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(54) Title: A METABOLITE PANEL FOR IMPROVED SCREENING AND DIAGNOSTIC TESTING OF CYSTIC FIBROSIS

(57) Abstract: A novel method of diagnosing cystic fibrosis in a human subject is provided. The method includes the steps of: i) determining in a biological sample from the subject the level of one or more metabolic biomarkers; ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and iii) determining that the subject has cystic fibrosis or a related disorder when the difference in the level of the biomarker in the sample is statistically different from the control level.

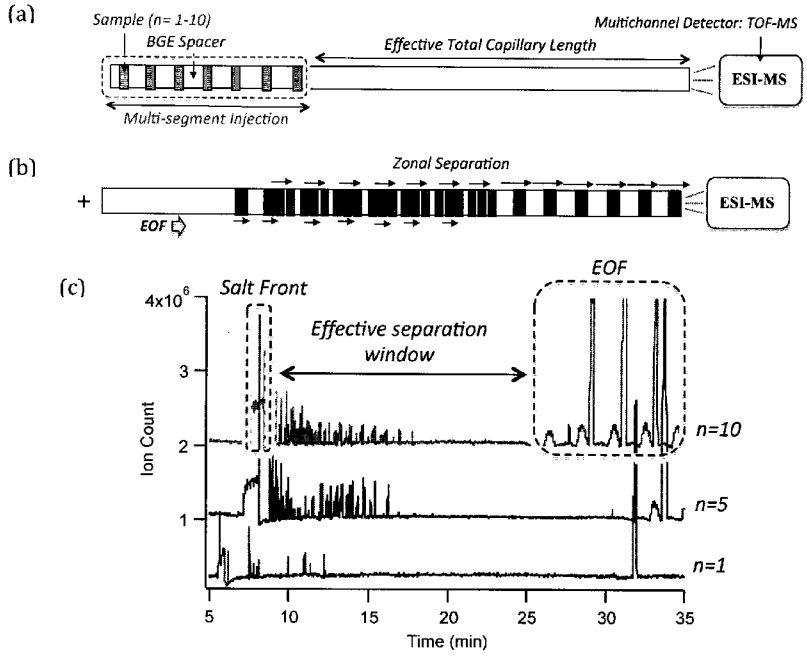


Figure 2.

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A METABOLITE PANEL FOR IMPROVED SCREENING AND DIAGNOSTIC TESTING OF CYSTIC FIBROSIS

Field of Invention

[0001] The present invention generally relates to a method for population-based screening and diagnostic testing of cystic fibrosis, and more particularly relates to a diagnostic method utilizing metabolites for the screening and/or diagnosis of cystic fibrosis.

Background of the Invention

[0002] Cystic fibrosis (CF) is one of the most frequent recessive genetic diseases in the western world caused by mutations to the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). More than 2,000 different mutations of the CFTR gene have been identified to date, however only a few dozen variants are of known clinical significance with ΔF508 occurring in about 70% of CF patients in North America. Mutations in the CFTR gene can lead to protein deficiency and/or loss in chloride transport function causing the formation of thick mucous at tissue surfaces. There is great clinical heterogeneity in the expression of CF that can include progressive respiratory failure, recurrent lung infections, pancreatic insufficiency, and failure to thrive, that reduces overall life expectancy. Recent evidence supports the benefits of universal CF screening of neonates (before 2 months of age), since early nutritional supplementation contributes to fewer hospitalizations, lower rates of complications with improved physical and cognitive development in children. CF patients diagnosed by newborn screening are associated with better lung function, improved nutritional status and longer survival relative to non-screened subjects. For these reasons, CF is now included within a panel of genetic diseases in newborn screening programs for pre-symptomatic diagnosis of infants at an earlier onset that results in positive clinical outcomes and lower healthcare expenditures.

[0003] A "two-tiered" approach is currently used for population-based CF screening using an algorithm based on elevated immunoreactive trypsinogen (IRT) followed by a DNA mutation panel (Parad et al. *J. Pediatrics* **2005**, 147: 878), however the exact IRT threshold and total number of CFTR mutations used varies by jurisdiction (Massie et al. *Med. J. Australia* **2012**, 196: 67). Limitations of this primary screening strategy include a high rate of false

positives (≈ 85-90%) as confirmed by low chloride sweat test results (< 30 mM), the identification of carriers with CFTR allele variants who do not express the disease, and potential false-negatives for individuals with rare CFTR mutations within diverse populations (Castellani et al. *Curr. Opin. Pulmon. Med.* **2010**, 16: 584). Furthermore, ethical concerns are related to asymptomatic carrier identification that increases costs due to referrals for diagnostic testing, genetic counseling and follow-up patient monitoring. As a result, the pilocarpine-stimulated iontophoresis sweat test remains the "gold standard" for confirmatory diagnosis of CF based on functional assessment of residual CFTR activity in terms of chloride absorptivity from skin. Since IRT and/or mutation screens provide only *probable or inconclusive information regarding disease status*, only 10-15% of screen-positive CF infants have elevated sweat chloride (> 60 mM) with the majority of patients having low/normal chloride (< 29 mM) or borderline chloride (30-59 mM). The latter outcome corresponds to an *ambiguous diagnostic test result* with mild/late-onset phenotypes and poorly understood natural clinical histories requiring repeat sweat testing and on-going clinical assessment.

[0004] Thus, there is a need for an improved method for the screening and diagnosis of cystic fibrosis in affected individuals which overcomes at least one of the disadvantages of prior methods.

Summary of the Invention

[0005] A novel method for screening and diagnosing cystic fibrosis has now been developed which is based on one or more CF-specific metabolites measured in biological specimens.

[0006] Thus, in one aspect of the invention, a method of diagnosing cystic fibrosis or a related disorder in a human subject is provided comprising:

i) determining in a biological sample from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-tryptophan, *L*-citrulline, *L*-glutamine, oxidized glutathione disulfide, β-alanine, *L*-proline, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-kynurenine, *L*-lysine, *L*-isoleucine, *L*-asparagine, *L*-aspartic acid, 3-hydroxy-*L*-proline, glycine, trimethylamine *N*-oxide, asymmetric *N*-dimethyl-

L-arginine, 5-hydroxy-*L*-lysine, adenine, guanine, hypoxanthine, 2-deoxyadenosine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 238.095 [MH^+] and a RMT of 1.108; a metabolite having a *m/z* of 180.087 [MH^+] and a RMT of 0.730; a metabolite having a *m/z* of 252.109 [MH^+] and a RMT of 1.274; a metabolite having a *m/z* of 298.053 [MH^+] and a RMT of 0.794; a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 1.152; a metabolite having a *m/z* of 176.071 [MH^+] and a RMT of 0.694; a metabolite having a *m/z* of 104.071 [MH^+] and a RMT of 0.772; and a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 0.872;

ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and

iii) determining that the subject has cystic fibrosis when the difference in the level of the biomarker in the sample is statistically different from the control level.

[0007] In another aspect of the invention, a method of diagnosing cystic fibrosis or a related disorder in a human subject is provided comprising:

i) determining in a sweat sample obtained from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-citrulline, pilocarpic acid, 3-hydroxy-*L*-proline, *L*-arginine, *L*-tyrosine, *L*-phenylalanine, hypoxanthine, pilocarpine, and *L*-lysine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 280.078 [$M\text{-}H^-$] and a RMT of 0.967; a metabolite having a *m/z* of 115.040 [$M\text{-}H^-$] and a RMT of 1.076; a metabolite having a *m/z* of 160.062 [$M\text{-}H^-$] and a RMT of 0.917; a metabolite having a *m/z* of 201.077 [$M\text{-}H^-$] and a RMT of 0.849; a metabolite having a *m/z* of 129.056 [$M\text{-}H^-$] and a RMT of 1.019; a metabolite having a *m/z* of 257.114 [$M\text{-}H^-$] and a RMT of 0.835; a metabolite having a *m/z* of 235.118 [MH^+] and a RMT of 0.768; a metabolite having a *m/z* of 194.138 [MH^+] and a RMT of 0.721; a metabolite having a *m/z* of 370.053 [MH^+] and a RMT of 0.820; a metabolite having a *m/z* of 247.037 [MH^+] and a RMT of 0.565; a metabolite having a *m/z* of 139.050 [MH^+] and a RMT of 0.686; and a metabolite having a *m/z* of 280.024 [MH^+] and a RMT of 0.687;

ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and

iii) determining that the subject has cystic fibrosis when the difference in the level of the biomarker in the sample is statistically different from the control level.

[0008] In a further aspect, a method of monitoring disease progression and/or response to treatment in a human subject with cystic fibrosis is provided. The method comprises:

i) determining in a biological sample from the human subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-tryptophan, *L*-citrulline, *L*-glutamine, oxidized glutathione disulfide, β-alanine, *L*-proline, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-kynurenine, *L*-lysine, *L*-isoleucine, *L*-asparagine, *L*-aspartic acid, 3-hydroxy-*L*-proline, glycine, trimethylamine *N*-oxide, asymmetric *N*-dimethyl-*L*-arginine, 5-hydroxy-*L*-lysine, adenine, guanine, hypoxanthine, 2-deoxyadenosine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 238.095 [MH^+] and a RMT of 1.108; a metabolite having a *m/z* of 180.087 [MH^+] and a RMT of 0.730; a metabolite having a *m/z* of 252.109 [MH^+] and a RMT of 1.274; a metabolite having a *m/z* of 298.053 [MH^+] and a RMT of 0.794; a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 1.152; a metabolite having a *m/z* of 176.071 [MH^+] and a RMT of 0.694; a metabolite having a *m/z* of 104.071 [MH^+] and a RMT of 0.772; and a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 0.872 in a first instance;

ii) comparing the level of the biomarker to a control level and determining the first difference between the biomarker level and the control level;

iii) determining the level of the biomarker at a second subsequent instance and determining the second difference between the biomarker level at the first instance and at the second instance;

iv) comparing the first and second differences and determining that the disease is progressing if the second difference is greater than the first difference, or determining that the subject is responding to treatment if the second difference is less than the first difference.

[0009] In a further aspect, a method of monitoring disease progression and/or response to treatment in a human subject with cystic fibrosis is provided. The method comprises:

i) determining in a sweat sample obtained from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-citrulline, pilocarpic acid, 3-hydroxy-*L*-proline, *L*-arginine, *L*-tyrosine, *L*-phenylalanine, hypoxanthine, pilocarpine, and *L*-lysine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 280.078 [M-H$^-$] and a RMT of 0.967; a metabolite having a *m/z* of 115.040 [M-H$^-$] and a RMT of 1.076; a metabolite having a *m/z* of 160.062 [M-H$^-$] and a RMT of 0.917; a metabolite having a *m/z* of 201.077 [M-H$^-$] and a RMT of 0.849; a metabolite having a *m/z* of 129.056 [M-H$^-$] and a RMT of 1.019; a metabolite having a *m/z* of 257.114 [M-H$^-$] and a RMT of 0.835; a metabolite having a *m/z* of 235.118 [MH$^+$] and a RMT of 0.768; a metabolite having a *m/z* of 194.138 [MH$^+$] and a RMT of 0.721; a metabolite having a *m/z* of 370.053 [MH$^+$] and a RMT of 0.820; a metabolite having a *m/z* of 247.037 [MH$^+$] and a RMT of 0.565; a metabolite having a *m/z* of 139.050 [MH$^+$] and a RMT of 0.686; and a metabolite having a *m/z* of 280.024 [MH$^+$] and a RMT of 0.687;

ii) comparing the level of the biomarker to a control level and determining the first difference between the biomarker level and the control level;

iii) determining the level of the biomarker at a second subsequent instance and determining the second difference between the biomarker level at the first instance and at the second instance;

iv) comparing the first and second differences and determining that the disease is progressing if the second difference is greater than the first difference, or determining that the subject is responding to treatment if the second difference is less than the first difference.

[0010] These and other aspects of the invention will become apparent from the detailed description that follows and by referenced to the following figures.

Brief Description of the Figures

[0011] FIGURE 1. Overview of the population-based screening of CF based on a two-tiered IRT and DNA mutation panel followed by confirmatory sweat chloride test used previously iin most jurisdictions in North America;

[0012] FIGURE 2 depicts multi-segment injection-capillary electrophoresis (MSI-CE-MS) that is used as a multiplexed separation platform for untargeted metabolite profiling of complex biological samples that increases sample throughput by over one order of magnitude;

[0013] FIGURE 3 depicts a wide array of polar metabolites from dried blood spot extracts that can be analyzed by MSI-CE-MS with high selectivity, including various amino acids and acylcarnitines used as primary or secondary markers of specific in-born errors of metabolism;

[0014] FIGURE 4 outlines an accelerated workflow in metabolomics for biomarker discovery that takes advantage of multiplexed separations by MSI-CE-MS with higher sample throughput and data fidelity;

[0015] FIGURE 5 depicts an asymmetric signal pattern recognition approach used in MSI-CE-MS for unambiguous identification of putative markers associated with in-born errors of metabolism (IEM) relative to a pooled healthy sample as quality control (QC), such as elevation in *L*-phenylalanine that is associated with phenylketonuria (PKU);

[0016] FIGURE 6 depicts multivariate statistical analysis of 36 authentic metabolites measured in dried blood spot (DBS) extracts by MSI-CE-MS for the discovery of putative markers differentially expressed in affected CF infants (n=4) relative to healthy/screen-negative controls (n=4) when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for single or ratiometric CF-specific markers;

[0017] FIGURE 7 depicts multivariate statistical analysis of 82 authentic metabolites measured in dried blood spot (DBS) extracts by MSI-CE-MS for the discovery of putative markers differentially expressed in affected CF adults (n=3) relative to healthy controls (n=3)

when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for single or ratiometric CF-specific markers;

[0018] FIGURE 8 depicts a strategy for structural elucidation of metabolites differentially expressed in dried blood spot extracts from CF subjects using accurate mass, spiking with authentic standards and tandem MS (MS/MS), such as a metabolite with a *m/z* of 238.095 [MH^+] and a RMT of 1.108;

[0019] FIGURE 9 demonstrates a sub-group comparison of metabolites associated with the urea cycle/nitric acid pathway differentially expressed in pooled sweat specimens from screen-positive CF infants with low (< 30 mM), intermediate (30-59 mM) and high (> 60 mM) sweat chloride results indicative of *L*-arginine deficiency for affected CF subjects;

[0020] FIGURE 10 depicts multivariate statistical analysis of 75 authentic metabolites measured in pilocarpine-stimulated sweat specimens by MSI-CE-MS for the discovery of markers differentially expressed in affected infants with classic CF (n=4, heterozygotes with delF508 CFTR mutation with a high sweat chloride result > 60 mM) relative to screen-positive/unaffected carriers (n=10 with a low sweat chloride result < 30 mM) when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for ratiometric CF-specific markers detected in residual sweat specimens.

