00105] GlycA has a positive correlation with death. To evaluate a risk of all-cause death, other parameters can be evaluated using an *in vitro* biosample (the same biosample used to evaluate the GlycA or a different biosample). One or more of the other parameters can have negative correlations with death. Thus, embodiments of the invention employ a multi-parameter risk equation and/or an all cause death (ACD) risk ratio that includes GlycA and at least one parameter that has a negative correlation with death, such as, for example valine and/or HDL-P. In this embodiment, the risk evaluation equation can comprise a ratio of HDL-P/GlycA and/or Valine/GlycA, for example.

[00106] Embodiments of the invention use a multi-parameter equation that includes: (i) an NMR measure of GlycA; (ii) a measure of valine (which may also be measured by NMR or by other analytic tests); and (iii) at least one other lipoprotein (subclass) parameter which may also be measured by NMR or by other analytic tests). The calculation can provide a risk predictor for all-cause death. The at least one other lipoprotein parameter can be HDL-P.

[00107] The multi-parameter equation can be defined by Equation 1.

(HDL-P*Valine)/GlycA **Equation 1**

[00108] HDL-P can be in μmol/L units. Valine and/or GlycA can be in arbitrary units or one or each can be multiplied by a respective defined conversion factor to provide the number in units of μmol/L (*see*, e.g., **Figure 7A**).

[00109] It is contemplated that such a multi-parameter value can also be used to monitor subjects in clinical trials and/or on drug therapies and/or to monitor for changes in

risk status (positive or negative) that may be associated with a particular drug, a patient's lifestyle and the like, which may be patient-specific.

[00110] The flowcharts and block diagrams of certain of the figures herein illustrate the architecture, functionality, and operation of possible implementations of analysis models and evaluation systems and/or programs according to the present invention. In this regard, each block in the flow charts or block diagrams represents a module, segment, operation, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that in some alternative implementations, the functions noted in the blocks might occur out of the order noted in the figures. For example, two blocks shown in succession may in fact be executed substantially concurrently or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved.

[00111] **Figure 1** illustrates the resonant peak regions for GA associated with GlycA and GB associated with GlycB in the plasma NMR spectrum. One or both of these peak regions can include signal that can be defined as inflammation markers in the plasma NMR spectrum.

[00112] **Figures 2A/2B** illustrates the chemical structure of the carbohydrate portion of N-acetylglycosylated proteins showing the CH_3 group that gives rise to the GlycA NMR signal. **Figures 3A/3B** illustrates the chemical structure of the carbohydrate portion of N-acetylneuraminic acid (also called sialic acid) modified glycoproteins showing the CH3 group that gives rise to the GlycB NMR signal.

[00113] **Figure 4A** illustrates a chemical shift portion of the NMR spectrum between 2.080 and 1.845 ppm. **Figure 4A** also illustrates both the calculated C signal and the measured (composite) signal envelope Cm from the allylic protons of the lipids in VLDL, LDL and HDL, with underlying deconvolved GlycA and GlycB and other resonant peaks. GlycA can include contributions from 2-NAcGlc and 2-NAcGal methyl groups. GlycB includes signal from the N-acetyl methyl groups on the sialic acid moieties of glycoproteins.

[00114] A defined mathematical deconvolution model can be used to measure the GlycA and/or GlycB. The "composite" or measured signal envelope Cm can be deconvolved to quantify the signal contributions of GlycA and GlycB and other contributing components such as lipoprotein subclass components. The deconvolution calculates signal amplitudes of the components contributing to the measured signal shapes and calculates the sum of the components. A close match between the calculated signal C and the measured signal Cm

indicates the deconvolution successfully modeled the components that make up the NMR signal.

[00115] The peak region of the GlycA region GA and the peak of the GlycB region GB are shown by the peaks centered at 2.04 ppm and 2.00 ppm (at about 47 deg C sample temperature), respectively, underlying the composite (upper) envelope signal line Cm. In some embodiments, the peak regions for GlycA and GlycB can include adjacent smaller nearby signals in the deconvolution model to account for GlycA and GlycB signals of slightly different frequency.

[00116] The protein signal Ps includes "humps" or peaks P_{GA} and P_{GB} that align with GA and GB, respectively. GlycA can be calculated using the difference between total plasma GlycA signal or "GA" as given by the total peak area of the plasma GlycA signal and "P_{GA}", that portion of GlycA that may derive from the non-inflammatory glycoproteins in the protein (d>1.21 g/L) component of plasma. The deconvolution can be carried out to subtract out the (patient/subject) variable "clinically non-informative" part of the total NMR signal at the GA region to leave the more informative disease association measure of GlycA.

[00117] Stated differently, while not being bound to any particular theory, in some embodiments, the measured GlycA signal at 2.00 ppm can be referred to as GA, the deconvolution can separate it into 3 parts: 1) the part contributed to by the protein (d>1.21 g/L) chosen to be largely devoid of inflammatory proteins, 2) the part contributed to by the non-inflammatory lipoproteins (d<1.21 g/L), and 3) the inflammatory glycoproteins (both lipoprotein and protein), the latter modeled by the overlapping Lorentzians (LGA) or other curve fit functions. The clinically informative GlycA from the deconvolution can be defined as GA minus P_{GA} and minus the non-inflammatory lipoprotein components = LGA. GlycB can be determined in a similar manner using the GB minus P_{GB} signal contribution minus the non-inflammatory lipoprotein components.

[00118] The lineshape deconvolution can be achieved with a non-negative least squares fitting program (Lawson, CL, Hanson RJ, Solving Least Squares Problems, Englewood Cliffs, NJ, Prentice-Hall, 1974). This avoids the use of negative concentrations which can lead to error especially in low signal to noise spectra. Mathematically, a suitable lineshape analysis is described in detail for lipoproteins in the paper by Otvos, JD, Jeyarajah, EJ and Bennett, DW, Clin Chem, 37, 377, 1991. A synthetic baseline correction function may also be used to account for baseline offsets from residual protein components. This can take the form of a quadratic or other polynomial function. Weighting factors are determined and the fit can be optimized by minimizing the root mean squared deviation between the

experimental and calculated spectrum. *See, e.g.*, U.S. Patent Nos. U.S. Patent Nos. 4,933,844 and 6,617,167 for a description of deconvolving composite NMR spectra to measure subclasses of lipoproteins, the contents of which are hereby incorporated by reference as if recited in full herein. *See also,* U.S. Patent No. 7,243,030 for a description of a protocol to deconvolve chemical constituents with overlapping signal contribution, the contents of which are hereby incorporated by reference as if recited in full herein.

[00119] **Figures 4B** and **4C** illustrate the composite (measured) signal "Cm" of the NMR spectra of **Figure 4A** with a fitting region F_R corresponding to the NMR spectrum between 2.080 and 1.845 ppm. The fitting region F_R typically comprises 315 data points but more or less may be used, such as between about 200-400 data points, for example. The GlycA quantification model includes VLDL/chylos components, LDL components, and HDL components. **Table 1** shows various TRLs that may be quantified in a deconvolution model according to embodiments of the present invention.

