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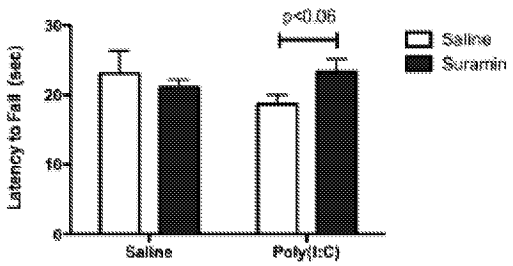

FIG. 18

(57) Abstract: The disclosure relates to biomarkers useful for diagnosing and predicting the development of chronic fatigue syndrome (CFS). The disclosure further provides methods to reset metabolism and facilitate healing in CFS patients by administering antipurinergic compounds.

DIAGNOSTIC AND METHODS OF TREATMENT FOR CHRONIC FATIGUE SYNDROME AND AUTISM SPECTRUM DISORDERS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119 from U.S. Provisional Application Serial No. 62/362,564, filed July 14, 2016, and U.S. Provisional Application Serial No. 62/464,369, filed February 27, 2017, the disclosures of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] The disclosure relates to biomarkers useful for diagnosing and predicting the development of chronic fatigue syndrome (CFS). The disclosure further provides methods to reset metabolism and facilitate healing in CFS patients by administering antipurinergic compounds as well as the treatment of mitochondrial diseases and disorders with antipurinergic compounds.

BACKGROUND

[0003] Recent studies have demonstrated that chronic fatigue is due to mitochondrial dysfunction. Mitochondrial dysfunction and defects in oxidative metabolism are found in many chronic illnesses including bipolar disorder, multiple sclerosis, Parkinson's disease, schizophrenia, depression, autism, and chronic fatigue syndrome. Elevated levels of reactive oxygen and nitrogen species together with elevated pro-inflammatory cytokines and reduced levels of glutathione impair oxidative metabolism result in mitochondrial dysfunction. Chronic fatigue syndrome is a complex and is characterized by profound fatigue and disability, episodes of cognitive dysfunction, sleep disturbance, autonomic abnormalities, chronic or intermittent pain syndromes, microbiome abnormalities, cerebral cytokine dysregulation, natural killer cell dysfunction, and other symptoms that are made worse by exertion of any kind.

[0004] Autism spectrum disorders (ASD) affect 1-2% of children in the US. Autism spectrum disorder (ASD) is the name for a group of developmental disorders. ASD includes a wide range, "a spectrum," of symptoms, skills, and levels of disability. People with ASD often have these characteristics: ongoing social problems that include difficulty communicating and interacting with others;

repetitive behaviors as well as limited interests or activities; symptoms that typically are recognized in the first two years of life; and symptoms that hurt the individual's ability to function socially, at school or work, or other areas of life. Some people are mildly impaired by their symptoms, while others are severely disabled. Early treatment for ASD and proper care can reduce individuals' difficulties while helping them learn new skills and make the most of their strengths. The very wide range of issues facing those "on the spectrum" means that there is no single best treatment for ASD.

[0005] In addition, complex diseases like chronic fatigue syndrome (CFS) are often difficult and expensive to diagnose. While individual tests may be affordable and possibly covered by medical insurance, many patients undergo a diagnostic odyssey that results in substantial personal expenditures that can exceed $100,000 over years of searching, absence from the workplace, and significant reductions in quality of life. The societal cost of chronic fatigue syndrome is estimated to be up to $24 billion annually.

SUMMARY

[0006] Chronic fatigue syndrome is a multi-system disease that can cause long-term pain and disability. It is difficult to diagnose because of its protean symptoms and the lack of a diagnostic laboratory test. The disclosure demonstrates that targeted, broad-spectrum metabolomics of plasma not only revealed a characteristic chemical signature, but also revealed an unexpected underlying biology. Metabolomics showed that chronic fatigue syndrome is a highly concerted hypometabolic response to environmental stress that traces to mitochondria and was similar to the classically studied developmental state of dauer state of the nematode.

[0007] The disclosure provides methods to reset metabolism and facilitate healing in subject with mitochondrial disorders including autism spectrum disorders and chronic fatigue syndrome by administering anti-purinergic compounds such as suramin. Although not wanting to be bound by a particular mechanism of action, it is believed that the antipurinergic compounds disclosed herein turn off the cell danger response (CDR) which is controlled by

purinergic signaling such as the biological mechanism of chronic fatigue syndrome.

[0008] In addition to treating mitochondrial disorders such as chronic fatigue syndrome, the disclosure also provides methods of diagnosis. For example, the disclosure provides a method to determine a subject's risk of having or developing chronic fatigue syndrome (CFS) comprising: detecting an amount of each of a plurality of metabolites in a biological sample obtained from the subject, the plurality of metabolites comprising at least seven metabolites, each of the at least seven metabolites being in a metabolic pathway selected from the group of pathways consisting of: a sphingolipid metabolic pathway, a phospholipid metabolic pathway, a glycosphingolipid metabolic pathway, a purine metabolic pathway, a microbiome metabolic pathway, a cholesterol metabolic pathway, a vitamin B2 metabolic pathway, a pyrroline-5-carboxylic acid metabolic pathway, an arginine metabolic pathway, a proline metabolic pathway, and a branch chain amino acid pathway; determining the presence or absence of an alteration in the metabolic pathways of the subject based upon comparing the amounts of the detected metabolites of the subject versus the amounts of the metabolites detected from a control population that does not have CFS; and indicating that the subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the subject are altered in comparison to the control population. In a further embodiment, the subject is a male subject, and the method further comprises: detecting an amount of each of a plurality of metabolites in a biological sample obtained from the male subject, the plurality of metabolites comprising at least three metabolites, each of the at least 3 metabolites being in a metabolic pathway selected from the group of pathways consisting of: a serine/1-carbon metabolic pathway, a S-adenosyl methionine pathway, a *S*-adenosylhomocysteine metabolic pathway, a methionine metabolic pathway, a very long chain fatty acid oxidation metabolic pathway, a propiogenic amino acid metabolic pathway, and a threonine metabolic pathway; determining the presence or absence of an alteration in the metabolic pathways of the male subject based upon comparing the amounts of the detected