Detailed Description of the Invention

[0021] A novel method of diagnosing cystic fibrosis in a human subject is provided comprising: i) determining in a biological sample from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-tryptophan, *L*-citrulline, *L*-glutamine, oxidized glutathione disulfide, β-alanine, *L*-proline, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-kynurenine, *L*-lysine, *L*-isoleucine, *L*-asparagine, *L*-aspartic acid, 3-hydroxy-*L*-proline, glycine, trimethylamine *N*-oxide, asymmetric *N*-dimethyl-*L*-arginine, 5-hydroxy-*L*-lysine, adenine, guanine, hypoxanthine, 2-deoxyadenosine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when

using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 238.095 [MH^+] and a RMT of 1.108; a metabolite having a *m/z* of 180.087 [MH^+] and a RMT of 0.730; a metabolite having a *m/z* of 252.109 [MH^+] and a RMT of 1.274; a metabolite having a *m/z* of 298.053 [MH^+] and a RMT of 0.794; a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 1.152; a metabolite having a *m/z* of 176.071 [MH^+] and a RMT of 0.694; a metabolite having a *m/z* of 104.071 [MH^+] and a RMT of 0.772; and a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 0.872; ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and iii) determining that the subject has cystic fibrosis when the difference in the level of the biomarker in the sample is statistically different from the control level.

[0022] The term "cystic fibrosis" is used herein to refer to an inherited autosomal disease associated with mutations to the gene encoding the cystic fibrosis transmembrane conductor regulator (CFTR). Commonly, CF is associated with homozygotes or compound heterozygotes having a delF508 mutation leading to CF symptoms such as pancreatic insufficiency, poor lung function and nutrient absorption. Cystic fibrosis represents a disease spectrum when considering compound heterozygotes, that results in related disorders referred to as CFTR-related metabolic syndrome (a CF-related metabolic syndrome or CFRMS), which may exhibit similar symptoms.

[0023] To conduct the method, a biological sample is obtained from a human subject. The term "biological sample" is meant to encompass any human sample that may contain relevant metabolites, including biological fluids such as, but not limited to, blood, plasma/serum, urine, sweat, saliva, sputum, cerebrospinal fluid, and for prenatal testing, amniotic fluid. Tissue biopsies from organs affected by CF may also be used, including, for example, lung, pancreas and tissue of the gastrointestinal tract. The sample is obtained from the subject in a manner well-established in the art.

[0024] Once a suitable biological sample is obtained, it is analyzed to determine the signal response or concentration of the selected biomarker(s) in the sample. Prior to analysis, the sample may be subject to processing such as extraction, filtration, centrifugation or other sample preparation techniques to provide a sample that is suitable for further analysis. For example, biological fluids may be filtered or centrifuged (e.g. ultracentrifugation) to remove solids from the sample to facilitate analysis. Tissue samples may be subject to extractions in order to

provide an analyzable sample. As one of skill in the art will appreciate, biomarker level may be determined using one of several techniques established in the art that would be suitable for detecting such biomarkers, e.g. polar metabolites, in a biological sample, including mass spectrometry, chromatographic techniques such as high performance liquid chromatography and gas chromatography, immunoassay or enzyme-based assays with colorimetric, fluorescence or radiometric detection. As one of skill in the art will appreciate, CF-specific markers may be analyzed directly or may be chemically derivatized for analysis, and may be analyzed by comparison against stable-isotope internal standards.

[0025] In a preferred embodiment, biomarker detection using a mass spectrometry (MS)-based method is used. Suitable MS-based methods for use include direct infusion-mass spectrometry, electrospray ionization (ESI)-MS, desorption electrospray ionization (DESI)-MS, direct analysis in real-time (DART)-MS, atmospheric pressure chemical ionization (APCI)-MS, electron impact (EI) or chemical ionization (CI), as well as MS-based methods coupled with a separation technique, such as liquid chromatography (LC-MS), gas chromatography (GC-MS), or capillary electrophoresis (CE-MS) mass spectrometry.

[0026] In other embodiments, the level of a biomarker in a sample may be measured by immunoassay using an antibody specific to the target biomarker. The antibody binds to the biomarker and bound antibody is quantified by measuring a detectable marker which may be linked to the antibody or other component of the assay, or which may be generated during the assay. Detectable markers may include radioactive, fluorescent, phosphorescent and luminescent (e.g. chemiluminescent or bioluminescent) compounds, dyes, particles such as colloidal gold and enzyme labels. The term "antibody" is used herein to refer to monoclonal or polyclonal antibodies, or antigen-binding fragments thereof, e.g. an antibody fragment that retains specific binding affinity for the target biomarker. Antibodies to the target biomarkers may be commercially available. Alternatively, antibodies to the target biomarkers may also be raised using techniques conventional in the art. For example, antibodies may be made by injecting a host animal, e.g. a mouse or rabbit, with the antigen (target biomarker), and then isolating antibody from a biological sample taken from the host animal. Alternative affinity ligands that bind to CF-specific metabolites may also be utilized for measurement of CF metabolites, such as

DNA or RNA-based aptamers derived from systematic evolution of ligands by exponential enrichment (SELEX).

[0027] Different types of immunoassay may be used to determine the level of target biomarkers in a sample, including indirect immunoassay in which the biomarker is non-specifically immobilized on a surface; sandwich immunoassay in which the biomarker is specifically immobilized on a surface by linkage to a capture antibody bound to the surface; and a competitive binding immunoassay in which a sample is first combined with a known quantity of biomarker antibody to bind biomarker in the sample, and then the sample is exposed to immobilized biomarker which competes with the sample to bind any unbound antibody. Enzyme Linked ImmunoSorbent Assay (ELISA) may also be used to determine the level of a biomarker in a sample. In this case, the biomarker to be analyzed is generally immobilized on a solid support, complexed with an antibody to the biomarker which is itself linked to an enzyme indicator, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), ß-galactosidase, acetylcholinesterase and catalase. Detection may then be accomplished by incubating this enzyme-complex with a substrate for the enzyme that yields a detectable product.

[0028] The method includes the quantitation of at least one metabolic biomarker from a biological sample. Preferably, the level of at least two or more biomarkers is determined to screen or diagnose cystic fibrosis, for example, the level of between two and ten biomarkers are determined in a sample, and more preferably, the level of between two and five biomarkers are determined in a sample, for use to screen or diagnose cystic fibrosis.

[0029] Once the level of the selected biomarker(s) is determined, the level is compared to a control level to determine the difference or statistical significance between the biomarker level and the control level. The level of some of the metabolite biomarkers is increased in cystic fibrosis in comparison to a control level. Examples of biomarkers that exhibit increased levels in cystic fibrosis relative to unaffected subjects (*i.e.* fold-change > 1) include *L*-tryptophan, *L*-citrulline, β-alanine, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-lysine, pilocarpic acid, 3-hydroxy-*L*-proline, and other metabolites as shown in Tables 1 and 2. Other metabolite biomarkers exhibit a reduced level in cystic fibrosis in comparison to unaffected subjects (*i.e.,* fold-change < 1), such as *L*-glutamine, *L*-arginine, oxidized glutathione

disulfide, *O*-butyryl-*L*-carnitine, *L*-kynurenine, and other metabolites as shown in Tables 1 and 2. The term "control level", as it is used herein, is the level of a selected metabolite biomarker detected in a sample from a healthy or screen-positive yet unaffected subject (*i.e.* a carrier) who does not have cystic fibrosis. Preferably the control value is a mean control value obtained from a healthy population of matched subjects (*e.g.* age-, gender- and/or ethnically-matched to a population).

[0030] In addition to the quantitation of selected biomarkers, a ratiometric determination between the levels of two biomarkers may be calculated, i.e. the ratio of the levels of two biomarkers from a sample, for comparison against a control value, i.e. the ratio of the control levels of the two selected biomarkers. For example, the ratio of the level of the biomarker, arginine, and the level of the biomarker, citrulline, may be determined in a biological sample, and compared to a control ratio of the levels of these two biomarkers, to determine the difference between the ratio in the sample and the control ratio. Preferred ratiometric determinations for use in the present method are between a metabolite biomarker that exhibits an increased level in cystic fibrosis, e.g. tryptophan, citrulline, ornithine, valine, lysine, asymmetric-dimethyl-L-arginine, beta-alanine, 3-hydroxy-L-proline, 5-hydroxy-L-lysine, histadine, and other metabolites that exhibit a fold-change of greater than 1 over a normal (control) level of the metabolite, and a metabolite biomarker that exhibits a reduced level in cystic fibrosis, e.g. arginine, C4, 5-deoxyadensosine, aspartic acid, and other metabolites that exhibit a fold-change of less than 1 over a normal (control) level of the metabolite. Such a ratio further amplifies the fold-change of the selected biomarkers and increases statistical significance (*p* value) for CF screening or diagnosis, while also correcting for differences in sample volume in a specimen analyzed (*e.g.* dried blood spot).

[0031] A human subject is determined to have cystic fibrosis when the difference in the level of one or more biomarkers in a biological sample is statistically different from the control levels of these biomarkers, and/or when the difference in a ratiometric determination between two biomarkers is statistically different from the control ratio between these biomarkers above a minimum control threshold established for a population. The determination of statistical significance is well-established in the art. Statistical significance is attained when a *p*-value is less than the significance level. The *p*-value is the probability of observing an effect given that

the null hypothesis is true whereas the significance or alpha (α) level is the probability of rejecting the null hypothesis given that it is true. Generally, a statistically significant difference, i.e. increase or decrease, in the level of a biomarker in accordance with the present method, is a difference in the level of the biomarker from the control level of at least about 5%, or greater, e.g. at least about 10%, 15%, 20%, 25% or more.

[0032] In another aspect of the present invention, a method of diagnosing cystic fibrosis or a related disorder in a human subject is provided comprising: i) determining in a sweat sample obtained from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-citrulline, pilocarpic acid, 3-hydroxy-*L*-proline, *L*-arginine, *L*-tyrosine, *L*-phenylalanine, hypoxanthine, pilocarpine, and *L*-lysine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 280.078 [M-H$^-$] and a RMT of 0.967; a metabolite having a *m/z* of 115.040 [M-H$^-$] and a RMT of 1.076; a metabolite having a *m/z* of 160.062 [M-H$^-$] and a RMT of 0.917; a metabolite having a *m/z* of 201.077 [M-H$^-$] and a RMT of 0.849; a metabolite having a *m/z* of 129.056 [M-H$^-$] and a RMT of 1.019; a metabolite having a *m/z* of 257.114 [M-H$^-$] and a RMT of 0.835; a metabolite having a *m/z* of 235.118 [MH$^+$] and a RMT of 0.768; a metabolite having a *m/z* of 194.138 [MH$^+$] and a RMT of 0.721; a metabolite having a *m/z* of 370.053 [MH$^+$] and a RMT of 0.820; a metabolite having a *m/z* of 247.037 [MH$^+$] and a RMT of 0.565; a metabolite having a *m/z* of 139.050 [MH$^+$] and a RMT of 0.686; and a metabolite having a *m/z* of 280.024 [MH$^+$] and a RMT of 0.687; ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and iii) determining that the subject has cystic fibrosis when the difference in the level of the biomarker in the sample is statistically different from the control level. The method may optionally include a ratiometric determination step between two of the biomarkers, and a comparison of the ratio to a control ratio, wherein the determination of a statistically significant difference in the ratio of biomarkers to the control ratio is indicative of cystic fibrosis.

[0033] Certain of the metabolite biomarkers in sweat exhibit an increased level in cystic fibrosis in comparison to a control level, e.g. citrulline, pilocarpic acid, 3-hydroxy-L-proline, tyrosine, phenylalanine, pilocarpine, lysine, and other metabolites that exhibit a fold-change of

greater than 1 over a normal (control) level of the metabolite. Other metabolite biomarkers exhibit a reduced level in cystic fibrosis in comparison to a control level, e.g. arginine, hypoxanthine, and a metabolite that exhibits a fold-change of less than 1 over a normal (control) level of the metabolite.

[0034] The present methods advantageously provide a means to screen and diagnose CF in a cost-effective manner while providing the necessary sensitivity with improved specificity as compared to conventional two-tiered screens. This method avoids identification of CF carriers, and thus, eliminates the ethical concerns of population-based CF screening in newborn screening programs that is limited by high rates of false positives. The present methods also enable differential diagnosis of the CF disease spectrum notably in cases associated with ambiguous or inconclusive sweat chloride test results. As a result, metabolite-based biomarkers associated with CF may be measured by mass spectrometry as a multiplexed instrumental platform already available in most clinical laboratories, eliminating the need for CF-specific immunoassays (*e.g.* IRT) and population-based genetic testing.

[0035] In addition to use to diagnose cystic fibrosis, the present methods may also be used to predict disease progression and/or monitor treatment response to therapy. In this regard, for a human subject determined to have cystic fibrosis, by the present or other diagnostic method, the present method may subsequently be used to determine the level of one or more of the metabolite biomarkers in a biological sample from the subject. The determination of the level of the metabolite biomarker is determined on at least two occasions. In this case, the difference in a first level of the biomarker from a control level (which may be a baseline level previously determined in the human subject) is determined (a first difference) and compared to a subsequent difference (second difference) which is the difference between a subsequent determined biomarker level and the control level. If the difference in biomarker level increases over time (difference 1 is less than difference 2), this indicates that the disease is progressing (or treatment is not effective), while no change in the difference of biomarker levels over time indicates that the disease is not progressing (or treatment may be effective), and a decrease in the difference of biomarker levels over time indicates disease remission (or treatment is effective).

[0036] Embodiments of the invention are described in the following examples which are not to be construed as limiting.

Example 1 – Determination of metabolites associated with cystic fibrosis

[0037] *Chemicals and Reagents.* Deionized water used for aqueous buffer and stock preparations were obtained from a Thermo Scientific Barnstead EasyPure II ultrapure water system (Cole Parmer, Vernon Hills, IL, USA). Ammonium acetate, formic acid, acetic acid, 3-chloro-*L*-tyrosine (Cl-Tyr), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and analyte standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (Caledon, Georgetown, ON, Canada) and HPLC-grade acetonitrile (Honeywell, Muskegon, MI, USA) were used to prepare sheath liquid and background electrolyte (BGE), respectively. Acylcarnitine standards were graciously provided by Dr. Murray Potter at McMaster University. Polar standards were prepared in water and stored at 4°C, whereas acylcarnitine standards were prepared in methanol and stored at -20°C.