Table 1: Characteristics of Triglyceride Rich Lipoprotein Subclasses Measured by *NMR LipoProfile®* Analysis

Subclass	TRL Subclass Components	NMR Chemical Shift (ppm)	Estimated Diameter (nm)
Chylomicrons	C-260	0.8477	260
Chylomicrons	C-250	0.8470	250
Chylomicrons	C-240	0.8464	240
Chylomicrons	C-225	0.8457	225
Chylomicrons	C-200	0.8443	200
Chylomicrons	C-190	0.8440	190
Chylomicrons	C-185	0.8436	185
Chylomicrons	C-180	0.8429	180
Chylomicrons	C-175	0.8422	175
Chylomicrons	C-170	0.8416	170
TRL V6	V6-140	0.8402	140
TRL V6	V6-120	0.8388	120
TRL V6	V6-100	0.8374	100
TRL V5	V5-80	0.8361	80
TRL V5	V5-70	0.8347	70
TRL V5	V5-60	0.8333	60

[00120] The term "TRL V6" refers to TRL (triglyceride rich lipoprotein) particles or sub-fractions having a diameter between about 90 nm up to as much as about 170 nm, more typically having diameters between about 100-140 nm. The term "TRL V6" can also be

defined with respect to the lipid methyl group NMR signal chemical shifts (ppm) corresponding to the estimated diameters as provided in **Table 1** above.

[00121] The term "TRL V5" refers to large TRL particles having a diameter of between about 60 nm and about 80 nm (see **Table 1** above for the associated NMR chemical shifts).

[00122] The terms "chylomicron" and "chylos" refer to very large TRL particles having diameters that are larger than TRL V6. As such chylomicrons reters to TRL particles or sub-fractions having a diameter between from about 170 nm up to about 260 nm (see Table 1 below for their associated NMR chemical shifts). There is not a clear demarcation between TRL V5 and TRL V6 nor between TRL V6 and chylomicrons, such that there is a distribution of particle sizes for each subgroup that overlaps in the range between about 80-90 nm for TRL V5-6 and between about 140-170 nm for TRL V6 & chylomicrons.

[00123] When the TRLs are quantified, the concentrations in particle concentration units (nmol/L) or triglyceride concentration units (mg/dL) can be expressed. Thus, for each of the different definitions of "large VLDL", either the particle concentrations or triglyceride concentrations could be used in the DRI model. Without wishing to be bound to any particular theory, based on linear regression analysis, the triglyceride concentration units may yield marginally better diabetes risk prediction.

[00124] **Figures 5A-5D** illustrate spectral overlaps from triglyceride rich lipoproteins as the TG (triglyceride) values increase which can be challenging to reliably deconvolve in a manner that provides precise and reliable GlycA and GlycB measurements.

[00125] The model provides sufficient HDL, LDL and VLDL/chylos components to be able to provide a good fit of the experimental signal as indicated by a close match between calculated signal C and experimental or measured composite signal Cm. Typically, the model will have more of the closely spaced VLDL/chylos components than either LDL or HDL components as these TRL contribute more signal to the left side of the spectrum. The model can include 20-50 VLDL/chylos components, typically about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40. In a preferred embodiment, the model includes 30 VLDL/chylos components.

[00126] The model can include a plurality "N" of (typically overlapping) curve fit components N that populate a sub-region Fs of the fitting region F_R that extends from a few data points (e.g., about 10 or less) to the right of the GlycA measurement region R_1 (e.g., starting at about 1.9 ppm or higher) to at least a few data points to the left of the GlycB region R_2 (and can extend to the end of the fitting region F_R to 2.080 ppm). Each component

N, in this embodiment, can be a Lorentzian-shaped signal with a linewidth about 1.4 Hz. Also, in particular embodiments, each data point can be about 0.275 Hz apart as determined by digital resolution of the spectrum. The tail portion of the region Fs on the left side may include more (Lorentzian) components than the tail portion on the right side. The number of components N in the region Fs n can be about 46 (e.g., about 46 Lorenztians) but more or less components "N" can be used. For example, the region Fs can include, but is not limited to, between 30-70 Lorenztians, or n=30, 35, 36. 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60. The curves N are typically Lorentzian functions with line widths at half-height of between 2-10 data points (0.55-2.75 Hz at 400 MHz), more typically between 4-6 data points, and are offset from each other by a defined amount, such as, for example, 2 data points (0.55 Hz).

[00127] The GlycA and GlycB Lorentzians (or other curve fitting components N) can have the same or different numbers of data points. The GlycB Lorentzians N can have the same, less or more data points than the GlycA Lorentzians N. The Lorentzian fit components "N" can have peak line widths (LW) of about 1.4 Hz (at half height). However, other LWs can be used including, but not limited to, 1.1, 1.2, 1.3, 1.5 and the like.

[00128] GlycA can be calculated using a defined subset of the number of curve fit components N that fill the entire region R_1 and GlycB can be calculated using a suitable number of curve fit (e.g., Lorentzian fit) components N that fill the entire region R_2. The region R_1 can be between 5-6 Hz. The GlycB region R_2 can be 7-8 Hz. Optionally, the GlycA components N can be offset by 2 data points while the GlycB components N can be offset by 4 data points.

[00129] GlycA can be calculated using a sum of adjacent Lorentzian components N, typically between 9-15, such as 9, 10, 11, 12, 13, 14 and 15 components. GlycB can be the sum of adjacent Lorentzian fit components N, with the same, more, or less, typically less, than that used for GlycA measurements, such as between about 5-10 components N, typically about 7, about 8 or about 9 components. The Lorentzians between R_1 and R_2 are not included in the quantified measurement of either GlycA or GlycB. **Figure 4B** illustrates the sum of 7 adjacent Lorentzians used to calculate the GlycB measurement and the sum of 10 (more narrow) Lorentzians can be used to calculate the GlycA measurements. **Figure 4C** illustrates the sum of 9 adjacent Lorentzians used to calculate the GlycB measurement and the sum of 12 (more closely spaced) Lorentzians can be used to calculate the GlycA measurements.

[00130] The number of HDL, LDL and VLDL components may vary. As shown, the HDL components can be 20 HDL components (spanning the range of HDL subclass

diameters), but more or less can be used, e.g., between about 10-26. As shown, the number of LDL components is 9 components (representing different LDL diameters), but more or less can be used, e.g., between about 5-20. As shown, the number of VLDLs/Chylos components is 30, but more or less can be used, e.g., 25-60 of different size ranges.

[00131] To be clear, while a preferred embodiment describes the curve fit components as Lorentzian fit components, other fitting components may be used including, but not limited to, experimental N-acetyl methyl group signals or Gaussian lineshape functions. Thus, any suitable curve fit function can be used.

[00132] **Figure 6A** is a table of different protein components (Protein 1, Protein 2 and Protein 3) that, when used in the Glyc deconvolution model, yields different GlycA concentrations and different GlycA associations with CHD events and All-Cause Death in MESA. **Figures 6B**, **6C** and **6D** illustrate the respective protein signal Ps in the deconvolved spectrum and the differences they exhibit in the amplitudes of the signals in the GlycA and GlycB peak regions. To optimize the calculated GlycA and/or GlycB measurement, in some embodiments, the deconvolution model includes a defined protein signal component as discussed above. This protein signal component Ps is for protein other than lipoproteins, e.g., other than HDL, LDL, VLDL/chylos, e.g., and may be associated with the >1.21 g/L density fraction of plasma obtained by ultracentrifugation (which includes albumin and other non-lipoprotein proteins in plasma).

[00133] The table in **Figure 6A** represents associations of GlycA with CHD events (n=289) and All Cause Death (n=346) that are from logistic regression analysis adjusted for age, gender, race, smoking, systolic blood pressure, hypertension medications, body mass index, diabetes, LDL-P and HDL-P. The likelihood ration χ^2 statistic gives a quantitative measure of the extent to which GlycA improves prediction of outcomes when added to the 10 covariates in the regression model.

