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(71) Applicant: METABOLON, INC. [US/US]; 617 Davis Drive, Suite 400, Durham, NC 27713 (US).

(72) Inventors: GALL, Walter; 312 West University Drive, Chapel Hill, NC 27516 (US). COBB, Jeffery, Edmond; 1015 Tallyho Trail, Chapel Hill, NC 27516 (US). PAPPAN, Kirk, Lane; 6015 Laws Farm Road, Rougemont, NC 27572 (US).

(74) Agents: ELLIOTT, Allyn, B. et al.; Brinks Gilson & Lione, P.O. Box 110285, Research Triangle Park, NC 27709 (US).

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(54) Title: BIOMARKERS RELATED TO INSULIN RESISTANCE PROGRESSION AND METHODS USING THE SAME

(57) Abstract: Biomarkers relating to insulin resistance and insulin resistance-related disorders are provided, as well as methods for using such biomarkers as biomarkers for insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease. In addition, methods for monitoring the respective disorders or conditions of a subject are also provided. Also provided are suites of small molecule entities as biomarkers for insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease.

## BIOMARKERS RELATED TO INSULIN RESISTANCE PROGRESSION AND METHODS USING THE SAME

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### Cross-Reference to Related Applications

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/758,924, filed January 31, 2013, the entire contents of which are hereby incorporated herein by reference.

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### Field

[0002] The invention generally relates to biomarkers for prediction of the progression of insulin resistance and susceptibility to and development of type 2 diabetes and cardiovascular disease in pre-diabetic individuals, methods for identifying biomarkers predictive of progression of insulin resistance, type 2 diabetes and cardiovascular disease and/or insulin resistance and insulin resistance-related disorders and methods based on the same biomarkers.

### Background

[0003] Diabetes is classified as either type 1 (early onset) or type 2 (adult onset), with type 2 comprising 90-95% of the cases of diabetes. Diabetes is the final stage in a disease process that begins to affect individuals long before the diagnosis of diabetes is made. Type 2 diabetes develops over 10 to 20 years and results from an impaired ability to utilize glucose (glucose utilization, glucose uptake in peripheral tissues) due to impaired sensitivity to insulin (insulin resistance).

[0004] Moreover, insulin resistance is central to development of a number of different diseases and conditions, such as cardiovascular disease, metabolic syndrome, nonalcoholic steatohepatitis (NASH), polycystic ovary syndrome (PCOS), and hypertension.

[0005] In pre-diabetes, insulin becomes less effective at helping tissues metabolize glucose. Pre-diabetics may be detectable as early as 20 years before diabetic symptoms become evident. Studies have shown that although patients show

very few overt symptoms, long-term physiological damage is already occurring at this stage. Up to 60% of these individuals will progress to type 2 diabetes within 10 years.

**[0006]** The American Diabetes Association (ADA) has recommended routine screening to detect patients with pre-diabetes. Current screening methods for pre-diabetes include the fasting plasma glucose (FPG) test, the oral glucose tolerance test (OGTT), the fasting insulin test and the hyperinsulinemic euglycemic clamp (HI clamp). The first two tests are used clinically whereas the latter two tests are used extensively in research but rarely in the clinic. In addition, mathematical means (e.g., HOMA, QUICKI) that consider the fasting glucose and insulin levels together have been proposed. However, normal plasma insulin concentrations vary considerably between individuals as well as within an individual throughout the day. Further, these methods suffer from variability and methodological differences between laboratories and do not correlate rigorously with HI clamp studies.

**[0007]** Worldwide, an estimated 194 million adults have type 2 diabetes and this number is expected to increase to 333 million by 2025, largely due to the epidemic of obesity in westernized societies. In the United States, it is estimated that over 54 million adults are pre-diabetic. There are approximately 1.5 million new cases of type 2 diabetes a year in the United States. The annual US healthcare cost for diabetes is estimated at \$174 billion. This figure has risen more than 32% since 2002. In industrialized countries such as the U.S., about 25% of medical expenditures treat glycemic control, 50% is associated with general medical care associated with diabetes, and the remaining 25% of the costs go to treat long-term complications, primarily cardiovascular disease. Considering the distribution of the healthcare costs and the fact that insulin resistance is a direct causal factor in cardiovascular disease and diabetes progression, it is no surprise that cardiovascular disease accounts for 70-80% of the mortality observed for diabetic patients. Detecting and preventing type 2 diabetes has become a major health care priority.

**[0008]** Diabetes may also lead to the development of other diseases or conditions, or is a risk factor in the development of conditions such as Metabolic Syndrome and cardiovascular diseases. Metabolic Syndrome is the clustering of a set of risk factors in an individual. According to the American Heart Association these risk factors include: abdominal obesity, decreased ability to properly process glucose (insulin resistance or glucose intolerance), dyslipidemia (high triglycerides, high LDL, low

HDL cholesterol), hypertension, prothrombotic state (high fibrinogen or plasminogen activator inhibitor-1 in the blood) and proinflammatory state (elevated C-reactive protein in the blood). Metabolic Syndrome is also known as syndrome X, insulin resistance syndrome, obesity syndrome, dysmetabolic syndrome and Reaven's

5 syndrome. Patients diagnosed with Metabolic Syndrome are at an increased risk of developing diabetes, cardiac and vascular disease. It is estimated that, in the United States, 20% of the adults (>50 million people) have metabolic syndrome. While it can affect anyone at any age, the incidence increases with increasing age and in individuals who are inactive, and significantly overweight, especially with excess

10 abdominal fat.

**[0009]** Type 2 diabetes is the most common form of diabetes in the United States. According to the American Diabetes Foundation over 90% of the US diabetics suffer from Type 2 diabetes. Individuals with Type 2 diabetes have a combination of increased insulin resistance and decreased insulin secretion that combine to cause

15 hyperglycemia. Most persons with Type 2 diabetes have Metabolic Syndrome.

**[0010]** The diagnosis for Metabolic Syndrome is based upon the clustering of three or more of the risk factors in an individual. A variety of medical organizations have definitions for the Metabolic Syndrome. The criteria proposed by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III), with

20 minor modifications, are currently recommended and widely used in the United States.

**[0011]** The American Heart Association and the National Heart, Lung, and Blood Institute recommend that the metabolic syndrome be identified as the presence of three or more of these components: increased waist circumference (Men—equal to or 25 greater than 40 inches (102 cm), Women—equal to or greater than 35 inches (88 cm); elevated triglycerides (equal to or greater than 150 mg/dL); reduced HDL (“good”) cholesterol (Men—less than 40 mg/dL, Women—less than 50 mg/dL); elevated blood pressure (equal to or greater than 130/85 mm Hg); elevated fasting glucose (equal to or greater than 100 mg/dL).

30 **[0012]** Type 2 diabetes develops slowly and often people first learn they have type 2 diabetes through blood tests done for another condition or as part of a routine exam. In some cases, type 2 diabetes may not be detected before damage to eyes, kidneys or other organs has occurred. A need exists for an objective, biochemical

evaluation (e.g. lab test) that can be administered by a primary care provider to identify individuals that are at risk of developing Metabolic Syndrome or Type 2 diabetes.

5 [0013] Newer, more innovative molecular diagnostics that reflect the mechanisms of the patho-physiological progression to pre-diabetes and diabetes are needed because the prevalence of pre-diabetes and diabetes is increasing in global epidemic proportions. Mirroring the obesity epidemic, pre-diabetes and diabetes are largely preventable but are frequently undiagnosed or diagnosed too late due to the asymptomatic nature of the progression to clinical disease.

10 [0014] Although insulin resistance plays a central role in the development of numerous diseases, it is not readily detectable using many of the clinical measurements for pre-diabetic conditions. Insulin resistance develops prior to the onset of hyperglycemia and is associated with increased production of insulin. Over time (decades) the ability of the cell to respond to insulin decreases and the subject 15 becomes resistant to the action of insulin (i.e., insulin resistant, IR). Eventually the beta-cells of the pancreas cannot produce sufficient insulin to compensate for the decreased insulin sensitivity and the beta-cells begin to lose function and apoptosis is triggered. Beta-cell function may be decreased as much as 80% in pre-diabetic subjects. As beta-cell dysfunction increases the production of insulin decreases 20 resulting in lower insulin levels and high glucose levels in diabetic subjects. Vascular damage is associated with the increase in insulin resistance and the development of type 2 diabetes.

25 [0015] Therefore there is an unmet need for diagnostic biomarkers and tests that can determine the risk of developing insulin resistance, type 2 diabetes, and cardiovascular disease in subjects within at least 3 years to at least 5 years and for progression of insulin resistance to type 2 diabetes and/or cardiovascular disease in non-diabetic subjects with insulin resistance. Insulin resistance biomarkers and diagnostic tests can better identify and determine the risk of cardiovascular disease and/or diabetes development in an at risk subject and/or a pre-diabetic subject, can 30 monitor disease development and progression and/or regression, can allow new therapeutic treatments to be developed and can be used to test therapeutic agents for efficacy on reversing insulin resistance and/or preventing insulin resistance and

related diseases. Further, a need exists for diagnostic biomarkers to more effectively assess the efficacy and safety of pre-diabetic and diabetic therapeutic candidates.

## Detailed Description

[0016] The present invention relates to biomarkers predictive of an individual's risk to develop insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease; methods of determining predisposition to insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease; methods of monitoring progression/regression of dysglycemia, type-2 diabetes, and cardiovascular disease; methods of assessing efficacy of treatments and compositions for treating insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease; methods of screening compositions for activity in modulating biomarkers of insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease; methods of treating insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease; methods of identifying subjects for treatment with insulin resistant, dysglycemia, type-2 diabetes, and cardiovascular disease therapies; methods of identifying subjects for inclusion in clinical trials of insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease therapies; as well as other methods based on biomarkers of insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease.

[0017] Current blood tests for insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease perform poorly for early detection of insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease or involve significant medical procedures.

[0018] In one embodiment, groups (also referred to as "panels") of metabolites that can be used in a simple blood, urine, etc. test to predict insulin resistance, dysglycemia, type-2 diabetes, and cardiovascular disease are identified using metabolomic analysis. Independent studies were carried out to identify a set of biomarkers that when used with a polynomic algorithm enables the early detection of changes in insulin sensitivity, dysglycemia, type-2 diabetes, and/or cardiovascular disease in a subject. The biomarkers of the instant disclosure can be used to provide a score indicating the probability of insulin resistance, dysglycemia, type-2 diabetes, and/or cardiovascular disease in a subject (e.g., "Risk Score"). The score can be based upon a clinically significant changed reference level for a biomarker and/or

combination of biomarkers. The reference level can be derived from an algorithm or computed from indices for impaired glucose tolerance and can be presented in a report. The Risk Score places the subject in the risk range of insulin resistance, dysglycemia, type-2 diabetes, and/or cardiovascular disease from normal (low risk) to 5 high risk and/or can be used to determine a probability that the subject has insulin resistance, dysglycemia, type-2 diabetes, or cardiovascular disease. Disease progression or remission can be monitored by periodic determination and monitoring of the Risk Score. Response to therapeutic intervention can be determined by monitoring the Risk Score. The Risk Score can also be used to evaluate drug efficacy 10 or to identify subjects to be treated with insulin resistance, dysglycemia, type-2 diabetes, and/or cardiovascular disease therapies, such as insulin sensitizers, or to identify subjects for inclusion in clinical trials.

**[0019]** Prior to describing this invention in further detail, however, the following terms will first be defined.

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Definitions:

**[0020]** “Biomarker” means a compound, preferably a metabolite, that is differentially present (i.e., increased or decreased) in a biological sample from a 20 subject or a group of subjects having a first phenotype (e.g., having a disease) as compared to a biological sample from a subject or group of subjects having a second phenotype (e.g., not having the disease). A biomarker may be differentially present at any level, but is generally present at a level that is increased by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, by at least 50%, by at least 55%, by at least 60%, by at least 65%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 100%, by at least 110%, by at least 120%, by at least 130%, by at least 140%, by at least 150%, or more; or is generally 25 present at a level that is decreased by at least 5%, by at least 10%, by at least 15%, by at least 20%, by at least 25%, by at least 30%, by at least 35%, by at least 40%, by at least 45%, by at least 50%, by at least 55%, by at least 60%, by at least 65%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, or by 100% (i.e., absent). A biomarker is preferably differentially present 30 at a level that is statistically significant (e.g., a p-value less than 0.05 and/or a q-value

of less than 0.10 as determined using either Welch's T-test or Wilcoxon's rank-sum Test). Alternatively, the biomarkers demonstrate a correlation with insulin resistance, or particular levels or stages of insulin resistance. The range of possible correlations is between negative (-) 1 and positive (+) 1. A result of negative (-) 1 means a perfect 5 negative correlation and a positive (+) 1 means a perfect positive correlation, and 0 means no correlation at all. A "substantial positive correlation" refers to a biomarker having a correlation from +0.25 to +1.0 with a disorder or with a clinical measurement (e.g., Rd), while a "substantial negative correlation" refers to a correlation from -0.25 to -1.0 with a given disorder or clinical measurement. A 10 "significant positive correlation" refers to a biomarker having a correlation of from +0.5 to +1.0 with a given disorder or clinical measurement (e.g., Rd), while a "significant negative correlation" refers to a correlation to a disorder of from -0.5 to -1.0 with a given disorder or clinical measurement.

15 [0021] The "level" of one or more biomarkers means the absolute or relative amount or concentration of the biomarker in the sample.

20 [0022] "Sample" or "biological sample" or "specimen" means biological material isolated from a subject. The biological sample may contain any biological material suitable for detecting the desired biomarkers, and may comprise cellular and/or non-cellular material from the subject. The sample can be isolated from any suitable biological tissue or fluid such as, for example, adipose tissue, aortic tissue, liver tissue, blood, blood plasma, saliva, serum, cerebrospinal fluid, cystic fluid, exudates, or urine.

25 [0023] "Subject" means any animal, but is preferably a mammal, such as, for example, a human, monkey, non-human primate, rat, mouse, cow, dog, cat, pig, horse, or rabbit.

30 [0024] A "reference level" of a biomarker means a level of the biomarker that is indicative of a particular disease state, phenotype, or lack thereof, as well as combinations of disease states, phenotypes, or lack thereof. A "positive" reference level of a biomarker means a level that is indicative of a particular disease state or phenotype. A "negative" reference level of a biomarker means a level that is indicative of a lack of a particular disease state or phenotype. For example, an "insulin resistance-positive reference level" of a biomarker means a level of a biomarker that is indicative of a positive diagnosis of insulin resistance in a subject,

and an “insulin resistance-negative reference level” of a biomarker means a level of a biomarker that is indicative of a negative diagnosis of insulin resistance in a subject. As another example, an “insulin resistance-progression-positive reference level” of a biomarker means a level of a biomarker that is indicative of progression of insulin

5 resistance in a subject, and an “insulin resistance-regression-positive reference level” of a biomarker means a level of a biomarker that is indicative of regression of insulin resistance. A “reference level” of a biomarker may be an absolute or relative amount or concentration of the biomarker, a presence or absence of the biomarker, a range of amount or concentration of the biomarker, a minimum and/or maximum amount or

10 concentration of the biomarker, a mean amount or concentration of the biomarker, and/or a median amount or concentration of the biomarker; and, in addition, “reference levels” of combinations of biomarkers may also be ratios of absolute or relative amounts or concentrations of two or more biomarkers with respect to each other. A “reference level” may also be a “standard curve reference level” based on

15 the levels of one or more biomarkers determined from a population and plotted on appropriate axes to produce a reference curve (e.g. a standard probability curve).

Appropriate positive and negative reference levels of biomarkers for a particular disease state, phenotype, or lack thereof may be determined by measuring levels of desired biomarkers in one or more appropriate subjects, and such reference levels may

20 be tailored to specific populations of subjects (e.g., a reference level may be age-matched so that comparisons may be made between biomarker levels in samples from subjects of a certain age and reference levels for a particular disease state, phenotype, or lack thereof in a certain age group). A standard curve reference level may be determined from a group of reference levels from a group of subjects having a

25 particular disease state, phenotype, or lack thereof (e.g. known glucose disposal rates) using statistical analysis, such as univariate or multivariate regression analysis, logistic regression analysis, linear regression analysis, and the like of the levels of such biomarkers in samples from the group. Such reference levels may also be tailored to specific techniques that are used to measure levels of biomarkers in

30 biological samples (e.g., LC-MS, GC-MS, etc.), where the levels of biomarkers may differ based on the specific technique that is used.