metabolites from the male subject versus the amounts of the metabolites detected from a control population of male subjects that do not have CFS; and indicating that the male subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the male subject are altered in comparison to the control population of male subjects. In yet a further embodiment, the metabolites of the male subject that are detected are phosphatidyl choline PC(16:0/16:0), glucosylceramide GC(18:1/16:0), 1-pyrroline-5-carboxylate (P5C), flavin adenine dinucleotide (FAD), pyroglutamic acid, 2-hydroxyisoccaproic acid, L-serine, and lathosterol. In alternate embodiment, the subject is a female subject, and the method further comprises: detecting an amount of each of a plurality of metabolites in a biological sample obtained from the female subject, the plurality of metabolites comprising at least three metabolites, each of the at least 3 metabolites being in a metabolic pathway selected from the group of pathways consisting of: a fatty acid oxidation metabolic pathway, a vitamin C/collagen metabolic pathway, a bile acid metabolic pathway; an endocannabinoid metabolic pathway, a vitamin B12 metabolic pathway; and an amino sugar metabolic pathway; determining the presence or absence of an alteration in the metabolic pathways of the female subject based upon comparing the amounts of the detected metabolites from the female subject versus the amounts of the metabolites detected from a control population of female subjects that do not have CFS; and indicating that the female subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the female subject are altered in comparison to the control population of female subjects. In a further embodiment, the metabolites that are detected are trihexosylceramide THC(18:1/24:0), phosphatidyl choline PC(16:0/16:0), hydroxyproline, ceramide(d18:1/22:2),lathosterol, adenosine, PI(16:0/16:0), flavin adenine dinucleotide (FAD), 2-octenoylcarnitine, phosphatidyl choline plasmalogen PC(22:6/P18:0), phosphatidyl choline PC(18:1/22:6), 1-pyrroline-5-carboxylate, and chenodeoxycholic acid. In another embodiment, the metabolites that are detected are selected from group consisting of PC(16:0/16:0), ceramide(d18:1/24:2), GC(18:1/16:0), ceramide (d18:1/16:0),

THC(d18:1/24:0), PI(38:4), DHC(18:1/16:0), PA(16:0/16:0), 1-pyrroline-5-carboxylic acid, SM(d18:1/24:2), ceramide(d18:1/16:1OH), SM(d18:1/22:0), SM(d18:1/23:0), ethanolamine, 4-hydroxyphenyllactic acid, FAD, ceramide(d18:1/16:1), ceramide(d18:1/18:0), ceramide(d18:1/26:2), ceramide(d18:1/22:2), L-serine, methionine sulfoxide, ceramide(d18:1/22:1), ceramide(d18:1/25:0), SM(d18:1/18:2OH), ceramide(d18:1/24:1), arginine, SM(d18:1/16:0), behenic acid, hydroxyisocaproic acid, uric acid, lathosterol, PC(16:0/20:4), PC(18:1/22:6), and SM(d18:1/24:0). In an alternate embodiment, the metabolites that detected are selected from the group consisting of ceramide(d18:1/25:0), THC(d18:1/24:0), PC(16:0/16:0), lathosterol, hydroxyproline, PI(16:0/16:0), ceramide(d18:1/22:2), adenosine, ceramide(d18:1/24:2), THC(d18:1/16:0), 2-octenoylcarnitine, GC(18:1/16:0), phenyllactic acid, ceramide(d18:1/26:0), ceramide (d18:1/24:0), DHC(d18:1/16:0), ceramide(d18:1/26:2), FAD, 1-pyrroline-5-carboxylic acid, ceramide(d18:1/16:0), SM(d18:1/22:2), adenosine monophosphate, PC(40:6), PI(38:3), PC(22:6/P-18:0), PC(36:0), PC(22:6/P-18:0), PC(36:0), chenodeoxycholic acid, ceramide(d18:1/20:0), PC(18:1/22:6), ceramide(d18:1/23:0), ceramide(d18:1/18:2OH), ceramide(d18:1/22:0), ceramide(d18:1/18:0), ceramide(d18:1/16:1OH), and ceramide(d18:1/24:2OH). In yet another embodiment, the at least 7 metabolites comprise a metabolite in each of at least 7 of the group of metabolic pathways. In a certain embodiment, an alteration in a metabolic pathway is determined by detecting a reduced or elevated amount of a metabolite from the subject in comparison to the control population. In another embodiment, the metabolites from the subject are converted to a non-naturally occurring by-product that is analyzed. In a further embodiment, the non-naturally occurring by-product is a mass fragment. In yet a further embodiment, the metabolites are detected by using one or more of the following: HPLC, TLC, electrochemical analysis, mass spectroscopy, refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, gas chromatography (GC), radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), and Light Scattering analysis (LS).

In a particular embodiment, the biological sample is selected from the group consisting of cells, cellular organelles, interstitial fluid, blood, blood-derived samples, cerebral spinal fluid, and saliva. In a further embodiment, the biological sample is a fluid sample. In another embodiment, the fluid sample is a serum sample. In an alternate embodiment, the fluid sample is a urine sample. In a further embodiment, the metabolites are detected by using at least mass spectroscopy. In yet a further embodiment, the metabolites are detected by using a combination of high performance liquid chromatography (HPLC) and mass spectroscopy (MS). In a further embodiment, each of the metabolites is measured based on a single run or injection. In another embodiment, the metabolites are detected by extracting from the biological sample each of the metabolites from each of the metabolic pathways. In yet another embodiment, an elevation or reduction in the detected amount of the metabolite from the subject of at least 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% in comparison to the amount of the same metabolite from the control population indicates an alteration in a metabolic pathway.

[0009] In a particular embodiment, the disclosure further provides a method of treatment comprising: administering an antipurinergic compound to a subject that has been indicated as having CFS. In a further embodiment, the antipurinergic compound is a P2Y inhibitor compound. In yet a further embodiment, the P2Y inhibitor is suramin. In another embodiment, the subject has been indicated as having CFS based upon carrying out a metabolomics method disclosed herein.

[0010] In a certain embodiment, the disclosure also provides a method for detecting the effectiveness of a treatment for a subject that has CFS comprising: carrying out a metabolomics method disclosed herein prior to treatment; carrying out the same metabolomics post treatment; and comparing the number of alterations prior to treatment and post treatment in each of a plurality of the group of pathways in comparison to a control population that does not have CFS, wherein if post treatment if there is a reduction in the number of alterations in the metabolic pathways, then the treatment is deemed effective.

[0011] The disclosure also provides a method of treating a subject suffering from an inherited or acquired mitochondrial disease or disorder comprising administering a therapeutically effective amount of an antipurinergic compound, *e.g.*, suramin or derivative thereof. It is contemplated that administration of the antipurinergic compound, *e.g.*, suramin or derivative thereof increases sulfur levels or free thiols in mitochondrial disease/disorder patients.

[0012] In some embodiments, the disclosure provides a method of treating a subject suffering from an inherited or acquired mitochondrial disease or disorder comprising administering a antipurinergic compound, *e.g.*, suramin or derivative thereof.