[00134] This signal component "Ps" is shown in **Figures 4A-4C**. Surprisingly, although this protein signal Ps does include a peak (P_{GA}, P_{GB}, respectively) aligned with the peak at the chemical shift for both GlycA and GlycB, eliminating this portion of the protein NMR signal from the deconvolution model (by, for example, digital manipulation or signal processing) was found to make the calculated GlycA and GlycB measurements relatively less clinically informative (weaker disease associations). At the other extreme, including in the deconvolution model a protein component with a relatively large signal at the GlycA and GlycB positions results in lower GlycA and GlycB concentrations that are also less clinically informative, as shown for Protein #2 and Protein #3 in **Figures 6C** and **6D**. Thus, by

selecting an appropriate protein component with an intermediate signal amplitude at the GlycA and GlycB positions, such as Protein #1 in **Figure 6B**, the deconvolution model may be "tuned" to produce GlycA and GlycB concentrations that are improved and/or optimized with respect to their clinical associations with inflammation and related disease states.

[00135] Thus, in some embodiments, it is contemplated that the GlycA measurement will provide a better clinical indicator if it does not include the lipoprotein signal (accounted for in the deconvolution model with the VLDL/chylo, LDL and HDL components) and if it includes only a portion of the remaining NMR signal, e.g., it does not include all other NMR protein signal at the GlycA peak region. This subset of the NMR signal at the GlycA peak region may be more reflective of inflammatory protein activity, e.g., N-acetyl methyl signals from glycosylated acute phase proteins.

[00136] **Figure 7A** is a screen shot of the deconvolution of a 10 mmol/L reference standard sample of N-acetylglucosamine, from which a conversion factor of 17.8 was determined to transform signal area concentrations of GlycA and GlycB to μmol/L glycoprotein N-acetyl methyl group concentrations. In some embodiments, according to MESA subjects, first to fourth quartile (mean) levels of GlycA (**Figure 14**) can be: Q1: 21.6*17.8, Q2: 25.8*17.8, Q3: 29.3*17.8 and Q4: 35.3*17.8.

[00137] GlycA measurement precision using the model shown in **Figure 4B** was shown to be good. A within-run (5 pools from 2009) analysis of lowest GlycA=40.5 (CV =2.47%) and highest GlycA =58.4 (CV=1.6%). Within-lab results from 13 pools from 2010 and 2011 had a lowest GlycA =25.6 (CV=4.08%) and highest GlycA=69.1 (CV=1.87%). These concentrations are expressed as "arbitrary units" of NMR signal areas and can be multiplied by 17.8 to convert them to μmol/L N-acetyl methyl group concentrations.

[00138] **Figure 7B** is a table of components that may be used in a GlycA/B deconvolution model. Metabolite A is one component that can be measured in a GlycA/B deconvolution model and may be used clinically. As illustrated in **Figures 7C** and **7D**, metabolite A can be present in a spectrum as a singlet peak and is typically present in a sample at low concentrations (**Figure 7C**), but a high concentration of metabolite A may be present in a sample (**Figures 7D**). A plurality of curve fitting functions for the metabolite A peak region can be used to quantitatively evaluate a level of metabolite A and/or to deconvolve the NMR spectrum for quantification of GlycA and/or GlycB, for example.

[00139] The deconvolving model components shown in **Figure 7B** list a plurality of curve fit functions Glyc1-Glyc46 that can be applied to a fitting region that includes the

GlycA peak region and extends to a GlycB peak region (typically with between about 40-50 curve fit functions, shown as with 46, but less or more such curve fit functions may be used, e.g., between 30-100). As discussed above, the GlycA measurement can be carried out by summing values of a defined first subset of the curve fit functions, values associated with all or some of the Glyc1-Glyc 26 components, for example. The GlycB measurement can be carried out by summing values of a second (typically smaller) defined subset of the curve fit functions, such as some or all components between Glyc27 and Glyc 46, for example.

[00140] **Figures 8A** and **8B** are histogram graphs of NMR-measured GlycA values from two different study populations. **Figure 8B** provides NMR measures of GlycA for 182 women with rheumatoid arthritis (RA), a known chronic inflammatory disease, while **Figure 8A** gives values from the MESA population of healthy men and women without known inflammatory disease.

[00141] It is believed that the measured amplitude of the GlycA signal in any one sample may have the advantage of providing a more stable and "time-integrated" measure of the patient's inflammation state than is provided by measurements of hs-CRP or other individual inflammatory proteins.

[00142] **Figure 9** is a chart of prospective associations of hs-CRP and NMR measured GlycA and Valine levels with various exemplary disease outcomes based on MESA data (n ≈5680). The chart was generated from logistic regression analyses adjusted for age, gender, race, smoking, systolic blood pressure, hypertension medications, body mass index, diabetes, LDL-P and HDL-P. The likelihood ratio statistic $\chi 2$ gives a quantitative measure of the extent to which the indicated variable improves disease prediction when added to the 10 covariates in the regression model. The analyses used GlycA measurement values from the deconvolution model shown in **Figure 4B**. The right side column shows that GlycA and Valine are additive in their associations with disease when they both have significant associations examined separately.

[00143] In some embodiments, the NMR measurement can include both GlycA and Valine, and each can be provided in a patient report for clinical consideration. **Figure 10A** is a graph showing a region of plasma NMR spectrum containing methyl signals from lipoproteins and branched-chain amino acids (leucine, valine and isoleucine). **Figure 10B** is an example of a deconvolved signal with a quartet of valine signals identified to generate a calculated C and measured Cm spectrum of the valine signals with two doublets of valine NMR signal (between about 0.90-1.07) that can be used to measure valine.

[00144] It is contemplated that NMR measurements of both GlycA and Valine of a single (blood/plasma) *in vitro* biosample can provide important clinical information and/or further improve a prediction or evaluation of a patient or subject's risk of having a clinical disease state or being at increased risk for same and/or to evaluate risk of all-cause death.

[00145] As is generally accepted, HDL-cholesterol and/or LDL-cholesterol levels provided by conventional lipid panels fail to sufficiently differentiate populations with and without elevated risk for CHD/CAD. As is known to those of skill in the art, the Framingham study proposed a relatively lengthy risk model that considers many factors such as age, gender, smoking habits, as well as cholesterol values. The research conducted in the Framingham Offspring Study also defined normative and at-risk population values from subjects in the study. *See* Wilson et al., *Impact of National Guidelines for Cholesterol Risk Factor Screening. The Framingham Offspring Study*, JAMA, 1989; 262: 41-44.

[00146] It is contemplated that inflammation is a separate contributor to heart attack risk. While not wishing to be bound to any particular theory of metabolic activity and risk, it is contemplated that, for example, if a patient has a stable form of atherosclerosis (the plaque or associated protein is stable within an arterial wall, for example), then the risk of a cardiac event may be less than if that same patient also exhibits inflammation, which may weaken a protein cover that makes the plaque susceptible to rupture, and hence present an increased risk of CHD and/or myocardial infarction (MI).

[00147] According to particular embodiments, NMR measures of GlycA, alone or with other defined analytes (e.g., one or more of GlycB, HDL-P, LDL-P, Mg or Valine), can provide a more robust and more readily measured health/death risk assessment over hs-CRP, the latter of which is present in a respective patient *in vitro* sample in very low levels.

