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(54) **Title:** METHOD FOR IDENTIFYING THE RISK OF PRETERM DELIVERY

(57) **Abstract:** Methods provided herein can identify subjects at risk for preterm delivery, including those at risk for preterm delivery with inflammation. In one embodiment, the methods utilize small molecule profiles of metabolites in pregnant subjects. For example, the presence, absence, or amount of amino acids and/or energy-producing carbon sources are determined and compared to amounts found in subjects that have undergone a full-term delivery. Changes in the presence, absence, or amount of these small molecules may indicate that the subject is at increased risk for having a preterm delivery, or a preterm delivery without inflammation.

WO 2006/086731 A2

METHOD FOR IDENTIFYING THE RISK OF PRETERM DELIVERY

PRIORITY

This claims the benefit of U.S. Provisional Application No. 60/650,977, filed February 9, 2006, which is incorporated herein by reference.

FIELD

This application relates to methods for identifying the risk of preterm delivery, specifically to methods that detect specific metabolites in a biological sample.

BACKGROUND

Premature birth is the leading cause of perinatal morbidity and mortality. Every year approximately 4.5 million premature babies are born worldwide, and, despite considerable advances in neonatal care, their mortality rate remains high. Moreover, survivors are at risk for long-term disabilities, including developmental delay, cerebral palsy, blindness, deafness, and chronic lung disease. Thus, the prevention of prematurity is an important challenge to obstetrics and perinatal medicine.

Intrauterine infection has emerged as a common and important cause of preterm delivery, as at least a third of all preterm births occur to mothers with microbial invasion of the amniotic cavity. Intrauterine infection often results in fetal infection with the development of the fetal inflammatory response syndrome, a risk factor for the impending onset of labor, short-term neonatal complications, and long-term handicaps, such as cerebral palsy and chronic lung disease. The accurate and rapid identification of a pregnant woman with subclinical intrauterine inflammation is an urgent priority for the development of rational therapy.

Despite the compelling evidence that infection is often causally linked to preterm delivery, antibiotic treatment of subjects with premature labor has not proven effective in preventing preterm delivery or neonatal morbidity in most trials. A potential explanation is that many subjects presenting with preterm labor do not have intrauterine infection and therefore may not benefit from antimicrobial therapy.

Moreover, intra-amniotic and fetal inflammation *per se* are linked to adverse outcome, even in the absence of microbiologically proven infection. Thus, the accurate and rapid identification of a pregnant woman with subclinical intrauterine inflammation is an urgent priority for the development of rational therapy.

Current evidence indicates that analysis of amniotic fluid, which is normally sterile, is the most accurate means of determining the presence or absence of infection and/or inflammation. Available tests have limited sensitivity and specificity, however, and the results of standard microbiologic techniques, such as microbial culture, take time and are not available for immediate management decisions.

There is a need for new methods for the analysis of samples from pregnant subjects, such as pregnant women, that can be used to determine the risk that the subject will deliver preterm, and can be used to identify an inflammatory process.

SUMMARY

The present disclosure relates to the analysis of small molecules in pregnant subjects and provides methods for identifying a subject at increased risk for preterm delivery. The methods can identify subjects at risk for preterm delivery as well as subjects at risk for preterm delivery with inflammation. In some embodiments, the methods utilize analyzing small molecule profiles of metabolites. In other embodiments, the methods utilize analyzing the presence of one or more specific metabolites.

In several embodiments, the methods include assaying the small molecule profile of a subject of interest, such as a pregnant woman, to identify the risk of preterm delivery. The methods can include assaying for changes in the amount of one or more small molecules associated with preterm delivery. In several examples, the method can include assaying for the presence of one or more energy-producing carbon sources, one or more amino acids, or both energy-producing carbon sources and amino acids in a sample from the subject. In some examples, a decrease in the amount of one or more energy-producing carbon sources associated with preterm delivery, as compared to a sample from a subject with a full-term delivery indicates that the subject is at increased risk for preterm delivery. In additional examples, a

decrease in the amount of one or more energy-producing carbon sources associated with preterm delivery, and an increase in the amount of one or more amino acids associated with preterm delivery, as compared to a sample from a subject with a full-term delivery, indicates that the subject is at increased risk for preterm delivery associated with inflammation.

In additional embodiments the methods include obtaining a small molecule profile from a pregnant subject. The small molecule profile from the subject is compared to a standard small molecule profile of a subject that has a full-term delivery, thereby identifying a subject at increased risk for preterm delivery. The small molecule profile includes the detection of the presence or absence, or amount of at least one small molecule associated with preterm delivery, including, but not limited to, amino acids, sugars, small organic acids and other energy-producing carbon sources. Examples of specific amino acids associated with preterm delivery include, but are not limited to leucine, tryptophan, phenylalanine, glycine, isoleucine, methionine, and glutamic acid. Examples of specific sugars associated with preterm delivery include, but are not limited to, galactose, mannose, glucose, alpha L-sorbopyranose, fructose, and glycerol. Examples of specific small organic acids include, but are not limited to, heptanedioic acid, butanedioic.acid, and citric acid. Other small molecules associated with preterm delivery include, but are not limited to, catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the supervised analysis of clinical variables in the prediction of clinical class. Since a Linear Discriminant Analysis is non-restricted and may use any rules necessary to achieve an appropriate classification, the inability of this analysis to find any permutation of available features capable of correctly classifying these patients is remarkable and clearly indicates the currently collected clinical data

cannot be used predictively. The clinical variables include cervical dilatation, cervical effacement, sonographic cervical length, history of preterm delivery, gestational age, frequency of uterine contractions, maternal temperature, maternal age, and parity.

FIG. 2 is a graph showing the supervised analysis of the amniotic fluid metabolome in the prediction of clinical class. In this case the ability of the Linear Discriminant Analysis achieves a reasonable separation, although it is not as predictive as a Random Forest Analysis.

FIG. 3 is a digital image showing the metabolic profile of 186 amniotic fluid samples obtained from pregnant subjects.

DETAILED DESCRIPTION

It is disclosed herein that an analysis of small molecules present in a sample obtained from a pregnant subject can be used to identify whether the subject is at risk for preterm delivery. The methods can identify the risk of preterm delivery as well as the risk of preterm delivery with inflammation. In some embodiments, the methods utilize analyzing small molecule profiles of metabolites. In other embodiments, the methods utilize analyzing the presence of one or more specific metabolites. In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

Amino acid: A molecule that contains both amino and carboxylic acid functional groups. In general, the term "amino acid" refers to alpha amino acids,

which are those amino acids in which the amino and carboxylate functionalities are attached to the same carbon, the so-called α–carbon.

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

Biomarker: An organic biomolecule, such as a small molecule, amino acid, sugar, carbon (energy) source, carbohydrate, or a polypeptide or protein, which is differentially present in a biological sample. In one example, the biomarker is present in a sample, such as serum or amniotic fluid taken from a subject who is, or may be at risk for, preterm complications. A biomarker can be differentially present in samples from a normal subject and one at-risk for preterm parturition, respectively, if it is present at an elevated level or a decreased level in latter samples as compared to samples of normal subjects. For example, biomarkers can be increased or decreased in a sample from a subject having intra-amniotic inflammation, as compared to a similar sample taken from a "normal" or "control" subject that did not experience preterm parturition subsequent to sampling.

Carbohydrate: A chemical compound that contains oxygen, hydrogen, and carbon atoms. Carbohydrates consist of monosaccharide sugars of varying chain lengths and that have the general chemical formula $C_n(H_2O)_n$ or are derivatives of such. Examples of carbohydrates include malic acid and succinic acid.

Diagnostic concentrations or diagnostic levels: A concentration (for example of a biomarker in amniotic fluid, blood or serum) that provides clinical information to detect a pathological condition or predict its course. An example of a diagnostic concentration is a concentration of a small molecule, such as an amino acid or energy-producing carbon source, including a carbohydrate, that differentiates probable normal parturition from probable preterm labor/parturition, and/or probable preterm labor/parturition with inflammation. Another example of a diagnostic concentration is one that detects the presence of probable inflammation.

Energy-producing carbon source: Any molecule containing at least one carbon atom that can be used in a biological process, such as the Krebs cycle, to produce energy (such as ATP). For example, carbohydrates and small organic acids

that have at least one carbon atom are considered energy-producing carbon sources if they can be used by a cell to produce energy.

Inflammation: A localized response elicited by injury or destruction of tissues, which can destroy, dilute or sequester an injurious agent, such as an infectious agent, and the injured tissue. Generally, inflammation is characterized in the acute form by the classical signs of pain, heat, redness, swelling and loss of function. Inflammation is often associated with an inflammatory response by the immune system. Histologically, it can involve dilatation of arterioles, capillaries and venules, with increased permeability and blood flow, exudation of fluids, including plasma proteins and leucocytic migration. Inflammation may be elicited by an infection of a host with a pathologic agent, but is not necessarily the result of an infection. Pathologic agents for mammalian subjects include viruses, bacteria, prions and fungi.

Linear Discriminant Function: Discriminant function analysis is used to determine which variables discriminate between two or more naturally occurring groups. Computationally, discriminant function analysis is very similar to analysis of variance (ANOVA). The basic idea underlying discriminant function analysis is to determine whether groups differ with regard to the mean of a variable, and then to use that variable to predict group membership (e.g., of new cases). One can ask whether or not two or more groups are significantly different from each other with respect to the mean of a particular variable. Usually, one includes several variables in a study in order to see which one(s) contribute to the discrimination between groups. In that case, there is a matrix of total variances and covariances; likewise, there is a matrix of pooled within-group variances and covariances. One can compare those two matrices via multivariate *F* tests in order to determine whether or not there are any significant differences (with regard to all variables) between groups. Step-wise discriminant analysis is a common application of discriminant function analysis is to include many measures in the study, in order to determine the ones that discriminate between groups.

In the two-group case, discriminant function analysis can also be thought of as (and is analogous to) multiple regression (the two-group discriminant analysis is also called Fisher linear discriminant analysis). Another major purpose to which

discriminant analysis is applied is the issue of predictive classification of cases. Specific methods for a linear discriminant analysis can be found, for example, on the StatSoft website (2005).

Metabolome: All of the small molecules present in a given organism. The metabolome includes both metabolites as well as products of catabolism. In one embodiment, the disclosure encompasses a small molecule profile of the entire metabolome of a species. Generally the metabolome or small molecule profile includes those molecules with a molecular weight of less than 2,000 Daltons Small molecules do not include large macromolecules, such as proteins (for example, proteins with molecular weights over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000 Daltons), large nucleic acids (such as 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 Daltons), or large polysaccharides (such as 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 Daltons). In another embodiment, the disclosure encompasses a computer database (as described below) of the entire metabolome of a species, such as a mammal, for example a mouse, rat, rabbit, pig, cow, horse, dog, cat, bear, monkey, or a human.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the fusion proteins herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary

substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

A "therapeutically effective amount" is a quantity of an agent that achieves a desired effect in a subject being treated. For instance, this can be the amount necessary to inhibit or prevent preterm labor or delivery. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations that has been shown to achieve an *in vitro* effect. Therapeutic agents can include chemical compounds, biological agents, nutritional supplements, and small molecules.

Preterm complications: Medical conditions that develop prior to full-term delivery of a fetus. In several examples, preterm complications include preterm parturition, preterm labor, preterm premature rupture of the membrane (PROM), intra-amniotic inflammation, and/or microbial invasion of the amniotic cavity (MIAC). The term "preterm PROM" or "preterm Premature Rupture of Membranes" refers to rupture of the amniotic sac prior to the onset of labor contractions and before the expiration of the normal gestational period.

Preterm delivery: Delivery of a fetus prior to the full term of gestation. For humans, a preterm delivery is a delivery that refers to delivery of offspring prior to the expiration of the normal gestational period, in humans before about the 37^{th} week of gestation. Preterm delivery can be associated with inflammation, but in some cases is not associated with inflammation. By "preterm labor" is intended the presence of uterine contractions (at least 3 in 10 minutes (min)) or advanced cervical dilatation at less than 37 weeks of gestation. "Spontaneous" preterm delivery is a vaginal delivery as a result of preterm labor (and is not due to Cesarean section).

Peptide/Protein/Polypeptide: All of these terms refer to a polymer of amino acids and/or amino acid analogs that are joined by peptide bonds or peptide bond mimetics. The twenty naturally-occurring amino acids and their single-letter and three-letter designations are as follows:

Amino Acid	Single-letter Symbol	Three-letter Symbol
Alanine	A	Ala
Cysteine	C	Cys
Aspartic Acid	D	Asp
Glutamic acid	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

Proteome: A "proteome" is, in simplest terms, the protein complement expressed by an organism. A "sub-proteome" is a portion or subset of the proteome; this sub-proteome is a subset of the proteins expressed by the organism. The proteome can be useful for obtaining quantitative information regarding the proteome of an organism or organisms and sub-proteomes thereof. Exemplary sub-proteomes include a set of proteins involved in a selected metabolic or signaling pathway, a set of proteins having a common enzymatic activity, or the proteins from a particular location in an organism or cell. Generally, the proteome is full-length

polypeptides expressed by an organism or cell and does not include the small molecules expressed by the organism or cell.