[0013] In various embodiments, the mitochondrial disease or disorder is selected from the group consisting of bipolar disorder, multiple sclerosis, Parkinson's disease, schizophrenia, depression, autism, chronic fatigue syndrome, Friedreich's Ataxia, Leber's hereditary optic neuropathy, myoclonic epilepsy and ragged-red fibers (MERRF), Mitochondrial encephalomyopathy, mitochondrial myopathy, mitochondrial neurogastrointestinal encephalopathy (MNGIE), lactic acidosis, and stroke-like syndrome (MELAS), Kearns-Sayre syndrome, Pearson marrow syndrome, neuropathy ataxia and retinitis pigmentosa (NARP), Polymerase gamma (POLG) disorders including but not limited to Alpers-Huttenlocher syndrome and ataxia neuropathy spectrum (ANS) disorders, and other mitochondrial DNA depletion syndromes, including but not limited to those caused by gene defects in TK2, Twinkle, DGUOK, RRMB2, SUCLA2, SUCLG1, MPV17, ANT1, MFN1, MFN2, and OPA1, subacute necrotizing encephalopathy (Leigh's Syndrome), and mitochondrial cardiomyopathies and other syndromes due to mitochondrial DNA depletion, including but not limited to those caused by gene defects in TK2, Twinkle, DGUOK, RRMB2, SUCLA2, SUCLG1, MPV17, ANT1, MFN1, MFN2, and OPA1. Other mitochondrial diseases include neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP), progressive external opthalmoplegia (PEO), and Complex I disease, Complex II disease, Complex III disease, Complex IV disease and Complex V disease, which relates to dysfunction of the OXPHOS complexes, and MEGDEL syndrome (3-methylglutaconic aciduria type IV

with sensorineural deafness, encephalopathy and Leigh-like syndrome.

[0014] In various embodiments, the total daily dose of an antipurinergic compound, *e.g.*, suramin or derivative thereof is about 100mg to 1 g per day (*e.g.*, about 10-20 mg/kg); typically administered weekly.

[0015] The suramin can be formulated as an immediate release or a delayed or extended release form. For example, the suramin can be formulated as a delayed or controlled release dosage form that provides increased delivery to the small intestine. For example, the formulation can comprise an enteric coating that releases the suramin or derivative thereof when the suramin or derivative thereof reaches the small intestine or a region of the gastrointestinal tract of a subject in which the pH is greater than about pH 4.5. For example, the coating can be selected from the group consisting of polymerized gelatin, shellac, methacrylic acid copolymer type CNF, cellulose butyrate phthalate, cellulose hydrogen phthalate, cellulose proprionate phthalate, polyvinyl acetate phthalate (PVAP), cellulose acetate phthalate (CAP), cellulose acetate trimellitate (CAT), hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate, dioxypropyl methylcellulose succinate, carboxymethyl ethylcellulose (CMEC), hydroxypropyl methylcellulose acetate succinate (HPMCAS), and acrylic acid polymers and copolymers, typically formed from methyl acrylate, ethyl acrylate, methyl methacrylate and/or ethyl methacrylate with copolymers of acrylic and methacrylic acid esters. The composition can be administered orally or parenterally.

[0016] In some embodiments, the subject is under oxidative stress. In another embodiment, the subject has decreased thiol levels compared to a non-affected subject.

[0017] In various embodiments, the administering results in improvement in mitochondrial activity markers compared to levels before administration of the suramin or derivative thereof. Exemplary mitochondrial activity markers include, but are not limited to, free thiol levels, glutathione (GSH), reduced glutathione (GSSH), total glutathione, advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), lactic

acid, pyruvic acid, lactate/pyruvate ratios, phosphocreatine, NADH(NADH+H^+) or NADPH(NADPH+H^+), NAD or NADP levels, ATP, anaerobic threshold, reduced coenzyme Q, oxidized coenzyme Q; total coenzyme Q, oxidized cytochrome C, reduced cytochrome C, oxidized cytochrome C/reduced cytochrome C ratio, etc.

[0018] In various embodiments, the administering results in increased thiol levels compared to levels before administration of the suramin or derivative thereof.

[0019] In various embodiments, the suramin or derivative thereof further comprises a pharmaceutically acceptable carrier. It is further contemplated that the suramin or derivative product is formulated as a sterile pharmaceutical composition.

[0020] In various embodiments, the disclosure provides that a suramin or derivative product or composition is administered with a second agent useful to treat inherited or acquired mitochondrial diseases or disorders. Exemplary second agents include, but are not limited to, cysteamine, cystamine, coenzyme Q10, coenzyme Q10 analogs, idebenone, decylubiquinone, Epi-743, resveratrol and analogs thereof, arginine, vitamin E, tocopherol, MitoQ, glutathione peroxidase mimetics, levo-carnitine, acetyl-L-carnitine, dichloroacetate, dimethylglycine, triacetyluridine (TAU) or its derivatives (see, *e.g.*, U.S. Pat. No. 8,748,408, incorporated herein by reference), and lipoic acid.

[0021] In various embodiments, the subject is a child or adolescent.

[0022] In one aspect, the methods of the disclosure also include use of a suramin or derivative product in preparation of a medicament for treatment an inherited or acquired mitochondrial disease, and use of a suramin or derivative product in preparation of a medicament for administration in combination with a second agent for treating an inherited or acquired mitochondrial disease. Also included is use of a second agent for treating an inherited or acquired mitochondrial disease.

DESCRIPTION OF DRAWINGS

[0023] **Figure 1A-E** presents a metabolomic diagnosis of chronic fatigue syndrome. **(A)** Males, **(B)** Females. Multivariate analysis using partial least squares discriminant analysis (PLSDA) clearly

distinguished controls and patients with chronic fatigue in both males and females. Biochemical Pathway Impact Analysis. **(C)** Males, the top five pathway disturbances in males were responsible for 82% of the metabolic impact. These were sphingolipids (49%), phospholipids (16%), pyrroline-5-carboxylate (P5C), arginine (Arg), proline (Pro) (7%), glycosphingolipids (6%), and cholesterol (4%). **(D)** Females, the top six pathway disturbances in females were responsible for 83% of the metabolic impact. These were sphingolipids (35%), phospholipids (26%), glycosphingolipids (9%), purines (5%), microbiome (5%), and pyrroline-5-carboxylate (P-5-C), arginine (Arg), proline Pro (3%). **(E)** Metabolic pathways disturbed in chronic fatigue syndrome, a total of 20 pathways were disturbed in males and females with chronic fatigue syndrome. Nine of these were common to both, and 11 showed gender differences.

[0024] **Figure 2A-B** provides for the characterization of total, low, and high metabolite abnormalities in chronic fatigue syndrome. **(A)** Males, **(B)** Females. Metabolites with Z-scores ≥ 2.0 are indicated in red. Metabolites with Z-scores ≤ -2.0 are indicated in green. Both the total number of metabolite abnormalities and the number of metabolites that were decreased were significantly increased in patients with chronic fatigue syndrome.

[0025] **Figure 3A-B** provides a rank order of distinguishing metabolite abnormalities in chronic fatigue syndrome. **(A)** Males, **(B)** Females. Partial least squares discriminant analysis (PLSDA) was used to rank the most significantly abnormal metabolites by variable importance in projection (VIP) scores. VIP scores ≥ 1.5 were considered significant. The top 35 most dysregulated metabolites are illustrated. See **Tables 1-4** for a complete listing of all 61 metabolites with VIP scores ≥ 1.5.

[0026] **Figure 4A-B** presents a cytoscape visualization of metabolite and pathway disturbances in chronic fatigue syndrome. **(A)** Males, **(B)** Females. The fractional contribution of each pathway is indicated as a percentage of the total variable importance in projection (VIP) score in black circles. The smaller circles indicate the measured metabolites in each pathway, quantified by z-score. Metabolites in red were increased and those

in green were decreased in chronic fatigue syndrome compared to controls.