[00148] As noted above, **Figure 7A** illustrates a conversion factor that can be used to calculate measurements of GlycA. The GlycA measurement can also be a unit less parameter as assessed by NMR by calculating an area under a peak region at a defined peak in NMR spectra. In any event, measures of GlycA with respect to a known population (such as MESA) can be used to define the level or risk for certain subgroups, e.g., those having values within the upper half of a defined range, including values in the third and fourth quartiles, or the upper 3-5 quintiles and the like.

[00149] **Figure 8A** illustrates a distribution of GlycA values for subjects from MESA. Bell et al., Assignment of resonances for 'acute-phase' glycoproteins in high resolution proton NMR spectra of human blood plasma, FEBS Letters, Vol. 215, No. 2, pp. 311-315 (1987) proposed that measurement of acute-phase reactive plasma glycoproteins could be of

considerable value in the detection, prognosis and therapeutic monitoring of patients with tissue damage and compared "normal" levels with patients having melanoma, rheumatoid arthritis, and monoclonal gammopathy. The contents of this reference are hereby incorporated by reference as if recited in full herein. Despite the foregoing, and the many years since the publication of this research, the inventors of the instant patent application have unexpectedly found that despite the non-specific nature of the general inflammation information provided by acute phase reactive plasma glycoproteins, increased levels of GlycA can be a robust biomarker for CHD risk, stronger than hs-CRP.

[00150] **Figure 11** is a Table of mean values of various characteristics of MESA subjects within each of four quartiles of NMR measured GlycA. The mean GlycA level of those in the 3rd quartile is 29.3. This table shows that people with higher GlycA levels have characteristics associated with higher inflammation (more smoking, hypertension, hs-CRP, etc). NMR signal area units can be called "arbitrary" units. The GlycA levels in this table are in these "arbitrary units" that may be converted to methyl group concentration units (umol/L) by multiplying by 17.8.

[00151] **Figure 12** is a chart of different models used to predict CHD events in MESA N=5607 with n=289 CHD events from Cox logistic regression models adjusted for age, sex, ethnicity, smoking, systolic blood pressure (SBP), hypertension medication treatment (HTNrx), BMI (body mass index), diabetes LDL-P and HDL-P status. The model $\chi 2$ statistic provides a quantitative measure of the extent to which the added variable in the model improves CHD risk prediction. The parameter/marker $\chi 2$ statistic and corresponding "p" value quantify the magnitude and statistical significance of the improved prediction given by the inflammation marker to the model. Notably, as shown in **Figure 12**, GlycA has a 10.4 χ^2 value, more than fibrinogen and hs-CRP (indeed, hs-CRP had the lowest of the three parameters, with a marker $\chi 2$ of 1.8).

[00152] **Figure 13** is a table of associations with All-Cause Death in MESA N=5607, 345 deaths. The Cox models were adjusted for age, sex, ethnicity, smoking, SPB, hypertension medication, BMI and diabetes. HDL-P, GlycA and a model with both HDL-P and GlycA parameters in the base model showed improved risk prediction.

[00153] **Figure 14** is a graph of NMR GlycA (signal area units) versus hs-CRP (mg/dL) based on MESA data. Linear regressions are shown for 2 hs-CRP ranges, 0-5 and >5 mg/dL. The steeper slope of the regression for lower values of CRP shows that GlycA is reflective of chronic inflammation status, but is less reflective of differences in acute inflammation that pertain to those with CRP levels >5 mg/dL.

[00154] **Figure 15A** is a graph of mean carotid IMT (microns) by quintile of LDL-P, GlycA and HDL-P from linear regression models adjusted for age, sex, ethnicity, SBP, hypertension medication, diabetes, BMI and smoking from unpublished MESA data. Elevated levels of both LDL-P and GlycA are associated with greater carotid IMT values, reflecting greater atherosclerotic risk.

[00155] **Figure 15B** is a graph of a single risk predictor of carotid IMT (related to CHD/CVD) based on the ratio of LDL-P/HDL-P multiplied by GlycA for carotid IMT (microns) versus quintile value based on unpublished data of MESA and the linear regression models discussed for **Figure 15A.**

[00156] Embodiments of the invention contemplate that patient biosamples can be analyzed for increased levels of GlycA relative to a patient's own "baseline" and/or relative to a population standard or "norm" or other defined elevated level associated with an increased medical "risk". GlycA may also or alternatively be used as one parameter in a multiple parameter risk prediction model for a clinical disease state. The multiple parameter model for CHD can include lipoprotein parameters.

[00157] **Figure 16** illustrates examples of lipoprotein subclass groupings, including those with concentrations that can be summed to determine HDL-P and LDL-P for CHD management/risk assessment according to some particular embodiments of the present invention. Embodiments of the invention classify lipoprotein particles into subclasses grouped by size ranges based on functional/metabolic relatedness as assessed by their correlations with lipid and metabolic variables. The evaluations can measure over 20 discrete subpopulations (sizes) of lipoprotein particles, typically between about 30-80 different size subpopulations (or even more). **Figure 16** also shows these discrete sub-populations can be grouped into defined subclasses, including three each for VLDL and HDL and two or three for LDL (if the former, with one of the three identified as IDL in the size range between large LDL and small VLDL). For the GlycA and/or GlycB measurement calculations, the discrete number of HDL and LDL groupings can be less than those used to quantitatively measure the lipoprotein subclasses.

[00158] HDL subclass particles typically range (on average) from between about 7 nm to about 15 nm, more typically about 7.3 nm to about 14 nm or 7.3-13.5 nm. The HDL-P concentration is the sum of the particle concentrations of the respective subpopulations of its HDL-subclasses, e.g., small HDL-P can include H1-H8 subpopulations. The large HDL subclasses can have a size range between 9.4-14 nm, typically between 9.7-13.5 nm. The HDL subclasses of different size can be quantified from the amplitudes of their

spectroscopically distinct lipid methyl group NMR signals. *See*, Jeyarajah et al., *Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy*, Clin Lab Med. 2006; 26: pp. 847-870, the contents of which are hereby incorporated by reference as if recited in full herein. The NMR derived HDL-P and LDL-P particle sizes noted herein typically refer to average measurements, but other size demarcations may be used.

[00159] As shown in **Figure 16**, the small LDL particles can include particles whose sizes range from between about 18.0 to about 20.5 nm, typically between 19-20 nm. The large LDL particles can include particles ranging in diameter between about 20.5-23.0 nm. It is noted that the LDL subclasses of particles can be divided in other size ranges. For example, small may be between about 19.0-20.5 nm, intermediate may be between about 20.5-21.2 nm, and large may be between about 21.2-23 nm. In addition, intermediate-density lipoprotein particles ("IDL" or "IDL-P"), which range in diameter from approximately 23.0-29.0 nm, can be included among the particles defined as LDL. Thus, LDL particles can include sizes between 19.0-28 nm, for example. VLDL can have sizes between 29-100 nm or 29-160 nm, with large VLDL between 60 nm and 100 or 160 nm, respectively and chylos between 100-260 nm or 160 nm to 260 nm, respectively

[00160] Referring now to **Figure 17**, it is contemplated that the GlycA and/or Glyc B measurement analysis can be carried out using a system **10** with an NMR clinical analyzer **22** as described, for example, with respect to **Figure 18** below and/or in U.S. Patent No. 8,013,602, the contents of which are hereby incorporated by reference as if recited in full herein. The analyzer **22** includes a spectrometer **22s** and sample handler system.