The term "proteomics" refers the study of the composition of the protein complement of an organism or organisms. "Quantitative proteomics" refers to the study of the relative or absolute amounts or concentrations of the proteins expressed by an organism or organisms, in one or more states.

Small molecule profile: A small molecule is an organic or inorganic molecule which is present in the cell, cellular compartment, or organelle. The term does not include large macromolecules, such as proteins (for example, proteins with molecular weights over 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000 Daltons), large nucleic acids (such as 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 Daltons), or large polysaccharides (such as 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 Daltons). Generally, small molecules are found free in solution in the cytoplasm or in other organelles of the cell, 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 molecule" 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, nucleotides, intermediates formed during cellular processes, and other small molecules found within the cell.

By "biologically active small molecule" or "biologically active metabolite" is intended a small molecule which modulates the activity of a biological system or pathway. For example, biologically active small molecules can be identified using *in vitro* or *in vivo* assays known in the art. Biologically active small molecules can also be identified by screening assays using target molecules *in vitro*, wherein the target molecule has been implicated in a disease state. In another example, biologically active small molecules can be identified using cell-based assays.

The term "isolated" includes molecules (such as small molecules) which are separated from other molecules which are present in the natural source of the molecules. In one embodiment, an "isolated" small molecule is free of other

molecules (both other small molecules and macromolecules) which naturally are present of the organism, cell or fluid from which the small molecules are derived.

A "small molecule profile" is an inventory of small molecules within a targeted cell, tissue, organ, organism, or any derivative fraction or sample thereof such as a cellular compartment. In one example, inventory includes both the quantity and type of small molecules present. A "small molecule profile," can be determined using a single technique for an intended use but can also be produced using several different techniques depending on a variety of factors, such as the disease state involved, the types of small molecules present in a particular targeted cellular compartment, the cellular compartment being assayed. The relevant information in a small molecule profile can depend on the intended use of the compiled information. For example, the amounts of a particular small molecule or a particular class of small molecules, such as amino acids, carbohydrates, and/or energy producing carbon sources can be relevant.

A "standard profile" is information regarding the small molecules of the profile that is necessary and/or sufficient to provide information to a user for its intended use within the methods described herein. The standard profile would include the quantity and/or type of small molecules present. In one example, a standard profile is obtained from one or more subjects with full term delivery.

Comparison of the standard profile to a profile from a pregnant subject that may be at risk for preterm delivery can be used to identify small molecules deregulated in the pregnant subject. These small molecules are referred to as "molecules associated with preterm delivery," and include amino acids and energy-producing carbon sources, such as those listed in Table 4. In one example, using Random Forest statistical methods, the splitting criterion for a molecule associated with preterm delivery is the "Gini index," thus the sum of all decreases in the forest due to a given variable, normalized by the number of trees, is used to define the Gini variable importance measure. This measure can reveal variables which can cause many small decreases summing up to a large contribution to model deviance reduction. In one example, in a small molecule profile of about 180 to 190 metabolites, such as 186 metabolites, a Gini index of about 0.8 or greater (such as

from about 0.8 to about 3.1) indicates that the small molecule is associated with preterm delivery.

The Gini coefficient is a measure of inequality usually used to measure income inequality, but can be used to measure any form of uneven distribution. The Gini coefficient is a number between 0 and 1, where 0 corresponds with perfect equality and 1 corresponds with perfect inequality. The Gini index is the Gini coefficient expressed in percentage form, and is equal to the Gini coefficient multiplied by 100.

Small organic acid: An acid capable of being burned for energy production. Small organic acids are utilized, for example, during the Krebs cycle.

Unknown 5: A small molecule with a chromatographic runtime of approximately 9.64 minutes using the methods described in Example 1, and with characteristic ion fragments that included (but were by no means exclusively) 256 amu (100%), 182 amu (24%).

Unknown 8: A small molecule with a chromatographic runtime of approximately 9.40 minutes using the methods described in Example 1, and with characteristic ion fragments that included (but were by no means exclusively) 246 amu (100%), 213 amu (28%), 287 amu (14%).

Unknown 128: A small molecule with a chromatographic runtime of approximately 10.14 minutes using the methods described in Example 1, and with a characteristic ion fragments that included (but were by no means exclusively) 227 amu (100%), 203 amu (60%), 317 amu (34%).

Unknown 289: A small molecule with a chromatographic runtime of approximately 7.58 minutes using the methods described in Example 1, and with characteristic ion fragments that included (but were by no means exclusively) 540 amu (100%).

Description of Several Embodiments

The methods disclosed herein can be used for identifying a subject at increased risk for preterm delivery. The subject can be any subject of interest, including a veterinary subject, a primate, or a human subject. The methods can identify subjects at risk for preterm delivery as well as subjects at risk for preterm

delivery with inflammation. In one example, the method can be used to identify a subject at risk for preterm delivery who has signs of preterm labor. In another example, the method can distinguish subjects at risk of spontaneous pre-term delivery not associated with inflammation from subjects who are not at risk of spontaneous preterm delivery.

In one embodiment, the method is used to asses the risk of preterm delivery in a subject undergoing preterm labor. Several methods known in the art have been used to identify subjects at risk or preterm labor associated with inflammation. For example, the white blood cell count, an analysis of T or B cells, expression of interleukins, and the amount of MMP-8 has been associated with the risk of preterm delivery associated with inflammation. The methods disclosed enable the identification of a subject who is at risk of preterm delivery, wherein the preterm delivery is not associated with inflammation.

For any of the methods disclosed herein, a biological sample is utilized from the subject of interest, such as a subject undergoing preterm labor. The biological sample can be any biological sample of interest, including an amniotic fluid sample, a blood sample (plasma, serum or otherwise), chorionic villous sample (CVS), or a urine sample. In one specific non-limiting example, the sample is an amniotic fluid sample. This amniotic fluid sample can be a mid-trimester amniotic fluid sample. The amniotic fluid sample can be obtained for genetic screening, or can be obtained when the subject is at risk of or is experiencing preterm labor.

The methods disclosed herein can include small molecule profiling. Generally, the methods include assaying for the presence of one or more small molecules, including energy-producing carbon sources, amino acids, or both energy-producing carbon sources and amino acids in a sample from the subject. The methods can include the analysis of the complete metabolic profile obtained from a biological sample. In one example, the methods do not include the use of proteomics, and do not include an analysis of the expression of full-length proteins. The methods can also include an analysis of one or more specific metabolites found in a biological sample obtained from the subject of interest, such as a pregnant subject undergoing preterm labor that could be at risk for preterm delivery.

In one example, a method for detecting the risk of preterm delivery for a pregnant subject is provided. The method includes determining the level of methyladenine, heptanedioic acid and N-acteyl glutamine from a biological sample obtained from the pregnant subject. It is then assessed as to whether the level of methyladenine, heptanedioic acid and N-acteyl glutamine differs from a corresponding small molecule profile from a subject with full term delivery. A change in the amount of one or more small molecules associated with preterm delivery in the small molecule profile in the sample, as compared to a sample from a subject with a full-term delivery indicates that the subject is at risk for preterm delivery (with or without inflammation).

In another example, the small molecule profile includes at least one of methlyadenine, heptanedioic acid, N-acetylglutamine, DL-beta-hydroxyphenylethylamine, unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, l-methionine, glycine, d-galactose, hexose cluster 3, catechol, salicylamide, butanedioic acid, unknown 5, d-mannose, unknown 8, glutamic acid, phenylalanine, alpha l-sorbopyranose, cholesterol, unknown 289, d-fructose, hexose cluster 2, l-tryptophan, and eicosanoic acid. In several examples, the small profile includes all of the small molecules listed in Table 4, or at least one, at least two, at least three, at least four, at least five, at least ten, at least 15, at least 20, at least 25, or all of the molecules listed in Table 4. Thus, the small molecule profile can include, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, or 30 of these small molecules. Thus, for example, the small molecule profile can include methlyadenine, heptanedioic acid, N-acetylglutamine, DL-beta-and hydroxyphenylethylamine. In another example, the small molecule profile can include those molecules with a Gini Index of greater than about 1.0, grate than aobut 1.4, greater than about 1.5, greater than about 2.0, greater than about 2.3 or greater than about 3.0. Additional examples are provided in the Examples section below. It should be noted that this is not intended to be limiting, methods are also provided herein for detecting preterm delivery that utilize the detection of a small molecules individually.

In one specific, non-limiting example, the sample is a sample of amniotic fluid, which can be obtained by amniocentesis. Amniocentesis is performed, for

example, by inserting a needle with a stylet (such as a 20-21 gauge needle) guided by an imaging device (such as ultrasound) through the abdominal wall of the subject and into the amniotic cavity. The stylet is removed and a sample of amniotic fluid (for example about 20 milliliters (ml)) is aspirated. To lessen the chance of maternal contamination, the initial 2 ml or so can be discarded. As noted above, the amniotic fluid sample can be obtained when a pregnant subject of interest is undergoing preterm labor. However, the amniotic fluid sample can also be a routine sample, such as a sample obtained for genetic screening. In other specific, non-limiting examples, the sample is a vaginal fluid sample, cervical fluid sample, maternal blood, maternal plasma, maternal urine, or chorionic villous sample.

It is disclosed herein that the level of energy-producing carbon sources are decreased in subjects with preterm deliveries, including preterm deliveries not associated with inflammation. The level of energy-producing carbon sources is also decreased in subjects wherein the preterm delivery is associated with inflammation. The level of energy-producing carbon sources can be decreased in subjects with preterm deliveries associated with inflammation. Generally, a "decrease" includes a decrease of more than about 10%, such as more than about 20%, about 30%, about 40% about 50%, about 60%, about 70%, about 80%, about 85%, about 90% or about 95% or more as compared to a control. In one example, a decrease in the amount of one or more energy-producing carbon sources as compared to a the amount identified sample from a subject with a full-term delivery indicates that the subject is at increased risk for preterm delivery. In another example, a decrease in the amount of one or more energy producing carbon source indicates that the subject is at risk for preterm delivery not associated with inflammation.

Energy-producing carbon sources include, but are not limited to, small molecules consumed directly or indirectly through the Krebs cycle, including citric, succinic, malic, oxaloacetic and pyruvic acids, or small molecules which may reasonably yield one of these by a normal metabolic route. Examples of specific sugars include, but are not limited to, galactose, mannose, glucose, alpha L-sorbopyranose, fructose, and glycerol. Examples of specific small organic acids include, but are not limited to, heptanedioic acid and butanedioic acid.

In several examples, a decrease in one or more of the sugars and one or more of the organic acids is associated with preterm delivery. In several examples, a decrease in one or more of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid and butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids is associated with pre-term delivery, such as a preterm delivery without inflammation. Thus, in one example, the method includes detecting a decrease in the amount of hepatnedioic acid and butanedioic acid, thereby identifying the subject as being at risk of preterm delivery, such as preterm delivery without inflammation. In another example, the method includes detecting a decrease in the amount of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, thereby identifying the subject as being at risk for preterm delivery, such as preterm delivery without inflammation. In a further example, the method includes detecting a decrease in the amount of citric, succinic, malic, oxaloacetic and pyruvic acids, thereby identifying the subject as being at risk of preterm delivery, such as preterm delivery without inflammation.

Other small molecules include, but are not limited to, catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid. Changes in the amount of one of more of these small molecules is associated with pre-term delivery and can be used to identify a subject at risk for preterm delivery, such as a preterm delivery without inflammation.

It is disclosed herein that higher amounts of one or more amino acids in a sample as compared to a control indicates that the subject is at risk of preterm delivery associated with inflammation. Thus, the methods disclosed herein can include assaying for the amount of one or more energy-producing carbon sources and/or one or more amino acids in a sample from the subject. An increase in the amount of one or more amino acids as compared to a sample from a subject with a full-term delivery, and/or a decrease in the amount of one or more energy-producing carbon sources as compared to the control, indicates that the subject is at increased risk for preterm delivery with inflammation. Generally, an "increase" includes an increase of about 10%, about 20%, about 30%, about 40% about 50%, about 60%, about 70%, about 80%, about 85%, about 90% or about 95% or more as compared to

a control. Amino acids of interest include, but are not limited to, leucine, isoleucine, tryptophan, phenylalanine, glycine, methionine, and glutamic acid.

In one example, a method is disclosed to identify a subject at risk for preterm delivery not associated with inflammation. The method includes assaying for the amount of one or more amino acids (as discussed above) and/or one or more energy-producing carbon sources (as discussed above). A decrease in the amount or one or more energy-producing carbon sources, and a lack of change in the amount of one or more amino acids, as compared to a control, indicates that the subject is at risk of preterm delivery without inflammation. The method can include assessing the small molecular profile of the subject, and comparing it to a control, such as the small molecule profile of a subject not at risk for preterm delivery. By a "lack of change" indicates that the amount in the sample of interest is not statistically different from the amount in the control sample. One of skill in the art can readily use statistical methods to determine if an amount in the sample of interest does not differ from the control. Exemplary amounts that indicate a "lack of change" are a no difference, or no more than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% or at most 10%.