[0038] *Blood Spot Collection and Sample Preparation.* Proficiency testing (PT) and quality assurance (QA) dried blood spot samples were acquired from the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Duplicate punches (technical replicates) of PT and QA dried blood spot specimens that simulate phenylketonuria (PKU), maple syrup urine disease (MSUD), citrullinemia (CIT), medium chain acyl-CoA dehydrogenase deficiency (MCADD) and glutaric acidemia type-1 (GA-1) were prepared by spiking in known amounts of primary biomarkers above accepted cut-off limits (McHugh et al. *Genet. Med.* **2011**, 13: 230) using reference standards to pooled blood from consenting adult donors. Duplicate punches of two cystic fibrosis (CF) samples were each made from the blood of a consenting adult with at least one CFTR mutation. A pooled, quality control (QC) sample was made at the time of analysis by combining the reconstituted filtrates of multiple, negative control dried blood spot samples derived from Newborn Screening Ontario at the Children's Hospital of Eastern Ontario (CHEO). Samples were stored at -80°C until analyzed. Samples were 3.2 mm disks corresponding to approximately ~ 3.4 µL of whole blood. Disks were placed in 0.5 mL centrifuge tubes containing 100 µL of methanol. Analytes were extracted from the disk via sonication for 10 min and then the methanolic extract was filtered

using a Nanosep 3K Omega 3kDa MWCO ultracentrifugation tube (Pall Life Sciences, MI, USA) at 14,500 rpm for 10 min. The resulting filtrate was dried down in a Vacufuge vacuum concentration at room temperature (Eppendorf, New York, USA) and reconstituted in 30 µL of sample solution (200 mM ammonium acetate, 25% acetonitrile, 25 µM Cl-Tyr, 25 µM HEPES, pH 5.0) prior to analysis.

[0039] *Sweat Specimen Collection and Sample Preparation.* Stimulated human sweat samples were collected from screen-positive infants at the Cystic Fibrosis Clinic at McMaster University. Sweating was induced by pilocarpine iontophoresis using a Webster Model 3700 Macroduct® Sweat Capillary Collection System (Wescor Inc. Logan, UT, USA) with electrodes composed of solid agar discs containing 0.5% w pilocarpine nitrate which were attached to the subject's right forearm for 5 min at 1.5 mA. Sweat samples (> 30 µL) were collected via a spiral capillary tube containing blue dye for visual confirmation prior to storage at -20°C. Chloride measurements for sweat were performed by a chloridometer via coulometric titration with silver ions using a temperature-controlled conductivity cell (Wescor Sweat-Check Model 3120). All sweat samples were diluted in de-ionized water with internal standards prior to analysis.

[0040] *Instrumentation and Conditions.* All CE-MS experiments were performed on an Agilent G7100A CE system (Agilent Technologies Inc., Mississauga, ON, Canada) with a coaxial sheath liquid dual Agilent Jetstream electrospray ion source interface to an Agilent 6230 TOF-MS. Acquisition was performed using Agilent MassHunter Workstation LC/MS Data Acquisition Software version B.05.01 and all data processing was performed using MassHunter Qualitative Analysis Software version B.06.00. All separations were performed using uncoated fused-silica capillaries (Polymicro Technologies, AZ, USA) with 50 uM ID and 120 cm length with an applied voltage of 30 kV at 25°C. The BGE was 1 M formic acid, with 15% *v* acetonitrile (pH 1.8). Two CE-MS configurations were used in this work depending on the requirement for high sample throughput or improved concentration sensitivity. The main configuration used for targeted metabolite profiling of known biomarkers and untargeted metabolomic studies of CF and normal dried blood spot extracts was based on multi-segment injection (MSI)-CE-MS (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664), whereas a series of hydrodynamic injections alternating between a sample (5 s at 100 mbar) and BGE/spacer (40 s at 100 mbar) was performed for a total of either seven or five sample segments analyzed

simultaneously within a single run. In cases when increased concentration sensitivity was needed for detection of low abundance metabolites of clinical relevance, on-line sample pre-concentration with CE-MS (Lee et al. *Anal. Chem.* **2007** 79: 403) was performed using a single extended hydrodynamic injection of sample (90 s at 100 mbar) followed by BGE (60 s at 100 mbar) prior to voltage application. Between runs, the capillary was flushed with BGE for 15 min (at 950 mbar) to avoid sample carry-over. The sheath liquid was 60:40 MeOH:H_2O with 0.1% formic acid supplied at a flow rate of 10 uL/min by an Agilent 1260 Infinity isocratic pump. Purine and hexakis(2,2,3,3-tetraflurorpropoxy)phosphazine (HP-921) were spiked into the sheath liquid at 0.02% *v* as reference ions at *m/z* 121.050873 and *m/z* 922.009798 for real-time internal mass calibration. The TOF-MS was operated in positive ion mode with a mass range of *m/z* 50-1700 with an acquisition rate of 2 Hz and an acquisition time of 500 ms. The dual AJS ESI settings were as follows: dry gas = 8 L/min at 300°C, nebulizer = 10 psi, sheath gas = 3.5 L/min at 195°C, VCap = 2000 V, Nozzle voltage = 2000 V, whereas the TOF-MS voltage settings were Fragmentor = 120 V, skimmer = 65 V and Oct 1 RF = 750 V.

[0041] *Sample Injection Configuration and Data Workflow.* Preliminary studies involving confirmatory analysis of known primary biomarkers of several IEMs made use of a seven-segment injection format in MSI-CE-MS to maximize sample throughput. In this case, duplicate injections of three diseases (analyst-blinded) was analyzed within a single run along with a pooled healthy/normal sample that served as a reference/negative control as well as internal QC when evaluating between-run variances. Asymmetric signal patterning was used to encode information temporally within a separation based on the specific dilution pattern used among pairs of samples. In this way, the origin of aberrant metabolite levels is readily identified based on the unique signal pattern detected for ions derived from a duplicate set of sample injections. This was useful particularly in cases in which ions were undetected in certain samples/diseases within the seven segment injection series. The dilution pattern used for pairs of samples comprised a signal pattern corresponding to 1:2, 1:1, 2:1 (as six sample segments) together with the QC (as seventh segment). The six IEMs were randomly split into two groups of three and one punch from each IEM was extracted and analyzed as previously described. The second punch was extracted and analyzed separately as a technical replicate for a total of four measurements (n=4). Follow-up studies on differentially expressed metabolites in CF were performed using a five-segment injection format in MSI-CE-MS to maximize resolution of any

isomeric or isobaric compounds. In this case, the asymmetric signal pattern used for analysis involved a two-way comparison of CF relative to healthy/normal dried blood spot extracts, which corresponded to 1:2 (CF), buffer blank, 1:1 (normal/healthy). The injection order was then reversed to negate any positional impacts on sample ionization. The same procedure was repeated for a second CF punch, for a total of eight runs (n=8) when using MSI-CE-MS. Instrumental duplicate analyses were performed for each CF punch using on-line sample pre-concentration with CE-MS along with an instrumental duplicate analysis of the QC when analyzing low abundance metabolites that were not detected by MSI-CE-MS.

[0042] *Calibration and Method Validation.* Calibration curves for metabolite standards were performed and analyzed in triplicate (n = 9) by serial dilution in 200 mM ammonium acetate, 25% acetonitrile, pH 5.0 with 25 μMCl-tyr as the internal standard. Linearity was measured over a 400-fold range for polar metabolites and over a 200-fold range for medium and long-chain acylcarnitines. Spike-recovery studies into a pooled QC sample were performed for primary markers of IEMs at three concentration levels, namely 50% of normal concentration, 75% of elevated disease concentration and the midpoint. Concentrations of spikes were adjusted slightly to ensure a significant response change relative to baseline levels detected in pooled dried blood spots from healthy infants. Percent recovery was calculated as [(*measured concentration - endogenous concentration)/added concentration* x 100]. Repeatability of the method was determined by analysis of three technical replicates of pooled dried blood spot extracts using a seven sample segment injection format in MSI-CE-MS (n=21).

[0043] *Statistical Analysis.* All electropherograms were generated using Igor Pro 5.0 software (Wavemetric Inc., Lake Oswego, OR). A dilution trend filter was used to rigorously filter out chemical and biochemical noise while characterizing authentic metabolites derived from dried blood spot extracts from healthy/normal infants (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664). Cationic metabolites were included in the final data matrix if their relative response ratio had acceptable precision (CV < 30%) and good linearity upon serial dilution (R^2 > 0.90) without a blank signal corresponding to a buffer filtrate sample. Complementary multivariate statistical analysis methods were used for classification of CF-specific biomarkers relative to normal/healthy controls comprising 80 cationic metabolites analyzed from two technical replicates of dried blood spot extracts analyzed as four replicate injections (n= 2 x 4 = 8) when

using MSI-CE-MS. Unsupervised data exploration was performed using volcano plots (fold-change > 2.0; Bonferroni-corrected $p < 1$ E-4) and supervised data analysis using partial least squares-discriminant analysis (PLS-DA) with *log*-transformed and autoscaled data using Metaboanalyst 2.0 (Xia et al. *Nucleic Acids Res.* **2012**, 40: W127), whereas receiver operating characteristic (ROC) curves were used for classification of single (untransformed data) or ratiometric (*log*-transformed data) biomarkers using ROCCET (Xia et al. *Metabolomics* **2013**, 9: 280).

Results

[0044] Figure 2 shows the injection sequence configuration used in MSI-CE-MS that involves a series of short injection segments of "seven identical" samples containing a standard mixture of polar metabolites that are each displaced by a spacer comprised of the buffer (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664). In this case, up to a third of the total capillary length is filled with multiple segments of samples and spacers in order to allow for sufficient resolution, desalting and peak capacity in the separation. To maximize sample throughout, short sample injection plugs are used with an optimum spacer length to allow for sufficient displacement of each sample segment that is needed for resolution of isomeric/isobaric ions prior to ionization. If greater sensitivity is desired, fewer sample plugs can be introduced but with a longer segment length (i.e., sample volume) injected to allow for on-line pre-concentration by CE (R. Lee et al. *Anal. Chem.* **2007** 79: 403). High throughput metabolite profiling is achieved by MSI-CE-MS since seven sample plugs (*i.e.*, dried blood spot extracts) are analyzed simultaneously within a single run with no loss in separation performance, such as resolution of three isomers of *L*-leucine (Leu, Ile, allo-Ile), whereas the high mass resolution of the time of flight (TOF)-MS permits resolution of co-migrating ions, such as *L*-phenylalanine (Phe) and *L*-citrulline (Cit).

[0045] **Figure 3** highlights the resolution of a wide variety of polar metabolites by CE-MS derived from methanol-based extracts of dried blood spots, including various amino acids and acylcarnitines that are used as primary, secondary or ratiometric markers of specific in-born errors of metabolism (IEM). CE provides a high efficiency separation platform for resolution of isobaric/isomeric ions in complex and highly saline biological samples prior to electrospray ionization-mass spectrometry as required for improving selectivity and quantitative performance.

[0046] **Figure 4** outlines an accelerated workflow in metabolomics for biomarker discovery that takes advantage of multiplexed separations by MSI-CE-MS, such as a seven-segment format which captures dynamic metabolomic responses to strenuous exercise for individual subjects, as well as their adaptive responses to exercise training (Kuehnbaum et al. *Electrophoresis* **2015**, doi: 10.1002/elps.201400604). For instance, hypoxanthine was identified as a plasma marker of energetic stress associated with untrained subjects that is related to the purine degradation cycle due to irreversible ATP hydrolysis with active muscle contraction. A similar approach for biomarker discovery was performed to identify metabolites as markers associated with cystic fibrosis (CF) in affected subjects (*e.g.* infant and adults) by untargeted metabolite profiling of dried blood spot extracts and pilocarpine-stimulated sweat specimens using MSI-CE-MS in conjunction with multivariate statistical methods.

[0047] **Figure 5** depicts an asymmetric signal pattern recognition approach used in MSI-CE-MS for unambiguous identification of pmarkers associated with in-born errors of metabolism (IEM) relative to a pooled healthy sample as quality control (QC), such as elevation in *L*-phenylalanine that is associated with phenylketonuria (PKU). In this case, three paired samples were analyzed in series, but were diluted in such a way as to generate a distinct signal pattern to facilitate biomarker discovery with high fidelity. For instance, there is no evidence of tyrosinemia since ion signals for *L*-tyrosine are not significantly different between pairs of samples (*i.e.,* disease controls), as well as QC (*i.e.,* healthy reference sample). In contrast, a significant elevation (fold-change > 4 relative to healthy/normal; $p < 1$ E-4) of *L*-phenylalanine above acceptable cut-off limits is indicative of PKU that is associated with the middle pair of samples appearing as a "doublet". In all cases, the same ion is directly compared with other diseases and a pooled normal/healthy sample (QC) within a single run. Thus, MSI-CE-MS serves as a multiplexed separation platform for confirmatory testing of screen-positive samples identified by MS/MS due to its high selectivity, excellent precision and good quantitative performance while using a single non-isotope labeled internal standard. This approach was applied to several other IEM (*e.g.,* citrullinemia, maple syrup urine disease etc.) by measuring known markers in dried blood spot extracts prior to performing metabolomic studies for biomarker discovery in CF.

[0048] **Figure 6** depicts multivariate statistical analysis of 36 authentic metabolites measured in dried blood spot (DBS) extracts by MSI-CE-MS for the discovery of markers differentially expressed in affected CF infants (n=4) relative to healthy/screen-negative controls (n=4) when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for single or ratiometric CF-specific markers. Excellent discrimination of infants confirmed with CF (*i.e.,* sweat chloride > 60 mM) from screen-negative healthy controls is feasible when analyzing a panel of polar metabolites derived from methanol-based dried blood spot extracts with good accuracy (R^2 =0.999) and robustness (Q^2 = 0.675) when using leave-one-out-at-a-time cross-validation. The VIP ranking summarizes the most significant metabolites associated with CF based on their measured normalized responses after *log*-transformation and autoscaling, such as *L*-glutamine and *L*-kynurenine (both lower in CF), as well as *L*-carnitine and a metabolite with a *m/z* of 238.095 and a RMT of 1.108 (both higher in CF). Alternatively, ROC curves can be used to select and rank CF-specific markers based on their ability to classify infants with CF with high sensitivity and specificity (in both cases area under the curve, AUC > 1.00) based on an optimum threshold cut-off value including single and ratiometric markers, such as *L*-glutamine and *L*-glutamine:*L*-carnitine ratio, respectively. Metabolites that have a high AUC value, a high average fold-change and/or a low p-value are considered markers for CF screening.

[0049] **Figure 7** depicts multivariate statistical analysis of 82 authentic metabolites measured in dried blood spot (DBS) extracts by MSI-CE-MS for the discovery of markers differentially expressed in affected CF adults (n=3) relative to healthy controls (n=3) when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for single or ratiometric CF-specific markers. Excellent discrimination of adults with CF from healthy controls was feasible when analyzing a panel of polar metabolites derived from methanol-based dried blood spot extracts with good accuracy (R^2 =0.999) and robustness (Q^2 = 0.675) when using leave-one-out-at-a-time cross-validation. The VIP ranking summarizes the most significant metabolites associated with CF based on their measured normalized responses after *log*-transformation and auto-scaling, such as two metabolites, one with a *m/z* of 252.109 and a RMT of 1.152, and another with a *m/z* 238.095 and

a RMT of 1.108 (both lower in CF), as well as β-alanine and *L*-kynurenine (both higher in CF). Alternatively, ROC curves can be used to select and rank CF-specific markers based on their ability to classify infants with CF with high sensitivity and specificity (in both cases area under the curve, AUC > 1.00) based on an optimum threshold cut-off value, including single and ratiometric markers, such as the metabolite with a *m/z* of 238.095 and a RMT of 1.108 and *L*-citrulline:metabolite (*m/z* of 238.095) ratio, respectively. Metabolites that have a high AUC value, a high average fold-change and/or a low *p*-value are considered markers for CF screening.