[00161] The system **10** can include a GlycA analysis module and/or circuit **20** that can be onboard the analyzer **22** or at least partially remote from the analyzer **22**. If the latter, the analysis module or circuit **20** can reside totally or partially on a server **150**. The server **150** can be provided using cloud computing which includes the provision of computational resources on demand via a computer network. The resources can be embodied as various infrastructure services (e.g. computer, storage, etc.) as well as applications, databases, file services, email, etc. In the traditional model of computing, both data and software are typically fully contained on the user's computer; in cloud computing, the user's computer may contain little software or data (perhaps an operating system and/or web browser), and may serve as little more than a display terminal for processes occurring on a network of external computers. A cloud computing service (or an aggregation of multiple cloud resources) may be generally referred to as the "Cloud". Cloud storage may include a model of networked computer data storage where data is stored on multiple virtual servers, rather than being

hosted on one or more dedicated servers. Data transfer can be encrypted and can be done via the Internet using any appropriate firewalls to comply with industry or regulatory standards such as HIPAA. The term "HIPAA" refers to the United States laws defined by the Health Insurance Portability and Accountability Act. The patient data can include an accession number or identifier, gender, age and test data.

[00162] The results of the analysis can be transmitted via a computer network, such as the Internet, via email or the like to a patient, clinician site **50**, to a health insurance agency **52** or a pharmacy **51**. The results can be sent directly from the analysis site or may be sent indirectly. The results may be printed out and sent via conventional mail. This information can also be transmitted to pharmacies and/or medical insurance companies, or even patients that monitor for prescriptions or drug use that may result in an increase risk of an adverse event or to place a medical alert to prevent prescription of a contradicted pharmaceutical agent. The results can be sent to a patient via email to a "home" computer or to a pervasive computing device such as a smart phone or notepad and the like. The results can be as an email attachment of the overall report or as a text message alert, for example.

[00163] Referring now to **Figure 18**, a system **207** for acquiring and calculating the lineshape of a selected sample is illustrated. The system **207** includes an NMR spectrometer **22s** for taking NMR measurements of a sample. In one embodiment, the spectrometer **22s** is configured so that the NMR measurements are conducted at 400 MHz for proton signals; in other embodiments the measurements may be carried out at between 200 MHz to about 900 MHz or other suitable frequency. Other frequencies corresponding to a desired operational magnetic field strength may also be employed. Typically, a proton flow probe is installed, as is a temperature controller to maintain the sample temperature at 47 +/- 0.5 degrees C. The spectrometer **22** is controlled by a digital computer **214** or other signal processing unit. The computer **211** should be capable of performing rapid Fourier transformations. It may also include a data link **212** to another processor or computer **213**, and a direct-memory-access channel **214** which can connects to a hard memory storage unit **215**.

[00164] The digital computer **211** may also include a set of analog-to-digital converters, digital-to-analog converters and slow device I/O ports which connect through a pulse control and interface circuit **216** to the operating elements of the spectrometer **22s**. These elements include an RF transmitter **217** which produces an RF excitation pulse of the duration, frequency and magnitude directed by at least one digital signal processor that can be onboard or in communication with the digital computer **211**, and an RF power amplifier **218** which amplifies the pulse and couples it to the RF transmit coil **219** that surrounds sample

cell **220** and/or flow probe **220p**. The NMR signal produced by the excited sample in the presence of a 9.4 Tesla polarizing magnetic field produced by superconducting magnet **221** is received by a coil 222 and applied to an RF receiver **223**. The amplified and filtered NMR signal is demodulated at **224** and the resulting quadrature signals are applied to the interface circuit **216** where they are digitized and input through the digital computer **211**. The lipoprotein measurement and/or GlycA analyzer circuit **20** and/or module **350** (**Figures 18-19**) can be located in one or more processors associated with the digital computer **211** and/or in a secondary computer **213** or other computers that may be on-site or remote, accessible via a worldwide network such as the Internet **227**.

[00165] After the NMR data are acquired from the sample in the measurement cell **220**, processing by the computer **211** produces another file that can, as desired, be stored in the storage **215**. This second file is a digital representation of the chemical shift spectrum and it is subsequently read out to the computer **213** for storage in its storage **225** or a database associated with one or more servers. Under the direction of a program stored in its memory or accessible by the computer **213**, the computer **213**, which may be a laptop computer, desktop computer, workstation computer, electronic notepad, electronic tablet, smartphone or other device with at least one processor or other computer, processes the chemical shift spectrum in accordance with the teachings of the present invention to generate a report which may be output to a printer **226** or electronically stored and relayed to a desired email address or URL. Those skilled in this art will recognize that other output devices, such as a computer display screen, electronic notepad, smartphone and the like, may also be employed for the display of results.

[00166] It should be apparent to those skilled in the art that the functions performed by the computer **213** and its separate storage **225** may also be incorporated into the functions performed by the spectrometer's digital computer **211**. In such case, the printer **226** may be connected directly to the digital computer **211**. Other interfaces and output devices may also be employed, as are well-known to those skilled in this art.

[00167] Certain embodiments of the present invention are directed at providing methods, systems and/or computer program products that use GlycA evaluations that may be particularly useful in automated screening tests of clinical disease states and/or risk assessment evaluations for screening of *in vitro* biosamples.

[00168] Embodiments of the present invention may take the form of an entirely software embodiment or an embodiment combining software and hardware aspects, all generally referred to herein as a "circuit" or "module."

[00169] As will be appreciated by one of skill in the art, the present invention may be embodied as an apparatus, a method, data or signal processing system, or computer program product. Accordingly, the present invention may take the form of an entirely software embodiment, or an embodiment combining software and hardware aspects. Furthermore, certain embodiments of the present invention may take the form of a computer program product on a computer-usable storage medium having computer-usable program code means embodied in the medium. Any suitable computer readable medium may be utilized including hard disks, CD-ROMs, optical storage devices, or magnetic storage devices.

[00170] The computer-usable or computer-readable medium may be, but is not limited to, an electronic, magnetic, optical, electromagnetic, infrared, or semiconductor system, apparatus, device, or propagation medium. More specific examples (a non-exhaustive list) of the computer-readable medium would include the following: an electrical connection having one or more wires, a portable computer diskette, a random access memory (RAM), a read-only memory (ROM), an erasable programmable read-only memory (EPROM or Flash memory), an optical fiber, and a portable compact disc read-only memory (CD-ROM). Note that the computer-usable or computer-readable medium could even be paper or another suitable medium, upon which the program is printed, as the program can be electronically captured, via, for instance, optical scanning of the paper or other medium, then compiled, interpreted or otherwise processed in a suitable manner if necessary, and then stored in a computer memory.

[00171] Computer program code for carrying out operations of the present invention may be written in an object oriented programming language such as Java7, Smalltalk, Python, Labview, C++, or VisualBasic. However, the computer program code for carrying out operations of the present invention may also be written in conventional procedural programming languages, such as the "C" programming language or even assembly language. The program code may execute entirely on the user's computer, partly on the user's computer, as a stand-alone software package, partly on the user's computer and partly on a remote computer or entirely on the remote computer. In the latter scenario, the remote computer may be connected to the user's computer through a local area network (LAN) or a wide area network (WAN), or the connection may be made to an external computer (for example, through the Internet using an Internet Service Provider).

[00172] The flowcharts and block diagrams of certain of the figures herein illustrate the architecture, functionality, and operation of possible implementations of analysis models and evaluation systems and/or programs according to the present invention. In this regard,

each block in the flow charts or block diagrams represents a module, segment, operation, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that in some alternative implementations, the functions noted in the blocks might occur out of the order noted in the figures. For example, two blocks shown in succession may in fact be executed substantially concurrently or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved.