**[0025]** “Non-biomarker compound” means a compound that is not differentially present in a biological sample from a subject or a group of subjects having a first

phenotype (e.g., having a first disease) as compared to a biological sample from a subject or group of subjects having a second phenotype (e.g., not having the first disease). Such non-biomarker compounds may, however, be biomarkers in a biological sample from a subject or a group of subjects having a third phenotype (e.g., 5 having a second disease) as compared to the first phenotype (e.g., having the first disease) or the second phenotype (e.g., not having the first disease).

**[0026]** “Metabolite”, or “small molecule”, means organic and inorganic molecules which are present in a cell. The term does not include large macromolecules, such as 10 large proteins (e.g., proteins with molecular weights over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000), large nucleic acids (e.g., nucleic acids with molecular weights of over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000), or large polysaccharides (e.g., polysaccharides with a molecular weights of over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000). The small 15 molecules of the cell are generally found free in solution in the cytoplasm or in other organelles, such as the mitochondria, where they form a pool of intermediates which can be metabolized further or used to generate large molecules, called macromolecules. The term “small molecules” includes signaling molecules and intermediates in the chemical reactions that transform energy derived from food into usable forms. Examples of small molecules include sugars, fatty acids, amino acids, 20 nucleotides, intermediates formed during cellular processes, and other small molecules found within the cell.

**[0027]** “Metabolic profile”, or “small molecule profile”, means a complete or partial inventory of small molecules within a targeted cell, tissue, organ, organism, or fraction thereof (e.g., cellular compartment). The inventory may include the quantity 25 and/or type of small molecules present. The “small molecule profile” may be determined using a single technique or multiple different techniques.

**[0028]** “Metabolome” means all of the small molecules present in a given organism.

**[0029]** “Diabetes” refers to a group of metabolic diseases characterized by high 30 blood sugar (glucose) levels which result from defects in insulin secretion or action, or both.

**[0030]** “Type 2 diabetes” or “T2D” refers to one of the two major types of diabetes, the type in which the beta cells of the pancreas produce insulin, at least in

the early stages of the disease, but the body is unable to use it effectively because the cells of the body are resistant to the action of insulin. In later stages of the disease the beta cells may stop producing insulin. Type 2 diabetes is also known as insulin-resistant diabetes, non-insulin dependent diabetes and adult-onset diabetes.

5 [0031] “Pre-diabetes” refers to one or more early diabetes-related conditions including impaired glucose utilization, abnormal or impaired fasting glucose levels, impaired glucose tolerance, impaired insulin sensitivity and insulin resistance.

[0032] “Insulin resistant” refers to the condition when cells become resistant to the effects of insulin — a hormone that regulates the uptake of glucose into cells — or 10 when the amount of insulin produced is insufficient to maintain a normal glucose level. Cells are diminished in the ability to respond to the action of insulin in promoting the transport of the sugar glucose from blood into muscles and other tissues (i.e. sensitivity to insulin decreases). Eventually, the pancreas produces far more insulin than normal and the cells continue to be resistant. As long as enough 15 insulin is produced to overcome this resistance, blood glucose levels remain normal. Once the pancreas is no longer able to keep up, blood glucose starts to rise, resulting in diabetes. Insulin resistance ranges from normal (insulin sensitive) to insulin resistant (IR).

[0033] “Insulin sensitivity” refers to the ability of cells to respond to the effects of 20 insulin to regulate the uptake and utilization of glucose. Insulin sensitivity ranges from normal (insulin sensitive) to Insulin Resistant (IR).

[0034] The “Risk Score” or “Disease Risk (DR) Score” is a measure of the probability of insulin resistance, dysglycemia, type-2 diabetes, and/or cardiovascular disease in a subject. As used herein, the term Disease Risk Score or DR Score is used 25 generically to exemplify the invention and may refer to risk of any of the diseases associated with insulin resistance and pre-diabetes while a specific Risk Score refers to that specific disease. For example, an “IR Risk Score” is based upon the predicted glucose disposal rate calculated using the insulin resistance biomarkers (e.g. along with models and/or algorithms) that will allow a physician to determine the probability that a subject is insulin resistant. Risk Scores for determining the 30 probability that a subject is dysglycemic (e.g., “Dysglycemia Risk Score”), type-2 diabetic (e.g., “T2D Risk Score”), and/or has cardiovascular disease (e.g., “CVD Risk Score”) are based upon measuring the levels of biomarkers for dysglycemia, type-2

diabetes or cardiovascular disease and using said measurements in a mathematical model (e.g. statistical model, algorithm).

5 [0035] “Glucose utilization” refers to the absorption of glucose from the blood by muscle and fat cells and utilization of the sugar for cellular metabolism. The uptake of glucose into cells is stimulated by insulin.

[0036] “Rd” refers to glucose disposal rate (Rate of disappearance of glucose), a metric for glucose utilization. The rate at which glucose disappears from the blood (disposal rate) is an indication of the ability of the body to respond to insulin (i.e. insulin sensitive). There are several methods to determine Rd and the 10 hyperinsulinemic euglycemic clamp is regarded as the “gold standard” method. In this technique, while a fixed amount of insulin is infused, the blood glucose is “clamped” at a predetermined level by the titration of a variable rate of glucose infusion. The underlying principle is that upon reaching steady state, by definition, glucose disposal is equivalent to glucose appearance. During hyperinsulinemia, 15 glucose disposal (Rd) is primarily accounted for by glucose uptake into skeletal muscle, and glucose appearance is equal to the sum of the exogenous glucose infusion rate plus the rate of hepatic glucose output (HGO). The rate of glucose infusion during the last 30 minutes of the test determines insulin sensitivity. If high levels of glucose (Rd = 7.5 mg/kg/min or higher) are required, the patient is insulin-sensitive. 20 Very low levels (Rd = 4.0 mg/kg/min or lower) of required glucose indicate that the body is resistant to insulin action. Levels between 4.0 and 7.5 mg/kg/min (Rd values between 4.0 mg/kg/min and 7.5 mg/kg/min) of required glucose are not definitive and suggest sensitivity to insulin is impaired and that the subject may have “impaired glucose tolerance,” which may sometimes be a sign of insulin resistance.

25 [0037] “Mffm” and “Mwbm” refer to glucose disposal (M) calculated as the mean rate of glucose infusion during the past 60 minutes of the clamp examination (steady state) and expressed as milligrams per minute per kilogram of fat free mass (ffm) or whole body mass (wbm). Subjects with an Mffm less than 45 umol/min/kg ffm are generally regarded as insulin resistant. Subjects with an Mwbm of less than 5.6 30 mg/kg/min are generally regarded as insulin resistant.

[0038] “Impaired fasting glucose (IFG)” and “impaired glucose tolerance (IGT)” are the two clinical definitions of “pre-diabetes”. IFG is defined as a fasting blood glucose concentration of 100-125 mg/dL. IGT is defined as a postprandial (after

eating) blood glucose concentration of 140-199 mg/dL. It is known that IFG and IGT do not always detect the same pre-diabetic populations. Between the two populations there is approximately a 60% overlap observed. Fasting plasma glucose levels are a more efficient means of inferring a patient's pancreatic function, or insulin secretion, 5 whereas postprandial glucose levels are more frequently associated with inferring levels of insulin sensitivity or resistance. IGT is known to identify a greater percentage of the pre-diabetic population compared to IFG. The IFG condition is associated with lower insulin secretion, whereas the IGT condition is known to be strongly associated with insulin resistance. Numerous studies have been carried out 10 that demonstrate that IGT individuals with normal FPG values are at increased risk for cardiovascular disease. Patients with normal FPG values may have abnormal postprandial glucose values and are often unaware of their risk for pre-diabetes, diabetes, and cardiovascular disease.

15 [0039] “Fasting plasma glucose (FPG) test” is a simple test measuring blood glucose levels after an 8 hour fast. According to the ADA, blood glucose concentration of 100-125 mg/dL is considered IFG and defines pre-diabetes whereas  $\geq 126$  mg/dL defines diabetes. As stated by the ADA, FPG is the preferred test to diagnose diabetes and pre-diabetes due to its ease of use, patient acceptability, lower cost, and relative reproducibility. The weakness in the FPG test is that patients are 20 quite advanced toward Type 2 Diabetes before fasting glucose levels change.

25 [0040] “Oral glucose tolerance test (OGTT)”, a dynamic measurement of glucose, is a postprandial measurement of a patient's blood glucose levels after oral ingestion of a 75 g glucose drink. Traditional measurements include a fasting blood sample at the beginning of the test, a one hour time point blood sample, and a 2 hour time point blood sample. A patient's blood glucose concentration at the 2 hour time point defines the level of glucose tolerance: Normal glucose tolerance (NGT)  $\leq 140$  mg/dL blood glucose; Impaired glucose tolerance (IGT) = 140-199 mg/dL blood glucose; Diabetes  $\geq 200$  mg/dL blood glucose. As stated by the ADA, even though the OGTT is known to be more sensitive and specific at diagnosing pre-diabetes and diabetes, it 30 is not recommended for routine clinical use because of its poor reproducibility and difficulty to perform in practice.

[0041] “Fasting insulin test” measures the circulating mature form of insulin in plasma. The current definition of hyperinsulinemia is difficult due to lack of

standardization of insulin immunoassays, cross-reactivity to proinsulin forms, and no consensus on analytical requirements for the assays. Within-assay CVs range from 3.7%-39% and among-assay CVs range from 12%-66%. Therefore, fasting insulin is not commonly measured in the clinical setting and is limited to the research setting.

5 [0042] The “hyperinsulinemic euglycemic clamp (HI clamp)” is considered worldwide as the “gold standard” for measuring insulin resistance in patients. It is performed in a research setting, requires insertion of two catheters into the patient and the patient must remain immobilized for up to six hours. The HI clamp involves creating steady-state hyperinsulinemia by insulin infusion, along with parallel glucose 10 infusion in order to quantify the required amount of glucose to maintain euglycemia (normal concentration of glucose in the blood; also called *normoglycemia*). The result is a measure of the insulin-dependent glucose disposal rate (Rd), measuring the peripheral uptake of glucose by the muscle (primarily) and adipose tissues. This rate of glucose uptake is notated by M, whole body glucose metabolism by insulin action 15 under steady state conditions. Therefore, a high M indicates high insulin sensitivity and a lower M value indicates reduced insulin sensitivity, i.e. insulin resistant. The HI clamp requires three trained professionals to carry out the procedure, including simultaneous infusions of insulin and glucose over 2-4 hours and frequent blood sampling every 5 minutes for analysis of insulin and glucose levels. Due to the high 20 cost, complexity, and time required for the HI clamp, this procedure is strictly limited to the clinical research setting.

[0043] “Obesity” refers to a chronic condition defined by an excess amount body fat. The normal amount of body fat (expressed as percentage of body weight) is between 25-30% in women and 18-23% in men. Women with over 30% body fat and 25 men with over 25% body fat are considered obese.

[0044] “Body Mass Index, (or BMI)” refers to a calculation that uses the height and weight of an individual to estimate the amount of the individual’s body fat. Too much body fat (e.g. obesity) can lead to illnesses and other health problems. BMI is the measurement of choice for many physicians and researchers studying obesity. 30 BMI is calculated using a mathematical formula that takes into account both height and weight of the individual. BMI equals a person’s weight in kilograms divided by height in meters squared. (BMI=kg/m<sup>2</sup>). Subjects having a BMI less than 19 are considered to be underweight, while those with a BMI of between 19 and 25 are

considered to be of normal weight, while a BMI of between 25 to 29 are generally considered overweight, while individuals with a BMI of 30 or more are typically considered obese. Morbid obesity refers to a subject having a BMI of 40 or greater.

[0045] “Insulin resistance-related disorder” refers to diseases or disorders 5 associated with insulin resistance and includes dysglycemia, type-2 diabetes, cardiovascular disease (including myocardial infarction, stroke).

## I. Biomarkers

[0046] The biomarkers described herein were discovered using metabolomic profiling techniques. Such metabolomic profiling techniques are described in more 10 detail in the Examples set forth below as well as in U.S. Patents No. 7,005,255 and 7,329,489 and U.S. Patent 7,635,556, U.S. Patent 7,682,783, U.S. Patent 7,682,784, and U.S. Patent 7,550,258, the entire contents of all of which are hereby incorporated herein by reference.

[0047] Generally, metabolic profiles may be determined for biological samples 15 from human subjects diagnosed with a condition such as being insulin resistant as well as from one or more other groups of human subjects (e.g., healthy control subjects with normal glucose tolerance, subjects with impaired glucose tolerance, subjects with insulin resistance, or having known glucose disposal rates, subjects with type 2 diabetes, subjects having cardiovascular disease (e.g., subjects having suffered 20 myocardial infarction or stroke), human subjects that develop type-2 diabetes within a time frame (e.g. those who develop type-2 diabetes within 3 years, those subjects who develop type-2 diabetes within 5 years), human subjects who do not develop type-2 diabetes, or human subjects that do not develop cardiovascular disease). The metabolic profile for biological samples from one group of subjects may then be 25 compared to the metabolic profile for biological samples from the one or more other groups of subjects. The comparisons may be conducted using models or algorithms, such as those described herein. Those molecules differentially present, including those molecules differentially present at a level that is statistically significant, in the metabolic profile of samples from subjects of one group as compared to another group 30 (e.g., insulin sensitive subjects that develop type 2 diabetes vs. insulin sensitive subjects that do not develop type 2 diabetes, insulin sensitive subjects that develop

cardiovascular disease vs. insulin sensitive subjects that do not develop cardiovascular disease) may be identified as biomarkers to distinguish those groups.

**[0048]** Biomarkers for use in the methods disclosed herein may be obtained from any source of biomarkers related to insulin resistance, dysglycemia, type-2 diabetes, 5 and/or cardiovascular disease. Biomarkers for use in methods disclosed herein relating to predicting development of insulin resistance include 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, 10 deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine and subsets thereof. Additional 15 biomarkers for use in combination with those disclosed herein include those disclosed in International Patent Application Publication No. WO 2009/014639 and U.S. Application No. 12/218,980, filed July 17, 2008, the entireties of which are hereby incorporated by reference herein. In one aspect, the biomarkers correlate to insulin 20 resistance.

**[0049]** Biomarkers for use in methods disclosed herein relating to predicting development of type 2 diabetes include those markers listed in Table 1 and Table 2 and subsets thereof. In one embodiment the biomarkers include 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro, mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, glycerol-3-phosphate, isoleucine, valine, erythrose, 3-hydroxy-2-oxovalerate, 4-methyl-2-oxopentanoate, 2-methylbutyrylcarnitine, 3-hydroxybutyrate, tyrosine, glycine, kynurename, xanthine, beta-hydroxypyruvate, 3-hydroxypropanoate, hexanoylcarnitine, urate, palmitoyl sphingomyelin, quinate, hippurate, catechol 25 sulfate, margarate, 5alpha-androstan-3beta, adrenate, alpha-hydroxyisovalerate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), deoxycholate, glutamine, 17alpha-diol disulfate and subsets thereof. Additional biomarkers for use 30 in combination with those disclosed herein include those disclosed in International

Patent Application Publication No. WO 2009/014639 and U.S. Application No. 12/218,980, filed July 17, 2008, the entireties of which are hereby incorporated by reference herein. In one aspect, the biomarkers correlate to type 2 diabetes.

**[0050]** Biomarkers for use in methods disclosed herein relating to predicting development of cardiovascular disease include those markers listed in Table 3 and Table 4 and subsets thereof. In one embodiment the biomarkers include 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta, 20alpha-diol disulfate, 5alpha-pregnan-3alpha,20beta-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperidine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, and subsets thereof. In one aspect the biomarkers correlate to cardiovascular disease. Additional biomarkers for use in combination with those disclosed herein include those disclosed in International Patent Application Publication No. WO 2009/014639 and U.S. Application No. 12/218,980, filed July 17, 2008, the entireties of which are hereby incorporated by reference herein. In an embodiment the cardiovascular disease is myocardial infarction and the biomarkers are selected from Table 3. In an embodiment the cardiovascular disease is stroke and the biomarkers are selected from Table 4. Additional biomarkers for use in combination with those disclosed herein include those disclosed in International Patent Application Publication No. WO 2009/014639 and U.S. Application No. 12/218,980, filed July 17, 2008, the entireties of which are hereby incorporated by reference herein.