[0050] **Figure 8** depicts a strategy for structural elucidation of metabolites differentially expressed in dried blood spot extracts from CF subjects using accurate mass, spiking with authentic standards and tandem MS (MS/MS), such as a metabolite with a *m/z* of 238.095 [MH^+] and a RMT of 1.108. In this case, the accurate mass of the ion corresponds to three putative molecular formulae, whereas the highest ranked molecular formula is associated with 229 possible chemical structures/isomers based on C9H11N5O3. A database search (*e.g.,* Human Metabolome Database) for the ion was biopterin as a putative chemical structure among several other isomers and isobars. However, spiking of biopterin available as a purified chemical standard into the dried blood sample extract resulted in its migration after the ion (*m/z* of 238.095 [MH^+]) since it has a characteristic RMT of 1.108. In addition, a comparison of tandem (MS/MS) spectra confirmed that biotperin did not correspond to the ion given the different fragment ions generated upon collision-induced dissociation and their relative intensities. Thus, identification of metabolite markers of CF may be determined by matching of RMT and MS/MS spectra with authentic chemical standards.

[0051] **Figure 9** demonstrates a sub-group comparison of metabolites associated with the urea cycle/nitric acid pathway differentially expressed in pooled sweat specimens from screen-positive CF infants with low (< 30 mM), intermediate (30-59 mM) and high (> 60 mM) sweat chloride results indicative of *L*-arginine deficiency for affected CF infants. Two specific metabolites in the urea cycle/NO biosynthesis were associated with CF subjects, including *L*-arginine and *L*-citrulline, as well as their ratio (Arg/Cit). In contrast, *L*-ornithine and L-arginninosuccinate do not exhibit a statistical significant difference in pooled sweat specimens from affected infants (*i.e.,* high or intermediate sweat chloride) and unaffected carriers (*i.e.,* low sweat chloride). Arginine deficiency is evident in infants with classic CF as demonstrated in the

asymmetric signal pattern recognition for *L*-arginine (an ion with a 175.119 *m/z* and a RMT of 0.560) using MSI-CE-MS when comparing pooled sweat specimens from screen-positive infants with different categories of sweat chloride results.

[0052] **Figure 10** depicts multivariate statistical analysis of 75 authentic metabolites measured in pilocarpine-stimulated sweat specimens by MSI-CE-MS for the discovery of putative markers differentially expressed in affected infants with classic CF (n=4, heterozygotes with delF508 CFTR mutation with a high sweat chloride result > 60 mM) relative to screen-positive/unaffected carriers (n=10 with a low sweat chloride result < 30 mM) when using (a) a 2D scores plot in partial-least squares discriminate analysis (PLS-DA) with markers annotated by their variable importance in projection (VIP) ranking and (b) receiver operating characteristic (ROC) curves for single or ratiometric CF-specific markers detected in residual sweat specimens. The VIP ranking summarizes the most significant metabolites associated with CF based on their measured normalized responses after *log*-transformation and autoscaling, such as thiocyanate (SCN) and a metabolite with a *m/z* of 267.129 and a RMT of 0.750 (both lower in classic CF with high sweat chloride), as well as 3-hydroxy-*L*-proline (HyP) and pilocarpic acid (PcpA) (both higher in CF with high sweat chloride). Alternatively, ROC curves can be used to select and rank CF-specific markers based on their ability to classify infants with CF with high sensitivity and specificity (in both cases area under the curve, AUC > 1.00) based on an optimum threshold cut-off value, such as ratiometric markers, including PcpA/SCN and HyP/metabolite (with a *m/z* of 282.005). Metabolites that have a high AUC value, a high average fold-change and/or a low p-value are considered markers for CF screening.

Discussion

[0053] Herein is reported for the first time an untargeted metabolomic study of DBS extracts (*i.e.*, whole blood specimens), as well as residual sweat samples from screen-positive infants for the determination of novel metabolite signatures indicative of CF. The major aim of metabolomics is to measure statistically different metabolite responses between two or more sample cohorts (*e.g.*, healthy/disease) based on their measured signal intensities when using nuclear magnetic resonance (NMR) or MS when coupled to various separation techniques (Kuehnbaum and Britz-McKibbin *Chem. Rev.* **2013**, 113: 2437). Complementary multivariate

statistical methods were then used to reduce data dimensionality and noise while ranking a subset of molecular features (*i.e.,* metabolites) that are associated with group classification (Kuehnbaum et al. *Sci. Reports* **2014**, 4: 6166). Nevertheless, a major trade-off of separations is the low sample throughput associated with solute elution and column pre-conditioning when using conventional GC, LC and/or CE-MS methods. Also, traditional data workflows in metabolomics require complicated data pre-processing algorithms to peak pick and align data that are time-consuming and prone to bias. In the present case, multi-segment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) was used as a high-throughput screening platform (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664) in metabolomics for biomarker determination that is applicable to characterization of volume-restricted samples from biorepositories (Nori de Macedo et al. *Anal. Chem.* **2014**, 86: 10010).

[0054] **Figure 2** highlights that seven or more sample segments can be serially injected into a single capillary to improve sample throughput up to one order of magnitude while retaining good separation efficiency, selectivity, desalting and quantitative performance prior to electrospray ionization (ESI)-MS. A major advantage of MSI-CE-MS is the increased sample throughput and data quality that is achieved via serial injection of multiple sample plugs within the same separation, including different biological samples from individual subjects and reference/quality controls (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664). This method was used to identify CF-specific markers in volume-restricted biological samples from affected CF infants and adults, including dried blood spot extracts and pilocarpine-stimulated sweat specimens. Indeed, residual sweat samples stored after sweat chloride measurements for confirmatory diagnosis of CF often contains less than 5 μL volume for subsequent analysis.

[0055] **Figure 3** shows that a wide range of polar/ionic metabolites can be analyzed from dried blood spot extracts when using CE-MS (Chalcraft and Britz-McKibbin *Anal. Chem.* **2009**, 81: 307), including primary or ratiometric markers associated with amino acid, fatty acid and organic acid disorders relevant to newborn screening programs (*e.g.,* amino acids and acylcarnitines). This was first performed in order to rigorously validate the specificity, precision and accuracy of MSI-CE-MS based on known markers (*e.g.,* primary, secondary or ratiometric markers) for different in-born errors of metabolism, such as elevated *L*-phenylalanine (Phe > 120

μM) or *L*-phenylalanine:*L*-tyrosine ratio (Phe/Tyr > 3) in dried blood spot extracts that are indicative of phenylketonuria (PKU).

[0056] **Figure 4** provides an overview of the data workflow in metabolomics for biomarker discovery that relies on unsupervised (*e.g.,* principal component analysis, hierarchical cluster analysis) and supervised multivariate statistical methods (*e.g.,* partial least squares-discriminate analysis, receiver operating characteristic curves) to reduce data dimensionality in metabolomics and classify sub-groups of subjects based on statistically significant molecular features (Kuehnbaum et al. *Electrophoresis* **2015**, doi: 10.1002/elps.201400604) related to CF in affected subjects as defined by their characteristic mass-to-charge ratio and relative migration time (*m/z*:RMT). These putative lead candidates were then identified by comparison with MS/MS spectral databases and co-migration after spiking with authentic standards followed by their accurate quantification.

[0057] An asymmetric signal pattern recognition strategy was applied when using MSI-CE-MS in order to unambiguously identify CF-specific metabolite signatures relative to healthy and disease controls. **Figure 5** depicts three pairs of proficiency DBS samples from the Centres for Disease Control (CDC) that were simultaneously analyzed by MSI-CE-MS. Duplicate extractions were analyzed for six different IEM while including a healthy control as reference. Serial injection of a specific dilution pattern for each pair of samples thus facilitated sample identification while providing greater quality fidelity. For instance, a statistically significant elevation of *L*-phenylalanine (Phe) is indicative of PKU (*i.e.,* second pair of samples injected) in contrast to other primary markers for IEM, such as *L*-tyrosine (Tyr) where ion peak intensities derived from all samples were not significantly different (*i.e.,* no evidence of tyrosinemia). This approach was then validated for four other IEM with known primary or secondary markers from DBS extracts, including medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD), glutaric academia (type 1), maple syrup urine disease, and citrullinemia (data not shown). A similar approach was adopted for analysis of DBS samples from three adult CF patients when using an untargeted metabolomics workflow in MSI-CE-MS (**Figure 4**) in order to identify CF-specific markers. In this case, a total of up to 82 cationic metabolites from DBS extracts and 75 cationic and anionic metabolites in sweat specimens were analyzed by MSI-CE-MS with adequate precision ($CV < 30\%$) that was not detected in sample blanks.

[0058] Univariate statistical methods were used to rank metabolites differentially expressed between CF patients and healthy controls when using a student's t-test based on the average fold-change (FC) in measured ion responses and its significance level (p-value) for each metabolite. **Table 1** summarizes twenty-one metabolites in methanol-derived dried blood spot extracts that were found to be statistically different in infant and/or adult CF patients ($p < 0.05$) relative to healthy controls, including several metabolites as defined by their characteristic *m/z*:RMT. CF-specific markers were derived from metabolic pathways associated with tryptophan/kynurenine pathway (*e.g.*, *L*-tryptophan, *L*-kynurenine), glutaminolysis/ammonia transport (*e.g.*, *L*-glutamine), urea cycle/nitric oxide biosynthesis (*e.g.*, *L*-citrulline, *L*-arginine), oxidative stress (*e.g.*, oxidized glutathione disulfide, *L*-proline), lysine catabolism/carnitine biosynthesis (*L*-lysine, *L*-carnitine), branched chain amino acids (*e.g.*, *L*-valine), mitochondrial β-oxidation (*O*-butyryl-*L*-carnitine) and protein turn-over (*e.g.*, 3-hydroxy-*L*-proline).

Table 1.

TOP 21 Putative CF-Specific Markers in Dried Blood Spot Extracts		***p-value***	***Fold-change***	
Name	***Metabolite (m/z:RMT)***	***Significance***	***CF/Normal***	***Metabolic Pathway***
1. L-Tryptophan (Trp)	205.097:0.910	1.89E-03	2.05	*Trptophan/kynurenine pathway*
2. L-Citrulline (Cit)	176.103:0.924	3.04E-03	1.4	*Urea cycle/NO biosynthesis*
3. *Unknown*	238.095:1.108	4.34E-03	0.0363	--
4. *Unknown*	180.087:0.730	4.75E-03	0.159	--
5. *Unknown*	252.109:1.274	5.58E-03	0.471	--
6. *Unknown*	298.053:0.794	5.90E-03	0.1	--
7. L-Glutamine (Gln)	147.076:0.894	6.29E-03	0.59	*Glutaminolysis/Ammonia transport*
8. *Unknown*	252.109:1.152	8.50E-03	0.0202	--
9. *Unknown*	176.071:0.694	9.10E-03	1.97	--
10. Oxidized glutathione (GSSG)	307.083:1.044	1.18E-02	0.656	*Oxidative stress*
11. Beta-Ala	90.055:0.562	1.23E-02	1.66	*Carnosine catabolism*
12. L-Proline (Pro)	116.071:0.887	1.42E-02	0.774	*Oxidative stress*
13. L-Ornithine (Orn)	133.097:0.538	1.87E-02	2.35	*Urea cycle/NO biosynthesis*
14. *Unknown*	104.071:0.772	2.21E-02	2.01	--
15. O-Butryl-L-Carnitine (C4)	232.154:0.758	3.22E-02	0.402	*Acylcarnitine/Beta-oxidation*
16. L-Arginine (Arg)	175.119:0.560	3.63E-02	0.527	*Urea cycle/NO biosynthesis*
17. *Unknown*	252.109:0.872	3.70E-02	0.644	--
18. L-Valine (Val)	118.0863:0.804	3.94E-02	1.81	*Branched-chain amino acid*
19. L-Carnitine (C0)	162.112:0.679	3.95E-02	1.28	*Acylcarnitine/Beta-oxidation*
20. L-Kynurenine (Kyn)	209.092:0.871	4.04E-02	0.63	*Trptophan/kynurenine pathway*
21. L-Lysine (Lys)	147.113:0.523	4.14E-02	2.89	*Lysine catabolism/carnitine biosynthesis*

** tentative identification based on database match, whereas all other metabolites were identified with authentic chemical standards*

[0059] In addition, complementary multivariate statistical methods were also applied to identify and rank CF-specific metabolites in dried blood spot extracts. **Figure 6a** depicts a 2D scores plot after partial-least squares-discriminant analysis (PLS-DA) and *log*-transformation and

autoscaling of the data matrix consisting from four affected CF infants (n=4) relative to a screen-negative/healthy infants (n=4) in dried blood spot extracts. Affected CF infants confirmed with high sweat chloride (> 60 mM) were readily distinguished from healthy controls on the same plot when using the first two principal components (PCs) representing about 54% of the explained data variance. **Figure 6a** shows that each CF subject can be readily distinguished from each other based on their characteristic metabolic phenotype that likely reflects their underlying disease phenotype heterogeneity.

[0060] Similarly, **Figure 7a** depicts a 2D scores plot after partial-least squares-discriminant analysis (PLS-DA) after *log*-transformation and autoscaling of the data matrix consisting from three adult CF patients (n=3) relative to healthy controls (n=3). In this case, adult CF patients were readily distinguished from healthy controls on the same plot when using the first two principal components (PCs) representing about 73% of the explained data variance. **Figure 7a** shows that each CF subject can be readily distinguished based on their characteristic metabolic phenotype that likely reflects their underlying disease phenotype heterogeneity and differential responses to therapy over a lifetime.

[0061] In addition, initial characterization of the sweat metabolome from affected CF infants was performed by MSI-CE-MS relative to unaffected screen-positive carriers. Indeed, this work represents the first reported study in metabolomics using authentic neonate sweat specimens collected by pilocarpine iontophoresis (Calderon-Santiago et al. *J Chromatogr A* **2014**,1333: 70). A major advantage of this work is the ability to directly analyze volume-restricted biofluids (≈ 3-5 µL) after chloride sweat testing without complicated sample workup. Over 80 cationic and anionic metabolites have been identified in pooled sweat samples when using a dilution trend filter in MSI-CE-MS (Kuehnbaum et al. *Anal. Chem.* **2013**, 85: 10664) to reject biochemical/chemical noise while authenticating metabolites based on three criteria to reduce false discoveries, namely a reproducible signal (CV < 30%) undergoing a linear decrease in response (R^2 >0.900) that lacks a blank signal. Amino acids, biogenic amines, organic acids and various other classes of metabolites were confirmed in pooled sweat samples, including pilocarpine (Pcp) and preservatives (*e.g.*, methylparaben) from the gel disk, as well as a blue dye (*i.e.*, Brilliant Blue FCF) used to visualize sweat collection in tubes. Three sub-sets of sweat samples were initially pooled together from screen-positive CF infants having low (< 30 mM),

intermediate (30-59 mM) and high (> 60 mM) sweat chloride test results. Differentially expressed metabolites between the three sweat sub-groups were identified by MSI-CE-MS for biomarker discovery based on asymmetric signal pattern recognition. One of the most significant findings was the discovery of alterations in sweat metabolites associated with urea cycle/nitric oxide pathway as reflected by *L*-arginine (Arg) depletion and lower Arg/*L*-citrulline (Arg/Cit) ratios in infants with high sweat chloride associated with classic CF who are frequently compound heterozygotes with a single del508 mutation for CFTR as shown in **Figure 9**. Indeed, these results are consistent with findings from dried blood spot extracts.