[0027] **Figure 5A-B** provides the distribution of diagnostic and personalized metabolic abnormalities in chronic fatigue syndrome. **(A)** Males, **(B)** Females.

[0028] **Figure 6A-B** presents the diagnostic performance of targeted metabolomics in chronic fatigue syndrome. Area Under the Receiver Operator Characteristic (AUROC) Curve Analysis. **(A)** Males, eight metabolites were selected and tested by bootstrap resampling as an example of one possible multi-analyte diagnostic classifier. Training set over-fitting was minimized by using random forest decision tree analysis. The 8 metabolites selected were phosphatidyl choline PC(16:0/16:0), glucosylceramide GC(18:1/16:0), 1-pyrroline-5-carboxylate (P5C), flavin adenine dinucleotide (FAD), pyroglutamic acid (also known as 5-oxoproline), 2-hydroxyisocaproic acid (HICA), L-serine, and lathosterol. The diagnostic accuracy measured as the area under the receiver operator characteristic curve was 0.94 (95% CI = 0.84-1.0). **(B)** Females, thirteen metabolites were selected as a diagnostic classifier in females as described above. The 13 metabolites were trihexosylceramide THC(18:1/24:0), phosphatidyl choline PC(16:0/16:0), hydroxyproline, ceramide(d18:1/22:2), lathosterol, adenosine, phosphatidylinositol PI(16:0/16:0), flavin adenine dinucleotide (FAD), 2-octenoylcarnitine, phosphatidyl choline plasmalogen PC(22:6/P18:0), phosphatidyl choline PC(18:1/22:6), 1-pyrroline-5-carboxylate (P5C), and chenodeoxycholic acid (CDCA). The diagnostic accuracy measured as the area under the receiver operator characteristic curve was 0.96 (95% CI = 0.86-1.0). N = 18 control males and 22 CFS males, and N = 21 control females and 23 CFS females.

[0029] **Figure 7** displays the mitochondrial control of redox, NADPH, nucleotide, and methylation Pathways. In embryonic cells and cancer, MTHFD2L is expressed and one-carbon units are efficiently converted to Formyl-THF and formate for cytosolic nucleotide synthesis. Under these conditions, fewer one-carbon units are available for SAM synthesis and DNA methylation. When MTHFD2L is turned down in differentiated cells, less mitochondrial formate is produced and one-carbon units are directed through

Methylene-THF toward increased SAM synthesis and increased DNA methylation. 1/2--Mitochondrial Bifunctional Enzyme (mBE, MTHFD2L): 1--NAD(P)+ Dependent Methylene Tetrahydrofolate Reductase, 2--Methenyl-THF Cyclohydrolase. 3--Formyl-THF Synthase (FTS), 3*--FTS can reverse directions in differentiated cells when MTHFD2L is decreased, 4--Mitochondrial Serine Hydroxymethyl Transferase (mSHMT), 5--Dimethylglycine Dehydrogenase, ETF--Electron Transfer Flavoprotein, 6--Sarcosine Dehydrogenase, 7--Glycine Cleavage System, 8--Methylene-THF Reductase (MTFR). 9--Thymidylate Synthase, 10--Dihydrofolate Reductase (DHFR). 11--Cytosolic Serine Hydroxymethyl Transferase (cSHMT). 11*--cSHMT reverse reaction, 12/13/14--Cytosolic Trifunctional Enzyme (cTE): 12/13/14--Cytosolic Trifunctional C1-THF Synthase (MTHFD1) [12--Formyl-THF Synthase, 13--Methenyl-THF Cyclohydrolase, 14--NADPH-dependent Methylene-THF Dehydrogenase], 15--Formyl-THF Dehydrogenase, 16--Homocysteine Methyl Transferase (Methionine Synthase, CblG). 17--Methionine Adenosyl Transferase (MAT). 18*--Multiple DNA-, RNA-, Protein--, Neurotransmitter, and Other Methyltransferase reactions in the nucleus, cytosol, and mitochondria. 19--S-Adenosyl Homocysteine Hydrolase (SAHH), 20--Cystathionine β-Synthase (CBS), 21—Cystathionase (Cystathionine γ-lyase), 22--γ-Glutamylcysteine Synthase (GCS), 23--Glutathione Synthase, 24--Nucleoside Diphosphate Kinase, 25--ATP Synthase (Complex V), 26--Propionyl CoA Carboxylase, 27--Methylmalonyl CoA Mutase, 28--Betaine Homocysteine Methyltransferase, 29--Choline Dehydrogenase, 30--Betaine Aldehyde Dehydrogenase, 31--S-Adenosylmethionine decarboxylase (adoMetDC, AMD1, SAMDC), 32--Spermidine synthase, 33--Spermine synthase, 34--Methylthioadenosine phosphorylase (MTAP), 35--Methionine synthase reductase (MSR, MTRR, CblE), 36--Delta Amino Levulinic Acid Synthase (dALAS), 37--Glutathione reductase, GAR--Glycinamide Ribonucleotide, AICAR--Aminoimidazole Carboxamide Ribonucleotide, FAICAR--Formaminoimidazole Carboxamide Ribonucleotide, Ado--Adenosine, NAC--N-Acetyl Cysteine, dcSAM--Decarboxylated S-Adenosyl Methionine, MTA--Methylthioadenosine, MTR-1P--Methylthioribose 1-phosphate, B6 (Pyridoxine, Pyridoxal phosphate, PLP), FAD--Flavin adenine dinucleotide, FMN--Flavin mononucleotide, GSH--reduced glutathione, GSSG--oxidized glutathione disulfide.

[0030] **Figure 8A-B.** Principal Components Analysis (PCA). **(A)** Males, **(B)** Females. Scree plots illustrate the cumulative (green line) and component-specific (blue line) fraction of variance explained by the top 5 principal components.

[0001] **Figure 9** provides a CONSORT flow chart of a suramin study design overview, including study recruitment, allocation and analysis. The two treatment groups were well matched by the 3 parameters that were the basis of the pairing (age, ADOS, and IQ), and were incidentally well matched for 7 other anthropometric criteria (see **Table 14**). Twenty families were interviewed. Four children were excluded for cause; two were taking prescription medications, one was 18 years old, and one lived 2.5 hours away. Sixteen children were eligible for enrollment. Ten children were matched into 5 pairs on the basis of age, non-verbal Leiter-3 IQ, and ADOS-2 scores. One child in each pair was then randomized to receive either saline or suramin (see also **Table 14**).

[0002] **Figure 10** provides a diagram of the suramin study design. Phone interviews, parent journals, and clinical observations revealed an unexpected pattern of response to the single infusion of suramin. Instead of showing maximum effect at 2-days after the infusion as would be predicted by classical pharmacology theory of maximum receptor occupancy, ASD symptoms continued to improve and catch-up development accelerated for the first 3 weeks after infusion began to gradually decrease for the next 2-4 weeks until 3 of the 5 children who received suramin had returned to close to their pre-infusion baseline. The two most verbal children continued to make advances for 7 weeks after the infusion (**Tables 15** and **17**). Despite not having formal observations scheduled at the 3-week time point, objective testing at 2-days and 6-weeks after the infusion documented improvements in all the core symptom domains of ASD. Saline-treated children did not exhibit these responses (see **FIG. 11, Tables 15, 17** and **21**).