A control includes a sample from a subject that has undergone full-term delivery. In general, decreased (as compared to control) amounts of a sugar or energy-providing organic acid indicate an abnormality in labor, and increased (as compared to control) amounts of any amino acid concentration is indicative of infection/inflammation.

It is also disclosed herein that changes in the amount of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and/or eicosanoic acid can identify a subject as being at risk for preterm delivery. Thus, the methods disclosed herein can include assaying for the amount of one or more of a small molecules selected from the group consisting of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1,

leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid, and comparing the amount of that small molecule to a control.

In several embodiments, the methods include obtaining a small molecule profile of a sample obtained from a pregnant subject, such as a human. The small molecule profile from the subject is compared to a control small molecule profile of a subject that has a full-term delivery. A difference in the small molecule profile of the subject as compared to the control identifies the subject as being at increased risk for preterm delivery. The small molecule can include energy-producing carbon sources and/or amino acids. The small molecule profile can include at least one small molecule selected from the group consisting of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid.

One of skill in the art can readily identify statistical methods and computer programs that can be used to identify an increase or a decrease in one or more metabolites, including differences in molecule profiles. Analysis of use includes linear discriminant analysis and Random Forest.

In one specific, non-limiting example, a biological sample is obtained from a subject of interest, and the small molecule profile of the biological sample is compared to a control profile, such a profile obtained from a subject not at risk of preterm delivery. The small molecule profile can be taken from any biological sample of interest, including, but not limited to, an amniotic fluid sample, a blood sample (plasma, serum or otherwise), or a urine sample. In one example, the biological sample is an amniotic fluid sample. In another example, a control small molecule profile is obtained from a pregnant subject that had or is having a full-term delivery.

Random Forest analysis is then used to identify changes in the amount of small molecules associated with preterm delivery, such as preterm delivery not associated with inflammation, as compared to the control. Changes in the amount of small molecules associated with preterm delivery without inflammation identifies the subject as being at risk.

Metabolomics and Detection Methods

Methods are disclosed herein for the identification of a subject at risk for preterm delivery. The methods include the identification of a subject at risk for preterm delivery associated with inflammation, such as inflammation associated with an infectious agent.

It is disclosed herein that metabolomics is specifically of use in the diagnosis and prevention of preterm delivery. Metabolomics is also of use in identifying preterm delivery that is (or is not) associated with inflammation. Metabolomics, or small molecule profiling of cells and organelles, can be used to study both genetic and non-genetically linked disease states. Small molecule profiling allows one to investigate the very biochemical pathway (such as cellular metabolites) involved in the disease state by comparing small molecule profiles of biological samples, biological fluids, cells, cellular compartments, or organelles with those of other biological samples, biological fluids, cells, cellular compartments, or organelles. See, for example, PCT Publication Nos. WO0178652 and WO2004038381 as well as U.S. Publication Nos. 2004146853 and 2005014132, all of which are herein incorporated by reference in their entirety.

Thus, this disclosure pertains, at least in part, to the generation of small molecule profiles of samples, biological fluids, cells, and cellular compartments. These small molecule profiles can be used to "fingerprint" the cell or cellular compartment and identify the presence, absence or relative quantity of small molecules. The small molecule profiles of the cells or cellular compartments can be obtained through, for example, a single technique or a combination of techniques for separating and/or identifying small molecules known in the art. Examples of separation and analytical techniques which can be used to separate and identify the compounds of the small molecule profiles include: HPLC, TLC, electrochemical

analysis, mass spectroscopy, refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), Light Scattering analysis (LS) and other methods known in the art. The methods can be used to detect electrically neutral as well as electrochemically active compounds. Detection and analytical techniques can be arranged in parallel to optimize the number of molecules identified.

As noted above, any biological sample can be used to produce a metabolic profile. In one embodiment, the biological sample can be a biological fluid (such as amniotic fluid, serum, blood, chorionic villous sample, or an umbilical cord blood sample). In several examples, the sample is obtained, for example, from the amniotic fluid or the blood, or a part of the blood, such as the plasma. In some examples, the samples are substantially free of macromolecules (e.g., large proteins and polynucleotides with molecular weights of greater than 10,000). In additional examples, the biological sample is a sample of amniotic fluid.

One of skill in the art, using the present disclosure and methods known in the art (such as HPLC, TLC, electrochemical analysis, mass spectroscopy, refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), Light Scattering analysis (LS)), can readily generate a small molecular profile. The small molecule profile from the sample of interest can then be compared with the small molecule profile from one or more subjects with preterm deliveries and/or with the small molecule profile from one or more subjects with a full-term delivery. These comparisons can be made by individuals or can be made using software designed to make such comparisons, such as a software program that provides a secondary output which provides useful information to a user. For example, a software program can be used to confirm a profile or can be used to provide a readout that identifies specific molecules within a large metabolic profile. The selection of an appropriate software program, such a pattern recognition software program, is within the ordinary skill of the art. An example of a statistical method of use is Random Forest (Breiman (2001) *L. Machine Learning* 45:5-32), which allows the identification of a "Random Forest

classifier." The classifier uses large number of individual decision trees and decides the class by choosing the mode (most frequently occurring) of the classes as determined by the individual trees. It should be noted that the comparison of the profiles can be done both quantitatively and qualitatively. In Random Forest, the splitting criterion is a "Gini index," thus the sum of all decreases in the forest due to a given variable, normalized by the number of trees, is used to define the Gini variable importance measure. This measure can reveal variables which can cause many small decreases summing up to a large contribution to model deviance reduction. In one example, in a small molecule profile of about 180 to 190, such as 186, a Gini index of about 0.8 or greater indicates that the small molecule is associated with preterm delivery.

In one embodiment, the small molecule profile of an organism is determined by using HPLC (Kristal *et al.* (1998) *Anal. Biochem.* 263:18-25), thin layer chromatography (TLC), or electrochemical separation techniques (see, PCT Publication No.WO 99/27361, PCT Publication No. WO 92/13273, U.S. Patent No. 5,290,420, U.S. Patent No. 5,284,567, U.S. Patent No. 5,104,639, U.S. Patent No. 4,863,873, and U.S. Patent No. RE32,920). Other techniques for determining the presence of small molecules or determining the identity of small molecules of the cell may also be used, such as refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), Light Scattering analysis (LS), mass spectroscopy, and other methods known in the art. The small molecule profiles can also be referred to as "metaboprints."

In one embodiment, HPLC columns equipped with coulometric array technology can be used to analyze the samples, separate the compounds, and/or create a small molecule profile of the samples. Such HPLC columns have been used extensively in the past for serum, urine and tissue analysis and are suitable for small molecule analysis (Acworth *et al.* (1999) *Methods Enzymol.* 300:297-313; Beal *et al.* (1990) *J. Neurochem.* 55:1327-1339; Matson *et al.* (1987) *Life Sci.* 41:905-908; Matson *et al.*, Basic, Clinical and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, vol II, pp. 513-516, Plenum, N.Y. 1990; LeWitt *et al.* (1992) *Neurology* 42:2111-2117; Milbury *et al.* (1998) *J. Wildlife Manag.*; Ogawa *et al.*

(1992) *Neurology* 42:1702-1706; Beal *et al.* (1992) *J. Neurol. Sci.* 108:80-87; Matson *et al.* (1984) *Clin. Chem.* 30:1477-1488; Milbury *et al.*, Coulometric Electrode Array Detectorsfor HPLC, pp. 125-141, VSP International Science Publication; Acworth *et al.* (1996) *Am. Lab* 28:33-38). HPLC columns equipped with coulometric arrays have been used for the simultaneous analysis of the majority of low-molecule weight, redox-active compounds in mitochondria. (Kristal *et al.* (1998) *Anal. Biochem.* 263:18-25).

Mass Spectroscopy (MS) Detectors can also be used. Generally, when MS is utilized, the sample, compound or molecule is ionized, passed through a mass analyzer, and the ion current is detected. There are various methods for ionization. Examples of these methods include electron impact (EI), where an electric current or beam created under high electric potential is used to ionize the sample migrating off the column, chemical ionization, where ionized gas is utilized to remove electrons from the compounds eluting from the column; and fast atom bombardment where Xenon atoms are propelled at high speed in order to ionize the eluents from the column.

Pyrolysis Mass Spectrometry can also be utilized. Pyrolysis is the thermal degradation of complex material in an inert atmosphere or vacuum. It causes molecules to cleave at their weakest points to produce smaller, volatile fragments called pyrolysate. Curie-point pyrolysis is a particularly reproducible and straightforward version of the technique, in which the sample, dried onto an appropriate metal is rapidly heated to the Curie-point of the metal. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass-to-charge ratio to produce a pyrolysis mass spectrum (Meuzelaar et al 1982) which can then be used as a "chemical profile" or fingerprint of the complex material analyzed. The combined technique is known as pyrolysis mass spectrometry (PyMS).

Nuclear Magnetic Resonance (NMR) can also be utilized. Certain nuclei with odd-numbered masses, including H and ^{13}C, spin about an axis in a random fashion. When they are placed between poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with parallel orientation favored since it is slightly lower energy. The nuclei are then irradiated with

electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state where they become in resonance with radiation. Different spectra will be produced depending on the location of the H or ^{13}C and on adjacent molecules or elements in the compound because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

Refractive index (RI) can also be utilized. In this method, detectors measure the ability of samples to bend or refract light. This property for each compound is called refractive index. For most RI detectors, light proceeds through a bi-modular flow to a photodetector. One channel of the flow-cell directs the mobile phase passing through the column while the other directs only the other directs only the mobile phase. Detection occurs when the light is bent due to samples eluting from the column, and is read as a disparity between the two channels. Laser based RI detectors have also become available.

Ultra-Violet (UV) detectors can be utilized. In this method, detectors measure the ability of a sample to absorb light. This could be accomplished at a fixed wavelength, usually 254 nm, or at variable wavelengths where one wavelength is measured at a time and a wide range is covered. Alternatively, Diode Arrays are capable of measuring a spectrum of wavelengths simultaneously. Sensitivity is in the 10<-8 > to 10<-9 > gm/ml range. Laser based absorbance or Fourier Transform methods have also been developed.

Fluorescent detectors can be utilized. This method measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths. Sensitivity is in the 10^{-9} to 10^{-11} gm/ml. Laser based fluorescence detectors are also available.

Radiochemical detection can be utilized. This method involves the use of radiolabeled material, for example, tritium (^{3}H) or carbon 14 (^{14}C). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research. The detector types include homogeneous method where addition of scintillation fluid to column effluent causes fluorescence, or

heterogeneous detection where lithium silicate and fluorescence by caused by beta-particle emission interact with the detector cell. Sensitivity is 10^{-9} to 10^{-10} gm/ml.

Electrochemical detection can be utilized. Detectors measure compounds that undergo oxidation or reduction reactions. This is usually accomplished by measuring gains or loss of electrons from migration samples as they pass between electrodes at a given difference in electrical potential. The sensitivity is 10^{-12} to 10^{-13} gms/ml.

Light scattering (LS) detectors can be utilized. This method involves a source which emits a parallel beam of light. The beam of light strikes particles in solution, and some light is then reflected, absorbed, transmitted, or scattered. Two forms of LS detection may be used to measure transmission and scattering.

Nephelometry is defined as the measurement of light scattered by a particular solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of background light or scatter since the detection occurs at a black or null background. Turbidimetry is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through particulate solution. Therefore, it quantifies the residual light transmitted. The sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete analyzer. Thus, the measurement of a decrease in transmitted light from a large signal of transmitted light is limited to the photometric accuracy and limitations of the instrument employed.

Near infrared scattering detectors operate by scanning compounds in a spectrum from 700-1100 nm. Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain wavelengths. This is a fast growing method which offers several advantages; speed, simplicity of preparation of sample, multiple analyses from single spectrum and nonconsumption of the sample.

Fourier transform infrared spectroscopy (FT-IR) can be utilized. This method measures dominantly vibrations of functional groups and highly polar bonds. The generated fingerprints are made up of the vibrational features of all the sample components. FT-IR spectrometers record the interaction of IR radiation with

experimental samples, measuring the frequencies at which the sample absorbs the radiation and the intensities of the absorptions. Determining these frequencies allows identification of the samples chemical makeup, since chemical functional groups are known to absorb light at specific frequencies. Both quantitative and qualitative analyses are possible using the FT-IR detection method.

Dispersive Raman spectroscopy can be utilized. Dispersive Raman Spectroscopy is a vibrational signature of a molecule or complex system. The origin of dispersive Raman spectroscopy lies in the inelastic collisions between the molecules composing say the liquid and photons, which are the particles of light composing a light beam. The collision between the molecules and the photons leads to an exchange of energy with consequent change in energy and hence wavelength of the photon.

Generally, to create a small molecule profile (or "Metaboprint") biological samples, such as blood, amniotic fluid, chorionic villous sample, plasma, cells, cellular compartments, or organelles can be homogenized using standard methods. Different fractionation procedures may be used to enrich the fractions for small molecules. The small molecules obtained can then be passed over several fractionation columns. The fractionation columns can employ a variety of detectors used in tandem or parallel to generate the small molecule profile for the biological sample, cell, cellular compartment, or organelle.