[0062] Untargeted metabolite profiling of sweat specimens from a group of screen positive CF infants with normal sweat chloride (*i.e.*, unaffected carriers < 30 mM) relative to four CF infants with high sweat chloride (*i.e.*, affected CF infants > 60 mM) in **Figure 10** shows clear group classification as reflected by the 2D scores plots when using partial least squares-discriminate analysis (PLS-DA). As expected, CF-affected infants had elevated levels of thiocyanate (SCN) present in sweat (Gibbs and Hutchings *Proc Soc Exp Biol Med.* **1961**,106: 368), but also higher levels of a number of other compounds as summarized **Table 2**, such as 3-hydroxy-*L*-proline (Hyp), as well as pilocarpic acid (PcpA) – the latter compound is a major metabolite of pilocarpine (Pcp) that was not detected in used gel pads after iontophoresis.

Table 2.

TOP 21 Putative CF-Specific Markers in Pilocarpine-stimulated Sweat		*p-value*	*Fold-change*	
Name	*Metabolite (m/z:RMT)*	*Significance*	*CF/Screen-negative*	*Metabolic Pathway*
1. *Unknown**	280.078:0.967	1.18E-09	0.00331	--
2. *Unknown**	115.040:1.076	8.07E-08	2.68	--
3. L-Citrulline (Cit)	176.103:0.924	4.26E-06	1.86	*Urea cycle/NO biosynthesis*
4. Pilocarpic acid (PcpA)	227.139:0.794	4.54E-06	3.25	*Hydrolysis product of drug stimulant (Pcp)*
5. *Unknown**	160.062:0.917	7.49E-06	2.54	--
6. 3-Hydroxy-L-Proline (Hyp)	132.066:1.024	1.51E-05	2.95	*Oxidative stress/collagen turn-over*
7. L-Arginine (Arg)	175.119:0.560	1.03E-04	0.0629	*Urea cycle/NO biosynthesis*
8. *Unknown**	201.077:0.840	3.37E-05	2.1	--
9. *Unknown**	129.056:1.019	1.03E-04	1.68	--
10. L-Tyrosine (Tyr)	182.081:0.952	1.64E-04	1.79	*Aromatic amino acid biosynthesis*
11. *Unknown**	257.114:0.835	3.41E-04	1.32	--
12. L-Phenylalanine (Phe)	166.086:0.917	3.95E-04	1.8	*Aromatic amino acid biosynthesis*
13. *Unknown*	235.118:0.768	7.16E-04	2.05	--
14. Hypoxanthine (HyX)	137.046:1.087	1.76E-03	0.35	*Purine degradation pathway*
15. *Unknown*	194.138:0.721	1.94E-03	1.52	--
16. *Unknown*	370.053:0.820	3.08E-03	1.56	--
17. Pilocarpine (Pcp)	209.129:0.713	3.08E-04	1.56	*Drug stimulant for sweat*
18. *Unknown*	247.037:0.565	9.36E-04	1.55	--
19. *Unknown*	139.050:0.686	1.23E-02	1.8	--
20. L-Lysine (Lys)	147.113:0.523	1.35E-02	2.21	*Lysine catabolism/carnitine biosynthesis*
21. *Unknown*	280.024:0.687	1.46E-02	1.62	--

** unknown molecular features detected under negative ion mode instead of positive ion mode conditions*

[0063] This preliminary data clearly highlights the wealth of "value-added" biochemical insights derived from secondary use of sweat beyond chloride that is needed for improved assessment of normal or indeterminate sweat chloride test results with ambiguous diagnoses despite having one or two CFTR mutations as in the case of CFTR-related metabolic disorder.

[0064] A panel of metabolites differentially expressed in adult/infant CF patients has been determined when analyzing both dried blood spot extracts and pilocarpine-stimulated sweat specimens. Multi-segment injection-capillary electrophoresis-mass spectrometry in conjunction with multivariate statistical methods was used for the identification of a panel of metabolites expressed in affected CF subjects that were associated with aberrant urea cycle/NO biosynthesis, tryptophan catabolism/kynurenine pathway, lysine catabolism/carnitine biosynthesis, fatty acid β-oxidation/branched chain amino acids and protein turn-over. Relevant portions of references referred to herein are incorporated by reference.

CLAIMS

1. A method of diagnosing cystic fibrosis in a human subject comprising:

i) determining in a biological sample from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-tryptophan, *L*-citrulline, *L*-glutamine, oxidized glutathione disulfide, β-alanine, *L*-proline, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-kynurenine, *L*-lysine, *L*-isoleucine, *L*-asparagine, *L*-aspartic acid, 3-hydroxy-*L*-proline, glycine, trimethylamine *N*-oxide, asymmetric *N*-dimethyl-*L*-arginine, 5-hydroxy-*L*-lysine, adenine, guanine, hypoxanthine, 2-deoxyadenosine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 238.095 [MH^+] and a RMT of 1.108; a metabolite having a *m/z* of 180.087 [MH^+] and a RMT of 0.730; a metabolite having a *m/z* of 252.109 [MH^+] and a RMT of 1.274; a metabolite having a *m/z* of 298.053 [MH^+] and a RMT of 0.794; a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 1.152; a metabolite having a *m/z* of 176.071 [MH^+] and a RMT of 0.694; a metabolite having a *m/z* of 104.071 [MH^+] and a RMT of 0.772; and a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 0.872;

ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and

iii) determining that the subject has cystic fibrosis when the difference in the level of the biomarker in the sample is statistically different from the control level.

2. The method of claim 1, wherein the level of at least two biomarkers is determined.

3. The method of claim 1, comprising the additional step of determining the ratio of the levels of two biomarkers, comparing the ratio to a control ratio, determining the difference between the ratio and the control ratio, and determining that the subject has cystic fibrosis when the difference in the ratio is statistically different from the control ratio.

4. The method of claim 1, wherein a determination that the biomarkers,

L-tryptophan, *L*-citrulline, β-alanine, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-lysine, pilocarpic acid, 3-hydroxy-*L*-proline, and metabolites that exhibit a fold-change of greater than 1 over a normal level of the metabolite, exhibit an increased level in comparison to a control level as indicative of cystic fibrosis.

5. The method of claim 1, wherein a determination that the biomarkers, arginine, C4, 5-deoxyadensosine, aspartic acid, and metabolites that exhibit a fold-change of less than 1 over a normal level of the metabolite, exhibit a reduced level in comparison to a control level as indicative of cystic fibrosis.

6. The method of claim 1, wherein the difference is at least 5% of the control level.

7. The method of claim 1, wherein the determination of the level of one or more biomarkers is a mass spectrometry (MS)-based method selected from direct infusion-mass spectrometry, electrospray ionization (ESI)-MS, desorption electrospray ionization (DESI)-MS, direct analysis in real-time (DART)-MS, atmospheric pressure chemical ionization (APCI)-MS, liquid chromatography- mass spectrometry (LC-MS), gas chromatography- mass spectrometry (GC-MS), or capillary electrophoresis- mass spectrometry (CE-MS).

8. A method of diagnosing cystic fibrosis or a related disorder in a human subject comprising:

i) determining in a sweat sample obtained from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-citrulline, pilocarpic acid, 3-hydroxy-*L*-proline, *L*-arginine, *L*-tyrosine, *L*-phenylalanine, hypoxanthine, pilocarpine, and *L*-lysine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 280.078 [M-H$^-$] and a RMT of 0.967; a metabolite having a *m/z* of 115.040 [M-H$^-$] and a RMT of 1.076; a metabolite having a *m/z* of 160.062 [M-H$^-$] and a RMT of 0.917; a metabolite having a *m/z* of 201.077 [M-H$^-$] and a RMT of 0.849; a metabolite having a *m/z* of 129.056 [M-H$^-$] and a RMT of 1.019; a metabolite having a *m/z* of 257.114 [M-H$^-$] and a RMT of 0.835; a metabolite having a *m/z* of 235.118 [MH$^+$] and a RMT of 0.768; a metabolite having a *m/z* of 194.138 [MH$^+$] and a RMT of 0.721; a metabolite having a *m/z* of 370.053 [MH$^+$] and a RMT of 0.820; a metabolite having a *m/z* of 247.037 [MH$^+$] and a RMT of

0.565; a metabolite having a *m/z* of 139.050 [MH^+] and a RMT of 0.686; and a metabolite having a *m/z* of 280.024 [MH^+] and a RMT of 0.687;

ii) comparing the level of the biomarker to a control level and determining the difference between the biomarker level and the control level; and

iii) determining that the subject has cystic fibrosis or a related disorder when the difference in the level of the biomarker in the sample is statistically different from the control level.

9. A method of monitoring disease progression and/or response to treatment in a human subject with cystic fibrosis comprising:

i) determining in a biological sample from the human subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-tryptophan, *L*-citrulline, *L*-glutamine, oxidized glutathione disulfide, β-alanine, *L*-proline, *L*-ornithine, *O*-butyryl-*L*-carnitine, *L*-arginine, *L*-valine, *L*-carnitine, *L*-kynurenine, *L*-lysine, *L*-isoleucine, *L*-asparagine, *L*-aspartic acid, 3-hydroxy-*L*-proline, glycine, trimethylamine *N*-oxide, asymmetric *N*-dimethyl-*L*-arginine, 5-hydroxy-*L*-lysine, adenine, guanine, hypoxanthine, 2-deoxyadenosine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 238.095 [MH^+] and a RMT of 1.108; a metabolite having a *m/z* of 180.087 [MH^+] and a RMT of 0.730; a metabolite having a *m/z* of 252.109 [MH^+] and a RMT of 1.274; a metabolite having a *m/z* of 298.053 [MH^+] and a RMT of 0.794; a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 1.152; a metabolite having a *m/z* of 176.071 [MH^+] and a RMT of 0.694; a metabolite having a *m/z* of 104.071 [MH^+] and a RMT of 0.772; and a metabolite having *m/z* of 252.109 [MH^+] and a RMT of 0.872 in a first instance;

ii) comparing the level of the biomarker to a control level and determining the first difference between the biomarker level and the control level;

iii) determining the level of the biomarker at a second subsequent instance and determining the second difference between the biomarker level at the first instance and at the second instance;

iv) comparing the first and second differences and determining that the disease is progressing if the second difference is greater than the first difference, or determining that the subject is responding to treatment if the second difference is less than the first difference.

10. A method of monitoring disease progression and/or response to treatment in a human subject with cystic fibrosis comprising:

i) determining in a sweat sample obtained from the subject the level of one or more metabolic biomarkers selected from the group consisting of *L*-citrulline, pilocarpic acid, 3-hydroxy-*L*-proline, *L*-arginine, *L*-tyrosine, *L*-phenylalanine, hypoxanthine, pilocarpine, and *L*-lysine, and metabolites exhibiting a mass-to-charge ratio (*m/z*) and relative migration time (RMT) when using capillary electrophoresis-mass spectrometry (CE-MS) selected from the group of a metabolite having a *m/z* of 280.078 [M-H$^-$] and a RMT of 0.967; a metabolite having a *m/z* of 115.040 [M-H$^-$] and a RMT of 1.076; a metabolite having a *m/z* of 160.062 [M-H$^-$] and a RMT of 0.917; a metabolite having a *m/z* of 201.077 [M-H$^-$] and a RMT of 0.849; a metabolite having a *m/z* of 129.056 [M-H$^-$] and a RMT of 1.019; a metabolite having a *m/z* of 257.114 [M-H$^-$] and a RMT of 0.835; a metabolite having a *m/z* of 235.118 [MH$^+$] and a RMT of 0.768; a metabolite having a *m/z* of 194.138 [MH$^+$] and a RMT of 0.721; a metabolite having a *m/z* of 370.053 [MH$^+$] and a RMT of 0.820; a metabolite having a *m/z* of 247.037 [MH$^+$] and a RMT of 0.565; a metabolite having a *m/z* of 139.050 [MH$^+$] and a RMT of 0.686; and a metabolite having a *m/z* of 280.024 [MH$^+$] and a RMT of 0.687;

ii) comparing the level of the biomarker to a control level and determining the first difference between the biomarker level and the control level;

iii) determining the level of the biomarker at a second subsequent instance and determining the second difference between the biomarker level at the first instance and at the second instance;

iv) comparing the first and second differences and determining that the disease is progressing if the second difference is greater than the first difference, or determining that the subject is responding to treatment if the second difference is less than the first difference.

A. Two-tiered Newborn Screening of CF



1. Immunoreactive trypsin (IRT) test

Poor specificity/positive predictive value (PPV) = 3-10%

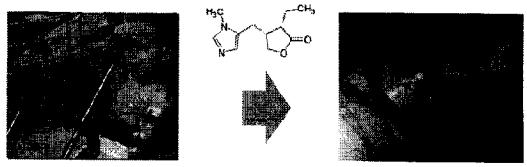
2. DNA test: panel of mutations

Large number of carriers/unknown or variable clinical history

Table 1. *Categories of positive CF screens*[1]

Category	IRT / DNA test	PPV (%)
A	IRT > 96th centile / 2 mutations	100
B	IRT > 96th centile / 1 mutation	1.6
C	IRT > 99.9th centile / no mutations	0.1

B. Confirmatory Chloride Sweat Test



3. Sweat stimulation by pilocarpine iontophoresis

Ambiguous sweat results for intermediate chloride

Sweat collection in microbore tube

Chloridometer analysis

Table 2. *Sweat chloride results*[2]

Cl^- (mM)	Result for CF
≤ 29	Negative
30 – 59	Inconclusive
≥ 60	Positive

5-10% of screen-positives:
- Asymptomatic carrier
- CF-related disease
- CF pancreatic sufficient

85-90% screen-positives have normal sweat chloride (< 29 mM)

Figure 1.