[0003] **Figure 11A-I** presents the results of safety monitoring with suramin administration. Suramin Safety Monitoring. **(A)** No change was observed in cortisol levels between saline and suramin treated infusion and decrease in the afternoon post-infusion were seen in both saline and suramin treated subjects. **(B)** Proteinuria

was unchanged by suramin treatment, **(C)** Creatinine was unchanged by suramin by 2-way ANOVA, but was normalized after 6-weeks in a paired analysis (see Figure 8B). **(D)** Hemoglobin subjects. Natural circadian elevations of cortisol in the mornings pre- was unchanged by suramin, **(E)** White blood cell count (WBC) was unchanged by suramin, **(F)** Platelets were unchanged by suramin; **(G)** Aspartate transaminase (AST) was unchanged by suramin; **(H)** Suramin rash, antecubital fossa; **(I)** Data were analyzed by 2-way ANOVA to test for treatment, time, and treatment x-time interaction effects. P and F values reflect the treatment effect. Only the rash was significantly different between suramin and placebo groups.

[0004] **Figure 12A-D** presents suramin pharmacokinetics. **(A)** 2-compartment model of suramin blood concentrations. The first 48 hours were dominated by the distribution phase. Over 90% of the model is described by the elimination phase. **(B)** Plasma suramin concentrations **(C)** A 2-compartment model correlated well with measured values **(D)** Pediatric PK parameters of suramin.

[0005] **Figure 13A-B** presents suramin pharmacometabolomics. **(A)** Metabolites and pathways changed at 6-weeks. **(B)** Shared biochemical pathways. 75% of the pathways that were altered by suramin in children with ASD were also altered in the mouse models. Asterisks (*) indicate pathways that were changed at 2-days, but not at 6-weeks after treatment.

[0006] **Figure 14** presents suramin pharmacometabolomics. Metabolites and pathways changed at 2-days.

[0007] **Figure 15A-B** provides for the visualization of the suramin pharmacometabolomics pathway. **(A)** After 2 days. **(B)** After 6 weeks. Metabolites indicated in red are increased, and those in green are decreased compared to controls (see z-score scale in upper right).

[0008] **Figure 16A-dd** demonstrates the various outcomes with suramin treatment versus placebo. **(A)** 6-week ADOS Comparison Scores by 2-Way ANOVA. **(B)** 6-Week ADOS Comparison Score Improvement after suramin. **(C)** 6-Week ADOS Social Affect Score Improvement after suramin. **(D)** 6-Week ADOS Restricted and Repetitive Behavior Score Improvement after Suramin. **(E)** 2-Day ADOS Comparison Scores were not changed. **(F)** No change in 6-Week ADOS Scores in subjects

receiving saline placebo. **(G)** No change in 6-Week ADOS Social Affect Scores in subjects receiving placebo. **(H)** No change in 6-Week ADOS Restricted and Repetitive Behavior Scores in subjects receiving placebo. **(I)** No change in 6-week Expressive One Word Picture Vocabulary scores. **(J)** 7-Day improvement in ABC stereotypy scores after suramin. **(K)** 6-week Improvement in ABC stereotypy scores after suramin. **(L)** 7-Day Improvement in ATEC total scores after suramin. **(M)** No change in 6-week EOWPVT scores after saline. **(N)** No change in 7-day ABC stereotypy scores after saline. **(O)** No change in 6-week ABC stereotypy scores after saline. **(P)** No change in 7-day ATEC total scores after saline. **(Q)** Improved ATEC speech, language, and communication scores 7-days after suramin. **(R)** Improved ATEC sociability scores 7-days after suramin. **(S)** Improved ATEC speech, language, and communication scores 6-weeks after suramin. **(T)** Improved ADOS comparison scores after dropping a subject who missed the 6-week visit (N = 4). **(U)** No change in 7-day ATEC speech, language, and communication after saline. **(V)** No change in 7-day ATEC sociability after saline. **(W)** No change in 6-week ATEC speech, language, and communication scores 6-weeks after saline **(X)** No change in EOWPVT scores after dropping subject who missed the 6-week visit (N = 4). **(Y)** No change in 2-day ADOS scores after suramin. **(Z)** No change in 6-week RBQ total scores after suramin. **(aa)** Improved core symptoms of ASD and other behaviors by CGI at 6-weeks after suramin. P values: * = 0.05; ** = 0.01; *** = 0.001. **(bb)** Top 3, most-changed symptoms named by parents in the 6-week CGI. **(cc)** No change in 2-day ADOS scores after saline. **(dd)** No change in 6-week RBQ total scores after saline.

[0009] **Figure 17** demonstrates that the social behavior in mice were improved by Antipurinergic Therapy with suramin. (N = 10 males per group). Social behavior change in females did not reach statistical significance with 10 animals per group. Results are the hand scored duration of contact in the first 5 minutes with a stranger mouse in a standard Crawley social approach chamber. PIC-SAL are ASD mice treated with saline. PIC-SUR are ASD mice treated with suramin.

[0010] **Figure 18** demonstrates a trend toward improved rotarod performance in Female ASD Mice. Two-way ANOVA with Bonferroni post hoc testing showed a trend toward improved performance in the Poly(IC)-exposed females treated with Suramin ($p<0.06$; n = 10 per group).

[0011] **Figure 19** shows that hyperactivity in male ASD mice was normalized by antipurinergic therapy with suramin. Two-way ANOVA with Bonferroni post hoc testing of center region entries in the first 10 minutes of exploration in the open field test. Suramin had no effect on the activity of normal control animals.

[0012] **Figure 20** demonstrates that antipurinergic therapy restores autonomic balance and normalizes stress-induced hyperthermia (SIH) in ASD mice with autism-like behaviors. This effect was observed in both males and females at 4- months of age.

[0013] **Figure 21A-B** shows the increased SIH Response in ASD mice is the result of a decreased basal body temperature and that treatment with antipurinergic therapy with suramin is corrective. **(A)** The post stress temperatures (dark bars) taken at 10 minutes after the baseline temperatures were not different. However, the pre-stress basal body temperature of the Poly(IC)-exposed controls (PIC-SAL; white bars) was significantly lower than the basal temperatures of the other 3 experimental groups. **(B)** Antipurinergic Therapy with Suramin Restores the Basal Body Temperature in Poly(IC) Exposed Animals, when compared to 16-week old Saline-exposed animals (N=9 males).

[0014] **Figure 22A-B** demonstrates that a maternal immune activation model of autism produces a state of chronic mild hypothermia. **(A)** The basal temperature of animals exposed *in utero* to Poly(IC) is typically 0.4 °C lower than saline exposed animals measured at 4 months of age (N = 10 males and 10 females). **(B)** The basal temperature of animals 1 hour after a purinergic stimulus given by an injection of ATP (0.5 mL of 25 mM ip) is 1.8 °C lower than saline injected controls.