For example, to generate a small molecule profile of water soluble molecules, the cell, cellular compartment, or organelle extracts may be fractionated on HPLC columns with a water soluble array. The water soluble small molecules may then be detected using fluorescence or ultra-violet (UV) detectors to generate the small molecule profiles. Alternatively, electrochemical detectors may be used with diads to pick up redox active compounds and the absorbance of active compounds. For generating detecting non water soluble molecules, hydrophobic columns may also be used to generate small molecule profiles. In addition, gas chromatography combined with mass spectroscopy, liquid chromatography combined with mass spectroscopy, MALDI combined with mass spectroscopy, ion spray spectroscopy combined with mass spectroscopy, capillary electrophoresis,

NMR and IR detection are among the many other combinations of separation and detection tools which may be used to generate small molecule profiles.

Additional Methods

The methods disclosed herein can be used for identifying a subject at increased risk for preterm delivery. Using the methods disclosed herein, the effectiveness of a therapeutic agent in clinical trials can be monitored. The methods include obtaining a small molecule profile from a subject in a clinical trial being treated with a therapeutic agent, and monitoring changes in the small molecule profile of the subject as an indication of the effectiveness of the therapeutic agent in preventing preterm delivery. In one embodiment, the small molecule profile of the subject can be compared to a predetermined standard (generally the profile of a pregnant subject having a full-term delivery). The methods also include assessing the amount of one or more metabolites in a subject in a clinical trial being treated with a therapeutic agent, and monitoring changes in the amount of the one or more metabolites. Specific non-limiting examples of metabolites of interest are disclosed in the above description and in the examples section. Increases and/or decreases in the amount of the one or more metabolites can be detected.

Methods are also provided for monitoring the effectiveness of a therapeutic agent during the course of a therapeutic treatment of a subject, such as a pregnant subject. The therapeutic treatment can include the use of one or more therapeutic agent(s), or can included an increased dose of a therapeutic agent currently being administered to the subject of interest. In one embodiment, the subject has a disease or a disorder, such as an autoimmune disorder and/or diabetes. In another embodiment, the subject is at increased risk of preterm labor delivery, such as preterm delivery associated with inflammation. For example, the subject can have one or more of the following risk factors: (1) preterm delivery in a previous pregnancy, (2) a history of ruptured membranes in a previous pregnancy, (3) a multipartite (twins, triplets, etc.), (4) miscarriages between 14 and 24 weeks in two prior pregnancies, (5) malformation of the uterus (including fibroids), (6) an excess of amniotic fluid (polyhydramnios), (7) previous surgery of the cervix (such as for cervical cancer), (8) substance abuse (such as alcohol or drugs), (9) smoking, (10)

experienced bleeding after about 14 weeks of gestation, and/or (11) has a short cervix (such as less than 2 cm at less than 24 weeks of gestation). In another example, the subject is experiencing preterm labor.

In one example, a sample is taken from a subject of interest prior to the initiation of a specific therapy. The small molecule profile of the sample taken prior to the initiation of therapy is then determined. This profile can serve as a control. The level, presence or absence of one or more specific metabolites can also be determined. This amount can serve as a control.

Following initiation of the therapy, a sample is taken from the subject, and the small molecule profile is determined. The small molecule profile of the sample prior to the initiation of therapy is then compared to the small molecule profile of the sample taken following the initiation of therapy. A change in the small molecule profile, such that is more similar to the profile of a pregnant subject that had a full-term delivery indicates that the therapy is effective. Similarly, the level, presence or absence of one or more specific small molecules associated with preterm delivery can be determined. A change in the amount of the one or more specific small molecules associated with preterm delivery, such that the amount is more similar to the amount of one or more specific small molecules associated with preterm delivery in a pregnant subject that had a full-term delivery indicates that the therapy is effective.

Conversely, the method disclosed herein can also be used to determine if a therapeutic regimen is ineffective. If the small molecule profile is the same after the onset of the therapeutic regimen (as compared to the profile obtained prior to the initiation of therapy), the treatment is discontinued. Similarly, the level, presence or absence of one or more specific small molecules associate with preterm delivery can be determined. A change in the amount of the one or more specific small molecules associated with preterm delivery indicates that the therapy is ineffective.

In yet another example, the small molecule profile of one or more samples taken from a subject during the course of therapeutic regimen can be used to determine an effective amount of a therapeutic agent (therapeutically effective dose). This method includes, for example, comparing the small molecule profile (or amount of one or more specific metabolites) of one or more samples following the

administration of a specific amount of a therapeutic agent to those samples taken (1) prior to initiation of therapy, and/or to (2) samples taken after administration of a different dose of the therapeutic agent. If a positive effect is not seen at a specific amount of the therapeutic agent of interest, the amount can be increased. An additional sample can be obtained, and the small molecule profile (or the amount of one or more specific small molecules associated with preterm delivery) of the sample is determined. This amount of therapeutic agent can be increased. An additional sample can be obtained, and the small molecule profile (or the amount of one or more the amount of one or more specific small molecules associated with preterm delivery) can then be compared to a control, such as the small molecule profile obtained prior to the initiation of therapy. The small molecule profile (or the amount of one or more the amount of one or more specific small molecules associated with preterm delivery) from a sample obtained when the subject was administered the lower dose of the therapeutic agent can also be used as a control. The amount of the therapeutic agent can be adjusted more than one time; this method can be repeated. Methods are provided, therefore, to determine the maximum (or minimum) dose that has a therapeutic effect, and does not cause intolerable side effects.

In a further example, methods are provided to determine the length of time a therapeutic regimen should continue. Specifically, samples are obtained at time intervals (regular or irregular), during a therapeutic regimen. For example, samples can be obtained daily, bi-weekly, weekly, bi-monthly, or monthly and analyzed. As long as a change in the small molecule profile (as compared to the small molecule profile obtained prior to the onset of treatment) is seen, the therapeutic regimen is continued. Once a change in the small molecule profile is no longer apparent (or if the level of a metabolite of interest drops below a threshold level), therapy can be discontinued.

Monitoring the influence of agents (for example, drugs, biological molecules or other therapeutic molecules, such as nutritional supplements) on the small molecule profile can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase levels of energy-producing carbon sources can be

monitored in clinical trails of subjects exhibiting decreased levels of energy-producing carbon sources. Alternatively, the effectiveness of an agent determined by a screening assay to decrease levels of amino acids can be monitored in clinical trails of subjects exhibiting increased levels of the amino acids. In such clinical trials, the level of the certain small molecule and, preferably, the remainder of the small molecule profile can be used as a "read out" of the disease state of the particular subject.

For example, and not by way of limitation, small molecules that are modulated in subjects by treatment with an agent (such as a compound, drug or small molecule) can be identified in screening assays. The effect of agents on preventing preterm delivery, for example, can be studied in a clinical trial. In addition or alternatively, the effect of agents on preventing preterm delivery without (or with) inflammation can be studied. For example, samples from the subjects can be isolated and small molecule profiles can be taken. In this way, the small molecule profile can serve as a marker, indicative of the physiological response of the subject to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the subject with the agent.

In an embodiment, a method is provided for monitoring the effectiveness of treatment of a subject with an agent (such as a peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the small molecule profile of the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the small molecule profile of the post-administration samples; (v) comparing the small molecule profile of the pre-administration sample with the small molecule profile of the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of certain agents may be desirable to increase the level of certain energy-producing carbon sources to higher levels than detected, or to decrease the level of amino acids to lower levels than detected, in order to increase the effectiveness of the agent.

In another embodiment, methods are provided (also referred to herein as a "screening assay") for identifying molecules which bind to the small molecules identified herein (also called the "target" molecule) through small molecule profiling. Methods are also provided for identifying molecules which have a stimulatory or inhibitory effect on the small molecules identified herein.

In an embodiment, the invention provides assays for screening libraries to identify samples which bind to or modulate the activity of a small molecule identified herein. Libraries of samples may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), on beads (Lam (1991) *Nature* 354:82-84), or chips (Fodor (1993) *Nature* 364:555-556). For example, in one embodiment, the samples in the library are prepared appropriately for an interaction with a specific target using a high throughput screen. The high throughput screen then is used to identify which of the samples, bind or otherwise interact with the target.

In one embodiment, an assay is a cell-based assay in which a cell which expresses a target (e.g., an energy-producing carbon source or amino acid identified herein) is contacted with a sample of the library and the ability of the sample to modulate the target's activity is determined. Determining the ability of the sample to modulate the target's activity can be accomplished by methods suitable for the particular target. Determining the ability of the sample to modulate the ability of a target to bind to its substrate can be accomplished, for example, by coupling the substrate with a radioisotope or enzymatic label such that binding of the target to its substrate can be determined by detecting the labeled substrate in a complex with the target. For example, substrates can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, substrates can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a target substrate with the unknown samples and determining the ability of the samples to modulate (e.g., stimulate or inhibit) the activity of the target.

Determining the ability of a target to bind to or interact with a target substrate can be accomplished by one of the methods described above for determining direct binding. In an embodiment, determining the ability of the target to bind to or interact with its substrate can be accomplished by determining the activity of the substrate. For example, the activity of the substrate can be determined by detecting induction of a cellular second messenger of the target, detecting catalytic/enzymatic activity of the target or its substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g., luciferase), or detecting a target-regulated cellular response.

In yet another embodiment, an assay is a cell-free assay in which a target is contacted with an unknown sample and the ability of the sample to bind to the target is determined. Binding of a sample to the target can be determined either directly or indirectly as described above. In a further embodiment, the assay includes contacting the target with a compound which is known to bind to the target to form an assay mixture, contacting the assay mixture with the sample, and determining the ability of the sample to interact with the target, wherein determining the ability of the sample to interact with the target comprises determining the ability of the sample to preferentially bind to the target as compared to a known compound.

Kits

This disclosure also encompasses kits for identifying a subject at increased risk for preterm delivery, with or without inflammation. The kit may comprise a labeled compound or agent capable of detecting the relevant small molecule in a biological sample and means for determining the amount of the relevant small molecule in the sample (such as an antibody against the relevant small molecule, or another molecular or chemical sensor). Kits may also include instructions for observing that the tested subject is at risk of preterm delivery if the amount of the relevant small molecule is above or below a normal level (that of a patient having a full-term delivery).

The kit may also comprise, for example, a buffering agent, a preservative, or a stabilizing agent. The kit may also comprise components necessary for detecting

the detectable agent (such as a substrate). The kit may also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Each component of the kit is generally enclosed within an individual container and all of the various containers are generally within a single package along with instructions for observing whether the tested subject is at risk of preterm delivery, with or without inflammation.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Materials and Methods

Study population

A total of 114 amniotic fluid samples from distinct subjects were analyzed in this study. Ninety samples were used to establish proteomic patterns with diagnostic value, and 24 samples were used to test and validate the algorithm. Amniotic fluid was obtained by amniocentesis performed for the assessment of the microbiological status of amniotic cavity and/or fetal lung maturity. Samples from subjects at term

were obtained at the time of elective caesarian section. Preterm labor was defined as the presence of uterine contractions (at least 3 in 10 minutes (min)) or advanced cervical dilatation at less than 37 weeks of gestation. PROM was diagnosed by sterile speculum examination confirming leakage of amniotic fluid in the vagina, and positive ferning and nitrazine test results. Samples for research were collected under IRB protocols approved by Wayne State University after written informed consent was obtained. The utilization of these samples for research was approved by the IRB of the National Institute of Child Health and Human Development. Cultures for aerobic, anaerobic bacteria and genital Mycoplasmas, Gram stain, counts of white and red blood cells were performed immediately after collection. The remaining amniotic fluid was centrifuged at 700g at 4°C for 10 min., and was then stored in aliquots at -80°C until analysis.

Sample Preparation:

Samples were kept frozen until assays were performed. The sample preparation process was automated on the MicroLab STAR® from Hamilton Company. Sample preparation was conducted using a series of organic and aqueous extractions. A recovery standard was added at the first step in the extraction process for QC purposes. The resulting extract was divided into a liquid chromatography (LC) fraction and a (gas chromatography) GC fraction. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Samples destined for GC analysis were frozen and dried under vacuum. Samples were then processed for the appropriate instrument, either LC/MS (mass spectrometry) or GC/MS. Internal standards, recovery standards, processing standards, chromatography, and other specific compounds were introduced during sample preparation for post-analysis QC review. Additionally, as noted above, during the randomization process, standard samples were included for contemporaneous analysis.

Liquid chromatography/Mass Spectroscopy (LC/MS):

The LC/MS portion of the platform was based on a Surveyor HPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which has an ion-trap (IT) front end and a Fourier-Transform (FT) backend. It was set for continuously monitoring both

positive and negative ions. Some compounds were redundantly visualized across more than one of these data-streams. However, not only is the sensitivity and linearity vastly different from interface to interface but these redundancies, in some instances, were actually used as part of the QC program. By using this combination of detector conditions, it is believed the vast majority of metabolites in the sample can be detected.