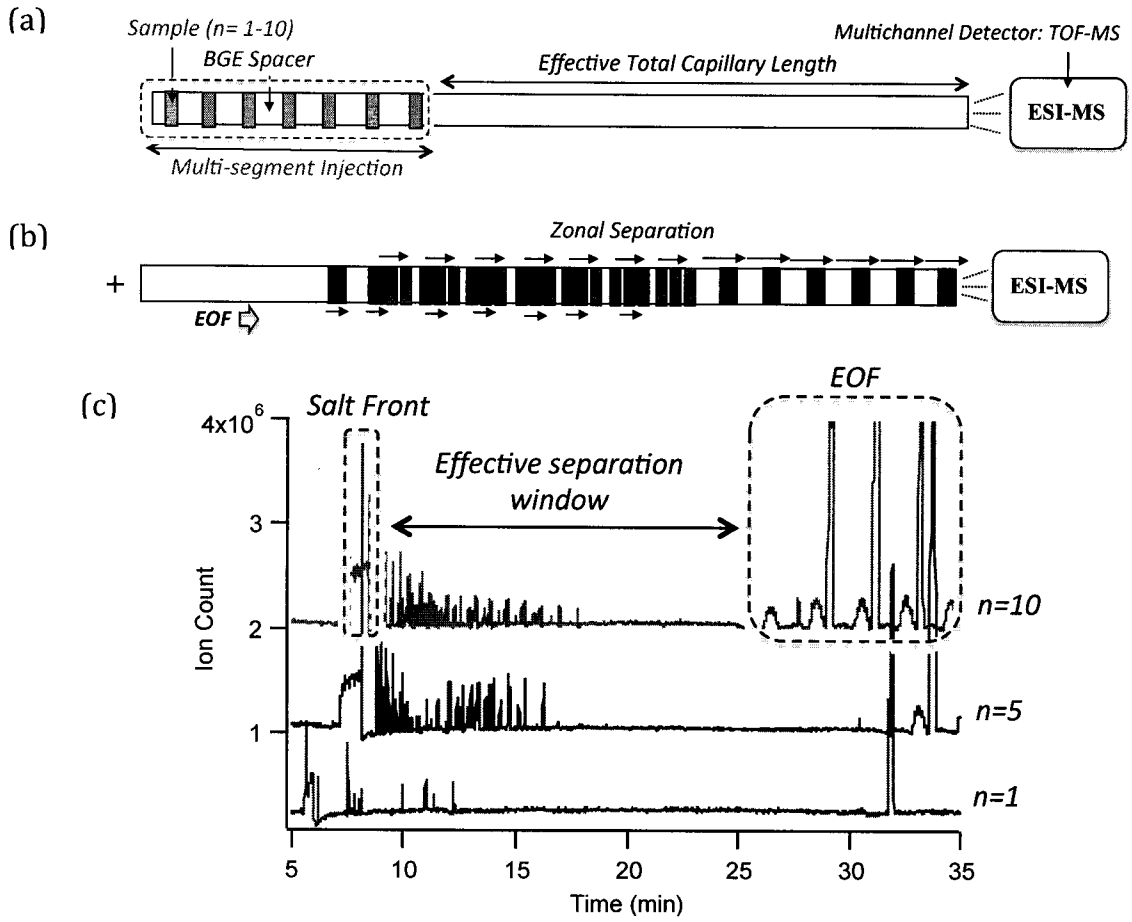

(a)
Sample (n= 1-10)
BGE Spacer
Multichannel Detector: TOF-MS
Effective Total Capillary Length
ESI-MS
Multi-segment Injection
(b)
Zonal Separation
+
EOF
ESI-MS
(c)
EOF
Salt Front
4x10^6
Effective separation window
3
2
1
Ion Count
n=10
n=5
n=1
5
10
15
20
25
30
35
Time (min)

Figure 2.

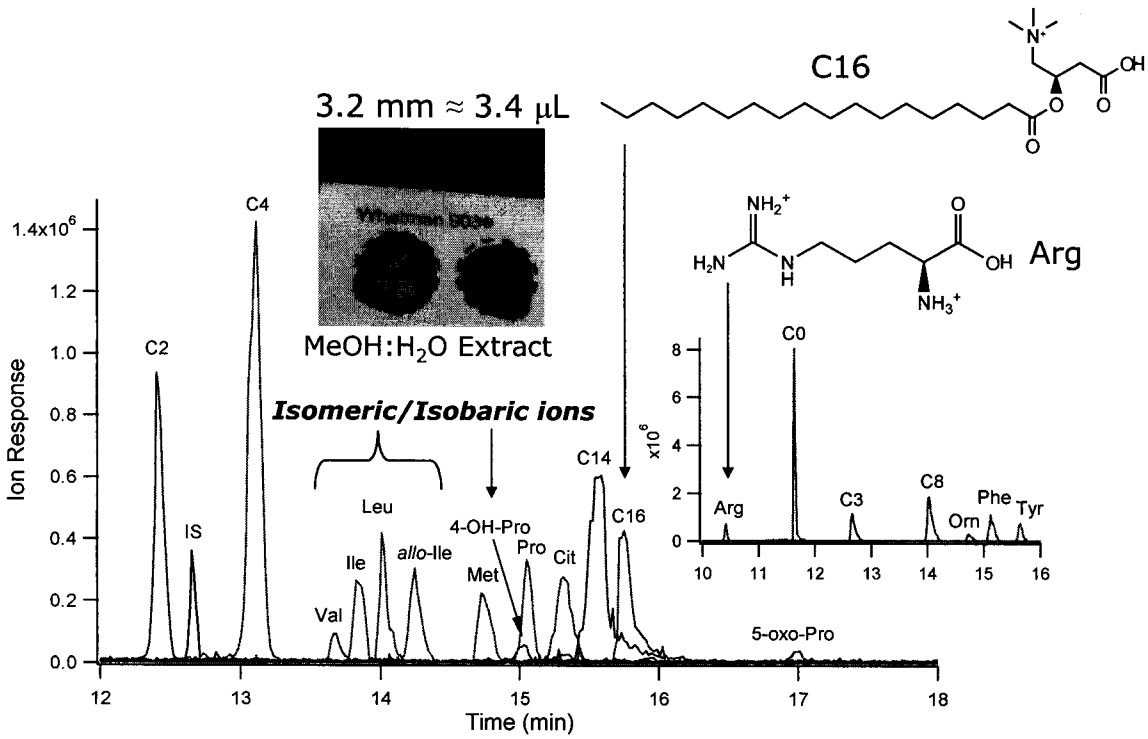

C16
3.2 mm ≈ 3.4 µL
MeOH:H2O Extract
Isomeric/Isobaric ions
Arg
Ion Response
Time (min)
C2
C4
IS
Val
Leu
Ile
allo-Ile
4-OH-Pro
Met
Pro
Cit
C14
C16
5-oxo-Pro
C0
C3
C8
Orn
Phe
Tyr

Figure 3.

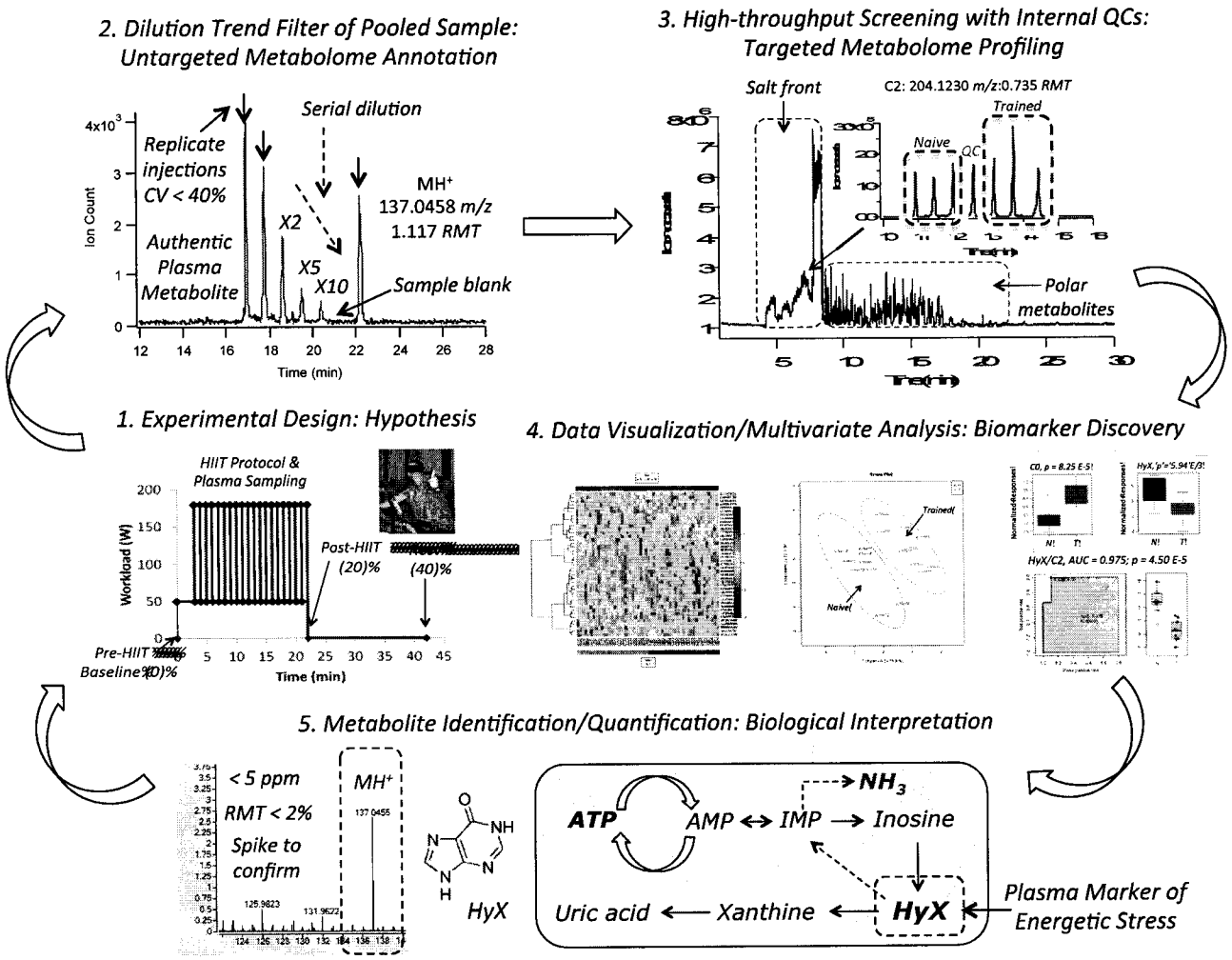

2. Dilution Trend Filter of Pooled Sample:
Untargeted Metabolome Annotation
Serial dilution
Replicate injections CV < 40%
Authentic Plasma Metabolite
X2
X5
X10
Sample blank
MH+ 137.0458 m/z 1.117 RMT
Ion Count
Time (min)
3. High-throughput Screening with Internal QCs:
Targeted Metabolome Profiling
Salt front
C2: 204.1230 m/z:0.735 RMT
Trained
Naive
QC
Polar metabolites
1. Experimental Design: Hypothesis
HIIT Protocol & Plasma Sampling
Workload (W)
Post-HIIT (20)%
(40)%
Pre-HIIT Baseline%(0)%
Time (min)
4. Data Visualization/Multivariate Analysis: Biomarker Discovery
HyX/C2, AUC = 0.975; p = 4.50 E-5
5. Metabolite Identification/Quantification: Biological Interpretation
< 5 ppm
RMT < 2%
Spike to confirm
MH+
HyX
ATP
AMP ↔ IMP → Inosine
NH3
Uric acid ← Xanthine ← HyX
Plasma Marker of Energetic Stress

Figure 4.

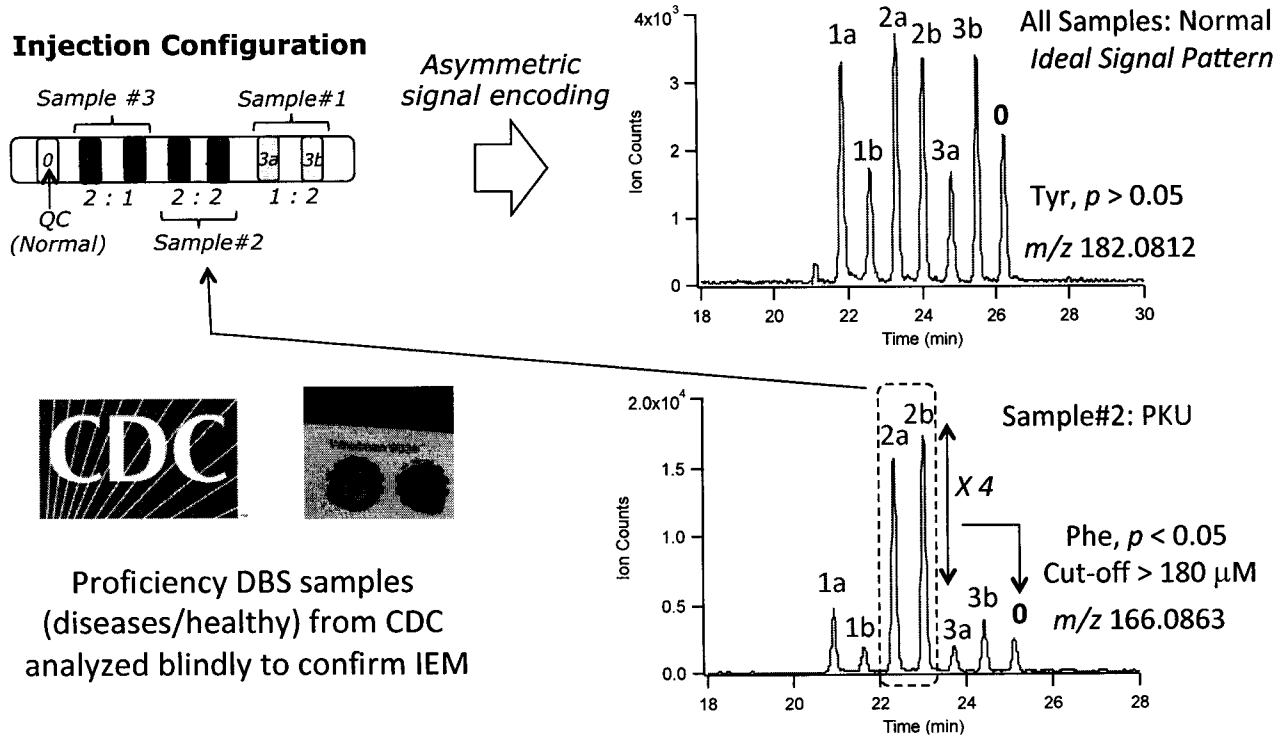
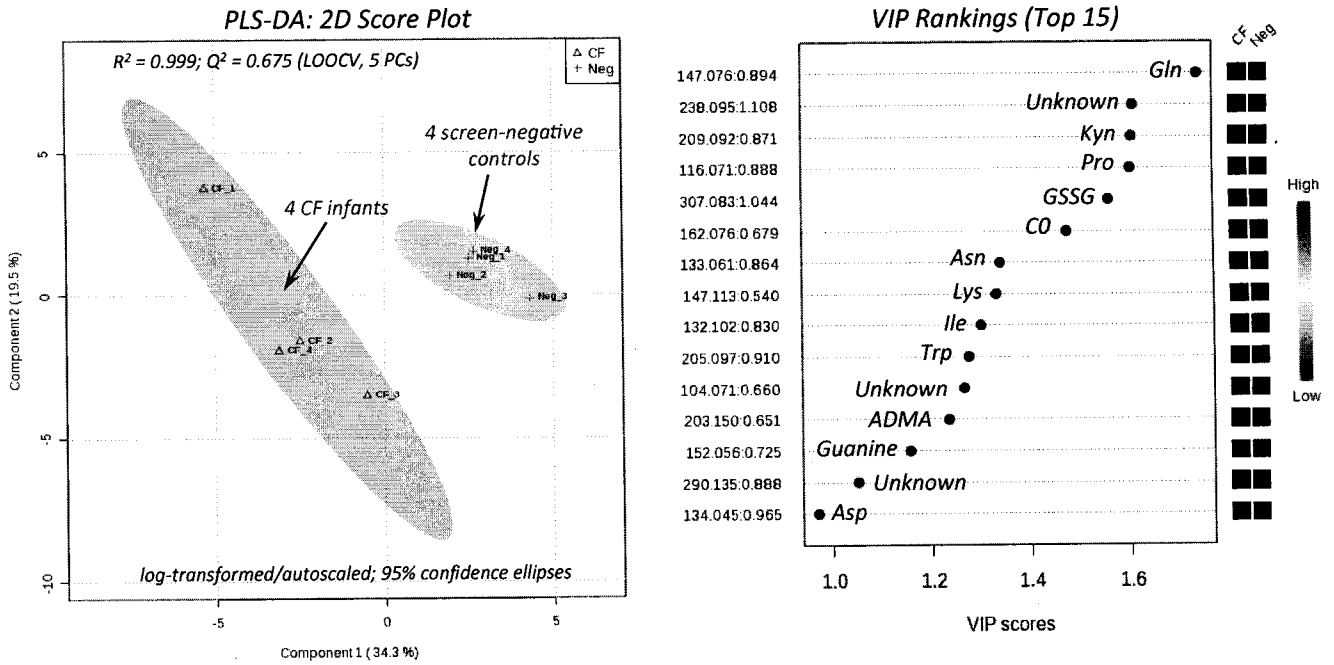