[00173] **Figure 19** is a block diagram of exemplary embodiments of data processing systems **305** that illustrates systems, methods, and computer program products in accordance with embodiments of the present invention. The processor **310** communicates with the memory **314** via an address/data bus **348**. The processor **310** can be any commercially available or custom microprocessor. The memory **314** is representative of the overall hierarchy of memory devices containing the software and data used to implement the functionality of the data processing system **305**. The memory **314** can include, but is not limited to, the following types of devices: cache, ROM, PROM, EPROM, EEPROM, flash memory, SRAM, and DRAM.

[00174] As shown in **Figure 19**, the memory **314** may include several categories of software and data used in the data processing system **305**: the operating system **352**; the application programs **354**; the input/output (I/O) device drivers **358**; a GlycA Evaluation Module **350**; and the data **356**. The GlycA Evaluation Module **350** can deconvolve NMR signal to reveal a defined NMR signal peak region in proton NMR spectra of a respective biosample to identify a level of GlycA. The system **305** may also include one or more of a Clinical Disease State Evaluation Module **370** or a Risk Prediction Module **375** that considers the level of the measured GlycA or generates a composite risk number or multi-parameter risk model. The system **305** may also or alternatively include an All Cause Death (ACD) Risk Evaluation Module **378**. The ACD Module can programmatically calculate at least one ratio using GlycA in the denominator.

[00175] The data **356** may include signal (constituent and/or composite spectrum lineshape) data **362** which may be obtained from a data or signal acquisition system **320** (e.g., NMR spectrometer **22s** and/or analyzer **22**). As will be appreciated by those of skill in the art, the operating system **352** may be any operating system suitable for use with a data processing system, such as OS/2, AIX or OS/390 from International Business Machines Corporation, Armonk, NY, WindowsCE, WindowsNT, Windows95, Windows98, Windows2000 or WindowsXP from Microsoft Corporation, Redmond, WA, PalmOS from

Palm, Inc., MacOS from Apple Computer, UNIX, FreeBSD, or Linux, proprietary operating systems or dedicated operating systems, for example, for embedded data processing systems.

[00176] The I/O device drivers **358** typically include software routines accessed through the operating system **352** by the application programs **354** to communicate with devices such as I/O data port(s), data storage **356** and certain memory **314** components and/or the image acquisition system **320**. The application programs **354** are illustrative of the programs that implement the various features of the data processing system **305** and can include at least one application, which supports operations according to embodiments of the present invention. Finally, the data **356** represents the static and dynamic data used by the application programs **354**, the operating system **352**, the I/O device drivers **358**, and other software programs that may reside in the memory **314**.

[00177] While the present invention is illustrated, for example, with reference to the Module **350** being an application program in **Figure 19**, as will be appreciated by those of skill in the art, other configurations may also be utilized while still benefiting from the teachings of the present invention. For example, the GlycA Module **350**, and, where used, one or more of the Clinical Disease State Evaluation Module **370**, the Risk Predictor Module **375** or the ACD Risk Evaluation Module **378** may also be incorporated into the operating system **352**, the I/O device drivers **358** or other such logical division of the data processing system **305**. Thus, the present invention should not be construed as limited to the configuration of **Figure 19**, which is intended to encompass any configuration capable of carrying out the operations described herein.

[00178] In certain embodiments, the Module **350** includes computer program code for providing a level of GlycA which may be used as an inflammation marker to assess a clinical disease state or risk and/or to indicate whether therapy intervention is desired and/or track efficacy of a therapy or even an unintended consequence of a therapy.

[00179] **Figure 20** is a flow chart of exemplary operations that can carry out embodiments of the present invention. A (measured) composite envelope NMR spectrum of NMR spectra of a fitting region of a biosample (e.g., blood plasma or serum) can be obtained (block **400**). The NMR composite signal envelope is electronically deconvolved using a defined model having HDL, LDL and VLDL/Chylos components and a plurality of curve fit (e.g., Lorentzian) functions associated with at least a GlycA peak region centered at a defined chemical shift location (e.g., 2.00 ppm) associated with GlycA (block **402**). A defined number of (e.g., Lorentzian and/or Gaussian) curve fit functions for the peak region

associated with GlycA can be summed (block **415**). A conversion factor can be applied to the summed functions to generate a calculated measurement of GlycA (block **420**).

[00180] The GlycA and/or GlycB measurement can be provided in a patient and/or clinical trial report (block **422**). The report can identify or alert as to whether he or she is at risk of having or developing a clinical disease state and/or whether additional screening may be appropriate, based at least in part on the GlycA measurement (block **424**).

[00181] The defined model can include a protein signal component at a density greater than about 1.21g/L, typically above 1.21 g/L, that can be deconvolved/ separated from the signal composite envelope (block **403**).

[00182] The subset of the fitting region can include or extend through a peak region centered at 2.04 ppm for GlycB (block **412**). A defined number of (e.g. Lorentzian and/or Gaussian) curve fit functions for the peak region associated with GlycB can be summed and the conversion factor can be applied to generate a calculated measurement of GlycB (block **414**).

[00183] **Figure 21A** is a flow chart of exemplary operations that can carry out embodiments of the present invention. GlycA in an *in vitro* biosample can be measured using a defined peak region in proton NMR spectra (block **500**). A patient's risk of CHD can be determined, at least in part, based on the GlycA measurement (block **510**).

[00184] The risk can be based on an *a priori* correlation of the measurement relative to a population norm or standard. The GlycA value and risk association may have a substantially continuous relationship. The increased risk can be associated with an NMR measured GlycA value above a defined population norm. It is contemplated that the risk value or range of values may be associated with values in quartiles 3 and 4, in tertile 3 or above or in quartile 4 or above, as defined by a Hazard Ratio.

[00185] The methods may also include calculating HDL-P (block **505**) and LDL-P (block **508**) using NMR subclass measurements. These parameters can also be evaluated for determining the patient's CHD risk.

[00186] The methods may include electronically providing a patient risk report with the GlycA measurement and an associated CHD risk summary (block **512**). The risk summary can provide a relative comparison to defined population norms.

[00187] **Figure 21B** is a flow chart of exemplary operations that can be used to carry out other embodiments of the present invention. As shown, GlycA in an *in vitro* blood sample is electronically measured using a using a defined deconvolution protocol and a defined peak in proton NMR spectra (block **515**). The method then determines whether a

patient is at increased risk for all cause death (ACD) based on the measured GlycA and at least one of (i) a measurement of valine or (ii) a measurement of HDL-P (block **520**). The measurements for the ACD evaluation can be obtained using NMR signal of the same *sample.*

[00188] The determination can be based on a calculation of ACD risk using a ratio of valine/GlycA and/or HDL-P/GlycA (box **516**).

[00189] The determination can be based on a calculation of ADC risk using a ratio of (valine*HDL-P)/GlycA (block **518**). The method may include placing the sample in an NMR spectrometer and collecting NMR spectra before the measuring step (block **517**).

[00190] The method may include providing a risk report with the ACD measurement and an associated risk summary (block **522**). The risk can be based on an *a priori* correlation of the measurement relative to a population norm or standard (e.g., MESA data or another defined study population). Increased risk of ACD can be associated with a ratio that is below a defined population norm. It is contemplated that the risk value or range of values may be associated with values in Q1 of the ratio (with a highest risk associated with a Q4 or Q5 value of GlycA and a Q1 value of valine or HDL-P and/or Q1 of (valine*HDL-P) as defined by a Hazard Ratio.