The vacuum-dried sample was resolubilized in 100 µl of an injection solvent that contained no less than five injection standards at fixed concentrations. The chromatography has been standardized and was never allowed to vary. The internal standards were used both to assure injection and chromatographic consistency. The chromatographic system runs as a gradient from 5% Acetonitrile (ACN) to 100% ACN over an 8 minute period, held at 100% for 8 min then reconditioned back to starting conditions. The columns (Perfluorobenzene, for instanceC-16) were maintained in temperature-controlled chambers during use and are exchanged, washed and reconditioned after every 50 injections. All columns were purchased from a single manufacturer's lot at the outset of these experiments. All solvents were similarly purchased in bulk from a single manufacturer's lot in sufficient quantity to complete all related experiments. The raw data files were tracked and processed by their identifiers and were archived to DVD at regular intervals.

Gas chromatography/Mass Spectroscopy (GC/MS):

The samples destined for GC were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The column was 5% phenyl and the temperature ramp was from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Mat-95 XP using electron impact ionization, and high resolution. The resulting fingerprint spectra were used for analysis of elemental composition and identification. The raw data files were identified by their identifiers and were archived to DVD at regular intervals.

Informatics and Data Analysis:

The Informatics system used for data analysis consisted of three major components:, the Laboratory Information Management System (LIMS); the data extraction systems (Metabolyzer™); QC and data processing tools (DataForge™) and a collection of Information Interpretation and Visualization tools for use by the Therapeutic Area Panels. The hardware and software foundations for these informatics components are the LAN backbone, and a database server running Oracle 9.2.0.1 Enterprise Edition. Tools used for the data analysis were commercial products such as SAS, Spotfire, and a number of basic tools for support of the data interpretation. Statistical techniques such as principle component analysis, discriminant analysis, hierarchical clustering, support vector machines, random forest, partitioning and similarity analyses to these datasets were used to asses the data (Simmons *et al.* (2004) *Int. Fed. Classification Soc.* 447- 457; Truong *et al.* (2004) *KDD*).

Example 2

Metabolites Identified by Random Forest to Identify Preterm Delivery

The study described below was performed to determine if metabolic profiling could be used to identify pregnant women who were at risk for preterm delivery. The study was designed as a retrospective analysis, using stored amniotic fluid samples. The samples were matched for gestational age at the time of sampling, within one week. All samples were obtained from women with a singleton pregnancy; samples from women with multiple fetuses were excluded from the study. A sample of 250 micro-liters of amniotic fluid was extracted for the detection of aqueous and non-polar compounds, and metabolic profiling was performed using mass spectrometry, gas chromatography (non-polar) and liquid chromatography (polar). Compound identification was performed using authentic standards, and for unknown identification. Amniotic fluid samples were obtained from 16 women who had full-term deliver, 19 women who had preterm delivery without inflammation, and 20 women who had preterm delivery accompanied by inflammation. Generally, inflammation was defined as a white blood cell count of

greater than 100/mm^3. The concentration of IL-6 may be used to determine if inflammation is present. An amniotic fluid culture may also be performed.

The following metabolites were identified as being predictive of the clinical classes (subjects at risk for preterm delivery, subjects at risk for preterm delivery with inflammation). A higher number under "probability" indicates a stronger correlation with risk for preterm delivery.

A supervised analysis of the clinical variables in the prediction of clinical class was performed using a Linear Discriminant Analysis (LDA). The following variables were assessed: cervical dilation, cervical effacement, sonograph cervical length, history of preterm delivery, gestational age, frequency of uterine contractions, maternal temperature, maternal age, and parity. Results are presented in FIG. 1. No correlation was found between the clinical variables and the risk for preterm delivery.

Using the methods described in Example 1, a supervised analysis (LDA) of the amniotic fluid metabolome (small molecule profile) in the prediction of clinical class was performed. The results are presented in FIG. 2. There was a clear correlation between the entire small molecule profile and the risk for preterm delivery.

The clinical class as predicted by small molecule profile was then compared to the actual clinical class (as determined by the outcome of the pregnancy). The results are presented in Table 1.

Table 1

Prediction of the clinical class according to the amniotic fluid metabolic profile

TRUE (down) vs. PREDICTED (across)	Term Delivery	Preterm Delivery with inflammation	Preterm Delivery without inflammation
Term delivery	15	0	1
Preterm Delivery with inflammation	0	19	1
Preterm Delivery without inflammation	0	0	19

Linear Discriminant Analysis Accuracy (53/55) = 96.36%

As presented in Table 1, the small molecule profile correctly predicted full term delivery, preterm delivery with inflammation, and preterm delivery without inflammation. There was only a single case wherein preterm delivery without inflammation was predicted, but a full term delivery was the actual clinical result. Similarly, there was only a single case wherein preterm delivery without inflammation was predicted, but a preterm delivery with inflammation was the actual clinical result.

Example 3

Second Study of the Small Molecule Profile as a Predictor of Preterm Delivery

The additional study described below was performed to determine if metabolic profiling could be used to identify pregnant women who were at risk for preterm delivery. The study was designed as a retrospective analysis, using stored amniotic fluid samples. The samples were matched for gestational age at the time of sampling, within one week. All samples were obtained from women with a singleton pregnancy; samples from women with multiple fetuses were excluded from the study. A sample of 250 micro-liters of amniotic fluid was extracted for the detection of aqueous and non-polar compounds, and metabolic profiling was performed using mass spectrometry, gas chromatography (non-polar) and liquid chromatography (polar). Compound identification was performed using authentic standards, and for unknown identification. Amniotic fluid samples were obtained from 40 women who had full-term deliver, 33 women who had preterm delivery without inflammation, and 40 women who had preterm delivery accompanied by inflammation. Preterm delivery without inflammation was defined as an amniotic fluid interleukin (IL)-6 concentration less than 2500 pg/ml. Preterm delivery with inflammation was defined as an amniotic fluid IL-6 concentration of greater than 2500 pg/ml.

A data analysis of the small molecule profile was performed using a Random Forest analysis (Breiman L. *Machine Learning* 45:5-32, 2001) with an internal validation method that included bootstrapping and out of bag methods. This method provides an unbiased estimation of the results and thus exceeds the reproducibility of the results over LDA. For the amniotic fluid small molecule profile

(metabolome), approximately 20,018 data points were analyzed. For each sample, approximately 186 metabolites were assessed. The small molecule profile of all 113 patients were compared. The prediction of the clinical class, as compared to the actual clinical result, is presented in Table 2

Table 2

Prediction of the clinical class according to the amniotic fluid metabolic profile

TRUE (down) vs. PREDICTED (across)	Term Delivery	Preterm Delivery with inflammation	Preterm Delivery without inflammation
Term delivery	39	1	0
Preterm Delivery with inflammation	7	32	1
Preterm Delivery without inflammation	2	2	29

Random Forest Accuracy (100/113) = 88.49%

As presented in Table 2, the small molecule profile correctly predicted full term delivery, preterm delivery with inflammation, and preterm delivery without inflammation. The Random Forrest analysis demonstrated that the small molecule profile prediction of the clinical class was accurate in almost 90% of the cases.

The ability of the diagnostic indices of the small molecule profile to predict clinical class was then assessed. The results are presented in Table 3. The results document that the small molecule profile of the amniotic fluid was both a sensitive and specific method in the predication of clinical class according to their traditional definitions.

Table 3

Diagnostic indices of amniotic fluid metabolome: prediction of clinical class

	Sensitivity	Specificity
Term delivery	98% (39/40)	88% (64/73)
Preterm Delivery with inflammation	80% (32/40)	96% (70/73)
Preterm Delivery without inflammation	88% (29/33)	99% (80/81)

The identity of the individual metabolites was then established. These metabolites can be identified in a small molecule profile, or can be identified in individual assays for the specific metabolite. With regard to the small molecule profile, the importance of the 30 most significant molecules, as estimated by Random Forest, is presented in Table 4.

Table 4

Importance of metabolites estimated by Random Forest (Gini Index) for the prediction of clinical classes

No.	Small molecule	Probability
1	Methyladenine	3.066
2	*Heptanedioic acid	3.005
3	*N Acetylglutamine	2.939
4	DL beta hydroxyphenylethylamine	2.698
5	Unknown 128[1]	2.389
6	Hexose cluster 5	1.878
7	Hexose cluster 1	1.874
8	Leucine	1.836
9	glycerol	1.729
10	Isoleucine	1.687
11	Inositol	1.541
12	L-methionine	1.506
13	Glycine	1.501
14	D-Galactose	1.464
15	Hexose cluster 3	1.463
16	Catechol	1.442
17	Salicylamide	1.431
18	Butanedioic.acid	1.41
19	Unknown 5[3]	1.263
20	D-mannose	1.22

No.	Small molecule	Probability
21	Unknown 8[4]	1.217
22	Glutamic acid	1.13
23	Phenylalanine	1.104
24	Alpha L-sorbopyranose	1.077
25	Cholesterol	1.045
26	Unknown 289[2]	1.039
27	D-fructose	1.031
28	Hexose cluster 2	0.995
29	L-tryptophan	0.883
30	Eicosanoic acid	0.816

[1]Unknown 128 had a chromatographic runtime of approximately 10.14 minutes and had characteristic ion fragments that included (but were by no means exclusively) 227 amu (100%), 203 amu (60%), 317 amu (34%).

[2]Unknown 289 had a chromatographic runtime of approximately 7.58 minutes and had characteristic ion fragments that included (but were by no means exclusively) 540 amu (100%).

[3]Unknown 5 had a chromatographic runtime of approximately 9.64 minutes and had characteristic ion fragments that included (but were by no means exclusively) 256 amu (100%), 182 amu (24%).

[4]Unknown 8 had a chromatographic runtime of approximately 9.40 minutes and had characteristic ion fragments that included (but were by no means exclusively) 246 amu (100%), 213 amu (28%), 287 amu (14%).

Both energy producing carbon sources, such as carbohydrates, and amino acids, were identified as being predictive of the clinical class. These results can be summarized as a decrease in the amount of carbohydrates was predictive a preterm delivery. A decrease in the amount of amino acids was predictive of preterm delivery that was associated with inflammation. The qualitative results are presented in Table 5.

These studies demonstrated that the small molecule profile of samples from pregnant patients can be used to predict the risk for preterm delivery and preterm delivery with inflammation. The small molecule profile predicted the risk for preterm delivery and preterm delivery with inflammation with a high degree of accuracy. In addition, the results were both sensitive and specific. The metabolites presented in Table 4 individually were associated with the risk of preterm delivery and preterm delivery with inflammation. Specifically, the metabolites N-

acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic.acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid were associated with preterm delivery. As disclosed in Table 5, the disclosed methods also permit detection of differentiation of preterm delivery with or without inflammation by detecting, with the small molecules listed in Table 4 or a subset thereof, a decrease in the energy producing carbon sources with or without a change in the level of amino acids. Specifically, preterm delivery without inflammation can be detected or differentiated using the small molecules listed in Table 4 or a subset thereof by a decrease in the energy producing carbon sources without a change in the level of amino acids. Preterm delivery with inflammation can be detected or differentiated using the small molecules listed in Table 4 or a subset thereof by a decrease in the energy producing carbon sources with a change in the level of amino acids.

Table 5

Levels of carbon sources and amino acids during delivery

	Energy-Producing Carbon Sources	Amino acids
Term delivery	Control Level	Control Level
Preterm delivery	Decreased Compared to Control Level	Same as Control Level
Preterm delivery with inflammation	Very Decreased Compared to Control Level	Increased Compared to Control Level

EXAMPLE 4

Identification of Risk of Preterm Delivery in a Subject Presenting with Preterm Labor

A subject is identified who presents in preterm labor. Specifically, the presence of uterine contractions (at least 3 in 10 minutes (min)) is detected in the subject. An amniotic fluid sample is obtained from the subject experiencing preterm labor.

Sample preparation is conducted using a series of organic and aqueous extractions. A recovery standard is added at the first step in the extraction process for QC purposes. The resulting extract is divided into a liquid chromatography (LC) fraction and a (gas chromatography) GC fraction. The sample is placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The samples destined for GC analysis is frozen and dried under vacuum. The sample is then processed for the appropriate instrument, either liquid chromatorgraph (LC)/MS (mass spectrometry) or gas chromatograph (GC)/MS.

The LC/MS portion of the platform is based on a Surveyor HPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which has an ion-trap (IT) front end and a Fourier-Transform (FT) backend. It is set for continuously monitoring both positive and negative ions. The vacuum-dried sample is resolubilized in 100 μl of an injection solvent that contains no less than five injection standards at fixed concentrations. The internal standards are used to assure injection and chromatographic consistency. The chromatographic system is run as a gradient from 5% Acetonitrile (ACN) to 100% ACN over an 8 minute period, held at 100% for 8 min then reconditioned back to starting conditions. The columns (Perfluorobenzene, for instanceC-16) are maintained in temperature-controlled chambers during use and are exchanged, washed and reconditioned after every 50 injections. The raw data files are tracked and processed by their identifiers and are archived to DVD at regular intervals.