Injection Configuration
Sample #3
Sample#1
0
3a
3b
2 : 1
2 : 2
1 : 2
QC
(Normal)
Sample#2
Asymmetric
signal encoding
4x10^3
Ion Counts
1a
2a
2b
3b
1b
3a
0
All Samples: Normal
Ideal Signal Pattern
Tyr, p > 0.05
m/z 182.0812
Time (min)
CDC
Proficiency DBS samples
(diseases/healthy) from CDC
analyzed blindly to confirm IEM
2.0x10^4
Sample#2: PKU
X 4
Phe, p < 0.05
Cut-off > 180 μM
m/z 166.0863

Figure 5.

(a)


PLS-DA: 2D Score Plot
R² = 0.999; Q² = 0.675 (LOOCV, 5 PCs)
Δ CF
+ Neg
4 screen-negative controls
4 CF infants
log-transformed/autoscaled; 95% confidence ellipses
Component 1 (34.3 %)
Component 2 (19.5 %)
VIP Rankings (Top 15)
147.076:0.894 Gln
238.095:1.108 Unknown
209.092:0.871 Kyn
116.071:0.888 Pro
307.083:1.044 GSSG
162.076:0.679 C0
133.061:0.864 Asn
147.113:0.540 Lys
132.102:0.830 Ile
205.097:0.910 Trp
104.071:0.660 Unknown
203.150:0.651 ADMA
152.056:0.725 Guanine
290.135:0.888 Unknown
134.045:0.965 Asp
CF
Neg
High
Low
VIP scores

(b)

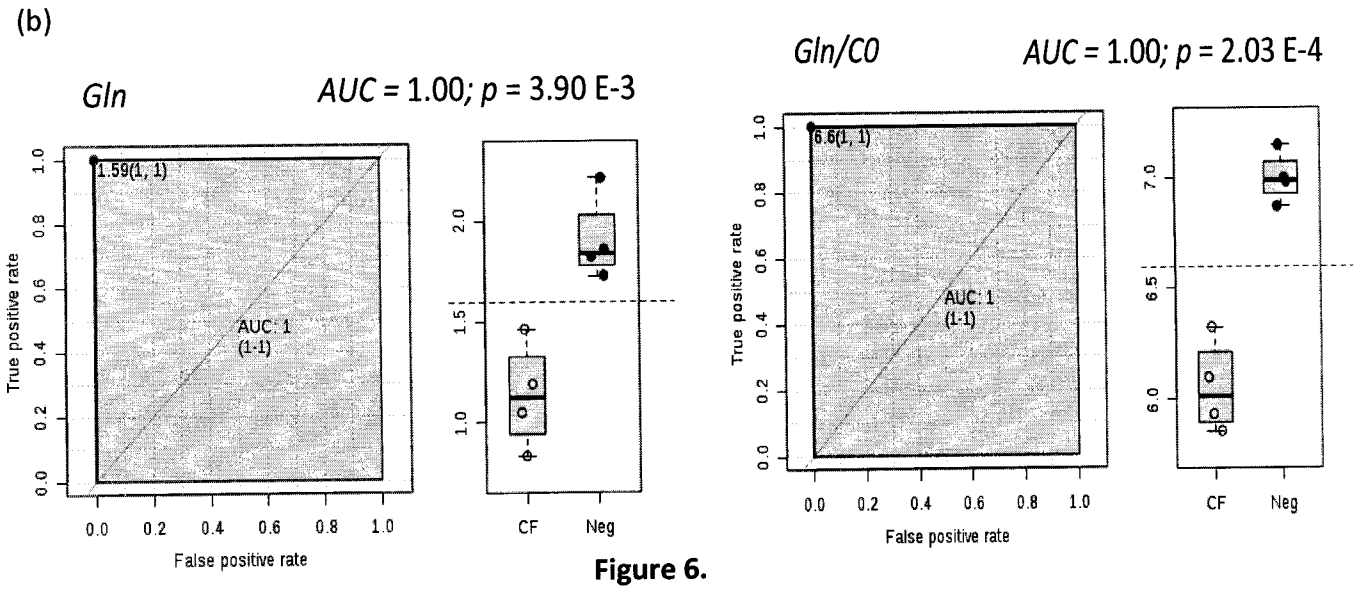
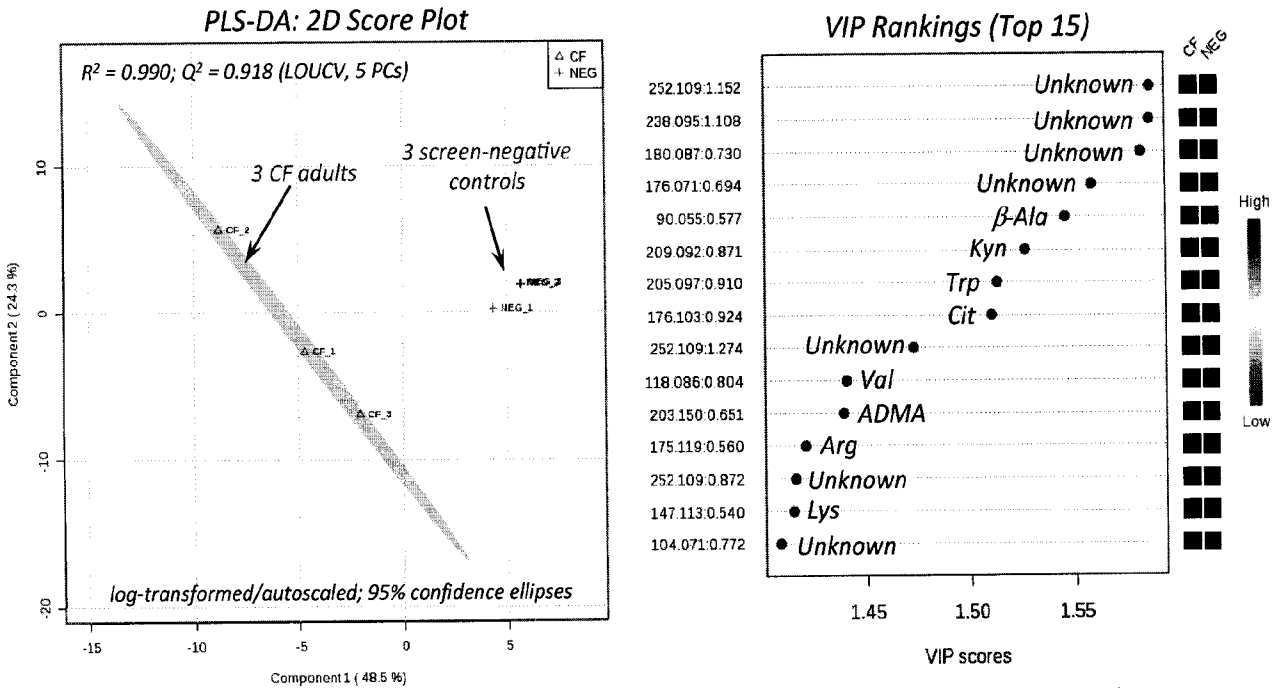

Gln
AUC = 1.00; p = 3.90 E-3
1.59(1, 1)
AUC: 1
(1-1)
True positive rate
False positive rate
CF
Neg
Gln/C0
AUC = 1.00; p = 2.03 E-4
6.6(1, 1)
AUC: 1
(1-1)
True positive rate
False positive rate
CF
Neg

Figure 6.

(a)


PLS-DA: 2D Score Plot
R² = 0.990; Q² = 0.918 (LOUCV, 5 PCs)
△ CF
+ NEG
3 CF adults
3 screen-negative controls
log-transformed/autoscaled; 95% confidence ellipses
Component 1 (48.5 %)
Component 2 (24.3 %)
VIP Rankings (Top 15)
252.109:1.152 Unknown
238.095:1.108 Unknown
180.087:0.730 Unknown
176.071:0.694 Unknown
90.055:0.577 β-Ala
209.092:0.871 Kyn
205.097:0.910 Trp
176.103:0.924 Cit
252.109:1.274 Unknown
118.086:0.804 Val
203.150:0.651 ADMA
175.119:0.560 Arg
252.109:0.872 Unknown
147.113:0.540 Lys
104.071:0.772 Unknown
CF
NEG
High
Low
VIP scores

(b)

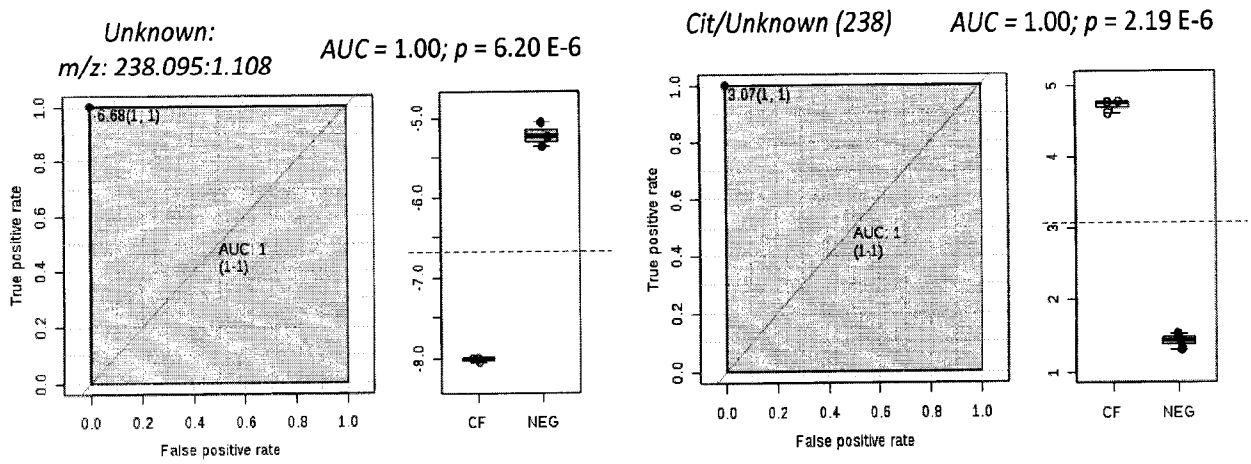

Unknown:
m/z: 238.095:1.108
AUC = 1.00; p = 6.20 E-6
AUC: 1
(1-1)
True positive rate
False positive rate
CF
NEG
Cit/Unknown (238)
AUC = 1.00; p = 2.19 E-6
AUC: 1
(1-1)
True positive rate
False positive rate
CF
NEG

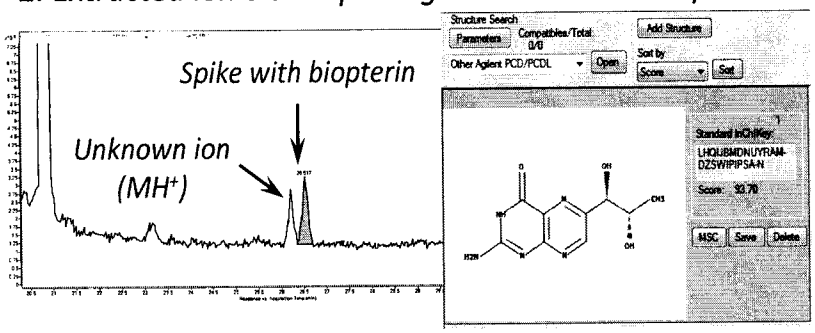
Figure 7.

1. Accurate Mass for Unknown Ion: 3 Empirical Formula

M = 237.0857; 3 formula candidates from MFG

	ID	Formula	Isomers	Taut. Grps	dM(ppm)	|dM(ppm)|	Product	Precur.	Overall
1	1	C9H11N5O3	229	145	2.0	2.0	100	99	99
2	2	C8H15NO7	87	42	-3.7	3.7	100	97	97
3	3	C9H19NO2S2	16	13	0.0	0.0	36	100	88

2. Extracted ion electropherogram: DBS extract spike



3. MS/MS Spectra: Product Ion Scan (Q-TOF)

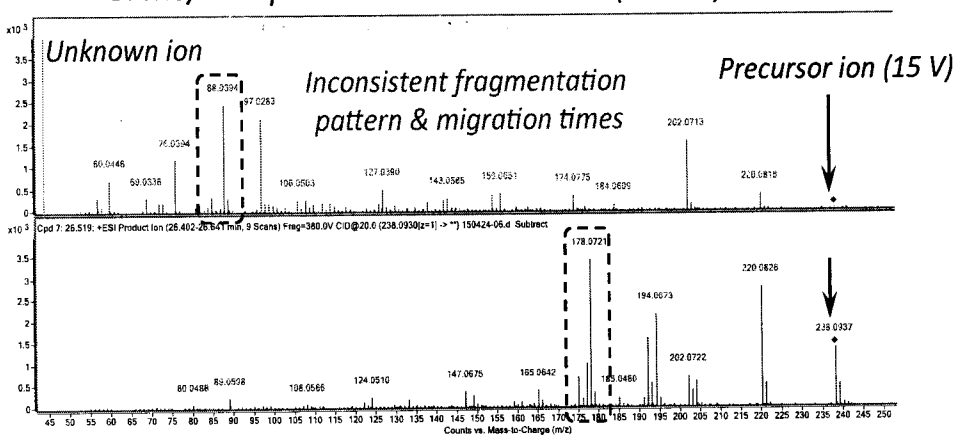


Figure 8.