[0015] **Figure 23** demonstrates that a single-dose of suramin corrected a short-term memory deficit in the Poly(IC) Mouse Model of ASD. A single dose of suramin given to 5 month-old animals corrected the memory deficit in this model.

[0031] **Figure 24** demonstrates that a single-dose of suramin corrected social abnormalities in a Poly(IC) mouse model of ASD. A single dose of suramin given to 5 month-old animals corrected the social approach abnormalities in this model.

DETAILED DESCRIPTION

[0032] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a biomarker" includes a plurality of such biomarkers and reference to "the metabolic feature" includes reference to one or more metabolic features and equivalents thereof known to those skilled in the art, and so forth.

[0033] Also, the use of "and" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0034] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0035] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although many methods and reagents are similar or equivalent to those described herein, the exemplary methods and materials are disclosed herein.

[0036] All publications mentioned herein are incorporated by reference in full for the purpose of describing and disclosing methodologies that might be used in connection with the description herein. Furthermore, with respect to any term that is presented in one or more publications that is similar to, or identical with, a term that has been expressly defined in this disclosure, the definition of the term as expressly provided in this disclosure will control in all respects.

[0037] The term "small molecules" includes organic and inorganic molecules, such as those present in a biological sample obtained

from a patient or subject. Examples of small molecules include sugars, fatty acids, amino acids, nucleotides, intermediates formed during cellular processes, and other small molecules found within a cell. In some embodiments, the small molecules are metabolites. In other embodiments a small molecule is a chemical compound, *e.g.*, a drug.

[0038] Autism affects 1-2% of children in the US. Dozens of single genes and chromosomal copy number variants (CNVs) increase the relative risk of autism spectrum disorder (ASD) nearly 5-50 times over the current background risk. Yet no single gene or CNV causes ASD in 100% of children who carry the mutation, and no single DNA mutation accounts for more than 1-2% of all ASD. Specific environmental factors have also been shown to increase the risk of ASD. However, no single child has all of the known genetic risk factors for ASD, or is exposed to all the same environmental risks. Although the non-core symptoms of ASD are highly heterogeneous from child to child, making each child unique, the same DSM-5 behavioral symptoms (abnormalities in social communication, restricted interests, repetitive behaviors, adherence to routine, and/or atypical sensory behaviors) are by definition expressed in every child.

[0039] "Chronic fatigue syndrome" or "CFS" is a complex, multi-organ system disease for which no single diagnostic test yet exists. The disease is characterized by profound fatigue and disability lasting for at least six months, episodes of cognitive dysfunction, sleep disturbance, autonomic abnormalities, chronic or intermittent pain syndromes, microbiome abnormalities, cerebral cytokine dysregulation, natural killer cell dysfunction, and other symptoms that are made worse by exertion of any kind. The Institute of Medicine recently published an update of the diagnostic criteria recommended for chronic fatigue syndrome:

Institute of Medicine Diagnostic Criteria for Chronic Fatigue Syndrome

Diagnosis requires that the patient have the following three symptoms:

1. A substantial reduction or impairment in the ability to engage in pre-illness levels of occupational, educational,

social, or personal activities, that persists for more than 6 months and is accompanied by fatigue, which is often profound, is of new or definite onset (not lifelong), is not the result of ongoing excessive exertion, and is not substantially alleviated by rest, and

2. Post-exertional malaise,* and
3. Unrefreshing sleep*

At least one of the two following manifestations is also required:

1. Cognitive impairment* or
2. Orthostatic intolerance

* Frequency and severity of symptoms should be assessed. The diagnosis should be questioned if patients do not have these symptoms at least half of the time with moderate, substantial, or severe intensity.

[0040] Complex diseases like chronic fatigue syndrome (CFS) are often difficult and expensive to diagnose. While individual tests may be affordable and possibly covered by medical insurance, many patients undergo a diagnostic odyssey that results in substantial personal expenditures that can exceed $100,000 over years of searching, absence from the workplace, and significant reductions in quality of life. The societal cost of chronic fatigue syndrome is estimated to be up to $24 billion annually. Health care professionals are also frustrated by the lack of an objective technology that can assist with diagnosis. Attempts to use a small number of biomarkers, whether analytes in blood, cerebrospinal fluid, or a handful of genetic loci, have not yielded diagnostically useful tests for CFS.

[0041] The term "disorder" as used herein is intended to be generally synonymous, and is used interchangeably with, the terms "disease," "syndrome," and "condition" (as in medical condition), in that all reflect an abnormal condition of the human or animal body or of one of its parts that impairs normal functioning, is typically manifested by distinguishing signs and symptoms. The disclosure provides, for example, a method of treating and/or preventing autism, autism-like disorders, and/or symptoms using suramin and derivatives thereof as therapeutic agents. Autism and

autism-like disorders include, for example, familial forms of autism, mental retardation, and/or Asperger syndrome. Other disorders that can be treated with the compositions and methods described herein are mitochondrial disorders and chronic fatigue syndrome.

[0042] "Mitochondrial disorders" or "-dysfunction" are defects in oxidative metabolism and are found in many chronic illnesses including bipolar disorder, multiple sclerosis, Parkinson's disease, schizophrenia, depression, autism, and chronic fatigue syndrome. Elevated levels of reactive oxygen and nitrogen species together with elevated pro-inflammatory cytokines and reduced levels of glutathione impair oxidative metabolism result in mitochondrial dysfunction. Chronic fatigue syndrome is a complex and is characterized by profound fatigue and disability, episodes of cognitive dysfunction, sleep disturbance, autonomic abnormalities, chronic or intermittent pain syndromes, microbiome abnormalities, cerebral cytokine dysregulation, natural killer cell dysfunction, and other symptoms that are made worse by exertion of any kind.

[0043] The term "small molecule metabolite profile" refers to the composition, amounts, and/or identity, of small molecule metabolites present in a biological sample, a cell, tissue, organ, or organism. The small molecule metabolite profile provides information related to the metabolism or metabolic pathways that are active in a cell, tissue or organism. Thus, the small molecule metabolite profile provides data for developing a "metabolomic profile" (also referred to as "metabolic profile") of active or inactive metabolic pathways in a cell, tissue, or subject. The small molecule metabolite profile includes, *e.g.*, the quantity and/or type of small molecules present. A "small molecule metabolite profile," can be obtained using a single measurement technique (*e.g.*, HPLC) or a combination of techniques (*e.g.*, HPLC and mass spectrometry). The type of small molecule to be measured will determine the technique to be used and can be readily determined by one of skill in the art.