The sample destined for GC/MS is re-dried under vacuum desiccation. The column is 5% phenyl and the temperature ramp is from 40° to 300° C in a 16 minute period. The sample is analyzed on a Thermo-Finnigan Mat-95 XP using electron impact ionization, and high resolution. The resulting fingerprint spectrum is used

for analysis of elemental composition and identification. The raw data file is identified by the identifiers and is archived to DVD at regular intervals.

The Informatics system that is used for data analysis consists of three major components:, the Laboratory Information Management System (LIMS); the data extraction systems (Metabolyzer™); QC and data processing tools (DataForge™) and a collection of Information Interpretation and Visualization tools for use by the Therapeutic Area Panels. The hardware and software foundations for these informatics components are the LAN backbone, and a database server running Oracle 9.2.0.1 Enterprise Edition. Tools used for the data analysis are a commercial products such as SAS and/or Spotfire. Statistical techniques such as random forest are used to asses the data (Simmons et al. (2004) Int. Fed. Classification Soc. 447-457; Truong et al. (2004) KDD). A metabolic profile is produced similar to that shown in FIG. 3.

A small molecule profile is obtained from the sample. In one set of analysis, the expression of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and/or eicosanoic acid is assessed. A statistical method is used to compare the sample to a standard small molecule profile obtained from a subject known not to be at risk for preterm delivery. The statistical program identifies the women as having a small molecule profile different from the standard small molecule profile, and smilar to the small molecule profile of a woman at risk for preterm delivery not associated with inflammation. Thus, the woman is at risk for preterm delivery.

EXAMPLE 5

Additional Small Molecule Profiles of Use

Additional small molecule profiles can be used to determine the risk of preterm delivery, with and/or without inflammation. Several exemplary profiles are set forth in Table 6, below.

Table 6

Exemplary Profiles

Small molecule	Profile 1	Profile 2	Profile 3	Profile 4
Methyladenine		+	+	
*Heptanedioic acid	+		+	+
*N Acetylglutamine	+	+		+
DL beta hydroxyphenylethylamine		+	+	
Unknown 128[1]		+	+[6]	
Hexose cluster 5				+
Hexose cluster 1				
Leucine	+	+	+	+[7]
glycerol				
Isoleucine	+	+[4]	+	+
Inositol		+	+	
L-methionine	+		+	+
Glycine	+	+	+	+
D-Galactose	+[1,3]			+
Hexose cluster 3				+
Catechol		+	+	
Salicylamide		+[5]	+	
Butanedioic.acid	+		+	+
Unknown 5		+	+[6]	
D-mannose	+[3]		+	+
Unknown 8		+	+[6]	
Glutamic acid	+[3]	+	+	+[7]
Phenylalanine	+		+	+
Alpha L-sorbopyranose	+		+	+
Cholesterol		+	+	
Unknown 289		+	+[6]	
D-fructose	+		+	+
Hexose cluster 2				+
L-tryptophan	+[2, 3]	+	+	+
Eicosanoic acid		+	+	

[1] Not included in profile 1A.
[2] Not included in profile 1B.
3 Not included in profile 1C.
4 Not included in profile 2A.
5 Not included in profile 2B.
6 Not included in profile 3A.
7. Not include in profile 4A.

As described above, an amniotic fluid (or other biological sample) is obtained from a pregnant woman at risk for preterm delivery, such as a subject

showing significant cervical dilation and effacement, or a subject with preterm labor. The amniotic fluid is processed for small molecule detection, such as using the methods described above in Example 1. It should be noted that each small molecule can also be detected individually, using any method known in the art. Alternatively, a small molecule profile can be obtained. In several examples, a small molecule profile is obtained for a subset of the small molecules disclosed in Table 4. Specific small molecule profiles are shown above, in Table 6, and are labeled profiles 1-4. Exemplary variant profiles, wherein less than the full set of small molecules are evaluated, are also indicated in Table 4.

When profile 1 is assessed, a statistically significant decrease in of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid, butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine shows a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

Variants of this profile eliminate one, two, three, four or five of the small molecules. Thus, an exemplary variant profile ("profile 1A") does not include galactose. Using profile 1A, a decrease in mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid, butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. An increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine a risk of preterm delivery without inflammation.

Another exemplary variant profile ("profile 1B") does not include L-tryptophan. A statistically significant decrease in galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid, butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term

delivery, including preterm delivery with and without inflammation. A statistically significant increase in glutamic acid, phenylalanine, glycine, isoleucine and leucine shows a risk of preterm delivery with inflammation. No statistically significant change in glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

A third exemplary profile ("profile 1C") does not include galactose or mannose, and does not include L-tryptophan or glutamic acid. A statistically significant decrease in mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid, butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant increase in phenylalanine, glycine, isoleucine and leucine shows a risk of preterm delivery with inflammation. No statistically significant change in phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

When profile 2 is assessed, a statistically significant change (either an increase or a decrease) in the level of catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid indicates the subject is at risk of preterm delivery. An increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine a risk of preterm delivery without inflammation.

An exemplary variant profile ("profile 2A") does not include isoleucine. A statistically significant change (either an increase or a decrease) in the level of catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid indicates the subject is at risk of preterm delivery. An increase in L-tryptophan, glutamic acid, phenylalanine, glycine and leucine indicates a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine a risk of preterm delivery without inflammation.

Another exemplary variant profile ("profile 2B") does not include salicylamide. When profile 2B is assessed, a statistically significant change (either an increase or a decrease) in the level of catechol, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid indicates the subject is at risk of preterm delivery. An increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine a risk of preterm delivery without inflammation.

When profile 3 is assessed, a statistically significant decrease in of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid, butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant change (either an increase or a decrease) in the level of catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknowns 5, 8, 128, and 289, inositol, and eicosanoic acid indicates the subject is at risk of preterm delivery. A statistically significant increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine shows a risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

An exemplary variant profile ("profile 3A") does not include Unknown 5, 8, 128 and 289. When profile 3 is assessed, a statistically significant decrease in of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid and butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant change (either an increase or a decrease) in the level of catechol, salicylamide, methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, inositol, and eicosanoic acid indicates the subject is at risk of preterm delivery. A statistically significant increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine shows a

risk of preterm delivery with inflammation. No statistically significant change in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

When profile 4 is assessed, a statistically significant decrease in of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid and butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant change in hexose cluster 2, hexose cluster 3 and hexose cluster 5 indicates that the subject is at risk of preterm delivery, with or without inflammation. A statistically significant increase in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine shows a risk of preterm delivery with inflammation. No statistically significant change in in L-tryptophan, glutamic acid, phenylalanine, glycine, isoleucine and leucine indicates a risk of preterm delivery without inflammation.

An exemplary variant profile ("profile 4A") does not include glutamic acid or leucine. When profile 4 is assessed, a statistically significant decrease in of galactose, mannose, glucose, alpha L-sorbopyranose, fructose, glycerol, heptanedioic acid and butanedioic acid, citric, succinic, malic, oxaloacetic and pyruvic acids indicates a risk of pre-term delivery, including preterm delivery with and without inflammation. A statistically significant change in hexose cluster 2, hexose cluster 3 and hexose cluster 5 indicates that the subject is at risk of preterm delivery, with our without inflammation. A statistically significant increase in L-tryptophan, phenylalanine, glycine, and isoleucine shows a risk of preterm delivery with inflammation. No statistically significant change in in L-tryptophan, phenylalanine, glycine, and isoleucine indicates a risk of preterm delivery without inflammation.

These profiles are exemplary only, and are not limiting. One, two or three of the small molecules from each profile can be eliminated, and the variant profiles can also be used to assess the risk of preterm delivery (with and without inflammation). Similarly, the amount of one, two, three, four or five additional small molecules listed in Table 4 can be added to any of profiles 1-4A, and this profile can then be used to assess the subjects risk for preterm delivery. These profiles are also of use

in identifying the subject as being is at risk for preterm delivery (with or without inflammation).

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

CLAIMS

1. A method for assessing the risk of spontaneous preterm delivery for a pregnant subject, comprising

a) determining the level of methyladenine, heptanedioic acid and N-acteyl glutamine from a biological sample obtained from the pregnant subject; and,

b) determining whether the level of methyladenine, heptanedioic acid and N-acteyl glutamine differs from a corresponding small molecule profile from a subject with full term delivery;

wherein detecting a change in the amount of one or more small molecules associated with preterm delivery in the small molecule profile in the sample, as compared to a sample from a subject with a full-term delivery indicates that the subject is at risk for spontaneous preterm delivery.

2. A method for detecting the risk of spontaneous preterm delivery for a pregnant subject, comprising

a) obtaining a small molecule profile from a biological sample obtained from the pregnant subject; and,

b) determining whether the small molecule profile differs from a corresponding small molecule profile from a subject with full term delivery;

wherein detecting a change in the amount of one or more small molecules associated with spontaneous preterm delivery in the small molecule profile in the sample, as compared to a sample from a subject with a full-term delivery indicates that the subject is at risk for spontaneous preterm delivery.

3. The method of claim 2, wherein the one or more small molecules associated with preterm delivery comprises methyladenine.

4. The method of claim 2 or 3, wherein the one or more small molecules associated with preterm delivery comprises heptanedioic acid, and wherein a decrease in heptanedioic acid identifies the subject as at risk for spontaneous preterm delivery.

5. The method of any one of claims 2-4, wherein the small molecule profile comprises N-acetylglutamine.

6. The method of any one of claims 1-3, wherein method comprises a method of detecting the risk of preterm delivery that is not associated with inflammation.

7. The method of any one of claims 1-6, wherein the biological sample is amniotic fluid.

8. The method of any one of claims 2-7, wherein obtaining the small molecule profile comprises obtaining the small molecule profile by one or more of mass spectrometry, liquid chromatograph, and gas chromatography.

9. The method of any one of claims 2-8, wherein the small molecule profile comprises at least one of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic.acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid.

10. The method of any one of claims 1-9, wherein the subject is a human.

11. The method of any one of claim 1-10, wherein the method comprises a method of distinguishing the risk of preterm delivery without inflammation from the risk of preterm delivery with inflammation or full-term delivery.

12. A method for identifying a subject at risk for spontaneous preterm delivery, comprising assaying for the presence of at least one energy-producing

carbon source associated with preterm delivery, at least one amino acid associated with preterm delivery, or both, in a sample from the subject, wherein a decrease in the amount of the at least one energy-producing carbon source, at least one amino acid, or both, as compared to a sample from a subject who as undergone a full-term delivery indicates that the subject is at risk for spontaneous preterm delivery.

13. The method of claim 12, wherein a the method comprises identifying a subject at risk for spontaneous preterm delivery without inflammation, wherein the method comprises

measuring the presence of at least one energy-producing carbon source in the sample, wherein an decrease in the amount of the energy-producing carbon source in the sample as compared to a sample from a subject who has undergone a full term delivery indicates that the subject is at risk for spontaneous preterm delivery without inflammation.

14. The method of claim 13, further comprising measuring the presence of at least one amino acid in the sample, and wherein the same amount of the at least one amino acid in the sample as compared to a sample from a subject who has undergone a full term delivery indicates that the subject is at risk for spontaneous preterm delivery without inflammation.

15. The method of any one of claims 11-14, wherein the carbon source comprises both at least one carbohydrate and at least one small organic acid.

16. The method of any one of claims 11-14, wherein the energy-producing carbon source is not a carbohydrate.

17. The method of any one of claim 11-16, wherein a decrease in the amount of one or more energy-producing carbon sources associated with spontaneous preterm delivery and no change in the amount of one or more amino acids associated with preterm delivery as compared to a sample from a subject with

a full-term delivery, indicates that the subject is at increased risk for spontaneous preterm delivery without inflammation.

18. The method of any one of claims 11-17, wherein the sample is an amniotic fluid sample.

19. The method of any one of claims 11-17, wherein the sample is a vaginal fluid, cervical fluid, maternal blood, plasma, serum, or chorionic villous sample.

20. The method of any one of claims 11-17, wherein the sample is an umbilical cord blood sample.

21. The method of any of claims 11-20, comprising assaying for the presence of one or more of methyladenine, N-acetylglutamine, beta hydroxyphenylethylamine, cholesterol, Unknown 5, Unknown 8, Unknown 128, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, inositol, or eicosanoic acid, or any combination thereof.

22. A method for identifying a subject at increased risk for preterm delivery, comprising

a) obtaining a small molecule profile from a sample from a pregnant subject, wherein the small molecule profile comprises at least one small molecule selected from the group consisting of methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic.acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid; and,

b) determining whether the small molecule profile from the subject differs from a corresponding standard small molecule profile that would be expected in a sample from a subject that has a full-term delivery,

wherein a difference in the small molecular profile of the subject as compared to the standard identifies the subject as being at increased risk for preterm delivery.

23. The method of claim 22, wherein the small molecule profile comprises methyladenine.

24. The method of claim 22 or 23, wherein the small molecule profile comprises heptanedioic acid.

25. The method of any one of claims 22-24, wherein the small molecule profile comprises N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic.acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid.