**[0051]** Biomarkers for use in methods disclosed herein correlating to insulin resistance, type 2 diabetes and/or cardiovascular disease, such as being impaired insulin sensitive, insulin resistant, or pre-diabetic include one or more of those listed in Tables 1, 2, 3, and/or 4 and subsets thereof. In an embodiment the biomarkers include a combination of the biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol

disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, 2-methylbutyrylcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, 5 erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine for use in the methods disclosed here. In one embodiment, the biomarkers for use in the disclosed methods include a combination of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, erythrose, glycerol-3-phosphate, isoleucine, valine, to predict the progression to type-2 diabetes. In another embodiment, the biomarkers for use in the disclosed methods include a 10 combination of adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta, 17alpha-diol disulfate, urate to determine progression to cardiovascular disease. Such combinations can also be combined with clinical 15 measurements or predictors of insulin resistance and/or type 2 diabetes, such as body mass index, fasting plasma insulin or C-peptide measurements. Examples of 20 additional combinations that can be used in the methods disclosed herein include those provided in the Examples below.

**[0052]** In one embodiment, biomarkers for use in identifying subjects for treatment by the administration of insulin resistance therapeutics include one or more 25 of those listed 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, 30 propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone. In still another

example, biomarkers for use in identifying subjects for admission into clinical trials for the administration of test compositions for effectiveness in treating insulin resistance or related conditions, include one or more of those listed 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone.

**[0053]** Additional biomarkers for use in the methods disclosed herein include metabolites related to the biomarkers listed 2-hydroxybutyrate, 3-hydroxy-butyrate, 3-methyl-2-oxo-butyric acid, arginine, betaine, 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone. In addition, such additional biomarkers may also be useful in combination and with clinical measures, for example as ratios of biomarkers and such additional clinical measures.

**[0054]** Any number of biomarkers may be used in the methods disclosed herein. That is, the disclosed methods may include the determination of the level(s) of one biomarker, two or more biomarkers, three or more biomarkers, four or more biomarkers, five or more biomarkers, six or more biomarkers, seven or more biomarkers, eight or more biomarkers, nine or more biomarkers, ten or more biomarkers, fifteen or more biomarkers, etc., including a combination of all of the biomarkers in Tables 1, 2, 3, 4 and/or 5. In another aspect, the number of biomarkers

for use in the disclosed methods include the levels of about twenty-five or less biomarkers, twenty or less, fifteen or less, ten or less, nine or less, eight or less, seven or less, six or less, five or less biomarkers. In another aspect, the number of biomarkers for use in the disclosed methods includes the levels of one, two, three, 5 four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, twenty, or twenty-five biomarkers.

[0055] The biomarkers disclosed herein may also be used to generate a risk score (“Disease Risk Score”) to predict a subject’s probability of being insulin resistant, type 2 diabetic, and/or having cardiovascular disease (including myocardial infarction 10 or stroke) for use in any of the disclosed methods. Any method or algorithm can be used to generate a Disease Risk Score based on the biomarkers in Tables 1, 2, 3, 4 and/or 5 for use in the methods of the present disclosure.

[0056] The biomarkers, panels, and algorithms may provide sensitivity levels for detecting or predicting predisposition to insulin resistance, type 2 diabetes, and/or 15 cardiovascular disease greater than conventional methods, such as the oral glucose tolerance test, fasting plasma glucose test, hemoglobin A1C (and estimated average glucose, eAG), fasting plasma insulin, fasting proinsulin, adiponectin, HOMA-IR, and the like. In some embodiments, the biomarkers, panels, and algorithms provided herein provide sensitivity levels greater than about 55%, 56%, 57%, 58%, 59%, 60% 20 or greater.

[0057] In other embodiments, the biomarkers, panels, and algorithms disclosed herein may provide a specificity level for detecting or predicting insulin resistance, type 2 diabetes and/or cardiovascular disease in a subject greater than conventional methods such as the oral glucose tolerance test, fasting plasma glucose test, 25 adiponectin, and the like. In some embodiments, the biomarkers, panels, and algorithms provided herein provide specificity levels greater than about 80%, 85%, 90%, or greater.

[0058] In addition, the methods disclosed herein using the biomarkers and models listed in the tables may be used in combination with clinical diagnostic measures of 30 the respective conditions. Combinations with clinical diagnostics (such as oral glucose tolerance test, fasting plasma glucose test, free fatty acid measurement, hemoglobin A1C (and estimated average glucose, eAG) measurements, fasting plasma insulin measurements, fasting proinsulin measurements, fasting C-peptide

measurements, glucose sensitivity (beta cell index) measurements, adiponectin measurements, uric acid measurements, systolic and diastolic blood pressure measurements, triglyceride measurements, triglyceride/HDL ratio, cholesterol (HDL, LDL) measurements, LDL/HDL ratio, waist/hip ratio, age, family history of diabetes

5 (T1D and/or T2D), family history of cardiovascular disease) may facilitate the disclosed methods, or confirm results of the disclosed methods, (for example, facilitating or confirming diagnosis, monitoring progression or regression, and/or determining predisposition to pre-diabetes, dysglycemia, type-2 diabetes, and/or cardiovascular disease).

10 **[0059]** Any suitable method may be used to detect the biomarkers in a biological sample in order to determine the level(s) of the one or more biomarkers. Suitable methods include chromatography (e.g., HPLC, gas chromatography, liquid chromatography), mass spectrometry (e.g., MS, MS-MS), enzyme-linked immunosorbent assay (ELISA), antibody linkage, other immunochemical techniques, 15 and combinations thereof (e.g. LC-MS-MS). Further, the level(s) of the one or more biomarkers may be detected indirectly, for example, by using an assay that measures the level of a compound (or compounds) that correlates with the level of the biomarker(s) that are desired to be measured.

10 **[0060]** In some embodiments, the biological samples for use in the detection of the biomarkers are transformed into analytical samples prior to the analysis of the level or detection of the biomarker in the sample. For example, in some 20 embodiments, protein extractions may be performed to transform the sample prior to analysis by, for example, liquid chromatography (LC) or tandem mass spectrometry (MS-MS), or combinations thereof. In other embodiments, the samples may be 25 transformed during the analysis, for example by tandem mass spectrometry methods.

#### **A. Diagnostic Methods**

**[0061]** The biomarkers described herein may be used to diagnose, or to aid in diagnosing, whether a subject has a disease or condition, such as being insulin resistant, dysglycemic, type 2 diabetic and/or having cardiovascular disease. For 30 example, biomarkers for use in diagnosing, or aiding in diagnosing, whether a subject is insulin resistant include one or more of those identified biomarkers in Tables 1, 2, 5. In one embodiment, the biomarkers include one or more of those identified in

Tables 1, 2, 5, and combinations thereof. Any biomarker listed in Tables 1, 2, 5 may be used in the diagnostic methods, as well as any combination of the biomarkers listed in Tables 1, 2, 5, or combinations thereof.

[0062] Methods for diagnosing, or aiding in diagnosing, whether a subject has a disease or condition, such as being insulin resistant or having an insulin resistance related disorder, may be performed using one or more of the biomarkers identified Table 1, 2, 3, 4, and/or 5. A method of diagnosing (or aiding in diagnosing) whether a subject has a disease or condition, such as being insulin resistant, comprises (1) analyzing a biological sample from a subject to determine the level(s) of one or more biomarkers of insulin resistance listed 2-hydroxybutyrate, 3-hydroxy-butyrate, 3-methyl-2-oxo-butyric acid, arginine, betaine, 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine in the sample and (2) comparing the level(s) of the one or more biomarkers in the sample to insulin-resistance-positive and/or insulin-resistance-negative reference levels of the one or more biomarkers in order to diagnose (or aid in the diagnosis of) whether the subject is insulin resistant. When such a method is used in aiding in the diagnosis of a disease or condition, such as insulin resistance or pre-diabetes, the results of the method may be used along with other methods (or the results thereof) useful in the clinical determination of whether a subject has a given disease or condition. Methods useful in the clinical determination of whether a subject has a disease or condition such as insulin resistance or pre-diabetes are known in the art. For example, methods useful in the clinical determination of whether a subject is insulin resistant or is at risk of being insulin resistant include, for example, glucose disposal rates (Rd, M-wbm, M-ffm), body weight measurements, waist circumference measurements, BMI determinations, waist/hip ratio, triglycerides measurements, cholesterol (HDL, LDL) measurements, LDL/HDL ratio,

triglyceride/HDL ratio, age, family history of diabetes (T1D and/or T2D), family history of cardiovascular disease, Peptide YY measurements, C-peptide measurements, Hemoglobin A1C measurements and estimated average glucose, (eAG), adiponectin measurements, fasting plasma glucose measurements (e.g., oral 5 glucose tolerance test, fasting plasma glucose test), free fatty acid measurements, fasting plasma insulin and pro-insulin measurements, systolic and diastolic blood pressure measurements, urate measurements and the like. Methods useful for the clinical determination of whether a subject has insulin resistance include the hyperinsulinemic euglycemic clamp (HI clamp).

10 [0063] Independent studies were carried out to identify a set of biomarkers that when used with a polynomic algorithm enables the early detection of changes in insulin resistance in a subject. In one aspect, the biomarkers provided herein can be used to provide a physician with a probability score ("Disease Risk (DR) Score") indicating the probability that a subject is insulin resistant. The score is based upon 15 clinically significant changed reference level(s) for a biomarker and/or combination of biomarkers. The reference level can be derived from an algorithm or computed from indices for impaired glucose disposal. The DR Score places the subject in the range of insulin resistance from normal (i.e. insulin sensitive) to insulin resistant to highly resistant. Disease progression or remission can be monitored by periodic 20 determination and monitoring of the DR Score. Response to therapeutic intervention can be determined by monitoring the DR Score. The DR Score can also be used to evaluate drug efficacy.

[0064] Thus, the disclosure also provides methods for determining a subject's Disease Risk score (DR score) that may be performed using one or more of the 25 biomarkers identified 2-hydroxybutyrate, 3-hydroxy-butyrate, 3-methyl-2-oxo-butyric acid, arginine, betaine, creatine, decanoyl carnitine, docosatetraenoic acid, glutamic acid, glycine, linoleic acid, linolenic acid, margaric acid, octanoyl carnitine, oleic acid, oleoyl-LPC, palmitate, palmitoleic acid, palmitoyl-LPC, serine, stearate, threonine, tryptophan, linoleoyl-LPC, 1,5-anhydroglucitol, stearoyl-LPC, glutamyl 30 valine, gamma-glutamyl-leucine, heptadecenoic acid, alpha-ketobutyrate, cysteine, urate or a model using such biomarkers. For example, a method for determining the DR score of a subject comprises the steps of: (1) analyzing a biological sample from a subject to determine the level(s) of one or more insulin resistance biomarkers 2-

hydroxybutyrate, 3-hydroxy-butyrate, 3-methyl-2-oxo-butyric acid, arginine, betaine, creatine, decanoyl carnitine, docosatetraenoic acid, glutamic acid, glycine, linoleic acid, linolenic acid, margaric acid, octanoyl carnitine, oleic acid, oleoyl-LPC, palmitate, palmitoleic acid, palmitoyl-LPC, serine, stearate, threonine, tryptophan, 5 linoleoyl-LPC, 1,5-anhydroglucitol, stearoyl-LPC, glutamyl valine, gamma-glutamyl-leucine, heptadecenoic acid, alpha-ketobutyrate, cysteine, urate in the sample, and (2) comparing the level(s) of the one or more insulin resistance biomarkers in the sample to insulin resistance reference levels of the one or more biomarkers in order to determine the subject's insulin resistance score. The method may employ any number 10 of markers selected from those listed 2-hydroxybutyrate, 3-hydroxy-butyrate, 3-methyl-2-oxo-butyric acid, arginine, betaine, creatine, decanoyl carnitine, docosatetraenoic acid, glutamic acid, glycine, linoleic acid, linolenic acid, margaric acid, octanoyl carnitine, oleic acid, oleoyl-LPC, palmitate, palmitoleic acid, palmitoyl-LPC, serine, stearate, threonine, tryptophan, linoleoyl-LPC, 1,5-anhydroglucitol, stearoyl-LPC, glutamyl valine, gamma-glutamyl-leucine, 15 heptadecenoic acid, alpha-ketobutyrate, cysteine, urate, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more markers. Multiple biomarkers may be correlated with a given condition, such as being insulin resistant, by any method, including statistical methods such as regression analysis.

20 [0065] Any suitable method may be used to analyze the biological sample in order to determine the level(s) of the one or more biomarkers in the sample. Suitable methods include chromatography (e.g., HPLC, gas chromatography, liquid chromatography), mass spectrometry (e.g., MS, MS-MS), enzyme-linked immunosorbent assay (ELISA), antibody linkage, other immunochemical techniques, 25 and combinations thereof. Further, the level(s) of the one or more biomarkers may be measured indirectly, for example, by using an assay that measures the level of a compound (or compounds) that correlates with the level of the biomarker(s) that are desired to be measured.

[0066] After the level(s) of the one or more biomarker(s) is determined, the 30 level(s) may be compared to disease or condition reference level(s) or reference curves of the one or more biomarker(s) to determine a rating for each of the one or more biomarker(s) in the sample. The rating(s) may be aggregated using any algorithm to create a score, for example, a Disease Risk (DR) score, for the subject.

The algorithm may take into account any factors relating to the disease or condition, such as having a family history of type 2 diabetes, including the number of biomarkers, the correlation of the biomarkers to the disease or condition, etc.

[0067] In one example, the subject's predicted disease risk level may be used to 5 determine the probability that the subject is insulin resistant, type 2 diabetic, and/or has cardiovascular disease (i.e. determine the subject's DR Score). For example, using a standardized curve generated using one or more biomarkers (e.g., selected from 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3- 10 oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'- 15 methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, 20 quinate, and isoleucine), a subject predicted to have a DR level of 9, may have a 10% probability of being a type 2 diabetic. Alternatively, in another example, a subject predicted to have a DR level of 3 may have a 90% probability of being type 2 diabetic.

## B. Determining Predisposition to a Disease or Condition

[0068] The biomarkers identified herein may also be used in the determination of 25 whether a subject not exhibiting any symptoms of a disease or condition, such as insulin resistance, dysglycemia, type 2 diabetes and/or cardiovascular disease, is predisposed to developing such a condition. The biomarkers may be used, for example, to determine whether a subject is predisposed to developing or becoming, 30 for example, insulin resistant. Such methods of determining whether a subject having no symptoms of a particular disease or condition such as, impaired insulin sensitivity, being insulin resistant, or having type 2 diabetes, and/or cardiovascular disease, is

predisposed to developing a particular disease or condition comprise (1) analyzing a biological sample from a subject to determine the level(s) of one or more biomarkers listed in Tables 1, 2, 3, 4 and/or 5 and combinations of the biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrylcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine in the sample and (2) comparing the level(s) of the one or more biomarkers in the sample to disease- or condition-positive and/or disease- or condition-negative reference levels of the one or more biomarkers in order to determine whether the subject is predisposed to developing the respective disease or condition. For example, the identification of biomarkers for cardiovascular disease allows for the determination of whether a subject having no symptoms of cardiovascular disease is predisposed to developing cardiovascular disease. A method of determining whether a subject having no symptoms of cardiovascular disease is predisposed to developing cardiovascular disease comprises (1) analyzing a biological sample from a subject to determine the level(s) of one or more biomarkers listed in Tables 3 and/or 4 and/or combinations of the biomarkers: adrenate; alpha-hydroxyisovalerate; glutamine; glycine; tyrosine; deoxycholate; cinnamoylglycine; dehydroisoandrosterone sulfate (DHEA-S); 5alpha-androstan-3beta,17alpha-diol disulfate; urate; 3-indoxylsulfate; propionylcarnitine; 3-dehydrocarnitine; acetylcarnitine; oleoylcarnitine; myo-inositol; 5alpha-pregnan-3beta,20alpha-diol disulfate; xanthine; trigonelline (N'-methylnicotinate); 2-hydroxyhippurate (salicylurate); piperine; 1-methylurate; 1,3-dimethylurate; 1,7-dimethylurate; 1,3,7-trimethylurate; kynurenine; in the sample and (2) comparing the level(s) of the one or

more biomarkers in the sample to cardiovascular disease-positive and/or cardiovascular disease-negative reference levels of the one or more biomarkers in order to determine whether the subject is predisposed to developing cardiovascular disease. The results of the method may be used along with other methods (or the 5 results thereof) useful in the clinical determination of whether a subject is predisposed to developing the disease or condition.