[0044] A "metabolomic profile" is a profile of pathway activity associated with the small molecule metabolites. The activity of

the pathways is an indication of metabolic health. For example, one or more small molecule metabolites can be measured in a specific pathway, the small molecule metabolites can include intermediates as well as the end product. The metabolomics profile identifies the pathway's "activity". If the pathway produced a normal amount of the metabolite, then the pathway is normal, however, if the pathway produces excessive or reduced amounts then the pathway has aberrant activity. Typically a disease state (or risk thereof) is identified by a plurality of aberrant pathways in a metabolomics profile. The pathway can be identified numerically, by color, by code or other symbols as being aberrant or normal. In the human body, a vast number of metabolic pathways are well characterized including substrates, intermediates, products, enzymes, genes and the like. One of skill in the art can readily identify the pathways and their metabolites and interconnectedness with other pathways. For example, Sigma-Aldrich has an on-line, interactive metabolic pathway for numerous species including humans (see, *e.g.*, [http://]www[.]sigmaaldrich.com/technical-documents/articles/biology/interactive-metabolic-pathways-map.html)(note that the foregoing has been modified with brackets to eliminate an active hyperlink). For particular disease states, the disclosure provides certain metabolomics profiles that are useful for diagnosis (*e.g.*, a "chronic fatigue syndrome metabolomics profile" and the like).

[0045] A small molecule metabolite profile and metabolomic profile can be obtained for a normal control (*e.g.*, a "control small molecule metabolite profile" or "control metabolomic profile") and would include an inventory of small molecules or metabolomic pathways that are active in similar cells, tissue or sample from a population of subject that are considered "normal" or "healthy" (*e.g.*, lack any disease or disorder traits or phenotypic characteristics relative to a specific disease or disorder being examined). For example, where CFS is to be determined or the risk of CFS is to be determined a "control small molecule metabolite profile" or "control metabolomic profile" would include the inventory and amounts of small molecules present (or metabolic

pathways active) in, *e.g.*, 70%, 80%, or 90%, but typically greater than 95% of a population that does not have any symptoms of CFS.

[0046] The term "subject" as used herein, refers to an animal, including, but not limited to, a primate (*e.g.*, human, monkey, chimpanzee, gorilla, and the like), rodents (*e.g.*, rats, mice, gerbils, hamsters, ferrets, and the like), lagomorphs, swine (*e.g.*, pig, miniature pig), equine, canine, feline, and the like. The terms "subject" and "patient" are used interchangeably herein. For example, a mammalian subject can refer to a human patient.

[0016] "Suramin" is a polysulfonated naphthylurea. "Suramin derivatives" and analogues for use in the disclosed methods are also provided. Derivatives of suramin are known in the art (See U.S. Pat. No. 5,173,509, Braddock, P S., *et al.* 1994; Dhar, S., *et al.* 2000; Firsching, A., *et al.* 1995; Gagliardi, A. R. T., *et al.* 1998; Kreimeyer, A., *et al.* 1998; Marchetti, D., *et al.* 2003; McCain, D. F., *et al.* 2004; Meyers, M. O., *et al.* 2000; which are each incorporated herein by reference). Suramin has many actions. One of its best-studied actions is as an inhibitor of purinergic signaling. It is the oldest member of a growing class of antipurinergic drugs (APDs) in development. Suramin was first synthesized in 1916, making it one of the oldest manmade drugs still in medical use. It is used to treat African sleeping sickness (trypanosomiasis), and remains on the World Health Organization list of essential medications. Concerns about the toxicity of high-dose suramin arose when the cumulative anti-trypanosomal dose was increased 5 times or more over several months to treat AIDS or kill cancer cells during chemotherapy. When blood levels were maintained over 150 μM for 3-6 months at a time to treat cancer, a number of dose-limiting side effects were described. These included adrenal insufficiency, anemia, and peripheral neuropathy. In contrast, mouse studies suggested that high-dose suramin was not necessary to treat autism-like symptoms. The disclosure shows that low-dose suramin that produced blood levels of about 5-10 μM was effective in treating autism spectrum disorders (ASD)-like symptoms and did not produce toxicity even when used for at least 4 months.

[0047] The term "therapeutically acceptable" refers to those compounds (or salts, prodrugs, tautomers, zwitterionic forms, etc.) which are suitable for use in contact with the tissues of subjects or patients without excessive toxicity, irritation, allergic response, immunogenicity, are commensurate with a reasonable benefit/risk ratio, and are effective for their intended use.

[0048] The disclosure provides for methods of treating mitochondrial-associated diseases and disorders including, but not limited to, autism spectrum disorders and chronic fatigue syndrome. Moreover, the disclosure also provides metabolomics diagnostics useful to diagnosing mitochondrial diseases and disorders including, but not limited to, autism spectrum disorders and chronic fatigue syndrome.

[0017] The disclosure provides for novel treatment options for to prevent or retard neuronal loss and/or improve short-term memory in a subject comprising administering to a subject in need thereof, a composition comprising an effective amount of a purinergic antagonist such as suramin or a derivative thereof. Purinergic antagonist are a new class of drugs that can be used safely for the treatment of autism and related spectrum disorders like chronic fatigue syndrome, fibromyalgia, obsessive compulsive disorder (OCD), generalized anxiety disorder (GAD), bipolar depression, schizophrenia, subacute therapy of Traumatic Brain Injury (TBI), post-traumatic stress disorder (PTSD), Chronic Traumatic Encephalopathy (CTE), certain disabling forms of attention deficit hyperactivity disorder (ADHD, ADD), acute therapy for Lupus enechalitis, and early Alzheimer dementia for the prevention of neuronal loss and improvement of short-term memory.

[0018] The only non-cardiovascular, antipurinergic drug currently approved for human use is suramin. More than a dozen others are in development around the world for a variety of indications, but none for mitochondrial diseases and disorders or for autism spectrum disorders or chronic fatigue syndrome. Suramin has been used to treat African sleeping sickness for nearly 100 years at a “medium dose” designed to produce blood levels of 50-100 μM for 1-3 months. This is generally well-tolerated, but side-effects can occur, particularly in fragile or malnourished

patients. Since the 1990s, suramin has been used in adjunct cancer chemotherapy protocols at a "high-dose" designed to produce blood levels of 150-270 µM for 3-6 months. High-dose regimens produce side effects in up to 10% of subjects per month, so that by 5 months, over half of patients has reported at least one side effect. These include adrenal insufficiency, anemia, and peripheral neuropathy. This disclosure teaches how "low-dose" suramin can be used safely to treat mitochondrial disorders, autism, chronic fatigue syndrome and related disorders.

[0019] The Cell Danger Theory of Autism teaches that autism is the net result of ecogenetic factors that ultimately lead to persistent increases in neuroinflammation, innate immunity, and durable metabolic disturbances. The root cause of this is increased purinergic signaling from extracellular nucleotides like ATP and UTP, resulting in dissipative losses of intracellular nucleotides for purposes of extracellular danger signaling and inflammation. Extracellular ATP (eATP) is well-known damage associated molecular pattern or DAMP2.