26. The method of claim 25, wherein the small molecule profile comprises methyladenine, heptanedioic acid, N-acetylglutamine, DL beta hydroxyphenylethylamine, Unknown 128, hexose cluster 5, hexose cluster 1, leucine, glycerol, isoleucine, inositol, L-methionine, glycine, D-galactose, hexose cluster 3, catechol, salicylamide, butanedioic.acid, Unknown 5, D-mannose, Unknown 8, glutamic acid, phenylalanine, alpha L-sorbopyranose, cholesterol, Unknown 289, D-fructose, hexose cluster 2, L-tryptophan, and eicosanoic acid.

27. The method of any one of claims 22-26, wherein the subject is a human.

28. The method of any one of claims 22-27, wherein the sample is amniotic fluid.

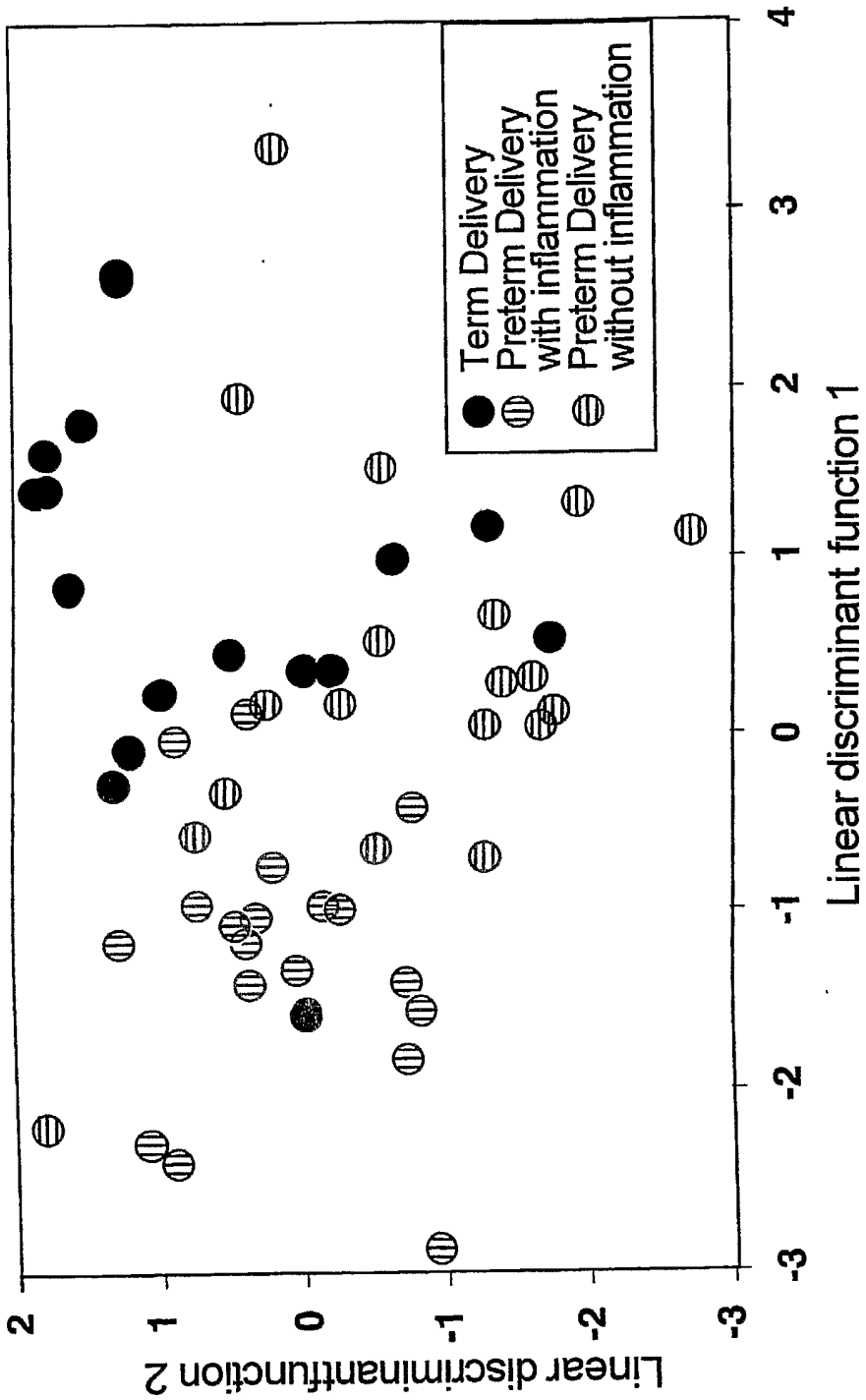
29. The method of any one of claims 22-27, wherein the sample is a vaginal fluid, cervical fluid, blood, serum, plasma, maternal urine sample or a chorionic villous sample.

30. The method of any one of claims 22-29, wherein said small molecule profile is obtained using one or more of the following: high performance liquid chromatography, thin layer chromatograph (TLC), electrochemical analysis, mass spectroscopy, refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), and Light Scattering analysis (LS).

31. The method of any one of claims 22-30, wherein the small molecule profile comprises amino acids.

32. The method of any one of claims 22-31, wherein the small molecule profile comprises an energy-producing carbon source.

33. The method of any one of claims 22-31, wherein the method assess the risk of preterm delivery without inflammation.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Preterm Delivery without inflammation
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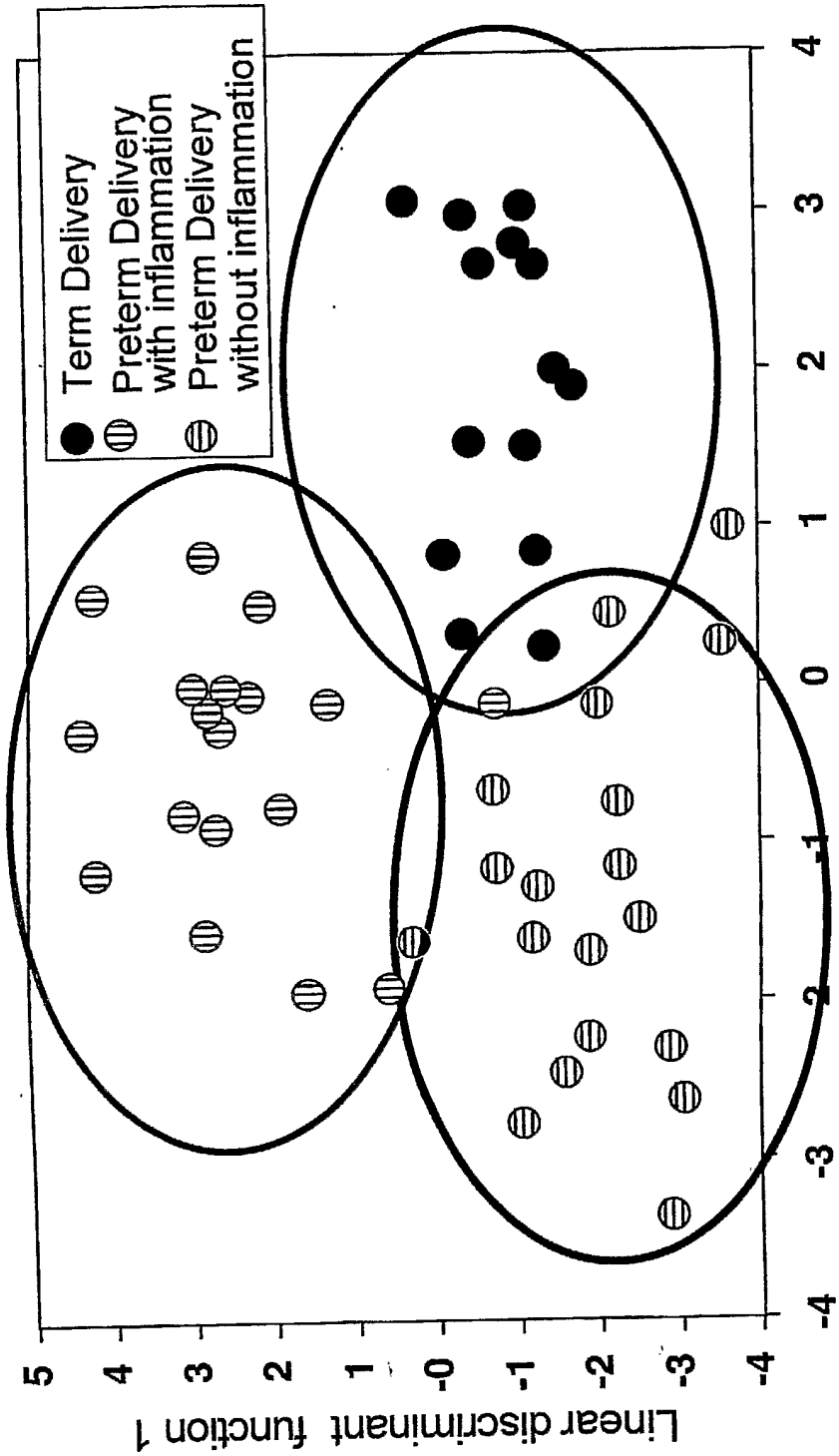
-2
-3

FIG. 1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Preterm Delivery without inflammation
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FIG. 2

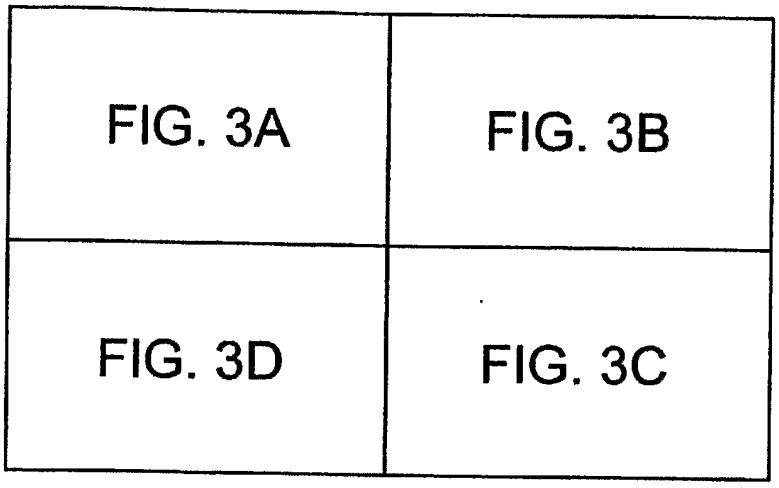
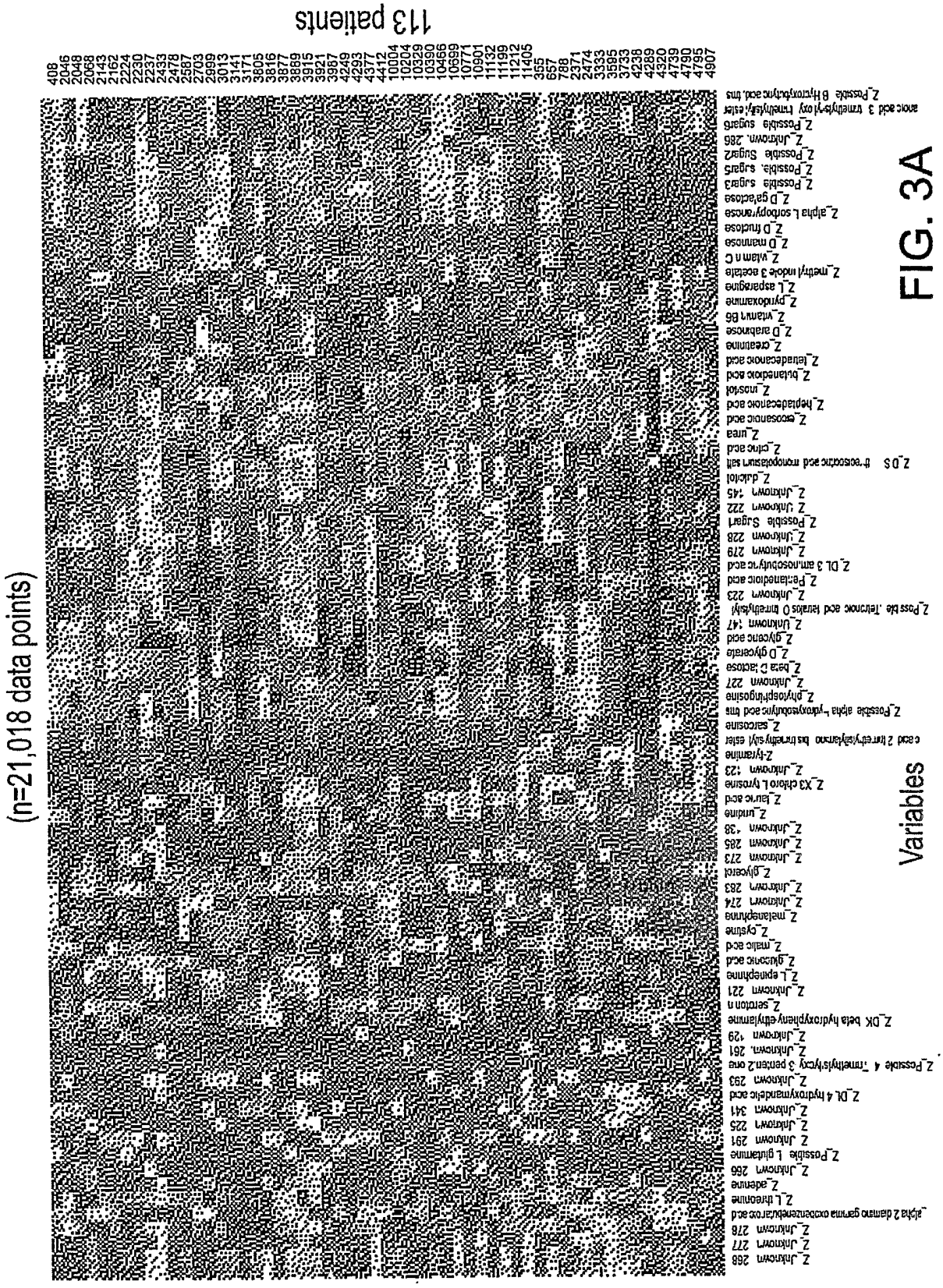