[0069] After the level(s) of the one or more biomarkers in the sample are determined, the level(s) are compared to disease- or condition-positive and/or disease- or condition-negative reference levels in order to predict whether the subject is 10 predisposed to developing a disease or condition such as insulin resistance, type-2 diabetes or cardiovascular disease. Levels of the one or more biomarkers in a sample corresponding to the disease- or condition-positive reference levels (e.g., levels that are the same as the reference levels, substantially the same as the reference levels, above and/or below the minimum and/or maximum of the reference levels, and/or 15 within the range of the reference levels) are indicative of the subject being predisposed to developing the disease or condition. Levels of the one or more biomarkers in a sample corresponding to disease- or condition-negative reference levels (e.g., levels that are the same as the reference levels, substantially the same as the reference levels, above and/or below the minimum and/or maximum of the reference levels, and/or within the range of the reference levels) are indicative of the subject not being 20 predisposed to developing the disease or condition. In addition, levels of the one or more biomarkers that are differentially present (especially at a level that is statistically significant) in the sample as compared to disease- or condition-negative reference levels may be indicative of the subject being predisposed 25 to developing the disease or condition. Levels of the one or more biomarkers that are differentially present (especially at a level that is statistically significant) in the sample as compared to disease- condition-positive reference levels are indicative of the subject not being predisposed to developing the disease or condition.

[0070] By way of example, after the level(s) of the one or more biomarkers in the 30 sample are determined, the level(s) are compared to cardiovascular disease-positive and/or cardiovascular disease-negative reference levels in order to predict whether the subject is predisposed to developing cardiovascular disease. Levels of the one or more biomarkers in a sample corresponding to the cardiovascular disease-positive

reference levels (e.g., levels that are the same as the reference levels, substantially the same as the reference levels, above and/or below the minimum and/or maximum of the reference levels, and/or within the range of the reference levels) are indicative of the subject being predisposed to developing cardiovascular disease. Levels of the one or more biomarkers in a sample corresponding to the cardiovascular disease-negative reference levels (e.g., levels that are the same as the reference levels, substantially the same as the reference levels, above and/or below the minimum and/or maximum of the reference levels, and/or within the range of the reference levels) are indicative of the subject not being predisposed to developing cardiovascular disease. In addition, levels of the one or more biomarkers that are differentially present (especially at a level that is statistically significant) in the sample as compared to cardiovascular disease -negative reference levels are indicative of the subject being predisposed to developing cardiovascular disease. Levels of the one or more biomarkers that are differentially present (especially at a level that is statistically significant) in the sample as compared to cardiovascular disease -positive reference levels are indicative of the subject not being predisposed to developing insulin resistance.

[0071] Furthermore, it may also be possible to determine reference levels specific to assessing whether or not a subject that does not have a disease or condition such as insulin resistance, type 2 diabetes, or cardiovascular disease, is predisposed to developing a disease or condition. For example, it may be possible to determine reference levels of the biomarkers for assessing different degrees of risk (e.g., low, medium, high) in a subject for developing a disease or condition. Such reference levels could be used for comparison to the levels of the one or more biomarkers in a biological sample from a subject.

25 **C. Monitoring Disease or Condition Progression / Regression**

[0072] The identification of biomarkers herein allows for monitoring progression/regression of insulin resistance or related conditions in a subject. A method of monitoring the progression/regression insulin resistance or related condition (i.e., type-2 diabetes, impaired glucose tolerance (IGT), cardiovascular disease (CVD)) in a subject comprises (1) analyzing a first biological sample from a subject to determine the level(s) of one or more biomarkers listed in Tables 1, 2, 3, 4 and/or 5 and combinations thereof including 3-phenylpropionate (hydrocinnamate),

3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N<sup>1</sup>-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, 2-methylbutyrylcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine, and combinations thereof, in the first sample obtained from the subject at a first time point, (2) analyzing a second biological sample from a subject to determine the level(s) of the one or more biomarkers, the second sample obtained from the subject at a second time point, and (3) comparing the level(s) of one or more biomarkers in the first sample to the level(s) of the one or more biomarkers in the second sample in order to monitor the progression/regression of the disease or condition in the subject. The results of the method are indicative of the course of insulin resistance, type 2 diabetes, and/or cardiovascular disease (i.e., progression or regression, if any change) in the subject.

**[0073]** In one embodiment, the results of the method may be based on a Disease Risk (DR) Score which is representative of the probability of, for example, insulin resistance in the subject and which can be monitored over time. By comparing the DR Score from a first time point sample to the DR Score from at least a second time point sample, the progression or regression of insulin resistance can be determined. Such a method of monitoring the progression/regression of insulin resistance, type 2 diabetes and/or cardiovascular disease in a subject comprises (1) analyzing a first biological sample from a subject to determine an DR score for the first sample obtained from the subject at a first time point, (2) analyzing a second biological sample from a subject to determine a second DR score, the second sample obtained from the subject at a second time point, and (3) comparing the DR score in the first sample to the DR score in the second sample in order to monitor the progression/regression of insulin resistance, type-2 diabetes and/or cardiovascular

disease in the subject. An increase in the probability of, for example, insulin resistance from the first to the second time point is indicative of the progression of insulin resistance in the subject, while a decrease in the probability from the first to the second time points is indicative of the regression of insulin resistance in the

5 subject.

**[0074]** Using the biomarkers and algorithm of the instant invention for progression monitoring may guide, or assist a physician's decision to implement preventative measures such as dietary restrictions, exercise, and/or early-stage drug treatment.

#### 10 **D. Monitoring Therapeutic Efficacy**

**[0075]** The biomarkers provided also allow for the assessment of the efficacy of a composition for treating a disease or condition such as insulin resistance, type 2 diabetes, or cardiovascular disease. For example, the identification of biomarkers for insulin resistance also allows for assessment of the efficacy of a composition for 15 treating insulin resistance as well as the assessment of the relative efficacy of two or more compositions for treating insulin resistance. Such assessments may be used, for example, in efficacy studies as well as in lead selection of compositions for treating the disease or condition. In addition, such assessments may be used to monitor the efficacy of surgical procedures and/or lifestyle interventions on insulin resistance in a 20 subject. Surgical procedures include bariatric surgery, while lifestyle interventions include diet modification or reduction, exercise programs, and the like.

**[0076]** Thus, in one such embodiment, provided are methods of assessing the efficacy of a composition for treating a disease or condition such as insulin resistance, type 2 diabetes or cardiovascular disease comprising (1) analyzing, from a subject (or 25 group of subjects) having a disease or condition such as insulin resistance, type 2 diabetes or cardiovascular disease and currently or previously being treated with a composition, a biological sample (or group of samples) to determine the level(s) of one or more biomarkers for insulin resistance, type 2 diabetes or cardiovascular disease selected from the biomarkers listed in Tables 1, 2, 3, 4 and/or 5 and the 30 biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine,

glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, 2-methylbutyrylcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine and (2) comparing the level(s) of the one or more biomarkers in the sample to (a) level(s) of the one or more biomarkers in a previously-taken biological sample from the subject, wherein the previously-taken biological sample was obtained from the subject before being treated with the composition, (b) disease- or condition-positive reference levels of the one or more biomarkers, (c) disease- or condition-negative reference levels of the one or more biomarkers, (d) disease- or condition-progression-positive reference levels of the one or more biomarkers, and/or (e) disease- or condition-regression-positive reference levels of the one or more biomarkers. The results of the comparison are indicative of the efficacy of the composition for treating the respective disease or condition.

20 [0077] In another embodiment, methods of assessing the efficacy of a surgical procedure for treating a disease or condition such as insulin resistance, or related condition comprising (1) analyzing, from a subject (or group of subjects) having insulin resistance, type 2 diabetes or cardiovascular disease, and having previously undergone a surgical procedure, a biological sample (or group of samples) to determine the level(s) of one or more biomarkers for insulin resistance selected from the biomarkers listed in Tables 1, 2, 3, and/or 4 and the biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-

methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine, and (2) comparing the level(s) of the one or more biomarkers in the sample to (a) level(s) of the one or more biomarkers in a previously-taken biological sample from the subject, wherein the previously-taken biological sample was obtained from the subject before undergoing the surgical procedure or taken 10 immediately after undergoing the surgical procedure, (b) insulin resistance-positive (or type 2 diabetes-positive, cardiovascular disease-positive) reference levels of the one or more biomarkers, (c) insulin resistance-negative (or type 2 diabetes-negative, cardiovascular disease-negative) reference levels of the one or more biomarkers, (d) insulin resistance-progression-positive (or type 2 diabetes-progression positive, 15 cardiovascular disease-progression-positive) reference levels of the one or more biomarkers, and/or (e) insulin resistance-regression-positive (or type 2 diabetes-regression positive, cardiovascular disease-regression-positive) reference levels of the one or more biomarkers. The results of the comparison are indicative of the efficacy of the surgical procedure for treating the respective disease or condition. In one 20 embodiment, the surgical procedure is a gastro-intestinal surgical procedure, such as bariatric surgery.

[0078] The change (if any) in the level(s) of the one or more biomarkers over time may be indicative of progression or regression of the disease or condition in the subject. To characterize the course of a given disease or condition in the subject, the 25 level(s) of the one or more biomarkers in the first sample, the level(s) of the one or more biomarkers in the second sample, and/or the results of the comparison of the levels of the biomarkers in the first and second samples may be compared to the respective disease- or condition-positive and/or disease- or condition-negative reference levels of the one or more biomarkers. If the comparisons indicate that the 30 level(s) of the one or more biomarkers are increasing or decreasing over time (e.g., in the second sample as compared to the first sample) to become more similar to the disease- or condition-positive reference levels (or less similar to the disease- or condition-negative reference levels), then the results are indicative of the disease's or

condition's progression. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time to become more similar to the disease- or condition-negative reference levels (or less similar to the disease- or condition-positive reference levels), then the results are indicative of the disease's or 5 condition's regression.

[0079] For example, in order to characterize the course of insulin resistance in the subject, the level(s) of the one or more biomarkers in the first sample, the level(s) of the one or more biomarkers in the second sample, and/or the results of the comparison of the levels of the biomarkers in the first and second samples may be compared to 10 insulin resistance-positive and/or insulin resistance-negative reference levels of the one or more biomarkers. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time (e.g., in the second sample as compared to the first sample) to become more similar to the insulin resistance-positive reference levels (or less similar to the insulin resistance-negative reference levels), 15 then the results are indicative of insulin resistance progression. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time to become more similar to the insulin resistance-negative reference levels (or less similar to the insulin resistance-positive reference levels), then the results are indicative of insulin resistance regression.

[0080] The second sample may be obtained from the subject any period of time 20 after the first sample is obtained. In one aspect, the second sample is obtained 1, 2, 3, 4, 5, 6, or more days after the first sample or after the initiation of the administration of a composition, surgical procedure, or lifestyle intervention. In another aspect, the second sample is obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more weeks after the first 25 sample or after the initiation of the administration of a composition, surgical procedure, or lifestyle intervention. In another aspect, the second sample may be obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more months after the first sample or after the initiation of the administration of a composition, surgical procedure, or lifestyle intervention.

[0081] The course of a disease or condition such as being insulin resistant, or 30 having type 2 diabetes, or cardiovascular disease in a subject may also be characterized by comparing the level(s) of the one or more biomarkers in the first sample, the level(s) of the one or more biomarkers in the second sample, and/or the

results of the comparison of the levels of the biomarkers in the first and second samples to disease- or condition-progression-positive and/or disease- or condition-regression-positive reference levels. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time (e.g., in the second 5 sample as compared to the first sample) to become more similar to the disease- or condition-progression-positive reference levels (or less similar to the disease- or condition-regression-positive reference levels), then the results are indicative of the disease or condition progression. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time to become more 10 similar to the disease- or condition-regression-positive reference levels (or less similar to the disease- or condition-progression-positive reference levels), then the results are indicative of disease or condition regression.

**[0082]** As with the other methods described herein, the comparisons made in the methods of monitoring progression/regression of a disease or condition such as being 15 insulin resistant, type 2 diabetic, or cardiovascular disease in a subject may be carried out using various techniques, including simple comparisons, one or more statistical analyses, and combinations thereof.

**[0083]** The results of the method may be used along with other methods (or the 20 results thereof) useful in the clinical monitoring of progression/regression of the disease or condition in a subject.

**[0084]** As described above in connection with methods of diagnosing (or aiding in the diagnosis of) a disease or condition such as being insulin resistant, type 2 diabetic, or cardiovascular disease, any suitable method may be used to analyze the biological samples in order to determine the level(s) of the one or more biomarkers in the 25 samples. In addition, the level(s) one or more biomarkers, including a combination of all of the biomarkers (Tables 1, 2, 3, 4, and/or 5, and/or the biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, 30 glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-

methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine) or any fraction thereof, may be determined and used in methods of monitoring progression/regression of the respective disease or condition in a subject.

5 [0085] Such methods could be conducted to monitor the course of disease or condition development in subjects, for example the course of insulin resistance to type-2 diabetes and/or cardiovascular disease in a subject having pre-diabetes, or could be used in subjects not having a disease or condition (e.g., subjects suspected of being predisposed to developing the disease or condition) in order to monitor levels of predisposition to the disease or condition.

10 [0086] Clinical studies from around the world have been carried out to test whether anti-diabetic therapies, such as metformin or acarbose, can prevent diabetes progression in pre-diabetic patients. These studies have shown that such therapies can prevent diabetes onset. From the U.S. Diabetes Prevention Program (DPP), metformin reduced the rate of progression to diabetes by 38% and lifestyle and 15 exercise intervention reduced the rate of progression to diabetes by 56%. Because of such successes, the ADA has revised its 2008 Standards of Medical Care in Diabetes to include the following statements in the section on Prevention/Delay of Type 2 Diabetes: *“In addition to lifestyle counseling, metformin may be considered in those who are at very high risk (combined IFG and IGT plus other risk factors) and who 20 are obese and under 60 years of age.”*

25 [0087] Pharmaceutical companies have carried out studies to assess whether certain classes of drugs, such as the PPAR $\gamma$  class of insulin sensitizers (e.g. muraglitazone), can prevent diabetes progression. Similar to the DPP trial, some of these studies have shown great promise and success for preventing diabetes, whereas 30 others have exposed a certain amount of risk associated with certain anti-diabetic pharmacologic treatments when given to the general pre-diabetic population as defined by current insulin resistance diagnostics. Pharmaceutical companies are in need of diagnostics that can identify and stratify high risk pre-diabetics so they can

assess the efficacy of their pre-diabetic therapeutic candidates more effectively and safely. In some embodiments, subjects that are identified as more insulin resistant or more predisposed to developing type 2 diabetes or cardiovascular disease may be more likely to respond to an insulin sensitizer composition.

5 [0088] Considering the infrequency of the oral glucose tolerance test (OGTT) procedures in the clinical setting, a new diagnostic test that directly measures insulin resistance, type 2 diabetes or cardiovascular disease in a fasted sample would enable a physician to identify and stratify patients who are moving toward the etiology of pre-diabetes, type-2 diabetes and cardiovascular disease much earlier.

10 **E. Identification of Responders and Non-responders to Therapeutic**

[0089] The biomarkers provided also allow for the identification of subjects in whom the composition for treating a disease or condition such as insulin resistance, pre-diabetes, or type-2 diabetes is efficacious (i.e. patient responds to therapeutic). For example, the identification of biomarkers for insulin resistance also allows for 15 assessment of the subject's response to a composition for treating insulin resistance as well as the assessment of the relative patient response to two or more compositions for treating insulin resistance. Such assessments may be used, for example, in selection of compositions for treating the disease or condition for certain subjects, or in the selection of subjects into a course of treatment or clinical trial.