[00191] **Figure 22A** is a schematic illustration of an exemplary patient test report **100** that can include various lipoprotein parameters such as one or more of Valine, HDL-P, LDL-P, VLDL and GlycA **101**. The GlycA number **101** can be presented with risk assessment summary **101s** correlated to population norms, typical ranges, and/or degree of risk (e.g., high, increased or low risk), shown as a sliding scale graph in **Figure 22B**. **Figure 22C** is an example of a report **100** with a report that includes the NMR measured GlycA value **101** with a risk summary **101s**, and one or both of a GlycA/GlycB ratio value **102**, and an NMR measure of Valine **103**. Ratios or other combinations of these and other analytes or parameters can be provided.

[00192] **Figure 22D** is an example of a report **100** with the NMR measured GlycA **101**, risk summary **101s**, and either (i) at least one other defined NMR measured analyte **101a** with a visual notified at-risk indication **101v** (either visual indicia of follow-up screening needed or a likelihood of a clinical disease state) or (ii) a notification of a contradicted therapy so as to alert a patient/clinician to avoid certain drug therapies.

[00193] **Figure 22E** is an example of a report **100** with a calculated ACD risk **101e** indicating a person at elevated ACD risk relative to a population norm.

[00194] However, other risk summary configurations may be used including ranges, high to low or low to high, or just noting whether the associated risk is low, medium or increased and/or high.

[00195] **Figure 23A** illustrates that a graph **130** of GlycA values over time can be provided to illustrate a change in patient health and/or inflammatory status over time due to age, medical intervention or a therapy according to some embodiments. Tracking this parameter may provide a clinical indicator of efficacy of a therapy and/or a better risk predictor for CHD other clinical disease states or all-cause death risk, for patients.

[00196] As shown in **Figure 23A**, the analysis can be used to monitor a patient over time to correlate known start or use of a drug or other therapy. Future drugs or uses of known drugs can be identified, screened or tested in patients identified using GlycA evaluations.

[00197] **Figure 23B** illustrates a graph of a calculated ACD risk ratio **101r** using GlycA in the denominator can be monitored over time similarly to the GlycA alone. It is contemplated that both values may also be monitored for changes over time reflective of a therapy or change in health status and/or to evaluate a patient's risk status and/or change in status, clinical efficacy of a therapy or even used for clinical trials or to contradict planned therapies.

[00198] **Figure 24** is a table showing prediction of all-cause death in MESA (n=5712). The prediction of all-cause death (n=346) for various parameters and combinations of parameters is from logistic regression analyses adjusted for age, gender, race, smoking, systolic blood pressure, hypertension medications, body mass index, diabetes, and LDL-P. The model likelihood ratio χ^2 statistic provides a quantitative measure of the risk prediction given by that model, allowing comparisons of how much each parameter improves prediction of incident death when added to the 9 covariates in the regression model. As shown, HDL-P, GlycA and valine are all strong predictors of ACD and each is independent of the other. Thus, alone each is better than the conventional TC/HDL-C ratio and combined these factors can provide an even more effective way to stratify people in terms of their ACD risk.

[00199] **Figure 25A** illustrates how risk of death differs as a function of the calculated risk predictor in quintiles of (HDL-P*Valine)/GlycA. **Figure 25A** is a graph of Hazard Ratio for all-cause death (n=346) from Cox proportional hazards regression analyses adjusted for age, gender, and smoking. The risk for values in the first quintile (Q1) are almost double (about 1.8 X) that in the second quintile (Q2).

[00200] **Figure 25B** is a three-dimensional graph (16 subgroups) of adjusted death rate (%)as a function of GlycA quartiles (x-axis) and HDL-P*Valine quartiles (y-axis). The lowest death rate (4.4%) is seen for those with low GlycA (Q1) and high HDL-P*Valine (Q4), whereas the highest death rate (14.1%) is observed for those with high GlycA (Q4) and low HDL-P*Valine (Q1).The death rate (%) is based on a 7-year follow-up period, adjusted for age, gender and smoking.

[00201] **Figures 26A-26C** are exemplary flow diagrams of operations that can be used to obtain NMR signal associated with valine according to embodiments of the present invention.

[00202] **Figure 26A** illustrates that a pre-analytical evaluation (block **610**) can occur before a valine region of the NMR signal is determined (block **625**), then deconvolved (block **650**). **Figure 26B** illustrates an exemplary pre-analytical evaluation **610** which includes delivery verification of the sample into the flow cell as either complete failure (block **612**) or partial injection failure (block **613**), shimming verification (block **615**), temperature verification (block **617**) and a citrate tube detection (failure) (block **619**), all using defined characteristics of signal associated with a defined diluent added to the sample.

[00203] Referring again to **Figure 26A**, once the defined parameters are confirmed within limits, the pre-analytical quality control analysis can end (block **620**) and the determination of the valine region can be identified (block **625**) and the spectrum deconvolved and valine level calculated (block **650**). Optionally, a post-analytical quality control can be electronically performed (block **655**) and the results output (block **660**). The results can be included in a test report with comments, visual indicia of high or low and the like (block **665**).

[00204] Referring to **Figure 26C**, a method/analysis **700** of operations that can be used to quantify valine are shown. NMR signal can be electronically obtained of an *in vitro* biosample with a defined added diluent (block **702**). The QC evaluation can be carried out (block **710**). The valine region is determined (block **725**). The valine region is deconvolved (block **750d**) and an NMR derived value of valine is calculated (**750c**).

[00205] The diluents can comprise calcium ethylenediamine tetraacetic acid (Ca EDta) (block **703**) or other suitable diluent that creates a reliable peak and behaves in a predictable manner. Well established chemical shift or quantitation references include, for example, formate, trimethylsilylpropionate (and isotopically labeled isomers), and EDTA.

[00206] The pre-analytical quality control evaluation **710** can be based on inspection of characteristics of the CaEDTA reference peak and the system or processor can be

configured not to perform the Valine test unless the NMR spectra have been acquired under specified conditions such as those shown in **Figure 26B**. The sample temperature can be 47 ± 0.5 °C in the flow cell for NMR scans/signal acquisition. The sample can comprise diluents in a 1:1 ratio (block **705**) or other defined ratio (e.g., more sample, less diluents or more diluent; less sample, e.g., 2:1 or more sample, less diluents, e.g. 1:2).

[00207] The test sample can be rejected with a defined error code if CaEDTA height > 140 for any acquired spectrum (block **719**). This high value is indicative of detection of the citrate peak in conjunction with the CaEDTA peak. The citrate peak is introduced by collection of the specimen in an improper citrate tube. By disrupting the ability to locate the exact position of the CaEDTA peak, the citrate peak can disrupt the process for determining the Valine region.

[00208] The Valine region is located upfield relative to the position of the CaEDTA peak. The broad peaks beneath Valine are various methyl (-CH_3-) protons of lipoproteins. The CaEDTA location can be determined at approximately 22258 ± 398 data points (block **721**). The Valine region can be determined independently for each acquired spectrum. The Valine signal can be modeled with suitable data points using, for example, 25 data points (center ± 12 data points) for each peak of the quartet or 300 data points for the valine region of both doublets, but other numbers of data points may be used. The measurement of Valine can be carried out using one, two, three or all four peaks of the valine peak quartet.

[00209] All basis set spectra can be linearly interpolated before utilized by the non-negative least squares algorithm. The spectra to be analyzed and the basis set spectra can have a zero baseline offset modification before utilized by the non-negative least squares algorithm.

[00210] The start of the Valine region can be at about 2196-4355 data points, typically the latter when including both doublets, (the "Valine region offset") upfield from the location of the CaEDTA peak (block **722**). In some embodiments, the start of the valine region is at 4355 data points upfield from the location of the CaEDTA peak.