FIG. 3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Z_cystine
Z_melanophrine
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Z_vitamin B6
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Z_vitamin C
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Z_Possible sugar5
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FIG. 3A

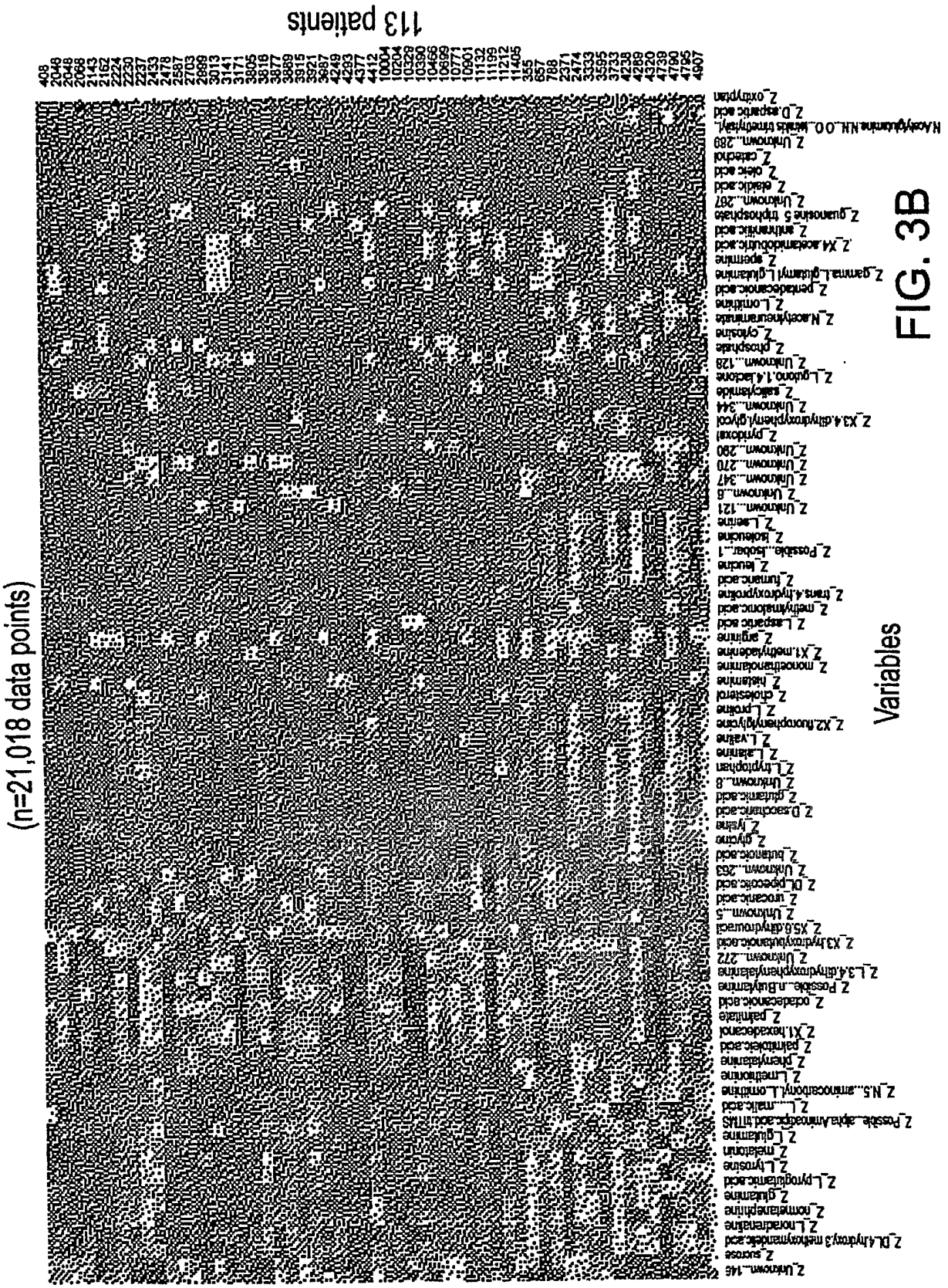
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FIG. 3B

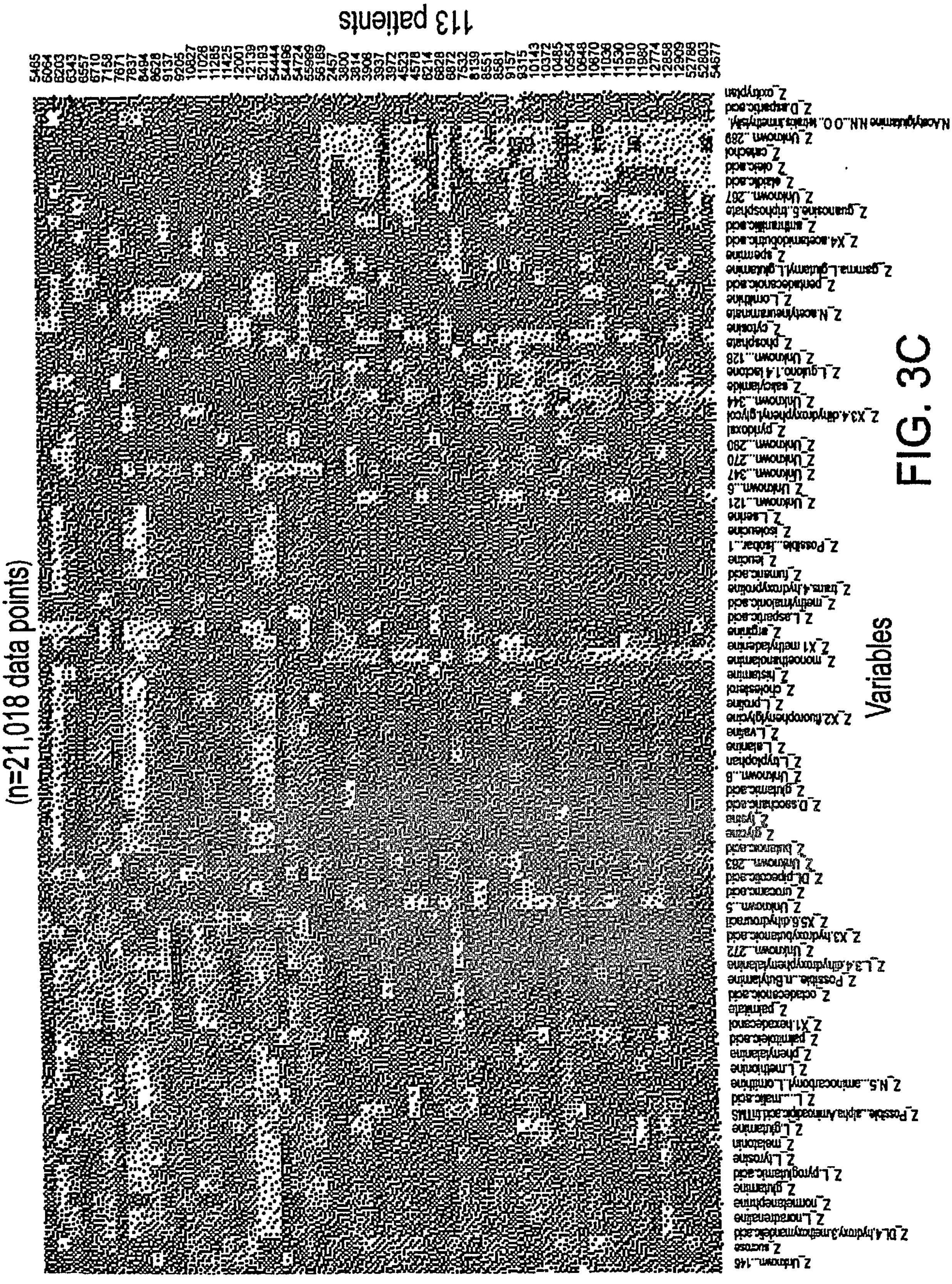

FIG. 3C

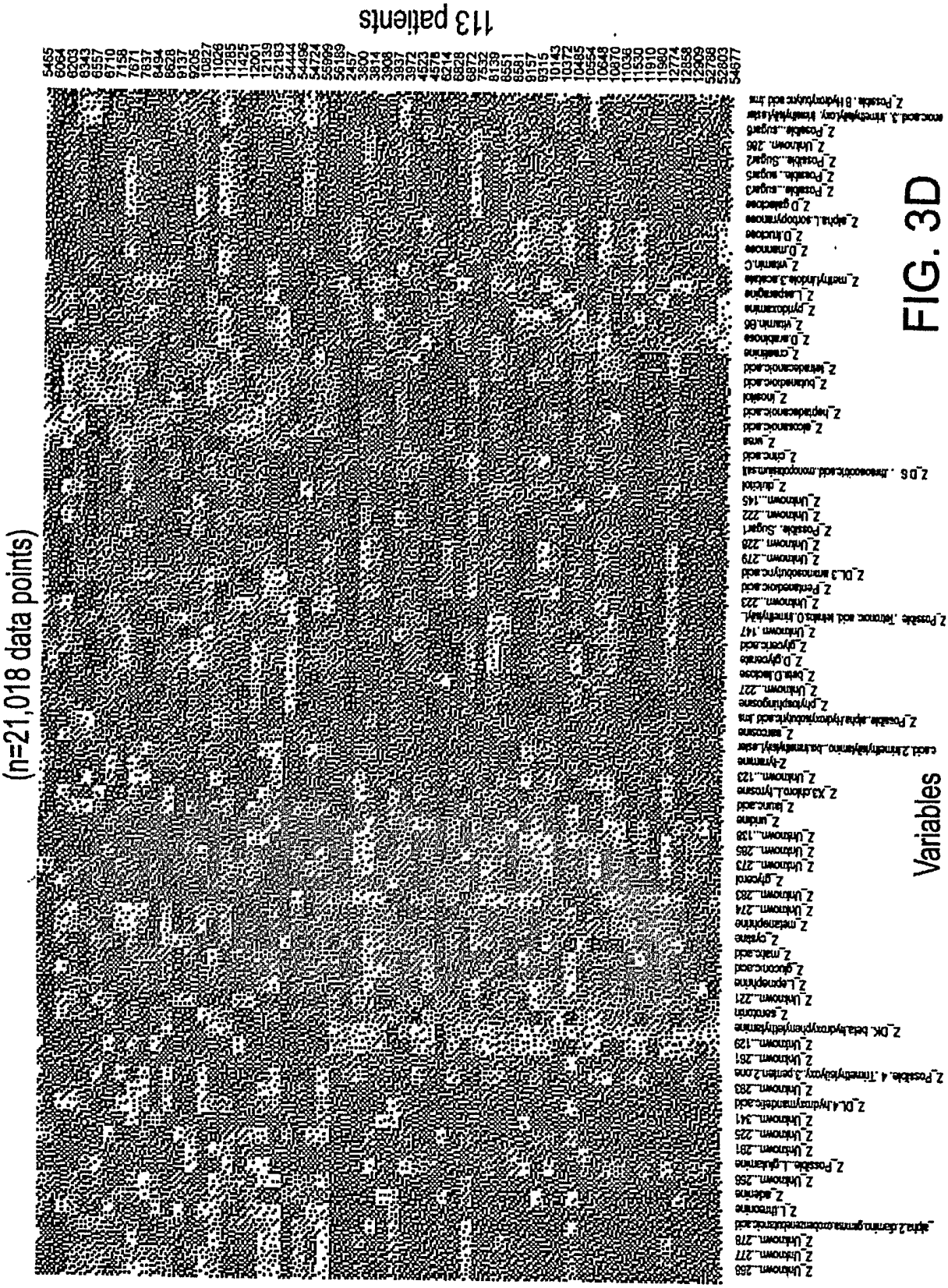
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FIG. 3D