20 [0090] Thus, also provided are methods of predicting the response of a patient to a composition for treating a disease or condition such as insulin resistance, pre-diabetes, or type-2 diabetes comprising (1) analyzing, from a subject (or group of subjects) having a disease or condition such as insulin resistance, pre-diabetes, or type-2 diabetes and currently or previously being treated with a composition, a biological 25 sample (or group of samples) to determine the level(s) of one or more biomarkers for insulin resistance selected from the biomarkers listed in Tables 1, 2, 3 and/or 4 and one or more selected biomarkers listed 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-30 hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine,

acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-  
5 hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine and (2) comparing the level(s) of the one or more biomarkers in the sample to (a) level(s) of the one or more biomarkers in a previously-taken biological sample from the subject, wherein the previously-taken  
10 biological sample was obtained from the subject before being treated with the composition, (b) disease- or condition-positive reference levels of the one or more biomarkers, (c) disease- or condition-negative reference levels of the one or more biomarkers, (d) disease- or condition-progression-positive reference levels of the one or more biomarkers, and/or (e) disease- or condition-regression-positive reference  
15 levels of the one or more biomarkers. The results of the comparison are indicative of the response of the patient to the composition for treating the respective disease or condition.

[0091] The change (if any) in the level(s) of the one or more biomarkers over time may be indicative of response of the subject to the therapeutic. To characterize the  
20 course of a given therapeutic in the subject, the level(s) of the one or more biomarkers in the first sample, the level(s) of the one or more biomarkers in the second sample, and/or the results of the comparison of the levels of the biomarkers in the first and second samples may be compared to the respective disease- or condition-positive and/or disease- or condition-negative reference levels of the one or more biomarkers.  
25 If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time (e.g., in the second sample as compared to the first sample) to become more similar to the disease- or condition-positive reference levels (or less similar to the disease- or condition-negative reference levels), then the results are indicative of the patient not responding to the therapeutic. If the comparisons  
30 indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time to become more similar to the disease- or condition-negative reference levels (or less similar to the disease- or condition-positive reference levels), then the results are indicative of the patient responding to the therapeutic.

[0092] For example, in order to characterize the patient response to a therapeutic for insulin resistance, the level(s) of the one or more biomarkers in the first sample, the level(s) of the one or more biomarkers in the second sample, and/or the results of the comparison of the levels of the biomarkers in the first and second samples may be 5 compared to insulin resistance-positive and/or insulin resistance-negative reference levels of the one or more biomarkers. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time (e.g., in the second sample as compared to the first sample) to become more similar to the insulin resistance-positive reference levels (or less similar to the insulin resistance-negative 10 reference levels), then the results are indicative of non-response to the therapeutic. If the comparisons indicate that the level(s) of the one or more biomarkers are increasing or decreasing over time to become more similar to the insulin resistance-negative reference levels (or less similar to the insulin resistance-positive reference levels), then the results are indicative of response to the therapeutic.

15 [0093] The second sample may be obtained from the subject any period of time after the first sample is obtained. In one aspect, the second sample is obtained 1, 2, 3, 4, 5, 6, or more days after the first sample. In another aspect, the second sample is obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more weeks after the first sample or after the initiation of treatment with the composition. In another aspect, the second sample 20 may be obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more months after the first sample or after the initiation of treatment with the composition.

[0094] As with the other methods described herein, the comparisons made in the methods of determining a patient response to a therapeutic for a disease or condition such as insulin resistance, pre-diabetes, or type-2 diabetes in a subject may be carried 25 out using various techniques, including simple comparisons, one or more statistical analyses, and combinations thereof.

[0095] The results of the method may be used along with other methods (or the results thereof) useful in determining a patient response to a therapeutic for the disease or condition in a subject.

30 [0096] As described above in connection with methods of diagnosing (or aiding in the diagnosis of) a disease or condition such as insulin resistance, pre-diabetes, or type-2 diabetes, any suitable method may be used to analyze the biological samples in order to determine the level(s) of the one or more biomarkers in the samples. In

addition, the level(s) one or more biomarkers, including a combination of all of the biomarkers 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, 5 glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine or any fraction thereof, may be determined and used in methods of monitoring progression/regression of the respective disease or condition in a subject.

**[0097]** Such methods could be conducted to monitor the patient response to a therapeutic for a disease or condition development in subjects, for example the course of pre-diabetes to type-2 diabetes in a subject having pre-diabetes, or could be used in subjects not having a disease or condition (e.g., subjects suspected of being predisposed to developing the disease or condition) in order to monitor levels of predisposition to the disease or condition.

**[0098]** Pharmaceutical companies have carried out studies to assess whether certain classes of drugs, such as the PPAR $\gamma$  class of insulin sensitizers, can prevent diabetes progression. Some of these studies have shown great promise and success for preventing diabetes, whereas others have exposed a certain amount of risk associated with certain anti-diabetic pharmacologic treatments when given to the general pre-diabetic population as defined by current IR diagnostics. Pharmaceutical companies are in need of diagnostics that can identify responders and non-responders in order to stratify high risk pre-diabetics to assess the efficacy of their pre-diabetic therapeutic candidates more effectively and safely. A new diagnostic test that discriminates non-responding from responding patients to a therapeutic would enable pharmaceutical companies to identify and stratify patients that are likely to respond to

the therapeutic agent and target specific therapeutics for certain cohorts that are likely to respond to the therapeutic.

#### **F. Other methods**

[0099] Other methods of using the biomarkers discussed herein are also contemplated. For example, the methods described in U.S. Patent Nos. 7,005,255; 5 7,329,489; 7,550,258; 7,550,260; 7,553,616; 7,635,556; 7,682,782; and 7,682,784 may be conducted using a small molecule profile comprising one or more of the biomarkers disclosed herein.

### **Examples**

#### **10 I. General Methods**

##### **A. Identification of Metabolic Profiles**

[00100] Each sample was analyzed to determine the concentration of several hundred metabolites. Analytical techniques such as GC-MS (gas chromatography-mass spectrometry) and LC-MS (liquid chromatography-mass spectrometry) were 15 used to analyze the metabolites. Multiple aliquots were simultaneously, and in parallel, analyzed, and, after appropriate quality control (QC), the information derived from each analysis was recombined. Every sample was characterized according to several thousand characteristics, which ultimately amount to several hundred chemical species. The techniques used were able to identify novel and chemically 20 unnamed compounds.

##### **B. Statistical Analysis:**

[00101] The data was analyzed using several statistical methods to identify molecules (either known, named metabolites or unnamed metabolites) present at differential levels in a definable population or subpopulation (e.g., biomarkers for 25 insulin resistant biological samples compared to control biological samples or compared to insulin sensitive patients) useful for distinguishing between the definable populations (e.g., insulin resistance and control, insulin resistance and insulin sensitive, insulin resistance and type-2 diabetes). Other molecules (either known, named metabolites or unnamed metabolites) in the definable population or 30 subpopulation were also identified.

[00102] Random forest analyses were used for classification of samples into groups (e.g. disease or healthy, insulin resistant or normal insulin sensitivity). Random forests give an estimate of how well we can classify individuals in a new data set into each group, in contrast to a t-test, which tests whether the unknown means for two populations are different or not. Random forests create a set of classification trees based on continual sampling of the experimental units and compounds. Then each observation is classified based on the majority votes from all the classification trees.

[00103] Regression analysis was performed using the Random Forest Regression method and the Univariate Correlation/Linear Regression method to build models that are useful to identify the biomarker compounds that are associated with disease or disease indicators (e.g. Rd) and then to identify biomarker compounds useful to classify individuals according to for example, the level of glucose utilization as normal, insulin impaired, or insulin resistant. Biomarker compounds that are useful to predict disease or measures of disease (e.g. Rd) and that are positively or negatively correlated with disease or measures of disease (e.g. Rd) were identified in these analyses. All of the biomarker compounds identified in these analyses were statistically significant (p<0.05, q<0.1).

[00104] Recursive partitioning relates a 'dependent' variable (Y) to a collection of independent ('predictor') variables (X) in order to uncover -- or simply understand -- the elusive relationship,  $Y=f(X)$ . The analysis was performed with the JMP program (SAS) to generate a decision tree. The statistical significance of the "split" of the data can be placed on a more quantitative footing by computing p-values, which discern the quality of a split relative to a random event. The significance level of each "split" of data into the nodes or branches of the tree was computed as p-values, which discern the quality of the split relative to a random event. It was given as LogWorth, which is the negative log10 of a raw p-value.

[00105] Statistical analyses were performed with the program "R" available on the worldwide web at the website cran.r-project.org and in JMP 6.0.2 (SAS® Institute, Cary, NC).

30 **Example 2: Biomarkers Useful for Predicting Disease Progression**

[00106] A number of biomarkers useful to determine the insulin sensitive (IS) individuals who will develop type 2 diabetes (T2D), myocardial infarction (MI) or

stroke were identified. The biomarkers were identified by collecting plasma samples at the time of study enrolment (baseline) from 543 non-diabetic, human subjects with a family history of diabetes who were followed for at least 7 to more than 10 years. Over the course of the study the participants fell into the following groups: 262 were 5 “non-progressors” (i.e., subjects who remained stable and did not progress to IR, T2D or CVD (e.g., MI or stroke)), 131 subjects developed T2D (“T2D progressors”), 105 subjects had myocardial infarction (MI, “MI progressors”), 45 subjects had a stroke (“stroke progressors”). In brief, the plasma samples were extracted and split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Proprietary 10 software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area integration. The resulting metabolomic profiles were statistically analyzed to identify the biomarkers that are differentially present between the groups using Welch’s Two Sample t-test analysis.

[00107] Biomarkers which, when measured in human plasma, predict the 15 development of type 2 diabetes within 3 years were identified. These biomarkers were present at different (higher or lower) levels at baseline in the plasma from participants that progressed to type 2 diabetes (N=131) within 3 years, compared to those subjects that did not progress to type 2 diabetes (“non-progressors”, N=262). The biomarkers for predicting the progression to Type 2 diabetes in 3 years is 20 presented in Table 1. Table 1 includes, for each listed biomarker, the biochemical name of the biomarker, an indication of the fold-change in the “Progressor” sample mean as compared to the “Non-Progressor” sample mean (values >1.0 represent an elevation in subjects who progress to T2D, and values <1.0 represent a lower level in the Progressors), the p-value, and the q-value determined in the statistical analysis of 25 the data concerning the biomarkers. Also included in Table 1 are: the internal identifier for that biomarker compound in the in-house chemical library of authentic standards (CompID); the identifier for that biomarker compound in the Kyoto Encyclopedia of Genes and Genomes (KEGG), if available; and the identifier for that biomarker compound in the Human Metabolome Database (HMDB), if available.

**Table 1. Biomarkers for predicting progression to Type 2 Diabetes in 3 years.**

Biomarker	T2D-3yr Progressor/ non-Progressor	p- value	q- value	ComplID	KEGG ID	HMDB ID
glycine	0.88	0.0452	0.4401	32338	C00037	00123
glutamine	0.64	0.0401	0.3991	53	C00064	00641
3-phenylpropionate (hydrocinnamate)	0.57	0.0176	0.2796	15749	C05629	00764
3-methyl-2-oxobutyrate	0.97	0.0246	0.3406	21047	C00141	00019
5-oxoproline	0.85	0.0308	0.3791	1494	C01879	00267
bradykinin, hydroxy-pro(3)	0.69	0.0271	0.3438	33962		11728
bradykinin, des-arg(9)	0.66	0.0395	0.3991	34420	C00306	04246
HGXAX	0.73	< 0.001	0.0111	31534		
HWESASXX	0.47	< 0.001	0.0017	32836		
XHWESASXXR	0.58	0.0096	0.2050	31538		
[H]HWESASLLR[OH]	0.62	0.0381	0.3991	33964		
phosphate	0.91	0.0383	0.3991	11438	C00009	01429
n-Butyl Oleate	0.70	0.0270	0.3438	36802		
1-linoleoylglycerophosphocholine	0.82	< 0.001	0.0256	34419	C04100	
palmitoyl sphingomyelin	0.88	0.0387	0.3991	37506		
5alpha-androstan- 3beta,17alpha-diol disulfate	0.81	< 0.001	0.0000	37187		
heme	0.30	0.0403	0.3991	32593	C00032	03178
iminodiacetate (IDA)	0.54	0.0064	0.1699	35837		11753
N-acetylthreonine	1.24	0.0052	0.1532	33939	C01118	
tyrosine	1.18	0.0355	0.3991	1299	C00082	00158
3-(4-hydroxyphenyl)lactate	1.34	0.0116	0.2291	32197	C03672	00755
indolelactate	1.26	0.0241	0.3406	18349	C02043	00671
valine	1.07	0.0395	0.3991	1649	C00183	00883
3-hydroxyisobutyrate	1.35	< 0.001	0.0304	1549	C06001	00336
2-hydroxybutyrate (AHB)	1.18	0.0145	0.2614	21044	C05984	00008
mannose	1.14	0.0167	0.2721	584	C00159	00169
trehalose	1.51	0.0064	0.1699	15573	C01083	00975
glucose	1.14	0.0029	0.1059	20488	C00293	00122
succinate	1.10	0.0140	0.2614	1437	C00042	00254
1-oleoylglycerophosphoinositol	1.07	0.0112	0.2291	36602		
urate	1.21	< 0.001	0.0087	1604	C00366	00289

[00108] Biomarkers which, when measured in human plasma, predict the development of T2D in 5 years were identified. These biomarkers were present at 5 different (higher or lower) levels at baseline in the plasma from participants who developed T2D in 5 years (“progressors”, N=131), compared to those subjects that did not (“non-progressors”, N=262). The biomarkers of progression to T2D in 5 years are

presented in Table 2. Table 2 includes, for each listed biomarker, the biochemical name of the biomarker, an indication of the fold-change in the “Progressor” sample mean as compared to the “Non-Progressor” sample mean (values >1.0 represent an elevation in subjects who progress to T2D, and values <1.0 represent lower levels in the Progressors), the p-value, and the q-value determined in the statistical analysis of the data concerning the biomarkers. Also included in Table 2 are: the internal identifier for that biomarker compound in the in-house chemical library of authentic standards (CompID); the identifier for that biomarker compound in the Kyoto Encyclopedia of Genes and Genomes (KEGG), if available; and the identifier for that biomarker compound in the Human Metabolome Database (HMDB), if available.