[00211] In some embodiments, the valine quantification is carried out by characterizing the valine resonances at between 0.0-1.01 ppm as two doublets. Three or more valine experimental spectra stepped by two data points can be used as basis sets to model valine signal. The center valine peaks can be located by sliding three valine components +/- 15 data points and determined through a least squares sum minimization. The valine signal can be modeled with a total of about 300 data points.

[00212] Each basis set, including those used for the baseline but excluding the DC offset, are offset such that the lowest value is subtracted from the function (making the lowest point equal to 0). This prevents inclusion of a DC offset in the shapes they represent.

[00213] The Valine region from each acquired spectrum is deconvolved with a series of analyte and baseline functions which have been treated to the same type of pre-processing as the acquired spectra. The deconvolution coefficient for each component can be multiplied by an associated conversion factor. The current Valine embodiment has a conversion factor of 2271 to report Valine in μM units; however, this value can vary by ± 10% without unduly affecting the reported value significantly.

Component Name	Filename	Conversion Factor	Basis Function Starting position relative to CaEDTA
Valine1	Valine318LB019.1r	2271	-4353
Valine2	Valine318LB019.1r	2271	-4355
Valine3	Valine318LB019.1r	2271	-4357

[00214] The resulting values are summed. Result values produced independently for each acquired spectrum can be averaged to generate final values to use in the measurement.

[00215] Data can be acquired using presaturation water suppression from a 1:1 diluted sample and can include between 5-20 scans, typically about 10 scans stored as 5 blocks of 2 (5 FIDs consisting of 2 scans each) (block **726**).

[00216] The pulse sequence used in conjunction with presaturation water suppression can optionally include a presaturation (water suppression) pulse and a suitable excitation pulse. FIDs can be acquired with 9024 data points with a sweep width of 4496.4 Hz. Each FID can be multiplied with a shifted Gaussian function:

$e^{-\left(\frac{(t-gfs)}{gf}\right)^2}$, or in computer terms, exp(–((t–gfs)/gf)^2), where gfs = 0.2 seconds and gf = 0.2 seconds.

[00217] This can be performed prior to Fourier transformation with zero-filling which yields the frequency-domain GM spectrum for each FID consisting of 16,384 data points (block **627**). The spectra can be phased using the calibration-specified phase value. The spectra can be scaled (multiplied) by a calibration-specified scaling factor. All basis set spectra can be linearly interpolated before utilized by the non-negative least squares

algorithm. The spectra to be analyzed and the basis set spectra can have a zero baseline offset modification before utilized by the non-negative least squares algorithm (*e.g.*, all components used for the model and the spectrum that will be analyzed can be linearly interpolated) (block **728**). To determine the center of the valine fitting region, the valine resonances between 0.9 and 1.0 as two doublets can be characterized and the center peaks can be identified by sliding three valine components ± 15 data points (block **729**).

Examples of Uses of GlycA and/or GlycB alone or with other parameters and/or analytes

[00218] All-Cause Death (ACD) Risk Prediction

[00219] ACD Risk Ratios

[00220] CHD Risk Evaluation

[00221] Diabetes Evaluation

[00222] CHD Risk Evaluation using GlycA and HDL-P and/or LDL-P

[00223] Mass Screening for At-Risk Medical Condition. The NMR GlycA measurement can be a standardized test parameter that can be generated from a blood plasma sample being analyzed for other test parameters including for example, magnesium, valine, lipoprotein particles such as HDL-P or LDL-P and the like.

[00224] Identification of a likelihood that the patient has or is at risks of having a Clinical Disease State.

[00225] Evaluating Drug Therapy for intended or unintended reactions and/or contradictions.

[00226] Evaluating Patient Response in Clinical Trials.

[00227] Evaluating Animal Response in Drug Development/Screening.

[00228] Use of GlycA/GlycB Ratio to identify risk or a clinical disease state.

[00229] Use of GlycA with one or more Secondary Analytes including HDL-P, LDL-P, Mg or Valine to identify risk or a clinical disease state.

[00230] Generating a risk predictor index using HDL-P/LDL-P*GlycA for CHD risk.

[00231] Monitoring Inflammation Status of a Patient over Time to indicate when medical intervention may be appropriate (additional screening or therapy).

[00232] Evaluating risk of developing or whether a person has Dementia using GlycA. The Dementia evaluation can use both GlycA and valine measurements as separate components and/or as a ratio.

[00233] Evaluating a patient's risk of stroke based on the measured level of GLycA and Mg.

[00234] Evaluating risk of colorectal cancer using GlycB.

[00235] The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. Although a few exemplary embodiments of this invention have been described, those skilled in the art will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the claims. In the claims, means-plus-function clauses, where used, are intended to cover the structures described herein as performing the recited function and not only structural equivalents but also equivalent structures. Therefore, it is to be understood that the foregoing is illustrative of the present invention and is not to be construed as limited to the specific embodiments disclosed, and that modifications to the disclosed embodiments, as well as other embodiments, are intended to be included within the scope of the appended claims. The invention is defined by the following claims, with equivalents of the claims to be included therein.

CLAIMS:

1. A method of measuring GlycA, an NMR biomarker derived from a measure of composite NMR signal from carbohydrate portions of acute phase reactant glycoproteins containing N-acetylglucosamine and/or N-acetylgalactosamine moieties, comprising:

obtaining a composite NMR spectrum of a fitting region of a blood plasma or serum sample of a subject;

deconvolving the composite NMR spectrum using a defined deconvolution model with a plurality of subclasses of high density lipoprotein (HDL) components, low density lipoprotein (LDL) components, VLDL (very low density lipoprotein)/chylomicron components, and a plurality of curve fit functions associated with at least a GlycA peak region; and

generating a measure of GlycA using the curve fit functions,

wherein the GlycA NMR biomarker is derived from NMR signal of the protons of N-acetylglucosamine and N-acetylgalactosamine methyl groups, and

wherein the GlycA peak region is centered at 2.00 ppm and the fitting region extends from 1.845 ppm to 2.080 ppm if the sample is at 47+/−0.2 degrees C. in an NMR spectrometer.

2. The method of claim 1, further comprising applying a conversion factor to the measure of GlycA to provide the measure in μmol/L.

3. The method of claim 1, wherein the curve fit functions are overlapping Lorentzian functions, and wherein the measure of GlycA is generated by summing a defined number of Lorentzian functions.

4. The method of claim 1, wherein the deconvolution model further comprises a protein signal component for protein having a density greater than 1.21 g/L.

5. The method of claim 2, wherein the deconvolution model further comprises a protein signal component for protein having a density greater than 1.21 g/L, and wherein the method further comprises generating a risk predictor comprising a mathematical equation of:

HDL-P/LDL-P*GlycA, HDL-P/GlycA, or Valine/GlycA,

wherein HDL-P is an NMR measure of HDL particle number and LDL-P is an NMR measure of LDL particle number and GlycA in the equation is the GlycA measurement, all in μmol/L.

6. The method of claim 1, wherein the plurality of curve fit functions includes between about 40-50 curve fit functions.

7. The method of claim 1, wherein the GlycA fitting region of the NMR spectrum is referenced internally to CaEDTA signal at 2.519 ppm.

8. The method of claim 1, further comprising obtaining a second measure of GlycA at a second point in time, wherein the GlycA values are compared to evaluate whether there is a change in inflammatory status over time due to age, medical intervention or a therapy.