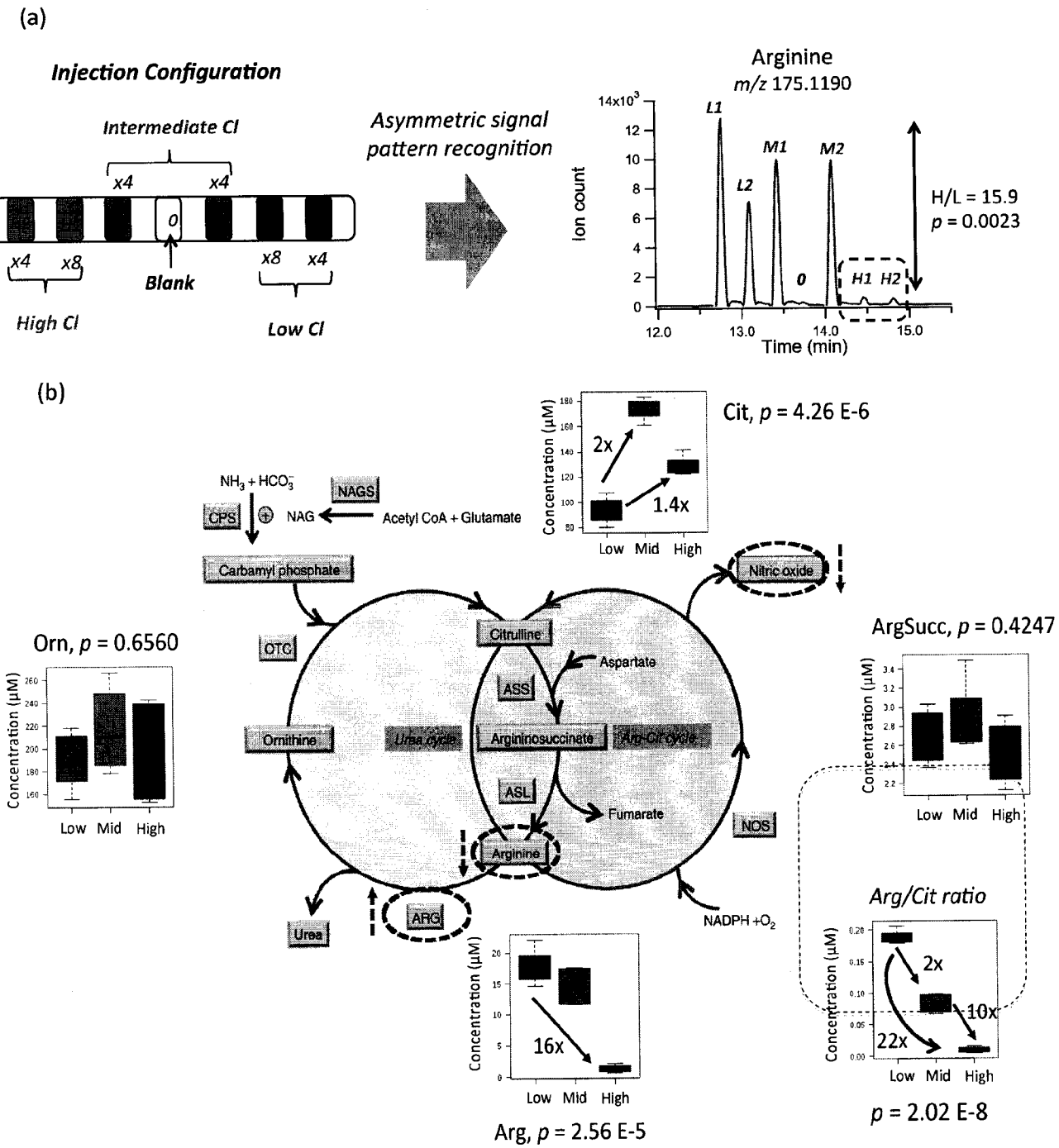

(a)
Injection Configuration
Intermediate CI
x4
x4
0
x4
x8
Blank
x8
x4
High CI
Low CI
Asymmetric signal pattern recognition
Arginine
m/z 175.1190
14x10^3
Ion count
L1
L2
M1
M2
0
H1 H2
H/L = 15.9
p = 0.0023
12.0
13.0
14.0
15.0
Time (min)
(b)
Cit, p = 4.26 E-6
Concentration (µM)
2x
1.4x
Low Mid High
NH3 + HCO3-
NAGS
CPS
NAG
Acetyl CoA + Glutamate
Carbamyl phosphate
Nitric oxide
Citrulline
Aspartate
OTC
ASS
Ornithine
Urea cycle
Argininosuccinate
Arg-Cit cycle
ASL
Fumarate
NOS
Arginine
ARG
Urea
NADPH +O2
Orn, p = 0.6560
Concentration (µM)
Low Mid High
ArgSucc, p = 0.4247
Concentration (µM)
Low Mid High
Arg/Cit ratio
Concentration (µM)
2x
10x
22x
Low Mid High
p = 2.02 E-8
Concentration (µM)
16x
Low Mid High
Arg, p = 2.56 E-5

Figure 9

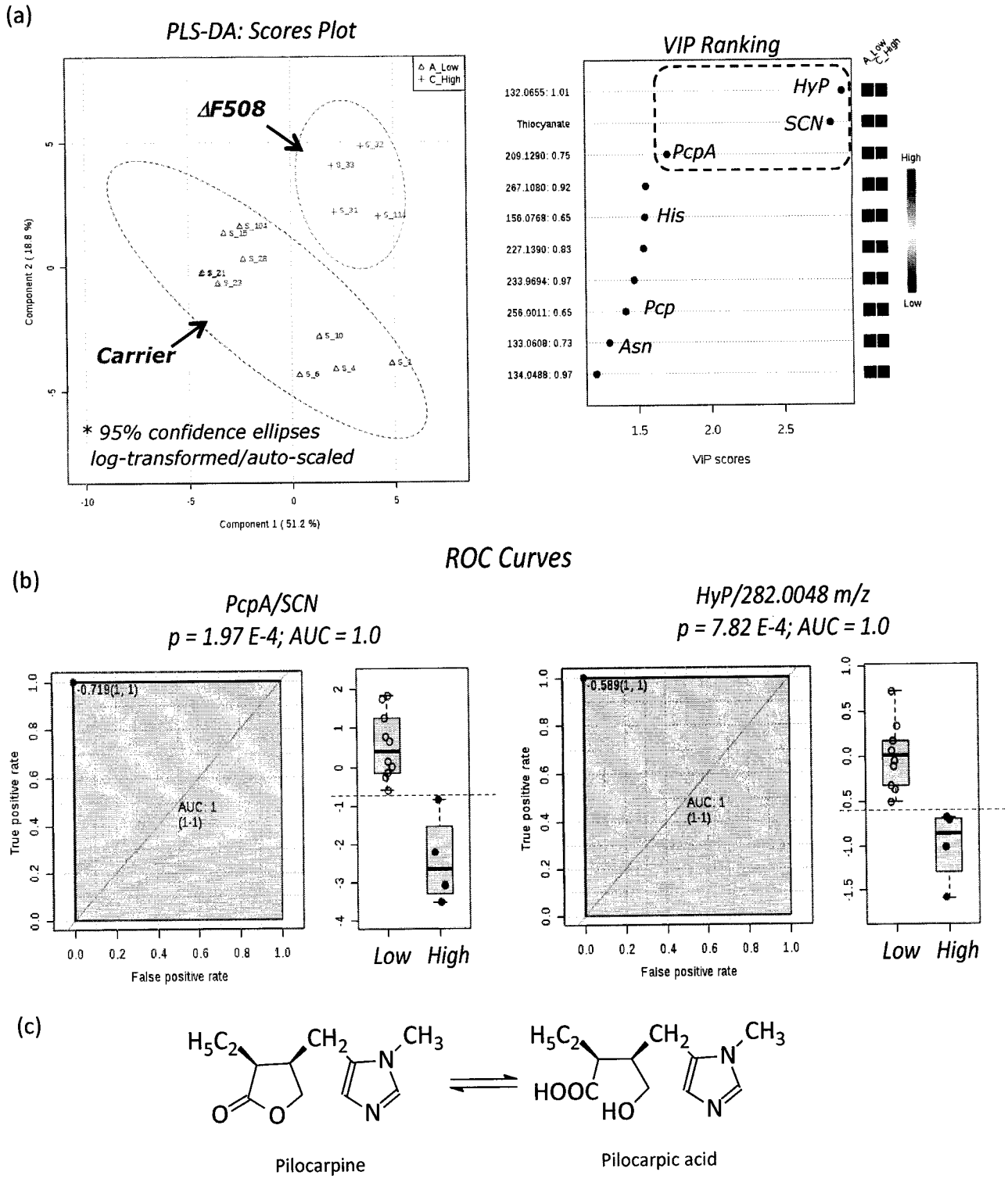

(a)
PLS-DA: Scores Plot
ΔF508
Carrier
* 95% confidence ellipses
log-transformed/auto-scaled
Component 1 (51.2 %)
Component 2 (18.8 %)
VIP Ranking
HyP
SCN
PcpA
His
Pcp
Asn
VIP scores
High
Low
ROC Curves
(b)
PcpA/SCN
p = 1.97 E-4; AUC = 1.0
HyP/282.0048 m/z
p = 7.82 E-4; AUC = 1.0
True positive rate
False positive rate
AUC: 1
(1-1)
Low
High
(c)
Pilocarpine
Pilocarpic acid

Figure 10.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2015/000344

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***G01N 33/48*** (2006.01) , ***G01N 33/53*** (2006.01) , *G01N 27/447* (2006.01) , *G01N 27/00* (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***G01N 33/48*** (2006.01) , ***G01N 33/53*** (2006.01) , *G01N 27/447* (2006.01) , *G01N 27/00* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, Questel Orbit, Google Scholar
Keywords: cystic fibrosis, citrulline, mass spectrometry,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRASEMANN et al "Decreased systemic bioavailability of L-arginine in patients with cystic fibrosis". *Respiratory Research* , 7(87), pages 1-7, 9 June 2006 (0-06-2006) *abstract, Table 1*	1-10
X	GRASEMANN et al "A randomized controlled trial of inhaled L-arginine in patients with cystic fibrosis". *Journal of Cystic Fibrosis*, 12(5), pages 468-474, 14 January 2013(14-01-2013) *see page 469*	1-10
X	MORRIS et al CA2587647 8 June 2006 (08-06-2006) *claims 1, 10, 23, and 25 and page 14*	1-10
X	CLARKE et al. "Components of sweat: Cystic fibrosis of the pancreas compared with controls". *JAMA*, 101(4), pages 490-500, 1961 *see the whole document*	1-10

☐ Further documents are listed in the continuation of Box C.

☑ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 10 August 2015 (10-08-2015)	Date of mailing of the international search report 28 August 2015 (28-08-2015)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Cecilia Alperin (819) 994-3009

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2015/000344

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.: 1-10 as they relate to citrulline

Remark on Protest

☐ The additional search fees were accompanied by the applicant=s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Group A - Claims 1-10 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-citrulline;
Group B - Claims 1-10 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is 3 hydroxy-L proline;
Group C - Claims 1-10 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-argigine;
Group D - Claims 1-10 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is hypoxanthine;
Group E - Claims 1-10 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-lysine.
Group F - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-tryptophan;
Group G - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-glutamine;
Group H - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-oxidized glutathione disulphide;
Group I - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is β-alanine;
Group J - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-proline;
Group K - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-ornithine;
Group L - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is O-butyryl-L-carnitine;
Group M - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-valine;
Group N - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-carnitine;
Group O - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-kynurenine;
Group P - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-isoleucine;
Group Q - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-asparagine;
Group R - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-aspartic acid;
Group S - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-glycine;
Group T - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-trimethylamine N-oxide;
Group U - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-asymmetric N-dimethyl-L-arginine;

Group W - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is adenine;
Group X - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is guanine;
Group Y - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is 2-deoxyadenosine;
Group Z - Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 238.095 [MH+] and a RMT of 1.108;
Group A1- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 180.085 [MH+] and a RMT of 0.730;
Group A2- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 252.109 [MH+] and a RMT of 1.274;
Group A3- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 298.053 [MH+] and a RMT of 0.794;
Group A4- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 252.109 [MH+] and a RMT of 1.152;
Group A5- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 176.071 [MH+] and a RMT of 0.694;
Group A6 Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 104.071 [MH+] and a RMT of 0.772;
Group A7- Claims 1-7 and 9 are directed to a method of diagnosing cystic fibrosis in a human subject comprising determining the level of one or more metabolic biomarkers in a sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 252.109 [MH+] and a RMT of 0.772;
Group A8 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is pilocarpic acid;
Group A9 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-tyrosine;
Group A10 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is L-phenylalanine;
Group A11 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is pilocarpine;
Group A12 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 280.078 [MH+] and a RMT of 0.967;
Group A13 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 115.040 [MH+] and a RMT of 1.076;

Group A14 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 160.062 [MH+] and a RMT of 0.917;

Group A15 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 201.077 [MH+] and a RMT of 0.849;

Group A16 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 129.056 [MH+] and a RMT of 1.019;

Group A17 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 257.114 [MH+] and a RMT of 0.835;

Group A18 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 235.118 [MH+] and a RMT of 0.768;

Group A19 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 194.138 [MH+] and a RMT of 0.721;

Group A20 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 370.053 [MH+] and a RMT of 0.820;

Group A21 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 247.037 [MH+] and a RMT of 0.565;

Group A22 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 139.050 [MH+] and a RMT of 0.686; and

Group A23 - Claims 8 and 10 are directed to a method of diagnosing cystic fibrosis or a related disorder in a human subject comprising determining the level of one or more metabolic biomarkers in a sweat sample, comparing the level of the biomarker to a control level and determining the difference between the two levels wherein the biomarker is a metabolite exhibiting a m/z of 280.024 [MH+] and a RMT of 0.687.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2015/000344

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
CA2587647A1	08 June 2006 (08-06-2006)	CA2587647A1	08 June 2006 (08-06-2006)
		AT517347T	15 August 2011 (15-08-2011)
		EP1825261A2	29 August 2007 (29-08-2007)
		EP1825261A4	07 January 2009 (07-01-2009)
		EP1825261B1	20 July 2011 (20-07-2011)
		US2006160236A1	20 July 2006 (20-07-2006)
		US7648840B2	19 January 2010 (19-01-2010)
		US2010196939A1	05 August 2010 (05-08-2010)
		US8309320B2	13 November 2012 (13-11-2012)
		WO2006060793A2	08 June 2006 (08-06-2006)
		WO2006060793A3	28 December 2006 (28-12-2006)