**Table 2. Biomarkers for predicting progression to Type 2 Diabetes in 5 years.**

Biomarker	T2D-5yr Progressor/ non-Progressor	p-value	q-value	CompID	KEGG ID	HMDB ID
3-phenylpropionate (hydrocinnamate)	0.54	< 0.001	0.0012	15749	C05629	00764
5-oxoproline	0.90	0.0189	0.1727	1494	C01879	00267
bradykinin, hydroxy-pro(3)	0.68	< 0.001	0.0255	33962		11728
phosphate	0.95	0.0319	0.2186	11438	C00009	01429
10-undecenoate (11:1n1)	0.90	0.0388	0.2431	32497		
2-hydroxypalmitate	0.93	0.0193	0.1729	35675		
glycerol 3-phosphate (G3P)	0.83	< 0.001	0.0190	15365	C00093	00126
glycerophosphorylcholine (GPC)	0.89	0.0058	0.0945	15990	C00670	00086
2-palmitoylglycerophosphoethanolamine	0.81	0.0334	0.2253	35688		
1-linoleoylglycerophosphoethanolamine	0.86	0.0353	0.2332	32635		11507
1-heptadecanoylglycerophosphocholine	0.86	0.0294	0.2154	33957		12108
1-oleoylglycerophosphocholine	0.93	0.0130	0.1465	33960		
1-linoleoylglycerophosphocholine	0.85	< 0.001	0.0012	34419	C04100	
1-arachidoylglycerophosphocholine	0.86	0.0251	0.1902	35623		10390
dehydroisoandrosterone sulfate (DHEA-S)	0.79	0.0142	0.1469	32425	C04555	01032
epiandrosterone sulfate	0.83	0.0167	0.1565	33973	C07635	00365
4-androsten-3beta,17beta-diol disulfate	0.86	0.0307	0.2158	37203		03818
5alpha-androstan-3beta,17alpha-diol disulfate	0.85	0.0011	0.0351	37187		
threonate	0.86	0.0139	0.1469	27738	C01620	00943
heme	0.37	0.0020	0.0544	32593	C00032	03178
biliverdin	0.82	0.0168	0.1565	2137	C00500	01008
catechol sulfate	0.83	0.0365	0.2355	35320	C00090	
glycerol 2-phosphate	0.86	0.0165	0.1565	27728	C02979, D01488	02520
thymol sulfate	0.61	0.0403	0.2489	36095	C09908	01878
cinnamoylglycine	0.75	0.0024	0.0622	38637		

tyrosine	1.08	0.0373	0.2371	1299	C00082	00158
3-(4-hydroxyphenyl)lactate	1.21	< 0.001	0.0266	32197	C03672	00755
indolelactate	1.13	0.0234	0.1865	18349	C02043	00671
isoleucine	1.10	< 0.001	0.0255	1125	C00407	00172
valine	1.07	0.0052	0.0908	1649	C00183	00883
alpha-hydroxyisovalerate	1.16	0.0065	0.1022	33937		00407
2-hydroxybutyrate (AHB)	1.17	< 0.001	0.0255	21044	C05984	00008
bradykinin, des-arg(9)	1.14	0.0211	0.1813	34420	C00306	04246
[H]HWESASLLR[OH]	1.30	0.0472	0.2721	33964		
erythrose	1.18	0.0048	0.0877	27722	C01796	02649
mannose	1.12	0.0032	0.0704	584	C00159	00169
glucose	1.15	< 0.001	0.0000	20488	C00293	00122
succinate	1.09	0.0092	0.1229	1437	C00042	00254
stearidonate (18:4n3)	1.17	0.0415	0.2497	33969	C16300	06547
adrenate (22:4n6)	1.15	0.0144	0.1469	32980	C16527	02226
deoxycholate	1.20	0.0414	0.2497	1114	C04483	00626
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	1.31	< 0.001	0.0255	36776	C17337	12458
urate	1.10	0.0040	0.0822	1604	C00366	00289

**[00109]** Biomarkers which, when measured in human plasma, predict the development of myocardial infarction (MI) were identified. These biomarkers were present at different (higher or lower) levels in the plasma at baseline from participants that had a myocardial infarction (MI) (“progressors”, N=105), compared to those subjects that did not suffer a myocardial infarction (MI) (“non-progressors”, N=262). The biomarkers of progression to myocardial infarction are presented in Table 3. Table 1 includes, for each listed biomarker, the biochemical name of the biomarker, an indication of the fold-change in the “Progressor” sample mean as compared to the “Non-Progressor” sample mean (values >1.0 represent an elevation in subjects who progress to MI, and values <1.0 represent a lower level in the Progressors), the p-value, and the q-value determined in the statistical analysis of the data concerning the biomarkers. Also included in Table 3 are: the internal identifier for that biomarker compound in the in-house chemical library of authentic standards (ComplID); the identifier for that biomarker compound in the Kyoto Encyclopedia of Genes and Genomes (KEGG), if available; and the identifier for that biomarker compound in the Human Metabolome Database (HMDB), if available.

**Table 3. Biomarkers for predicting progression to Myocardial Infarction.**

Biomarker	MI Progres- sor/non- Progres- sor	p-value	q-value	ComplID	KEGG ID	HMDB ID
glycine	0.94	0.0390	0.0986	32338	C00037	00123
N-acetylglycine	0.86	0.0196	0.0639	27710		00532
serine	0.96	0.0397	0.0996	32315	C00065	03406
glutamine	0.78	0.0081	0.0374	53	C00064	00641
HGX A	0.69	0.0039	0.0265	31534		
ADSGEGDFXAEGGGVR	0.86	0.0359	0.0935	33084		
glycerate	0.91	0.0019	0.0208	1572	C00258	00139
heptanoate (7:0)	0.95	0.0423	0.1050	1644	C17714	00666
caprylate (8:0)	0.93	0.0014	0.0187	32492	C06423	00482
caprate (10:0)	0.94	0.0069	0.0345	1642	C01571	00511
10-undecenoate (11:1n1)	0.93	0.0315	0.0853	32497		
laurate (12:0)	0.95	0.0020	0.0208	1645	C02679	00638
5-dodecenoate (12:1n7)	0.88	0.0184	0.0618	33968		00529
stearate (18:0)	0.95	0.0286	0.0811	1358	C01530	00827
methyl palmitate (15 or 2)	0.95	0.0262	0.0790	38768		
17-methylstearate	0.91	0.0085	0.0388	38296		
glycerophosphorylcholine (GPC)	0.93	0.0104	0.0442	15990	C00670	00086
1-linoleoylglycerophosphocholine	0.94	0.0286	0.0811	34419	C04100	
5alpha-pregnan-3beta,20alpha-diol disulfate	0.54	0.0101	0.0442	37198		
5alpha-pregnan-3alpha,20beta-diol disulfate	0.56	< 0.001	0.0121	37201		
piperine	0.74	0.0018	0.0208	33935	C03882	
betaine	1.09	0.0077	0.0369	3141	C00719	00043
N-acetylalanine	1.05	0.0484	0.1100	1585	C02847	00766
glutamate	1.04	0.0050	0.0297	57	C00025	03339
pyroglutamine	1.22	< 0.001	0.0067	32672		
phenyllactate (PLA)	1.10	0.0137	0.0502	22130	C05607	00779
phenylalanine	1.05	< 0.001	0.0053	64	C00079	00159
tyrosine	1.06	< 0.001	0.0121	1299	C00082	00158
3-(4-hydroxyphenyl)lactate	1.13	< 0.001	0.0067	32197	C03672	00755
phenylacetylglutamine	1.32	< 0.001	0.0067	35126	C05597	06344
<b>kynurenone</b>	1.08	0.0034	0.0257	15140	C00328	00684
indolelactate	1.10	0.0042	0.0270	18349	C02043	00671
C-glycosyltryptophan	1.09	< 0.001	0.0001	32675		
<b>3-indoxyl sulfate</b>	1.17	< 0.001	0.0136	27672		00682
alpha-hydroxyisocaproate	1.13	0.0167	0.0590	22132	C03264	00746
isoleucine	1.06	< 0.001	0.0083	1125	C00407	00172
leucine	1.05	0.0026	0.0241	60	C00123	00687
valine	1.03	0.0173	0.0590	1649	C00183	00883

2-methylbutyroylcarnitine	1.10	0.0035	0.0257	35431		00378
dimethylarginine (SDMA + ADMA)	1.05	0.0123	0.0475	36808	C03626	01539, 03334
ornithine	1.14	0.0032	0.0257	35832	C00077	03374
urea	1.12	0.0033	0.0257	1670	C00086	00294
citrulline	1.08	0.0113	0.0464	2132	C00327	00904
creatinine	1.04	0.0284	0.0811	513	C00791	00562
4-guanidinobutanoate	1.05	0.0457	0.1084	15681	C01035	03464
mannitol	1.18	0.0114	0.0464	15335	C00392	00765
1,5-anhydroglucitol (1,5-AG)	1.07	0.0154	0.0552	20675	C07326	02712
lactate	1.12	0.0054	0.0298	527	C00186	00190
succinate	1.07	0.0017	0.0208	1437	C00042	00254
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	1.29	0.0037	0.0257	31787		
<b>3-dehydrocarnitine</b>	1.06	0.0218	0.0677	32654	C02636	12154
palmitoylcarnitine	1.08	0.0334	0.0884	22189	C02990	00222
<b>oleoylcarnitine</b>	1.08	0.0280	0.0811	35160		05065
glycocholenate sulfate	1.12	0.0473	0.1084	32599		
choline	1.08	< 0.001	0.0002	15506	C00114	00097
<b>myo-inositol</b>	1.07	< 0.001	0.0121	19934	C00137	00211
2-palmitoylglycerophosphocholine	1.08	0.0270	0.0803	35253		
1-stearoylglycerophosphocholine	1.05	0.0319	0.0853	33961		
2-stearoylglycerophosphocholine	1.05	0.0463	0.1084	35255		
1-arachidoylglycerophosphocholine	1.11	0.0197	0.0639	35623		10390
1-stearoylglycerol (1-monostearin)	1.04	0.0220	0.0677	21188	D01947	
<b>5alpha-androstan-3beta,17beta-diol disulfate</b>	1.19	0.0102	0.0442	37190		00493
<b>xanthine</b>	1.29	0.0119	0.0472	3147	C00385	00292
pseudouridine	1.06	< 0.001	0.0053	33442	C02067	00767
<b>trigonelline (N'-methylnicotinate)</b>	1.30	0.0117	0.0471	32401	C01004	00875
2-hydroxyhippurate (salicylurate)	1.97	0.0054	0.0298	18281	C07588	00840
4-vinylphenol sulfate	1.24	0.0051	0.0297	36098	C05627	04072
<b>1-methylurate</b>	1.24	0.0011	0.0148	34395		03099
<b>1,3-dimethylurate</b>	1.21	0.0026	0.0241	32391		01857
<b>1,7-dimethylurate</b>	1.22	0.0173	0.0590	34400	C16356	11103
<b>1,3,7-trimethylurate</b>	1.18	0.0092	0.0411	34404	C16361	02123
erythritol	1.09	0.0376	0.0969	20699	C00503	02994

**[00110]** Biomarkers which, when measured in human plasma, predict the development of stroke were identified. These biomarkers were present at different (higher or lower) levels at baseline in the plasma from participants who developed a 5 stroke (“progressors”, N=45), compared to those subjects that did not (“non-progressors”, N=262). The biomarkers of progression to stroke are presented in Table 4. Table 4 includes, for each listed biomarker, the biochemical name of the

biomarker, an indication of the fold-change in the “Progressor” sample mean as compared to the “Non-Progressor” sample mean (values >1.0 represent an elevation in subjects who progress to Stroke, and values <1.0 represent a lower level in the Progressors), the p-value, and the q-value determined in the statistical analysis of the 5 data concerning the biomarkers. Also included in Table 4 are: the internal identifier for that biomarker compound in the in-house chemical library of authentic standards (CompID); the identifier for that biomarker compound in the Kyoto Encyclopedia of Genes and Genomes (KEGG), if available; and the identifier for that biomarker compound in the Human Metabolome Database (HMDB), if available.

10 **Table 4. Biomarkers for predicting progression to Stroke.**

Biomarker	Stroke Progressor/ non- Progressor	p-value	q-value	CompID	KEGG ID	HMDB ID
anthranilate	0.88	0.0184	0.1669	4970	C00108	01123
caproate (6:0)	0.91	0.0370	0.2217	32489	C01585	00535
heptanoate (7:0)	0.92	0.0096	0.1437	1644	C17714	00666
3-hydroxyoctanoate	0.91	0.0321	0.2062	22001		01954
8-hydroxyoctanoate	0.89	0.0439	0.2468	21239		00711
2-hydroxystearate	0.93	0.0403	0.2294	17945	C03045	
2-hydroxypalmitate	0.93	0.0258	0.1875	35675		
azelate (nonanedioate)	0.89	0.0253	0.1875	18362	C08261	00784
isovalerate	0.91	0.0319	0.2062	34732	C08262	00718
glycerophosphorylcholine (GPC)	0.91	0.0169	0.1669	15990	C00670	00086
dehydroisoandrosterone sulfate (DHEA-S)	0.86	0.0311	0.2059	32425	C04555	01032
5alpha-pregn-3beta,20alpha-diol disulfate	0.49	< 0.001	0.0451	37198		
5alpha-pregn-3alpha,20beta-diol disulfate	0.46	< 0.001	0.0135	37201		
pregn steroid monosulfate	0.82	0.0254	0.1875	32619	C18044	00774
arabonate	0.87	0.0150	0.1669	37516		00539
triethyleneglycol	0.82	0.0046	0.0903	27743		
alanine	1.05	0.0176	0.1669	32339	C00041	00161
N-acetylalanine	1.09	0.0015	0.0606	1585	C02847	00766
glutamate	1.04	0.0198	0.1669	57	C00025	03339
glutaroyl carnitine	1.10	0.0028	0.0690	35439		13130
C-glycosyltryptophan	1.07	0.0018	0.0682	32675		
ornithine	1.17	0.0085	0.1315	35832	C00077	03374
citrulline	1.10	0.0178	0.1669	2132	C00327	00904
4-acetamidobutanoate	1.06	0.0333	0.2110	1558	C02946	03681
lactate	1.21	< 0.001	0.0130	527	C00186	00190
citrate	1.11	0.0108	0.1437	1564	C00158	00094
acetylphosphate	1.08	0.0026	0.0690	15488	C00227	01494

<b>propionylcarnitine</b>	1.26	0.0391	0.2283	32452	C03017	00824
deoxycarnitine	1.05	0.0200	0.1669	36747	C01181	01161
<b>3-dehydrocarnitine</b>	1.16	< 0.001	0.0014	32654	C02636	12154
<b>acetylcarnitine</b>	1.72	0.0358	0.2186	32198	C02571	00201
<b>oleoylcarnitine</b>	1.23	0.0063	0.1146	35160		05065
glycerol	1.06	0.0338	0.2111	15122	C00116	00131
<b>myo-inositol</b>	1.08	< 0.001	0.0290	19934	C00137	00211
1-palmitoylglycerol (1-monopalmitin)	1.04	0.0267	0.1904	21127		
1-oleoylglycerol (1-monoolein)	1.13	0.0045	0.0903	21184		11567
palmitoyl sphingomyelin	1.13	< 0.001	0.0451	37506		
stearoyl sphingomyelin	1.12	0.0024	0.0690	19503	C00550	01348
lathosterol	1.36	0.0227	0.1822	33488	C01189	01170
cholesterol	1.10	0.0044	0.0903	63	C00187	00067
uridine	1.06	0.0197	0.1669	606	C00299	00296
pseudouridine	1.08	< 0.001	0.0014	33442	C02067	00767
bilirubin (E,E)	1.18	0.0250	0.1875	32586		
<b>trigonelline (N<sup>+</sup>-methylnicotinate)</b>	1.27	0.0142	0.1669	32401	C01004	00875
benzoate	1.07	0.0030	0.0690	15778	C00180	01870
4-vinylphenol sulfate	1.15	0.0139	0.1669	36098	C05627	04072
<b>1,3,7-trimethylurate</b>	1.28	0.0446	0.2475	34404	C16361	02123
erythritol	1.11	0.0297	0.2053	20699	C00503	02994

[00111] In an independent experiment with a different cohort, a number of biomarkers useful to determine the non-diabetic individuals who will develop type 2 diabetes (T2D) were identified. The biomarkers were identified by collecting plasma samples at the time of study enrollment (baseline) from 660 non-diabetic, human subjects who were followed for 5 years. Over the course of the study the participants fell into the following groups: 440 were “non-progressors” (i.e., subjects who remained stable and did not progress to T2D) and 220 developed T2D (“T2D progressors”). In brief, the plasma samples were extracted and split into equal parts for analysis on the GC/MS and LC/MS/MS platforms as described in General Methods. Proprietary software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantification by peak area integration. A total of 695 metabolites were measured of which the structural identities of 402 were determined (“Named”) and 293 were undetermined (“Unnamed”). The resulting metabolomic profiles were statistically analyzed to identify the biomarkers that are differentially present between the groups using Analysis of Variance (ANOVA) with contrasts and by Random Forest classification.

**[00112]** Biomarkers which, when measured in human plasma, predict the development of type 2 diabetes within 5 years were identified. At baseline, the biomarkers were present at different (higher or lower) levels in the plasma from participants that progressed to type 2 diabetes within 5 years (“Progressors”), 5 compared to those subjects that did not progress to type 2 diabetes (“non-progressors”). The biomarkers for predicting the progression to Type 2 diabetes in 5 years validate the earlier results presented in Table 2 and are presented in Table 5.

**[00113]** These plasma biomarkers were evaluated for the ability to predict the individuals who will progress to Type 2 Diabetes using regression analysis. The 10 biomarker candidates were ranked using Random Forest analyses according to their contribution to the ability to predict separation of participants that progressed to type 2 diabetes within 5 years (“progressors”) compared to those subjects that did not progress to type 2 diabetes (“non-progressors”). The top identified biomarkers for predicting the progression to Type 2 diabetes in 5 years according to Random Forest 15 classification are presented in Table 5. Table 5 includes, for each listed biomarker, the biochemical name of the biomarker, an indication of the fold-change in the “Progressor” sample mean as compared to the “Non-Progressor” sample mean (values >1.0 represent an elevation in subjects who progress to T2D, and values <1.0 represent a lower level in the Progressors), the p-value, and the q-value determined in 20 the statistical analysis of the data concerning the biomarkers. Also included in Table 5 are: the internal identifier for that biomarker compound in the in-house chemical library of authentic standards (CompID); the identifier for that biomarker compound in the Kyoto Encyclopedia of Genes and Genomes (KEGG), if available; and the identifier for that biomarker compound in the Human Metabolome Database 25 (HMDB), if available.

**Table 5. Biomarkers for predicting progression to Type 2 Diabetes in 5 years.**

Biomarker	Fold-of-change T2D-5yr Progress or/non- Progress or	p-value	q-value	ComplID	KEGG ID	HMDB ID
3-methyl-2-oxovalerate	1.13	2.00E-15	1.77E-13	15676	C00671	03736
2-methylbutyrylcarnitine (C5)	1.15	4.04E-11	7.69E-10	35431		00378
kynurename	1.15	0.0001	0.0003	1417	C01717	00715
mannose	1.19	0.00E+0	0.00E+00	584	C00159	00169
alpha-ketoglutarate	1.33	9.61E-09	7.75E-08	33453	C00026	00208
2-hydroxybutyrate (AHB)	1.24	1.44E-11	3.81E-10	21044	C05984	00008
4-methyl-2-oxopentanoate	1.13	2.00E-15	1.77E-13	22116	C00233	00695
alpha-ketobutyrate	1.32	6.44E-12	2.08E-10	42107	C00109	00005
xanthine	1.33	3.98E-11	7.69E-10	3147	C00385	00292
glycine	0.88	3.25E-11	7.34E-10	32338	C00037	00123
arachidonate (20:4n6)	1.13	1.35E-10	2.07E-09	1110	C00219	01043
valine	1.08	2.84E-09	2.61E-08	1649	C00183	00883
stearidionate (18:4n3)	1.24	3.51E-09	3.00E-08	33969	C16300	06547
creatine	1.24	2.25E-10	3.11E-09	27718	C00300	00064
3-hydroxyisobutyrate	1.18	3.84E-12	1.52E-10	1549	C06001	00336
tyrosine	1.11	2.63E-13	1.33E-11	1299	C00082	00158
carnitine	1.07	4.24E-11	7.69E-10	15500	C00318	
alpha-hydroxyisocaproate	1.17	2.25E-07	1.21E-06	22132	C03264	00746
glucose	1.13	0.00E+0	0.00E+00	20488	C00031	00122
isovalerylcarnitine	1.19	1.09E-11	3.18E-10	34407		00688
cyclo(leu-pro)	1.4	1.24E-10	2.07E-09	37104		
palmitate (16:0)	1.13	4.63E-10	5.60E-09	1336	C00249	00220
<b>palmitoyl sphingomyelin</b>	0.9	2.25E-09	2.25E-08	37506		
dihomo-linolenate (20:3n3 or n6)	1.14	1.30E-10	2.07E-09	35718	C03242	02925
beta-hydroxyisovalerate	1.12	8.14E-06	3.19E-05	12129		00754
hexanoylcarnitine	1.14	1.41E-08	1.08E-07	32328		00705
palmitoleate (16:1n7)	1.26	3.18E-09	2.80E-08	33447	C08362	03229
10-heptadecenoate (17:1n7)	1.2	8.96E-09	7.44E-08	33971		
N-acetylmethionine	0.92	4.99E-06	2.01E-05	1589	C02712	11745
pyruvate	1.17	1.24E-07	7.37E-07	599	C00022	00243
oleate (18:1n9)	1.12	5.75E-08	3.63E-07	1359	C00712	00207
isoleucine	1.07	1.15E-07	6.96E-07	1125	C00407	00172
piperine	1.42	4.35E-06	1.81E-05	33935	C03882	
leucine	1.07	1.25E-08	9.77E-08	60	C00123	00687
<b>beta-hydroxypyruvate</b>	1.2	0.0019	0.0038	15686	C00168	01352
cinnamoylglycine	0.69	5.48E-07	2.74E-06	38637		
3-methyl-2-oxobutyrate	1.07	0.8276	0.4012	21047	C00141	00019
docosapentaenoate (n6 DPA; 22:5n6)	1.22	1.87E-06	8.26E-06	37478	C16513	13123

gamma-glutamyltyrosine	1.1	3.15E-10	4.16E-09	2734		
glycerol 3-phosphate (G3P)	1.02	0.0005	0.0012	15365	C00093	00126
dihomo-linoleate (20:2n6)	1.17	1.43E-07	8.00E-07	17805	C16525	
3-(4-hydroxyphenyl)lactate	1.16	2.67E-09	2.58E-08	32197	C03672	00755
gamma-glutamylphenylalanine	1.1	1.66E-08	1.24E-07	33422		00594
<b>3-hydroxypropanoate</b>	1.14	1.67E-06	7.69E-06	42103	C01013	00700
pantothenate	1.15	2.33E-07	1.23E-06	1508	C00864	00210
glutamate	1.13	1.91E-08	1.35E-07	57	C00025	03339
alanine	1.11	2.66E-08	1.76E-07	1126	C00041	00161
urate	1.08	1.41E-07	8.00E-07	1604	C00366	00289
glycerol 2-phosphate	0.96	0.0009	0.0021	27728	C02979	02520
propionylcarnitine	1.13	1.79E-08	1.30E-07	32452	C03017	00824
tryptophan	1.05	1.43E-05	5.18E-05	54	C00078	00929
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	1.13	7.15E-07	3.52E-06	36776	C17337	12458
phenylalanine	1.05	4.07E-06	1.71E-05	64	C00079	00159
bradykinin, hydroxy-pro(3)	0.75	1.59E-09	1.71E-08	33962		11728
4-androsten-3beta,17beta-diol disulfate 1*	1.59	3.12E-06	1.35E-05	37202		03818
1-linoleoylglycerophosphocholine	0.9	5.35E-06	2.13E-05	34419	C04100	
<b>catechol sulfate</b>	0.84	4.58E-07	2.37E-06	35320	C00090	
quinate	0.79	0.0002	0.0007	18335	C00296	03072
docosapentaenoate (n3 DPA; 22:5n3)	1.18	1.88E-06	8.26E-06	32504	C16513	01976
2-linoleoylglycerophosphocholine	0.85	1.72E-05	6.10E-05	35257		
hippurate	0.81	1.71E-06	7.75E-06	15753	C01586	00714
glutamine	0.95	6.44E-05	0.0002	53	C00064	00641
2-aminobutyrate	1.11	1.29E-06	6.15E-06	32348	C02261	00650
tryptophan betaine	0.5	0.0011	0.0025	37097	C09213	
N-acetyltryptophan	1.16	1.53E-06	7.17E-06	33959	C03137	
eicosenoate (20:1n9 or 11)	1.15	7.82E-05	0.0002	33587		02231
butyrylcarnitine	1.22	1.32E-07	7.67E-07	32412		
3-phenylpropionate (hydrocinnamate)	0.75	2.21E-05	7.55E-05	15749	C05629	00764
lactate	1.1	5.26E-05	0.0002	527	C00186	00190
gluconate	1.08	0.0075	0.0118	587	C00257	00625
hydroxybutyrylcarnitine	1.24	3.60E-05	0.0001	33910		
hydrochlorothiazide	1.44	1.14E-05	4.19E-05	39625	C07041	01928
isovalerate	1.11	0.0104	0.0154	34732	C08262	00718
caffeine	1.31	7.33E-05	0.0002	569	C07481	01847
asparagine	0.95	0.0121	0.0173	34283	C00152	00168
stearate (18:0)	1.09	9.64E-06	3.68E-05	1358	C01530	00827
2-hydroxy-3-methylvalerate	1.27	9.24E-06	3.58E-05	36746		00317
1,3,7-trimethylurate	1.26	0.0007	0.0017	34404	C16361	02123
10-nonadecenoate (19:1n9)	1.16	1.13E-05	4.19E-05	33972		
linoleate (18:2n6)	1.06	0.0032	0.0059	1105	C01595	00673
gamma-glutamylglutamine	0.92	2.24E-05	7.56E-05	2730		11738

maltooltriose	1.46	0.0105	0.0154	15910	C02052	01296
3-dehydrocarnitine*	1.07	0.0118	0.017	32654	C02636	12154
3-hydroxy-2-ethylpropionate	1.09	0.0047	0.0081	32397		00396
taurocholate	1.6	0.0004	0.001	18497	C05122	00036
adrenate (22:4n6)	1.09	0.0004	0.001	32980	C16527	02226
fumarate	1.06	0.0012	0.0026	1643	C00122	00134
trigonelline (N'-methylnicotinate)	0.82	0.0022	0.0041	32401	C01004	00875
2-hydroxyhippurate (salicylurate)	5.02	0.3372	0.2298	18281	C07588	00840
3-hydroxyhippurate	0.8	0.0062	0.0101	39600		06116
campesterol	0.84	0.0002	0.0005	39511	C01789	02869
indolepropionate	0.79	0.0002	0.0005	32405		02302
2-hydroxyglutarate	1.06	0.0399	0.0462	37253	C02630	00606
methionine	1.03	0.0053	0.009	1302	C00073	00696
gamma-glutamylleucine	1.1	3.36E-06	1.43E-05	18369		11171
xylitol	1.08	0.2375	0.1879	4966	C00379	02917
margarate (17:0)	1.1	0.0002	0.0005	1121		02259
phenyllactate (PLA)	1.1	0.0112	0.0164	22130	C05607	00779
acetylcarnitine	1.09	0.0002	0.0005	32198	C02571	00201
1-arachidonoylglycerophosphoinositol	1.11	0.001	0.0023	34214		
lathosterol	1.06	0.4008	0.2519	39864	C01189	01170
dodecanedioate	0.98	0.085	0.0866	32388	C02678	00623
eicosapentaenoate (EPA; 20:5n3)	1.14	0.0011	0.0024	18467	C06428	01999
5-oxoproline	0.94	0.0115	0.0166	1494	C01879	00267
malate	1.09	0.0036	0.0065	1303	C00149	00156
pyroglutamine	0.89	0.0006	0.0014	32672		
deoxycholate	1.16	0.0019	0.0038	1114	C04483	00626
5alpha-pregnan-3beta,20alpha-diol disulfate	1.12	0.008	0.0124	37198		
1,3-dihydroxyacetone	1.13	0.0152	0.0209	35963	C00184	01882
linolenate [alpha or gamma; (18:3n3 or 6)]	1.07	0.0047	0.0081	34035	C06427	01388
indoleacrylate	0.9	0.0144	0.0199	22114		00734
inosine 5'-monophosphate (IMP)	1.08	0.6603	0.348	2133	C00130	00175
serotonin (5HT)	0.94	0.2798	0.2073	2342	C00780	00259
andro steroid monosulfate	1.31	0.0001	0.0004	32792	C04555	02759
N-acetylphenylalanine	1.14	0.0014	0.003	33950	C03519	00512
allantoin	0.97	0.1396	0.1267	1107	C02350	00462
phosphoethanolamine	0.91	0.0929	0.0926	12102	C00346	00224
3-hydroxybutyrate (BHBA)	1.03	0.448	0.2708	542	C01089	00357
leucylleucine	1.28	0.0204	0.0267	36756	C11332	
methyl-beta-glucopyranoside	0.58	0.0039	0.007	40480		
cortisone	1.05	0.0138	0.0193	1769	C00762	02802
glycolate (hydroxyacetate)	1.04	0.086	0.0871	15737	C00160	00115
4-hydroxyhippurate	0.8	0.0114	0.0166	35527		

octanoylcarnitine	1.03	0.0188	0.0247	33936	C02838	00791
myristate (14:0)	1.12	0.0001	0.0004	1365	C06424	00806
1,7-dimethylurate	1.09	0.0235	0.03	34400	C16356	11103
1-methylurate	0.93	0.2911	0.2129	34395		03099
nicotinamide	1.06	0.1676	0.147	594	C00153	01406
1-palmitoleoylglycerophosphoethanolamine	1.33	0.0119	0.0171	34565		
glycerophosphorylcholine (GPC)	0.91	0.0008	0.0018	15990	C00670	00086
kynurenine	1.06	0.0021	0.0041	15140	C00328	00684
1-linoleoylglycerophosphoethanolamine	0.94	0.025	0.0317	32635		11507
12-HETE	1.14	0.105	0.102	37536		06111
glycoursodeoxycholate	0.91	0.3843	0.2476	39379		00708

**Example 3 Selected, Priority Biomarkers:**

[00114] The biomarkers for cardiometabolic disease progression, such as Type 2 diabetes (T2D), Myocardial Infarction (MI), or stroke were selected based upon the following criteria: (a) biomarkers predictive of T2D progression with a >10% fold change observed in both T2D time intervals of 3 years and 5 years and having  $p<0.05$ ; (b) biomarkers predictive of T2D progression with a >15% fold change in at least one T2D time interval (3 years or 5 years) AND with statistical trending ( $p<0.1$ ) in other T2D time interval (e.g. alpha-hydroxyisovalerate) OR biomarker having physiological features relevant to said disease (e.g. erythrose); (c) biomarkers predictive of T2D progression with a >20% fold change in one T2D time interval (3 years or 5 years, e.g. trehalose, n-butyl oleate); (d) other statistical measures from our metabolomics screening and targeted data indicating biomarker significance (e.g. glycerol-3-phosphate; (e) biomarkers predictive of MI or stroke with a >15% fold observed change in analyte levels compared to “progressors” of said disease versus “non-progressors”. The selected priority biomarkers have  $p<0.05$ ,  $q<0.1$  and were ranked among the top 50 biomarker candidates in RF importance plots. These biomarkers are measured in plasma samples from subjects and the values obtained from the measurements are used in a mathematic model (e.g., logistic regression) to determine or aid in determining the progression of insulin resistance or pre-diabetes to the associated disorders of type 2 diabetes or cardiovascular disease (e.g., myocardial

infarction or stroke) and/or the susceptibility of the subject to develop these cardiometabolic diseases.

**3A. Selected Biomarkers for Development of or Progression to T2D:**

[00115] The biomarkers are: 3-phenylpropionate (hydrocinnamate), 3-(4-

5 hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, erythrose, glycerol-3-phosphate, isoleucine, valine.

10 **3B. Selected biomarkers for development of or progression to stroke/MI:**

[00116] The biomarkers are: 3-indoxylsulfate, propionylcarnitine, 3-

dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, 5alpha-pregnan-3alpha,20beta-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperidine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-54imethylurate, kynurenone.

15 **3C. Selected biomarkers for development of or progression to cardiovascular disease, IR/IGT, T2D:**

[00117] The biomarkers are: adrenate, alpha-hydroxyisovalerate, glutamine,

glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate

20 (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate.

**Example 4: Comparison of Biomarkers and Algorithms to Current Clinical Tests for Prediction of Progression to Type-2 Diabetes, Cardiovascular Disease**

[00118] The performance of IR Biomarkers Model was compared with the results

of the OGTT and FPG test in a cohort of 401 subjects. The IR Biomarkers Model had

25 better Sensitivity, Specificity, Positive Predictive Value and Negative Predictive

Value than either the Oral Glucose Tolerance Test (OGTT) or Fasting Plasma

Glucose (FPG), two widely and currently used clinical tests. The results of the

comparison of IR biomarkers with these clinical assays currently used to measure insulin resistance and type 2 diabetes are summarized in Table 6.

**Table 6. Comparison of IR Biomarkers in instant application with Clinical Assays currently used to measure insulin resistance and type 2 diabetes**

TEST	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<b>IR Biomarkers Model (AHB, LGPC, Oleate, BMI, insulin)</b>	<b>62.2</b>	<b>93.8</b>	<b>83.2</b>	<b>83.3</b>
OGTT	46.2	92.5	75.3	77.6
FPG	33.6	85.5	56.1	50.0

5 [00119] Plasma samples from a subset of the 401 subjects in the cohort that had data available for insulin, glucose disposal (Rd), adiponectin and results from the OGTT and HOMA-IR tests were evaluated for the correlation with Rd, the glucose disposal rate measurement obtained from the HI clamp. A total of 369 plasma samples from the 369 subjects where this data was available were analyzed. Subjects that had 10 missing values were not included; 14 subjects were missing Fasting Insulin values and 2 additional subjects were missing values for adiponectin. These results and the result obtained on the same 369 subjects with the IR Model: SQRTRD ~ BMI + 2 Hydroxybutyrate + Linoleate (x) + Linolyl\_GPC + decanoylcarnitine are shown in Table 14. The IR Model was significantly correlated (p-value = 2.01E-54) with Rd 15 and showed a better R value than did any of the other markers or models. The IR Model also had better diagnostic performance based upon the AUC, Sensitivity, Specificity, Negative Predictive Value and Positive Predictive Value than any of the other tests. In addition, the biomarkers and models provided herein demonstrate a similar correlation with glucose disposal than the HI clamp.

**Table 7. Comparison of IR model with other commonly used tests, algorithms and biomarkers to determine insulin sensitivity in a subject.**

Dx Test	N	R	P-value	AUC	Sens	Spec	NPV	PPV
IR Model	369	0.71	2.01E-54	74.8	59.5	90.1	75.8	81.1
OGTT	369	NA	NA	68.0	43.7	92.2	74.3	75.9
FPG	369	-0.16	0.002072	58.7	31.8	85.6	53.3	70.8
HOMA-IR	369	-0.56	1.44E-31	70.0	50.8	89.3	71.1	77.8
Adiponectin	369	0.31	7.44E-10	57.6	35.0	80.3	47.8	70.4

[00120] The ability of the biomarkers to predict individuals who will progress to Type 2 Diabetes (T2D), Myocardial infarction (MI) or stroke was evaluated using Random Forest regression analysis to classify the subjects as “Non-Progressors” or “Progressors” based on the baseline measured values of the biomarkers and/or the measured values of the clinical parameters of sex, age, Body Mass Index (BMI), fasting plasma glucose (FPG), and fasting insulin. These clinical parameters are used currently by clinicians to evaluate the risk of a subject developing T2D, MI or stroke. The Random Forest analyses for each outcome (T2D, MI, Stroke) was carried out using 1) the clinical factors alone, 2) all of the biomarkers in Tables 1, 2, 3, 4 and 5 alone, 3) the selected subset of the biomarkers alone, or 4) the selected subset of biomarkers plus the clinical factors. The cohort is described in Example 2. The results of the analyses are presented in Tables 8, 9 and 10. In Table 8, the results of the classification of the 131 subjects that progressed to T2D and the 262 subjects that did not progress (non-progressors) are presented. In Table 9, the results of the classification of the 105 subjects that progressed to MI and the 262 subjects that did not progress (non-progressors) are presented. In Table 10, the results of the classification of the 45 subjects that progressed to stroke and the 262 subjects that did not progress (non-progressors) are presented. In each analysis, the biomarkers correctly classified the subjects as “Progressors” or “Non-progressors” with better predictive accuracy than was achieved with the clinical parameters alone. Using the selected biomarkers in combination with the clinical factors improved predictive accuracy. The diagnostic parameters of sensitivity, specificity and the prediction accuracy are presented in the Tables.

**Table 8. T2D RF Analysis Summary**

Test	Sensitivity	Specificity	Accuracy (Error)
<b>Clinical factors only</b>	<b>63.3%</b>	<b>59.7%</b>	<b>61.1% (39.9)</b>
<b>All Biomarkers only</b>	<b>70%</b>	<b>66.3%</b>	<b>66.7% (33.3)</b>
<b>Selected biomarkers</b>	<b>76%</b>	<b>67%</b>	<b>67.9% (32.1)</b>
<b>Clinical factors + Selected biomarkers</b>	<b>71.4%</b>	<b>70.1%</b>	<b>70.7% (29.7)</b>

**Table 9. MI RF Analysis Summary**

Test	Sensitivity	Specificity	Accuracy (Error)
<b>Clinical factors only</b>	<b>64.2%</b>	<b>63%</b>	<b>63.3% (36.7)</b>
<b>All Biomarkers only</b>	<b>64%</b>	<b>65.6%</b>	<b>65.2% (34.8)</b>
<b>Selected biomarkers</b>	<b>62.4%</b>	<b>61.4%</b>	<b>61.7% (38.3)</b>
<b>Clinical factors + Selected biomarkers</b>	<b>63.4%</b>	<b>65.6%</b>	<b>65.1% (34.9)</b>

5 **Table 10. RF Analysis Stroke Summary**

Test	Sensitivity	Specificity	Accuracy (Error)
<b>Clinical factors only</b>	<b>61.4%</b>	<b>56.8%</b>	<b>57.3% (42.7)</b>
<b>All Biomarkers only</b>	<b>69%</b>	<b>67.8%</b>	<b>68% (32)</b>
<b>Selected biomarkers</b>	<b>63.8%</b>	<b>64%</b>	<b>64% (36)</b>
<b>Clinical factors + Selected biomarkers</b>	<b>68.4%</b>	<b>65.3%</b>	<b>65.6% (34.4)</b>

[00121] While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made without departing from the spirit and scope of the invention.

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## Claims

What is claimed is:

1. A method for determining the probability that a subject will progress from being normal to having one or more diseases selected from Insulin Resistance, dysglycemia, Type-2 diabetes, CVD, the method comprising:
  - 5 obtaining a biological sample from the subject;
  - determining the level(s) of one or more biomarkers selected from the group consisting of one or more biomarkers listed in Tables 1, 2, 3, 4 and/or 5 in the biological sample by directly measuring the level of the one or more biomarkers in the sample; and
    - 10 analyzing the levels of the one or more biomarkers in the sample by a statistical analysis to determine the probability that the subject will progress to CVD, Type 2 diabetes, dysglycemia.
2. The method of claim 1, wherein the analyzing step comprises generating a probability curve using a multivariate regression model based on the measured levels of one or more biomarkers listed in Tables 1, 2, 3, 4, and/or 5.
3. The method of claim 2, wherein the probability curve is generated using a multivariate regression model of all of the biomarkers listed in Tables 1, 2, 3, 4 and/or 5.
- 20 4. The method of claim 2, wherein the probability curve is generated using a multivariate regression model using the measured level of biomarkers selected from the group 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenone, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-

phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine.

5. The method of claim 1, wherein the statistical analysis comprises a logistic regression model.

5 6. The method of claim 1, wherein the one or more biomarkers are selected from the group 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate 10 (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine.

20 7. The method of claim 1, wherein the disease is Type-2 diabetes and the one or more biomarkers are selected from the group consisting of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, erythrose, glycerol-3-phosphate, isoleucine, valine, to predict the progression to type-2 diabetes.

25 8. The method of claim 1, wherein the disease is cardiovascular disease and the one or more biomarkers are selected from the group consisting of adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta, 17alpha-diol disulfate, urate to determine progression to cardiovascular disease.

30 9. A method of determining susceptibility of a subject to type-2 diabetes, the method comprising:

analyzing the biological sample from the subject to determine the level(s) of one or more biomarkers selected from the group consisting one or more biomarkers listed in Tables 1, 2, and/or 5; and

5 comparing the level(s) of the one or more biomarkers in the sample to diabetes-positive and/or diabetes-negative reference levels of the one or more biomarkers in order to determine whether the subject is susceptible to developing type-2 diabetes.

10. The method of claim 9 wherein the one or more biomarkers are selected from the group consisting of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, erythrose, glycerol-3-phosphate, isoleucine, valine.

15 11. The method of claim 9, wherein the comparing step comprises generating a type 2 diabetes risk score for the subject in order to determine the susceptibility to type 2 diabetes in the subject.

12. A method of determining susceptibility of a subject to cardiovascular disease, the method comprising:

20 analyzing the biological sample from the subject to determine the level(s) of one or more biomarkers selected from the group consisting one or more biomarkers listed in Tables 3 and/or 4; and

25 comparing the level(s) of the one or more biomarkers in the sample to diabetes-positive and/or diabetes-negative reference levels of the one or more biomarkers in order to determine whether the subject is susceptible to developing cardiovascular disease.

30 13. The method of claim 12 wherein the one or more biomarkers are selected from the group consisting of adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta, 17alpha-diol disulfate, urate.

14. The method of claim 12 wherein the cardiovascular disease is selected from the group consisting of myocardial infarction or stroke.

15. The method of claim 12, wherein the comparing step comprises generating a cardiovascular disease risk score for the subject in order to determine the susceptibility to cardiovascular disease in the subject.

16. A method of monitoring the progression or regression of insulin 5 resistance in a subject, the method comprising:

analyzing the biological sample from the subject to determine the level(s) of one or more biomarkers selected from the group consisting one or more biomarkers listed in Tables 1, 2, 3, 4, and/or 5; and

10 comparing the level(s) of the one or more biomarkers in the sample to insulin resistance progression and/or insulin resistance-regression reference levels of the one or more biomarkers in order to monitor the progression or regression of insulin resistance in the subject.

17. The method of claim 16, wherein the subject is selected from the group consisting of a subject being treated with a pharmaceutical composition, a subject 15 having undergone bariatric surgery, a subject undergoing an exercise modification, and a subject using a dietary modification.

18. The method of claim 16, wherein the analyzing step comprises transforming the biological sample by protein extraction.

19. The method of claim 16, wherein the comparing step comprises 20 generating a disease risk score for the subject in order to monitor the progression or regression of insulin resistance in the subject.

20. The method of Claim 19, wherein the method comprises analyzing the biological sample to determine the level of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), 25 mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, propionylcarnitine, 3-dehydrocarnitine, 30 acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrylcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate,

erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine .

21. The method of claim 19, wherein the disease risk score is a cardiovascular disease risk score.

5 22. The method of claim 21 wherein the one or more biomarkers are selected from the group consisting of adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta, 17alpha-diol disulfate, urate.

10 23. The method of claim 19, wherein the disease risk score is a type 2 diabetes risk score.

15 24. The method of claim 23 wherein the one or more biomarkers are selected from the group consisting of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate, indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta, 17alpha-diol disulfate, urate, erythrose, glycerol-3-phosphate, isoleucine, valine.

25. A method of monitoring the progression or regression of insulin resistance in a subject, the method comprising:

20 analyzing the biological sample from the subject to determine the level(s) of one or more biomarkers selected from the group consisting one or more biomarkers listed in Tables 1, 2, 3, 4 and/or 5; and

25 using the determined levels of the level(s) of the one or more biomarkers and a reference model based on the one or more biomarkers to monitor the progression or regression of insulin resistance in the subject.

26. The method of claim 25 wherein insulin resistance progresses to Type 2 diabetes and the biomarkers are selected from the group consisting of one or more biomarkers listed in Tables 1, 2, and/or 5.

30 27. The method of claim 25 wherein insulin resistance progresses to cardiovascular disease and the biomarkers are selected from the group consisting of one or more biomarkers listed in Tables 3 and/or 4.

28. The method of claim 25, wherein the biomarker is selected from the group consisting of 3-phenylpropionate (hydrocinnamate), 3-(4-hydroxyphenyl)lactate,

indolelactate, 5-oxoproline, bradykinin-hydroxy-pro(3), mannose, 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca), heme, adrenate, alpha-hydroxyisovalerate, glutamine, glycine, tyrosine, deoxycholate, cinnamoylglycine, dehydroisoandrosterone sulfate (DHEA-S), 5alpha-androstan-3beta,17alpha-diol disulfate, urate, 3-indoxylsulfate, 5 propionylcarnitine, 3-dehydrocarnitine, acetylcarnitine, oleoylcarnitine, myo-inositol, 5alpha-pregnan-3beta,20alpha-diol disulfate, xanthine, trigonelline (N'-methylnicotinate), 2-hydroxyhippurate (salicylurate), piperine, 1-methylurate, 1,3-dimethylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, kynurenine, 2-methylbutyrcarnitine, 3-hydroxy-2-oxovalerate, 3-hydroxybutyrate, 3-hydroxypropanoate, beta-hydroxypyruvate, catechol sulfate, erythrose, glycerol-3-phosphate, hexanoylcarnitine, hippurate, margarate, palmitoyl sphingomyelin, quinate, and isoleucine and combinations thereof.

29. A method of monitoring the efficacy of insulin resistance treatment, the method comprising:

15 analyzing the biological sample from a subject to determine the level(s) of one or more biomarkers selected from the group consisting of one or more biomarkers listed in Tables 1, 2, 3, 4 and/or 5; treating the subject for insulin resistance; analyzing a second biological sample from the subject to determine the level(s) of the 20 one or more biomarkers, the second sample obtained from the subject at a second time point after treatment; and comparing the level(s) of one or more biomarkers in the first sample to the level(s) of the one or more biomarkers in the second sample to assess the efficacy of the treatment for treating insulin resistance.

25 30. The method of claim 29, wherein the subject is treated by a method selected from the group consisting of administration of a therapeutic agent, a dietary change, an exercise program change, a surgical procedure, and combinations thereof.

31. The method of claim 29, wherein the comparing step comprises generating a disease risk score for the subject in order to assess the efficacy of the 30 treatment for insulin resistance.

32. The method of claim 31, wherein the disease risk score is a type 2 diabetes risk score for the subject in order to assess the efficacy of the treatment for insulin resistance.

33. The method of claim 31, wherein the disease risk score is a cardiovascular risk score for the subject in order to assess the efficacy of the treatment for insulin resistance.

34. A method for monitoring a subject's response to a course of treatment for insulin resistance, the method comprising:

analyzing a first biological sample from a subject to determine the level(s) of one or more biomarkers listed in Tables 1, 2, 3, 4, and/or 5, the first sample obtained from the subject at a first time point;

treating the subject for insulin resistance;

10 analyzing a second biological sample from the subject to determine the level(s) of the one or more biomarkers, the second sample obtained from the subject at a second time point after treatment;

comparing the level(s) of one or more biomarkers in the first sample to the level(s) of the one or more biomarkers in the second sample to assess the efficacy of the

15 treatment for treating insulin resistance.

35. The method of claim 34, wherein the treatment is selected from the group consisting of administration of a composition, a lifestyle modification, and a surgery.

36. The method of claim 35, wherein the lifestyle modification is selected from the group consisting of a dietary modification and an increase in activity or 20 exercise.

37. The method of claim 35, wherein the surgery is bariatric surgery.

38. The method of claim 34, wherein the comparing step comprises generating a disease risk score for the subject in order to assess the efficacy of the treatment for treating insulin resistance.

25 39. The method of claim 38, wherein the disease risk score is a cardiovascular disease risk score for the subject in order to assess the efficacy of the treatment for treating insulin resistance.

40. The method of claim 38, wherein the disease risk score is a type 2 diabetes risk score for the subject in order to assess the efficacy of the treatment for 30 treating insulin resistance.

## INTERNATIONAL SEARCH REPORT

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/011759

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/54 (2014.01)

USPC - 435/140

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61P 3/10; C12Q 1/54; G01N 33/66, 33/74 (2014.01)

USPC - 435/140; 436/501; 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
CPC - G01N 33/5091, 33/66, 2800/04, 2800/042, 2800/50, 2800/52, 2800/56 (2014.01)

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

Orbit, Google Patents, Google Scholar

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0208215 A1 (HU et al) 16 August 2012 (16.08.2012) entire document	1-17, 19-36, 38-40
Y		-----
Y	US 2010/0120629 A1 (ELLIS et al) 13 May 2010(13.05.2010) entire document	18, 37
Y	US 2011/0097807 A1 (MONTE et al) 28 April 2011(28.04. 2011) entire document	18
A	US 2012/0328594 A1 (McKENNA et al) 27 December 2012 (27.12.2012) entire document	37
A	US 2009/0012716 A1 (URDEA et al) 08 January 2009 (08.01.2009) entire document	1-40
A	US 2012/0122981 A1 (HU et al) 17 May 2012 (17.05.2012) entire document	1-40

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:	
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"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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Date of mailing of the international search report

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Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
P.O. Box 1450, Alexandria, Virginia 22313-1450  
Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300  
PCT OSP: 571-272-7774