[0020] It has been shown herein that "low-dose suramin", given at intervals of 3-8 weeks, for 2-12 months, and designed to maintain blood levels of 4-20 µM is safe and effective in treating the 3 core symptoms of autism. This treatment leads to improvements in language, social interaction, and the reduction in repetitive or restricted behaviors. There is currently no unifying theory of autism. There are no universally helpful medications in autism. Doses of suramin and bioequivalent doses of other antipurinergic drugs designed to produce blood levels of under 20 µM for weeks to months are safe and effective for the treatment of autism spectrum disorders, and are effective in treating several other pathophysiologically related disorders, including but not limited to: Autism, Chronic fatigue syndrome, Fibromyalgia, Obsessive compulsive disorder, Generalized anxiety disorder, Schizophrenia, Bipolar Depression, Subacute therapy for Traumatic Brain Injury (TBI), Post-Traumatic Stress Disorder (PTSD), Chronic Traumantic Encephalopathy (CTE), Disabling Attention Deficit Hyperactivity Disorder (ADHD, ADD), acute therapy of Lupus encephalitis, early

Alzheimer Disease—for the prevention of chronic neuronal loss and improvement in short term memory.

[0021] In an embodiment disclosed herein, a subject to be treated by a compound disclosed herein has a developmental disorder. In a further embodiment, a subject to be treated by a compound disclosed herein has a mental disorder. In a particular embodiment, the subject is an infant, child or adolescent.

[0022] The compounds of the disclosure can be formulated for deliver by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Mention may be made, as examples of pharmaceutically acceptable salts, of the addition salts with inorganic or organic acids (such as acetate, trifluoroacetate, propionate, succinate, benzoate, fumarate, maleate, oxalate, methanesulphonate, isethionate, theophyllinacetate, salicylate, methylenebis-β-oxynaphthoate, hydrochloride, sulphate, nitrate and phosphate), the salts with alkali metals (sodium, potassium or lithium) or with alkaline-earth metals (calcium or magnesium), the ammonium salt or the salts of nitrogenous bases (ethanolamine, trimethylamine, methylamine, piperidine, benzylamine, *N*-benzyl-α-phenethylamine, choline, arginine, leucine, lysine or *N*-methylglucamine).

[0023] The disclosure provides pharmaceutical compositions of an antipurinergic agent or their salts. The antipurinergic agent or their physiologically acceptable salts or solvates, may be formulated for administration for injection, or for oral, topical, nasal, inhalation, insufflation (either through the mouth or the nose) buccal, parenteral, rectal administration or other forms of administration. The disclosure provides pharmaceutical compositions comprising effective amounts of antipurinergic agent together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants, excipients and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, thimerosal, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). In one

embodiment, the antipurinergic agent is suramin or a derivative thereof.

[0024] The compositions may also be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, and the like or liposomes. Hyaluronic acid may also be used. Biocompatible absorbable polymers may be selected from the group consisting of aliphatic polyesters, copolymers and blends, which include, but are not limited to, homopolymers and copolymers of lactide (which include D-, L-, lactic acid and D-, L- and meso lactide), glycolide (including glycolic acid), epsilon-caprolactone, p-dioxanone (1,4-dioxan-2-one), alkyl substituted derivatives of p-dioxanone (*i.e.*, 6,6-dimethyl-1,4-dioxan-2-one), triethylene carbonate (1,3-dioxan-2-one), alkyl substituted derivatives of 1,3-dioxanone, delta-valerolactone, beta-butyrolactone, gamma-butyrolactone, epsilon-decala tone, hydroxybutyrate, hydroxyvalerate, 1,4-dioxepan-2-one and its dimer 1,5,8,12-tetraoxacyclotetradecane-7,14 dione, 1,5-dioxepan-2-one, and polymer blends thereof.

[0025] Such compositions may influence physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance. See, *e.g.*, Remington s Pharmaceutical Sciences, 18th ed., (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712). The compositions may be prepared in liquid form, or be in dried powder, such as lyophilized form.

[0026] Contemplated for use herein are oral solid dosage forms, which are disclosed generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate compositions. Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers. A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: Modern Pharmaceutics Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979). Because suramin has been demonstrated to increase gastric acid secretion, it may be advantageous to provide a formulation to bypass the stomach. In general, the formulation

will include an antipurinergic agent (*e.g.*, suramin and/or derivatives thereof) and inert ingredients (which allow for protection against the stomach environment and release of the biologically active material in the intestine).

[0027] To ensure full gastric resistance a coating impermeable to at least pH 5.0 is useful. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

[0028] A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings that make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic, *i.e.*, powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets may be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

[0029] The therapeutic can be included in the formulation as fine multi-particulates in the form of granules or pellets. The formulation of the material for capsule administration can also be as a powder, lightly compressed plugs or even as tablets. The therapeutic can also be prepared by compression.

[0030] Colorants and flavoring agents may all be included. For example, the peptide (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

[0031] One may dilute or increase the volume of the therapeutic with an inert material or filler. These diluents or fillers can include carbohydrates, especially mannitol, anhydrous lactose, cellulose (*e.g.*, microcrystalline cellulose), sucrose, calcium hydrogen phosphate modified dextrans and starch. Certain inorganic salts may also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially

available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

[0032] Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include, but are not limited to, starch (*e.g.*, potato starch or the commercial disintegrant based on starch, Explotab). Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

[0033] Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch (*e.g.*, pre-gelatinized maize starch) and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) can both be used in alcoholic solutions to granulate the therapeutic.

[0034] An anti-frictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes, talc and silica. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

[0035] Glidants that can improve the flow properties of the drug during formulation and to aid rearrangement during compression can be added. The glidants can include starch, talc, pyrogenic silica and hydrated silicoaluminate.

[0036] To aid dissolution of the therapeutic into the aqueous environment a surfactant can be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl

sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents can be used and can include benzalkonium chloride or benzethomium chloride. The list of potential non-ionic detergents that can be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty cid ester, methyl cellulose and carboxymethyl cellulose. These surfactants can be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

[0037] Additives that potentially enhance uptake of the agent are, for example, the fatty acids oleic acid, linoleic acid and linolenic acid.

[0038] Controlled release oral formulation may be desirable. The agent can be incorporated into an inert matrix that permits release by either diffusion or leaching mechanisms, *e.g.*, gums. Slowly degenerating matrices may also be incorporated into the formulation. Some enteric coatings also have a delayed release effect.

[0039] Other coatings may be used for the formulation. These include a variety of sugars that can be applied in a coating pan. The therapeutic agent can also be given in a film coated tablet and the materials used in this instance are divided into two groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

[0040] A mix of materials can be used to provide the optimum film coating. Film coating may be carried out in a pan-coater or in a fluidized bed or by compression coating.

[0041] Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable

additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

[0042] The compounds (*e.g.*, antipurinergic agents such as suramin and/or derivatives thereof) disclosed herein may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[0043] The compounds (*e.g.*, suramin and/or derivatives thereof) may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[0044] In addition to the formulations disclosed previously, the compounds disclosed herein may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds disclosed herein may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a soluble salt.

[0045] The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[0046] Although the disclosure provide dosage information and the dosing of suramin is well known, toxicity and therapeutic efficacy of the compounds disclosed herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animal/animal models (such as those described herein), *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0047] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0048] A compound disclosed herein and components of a therapeutic composition may be introduced parenterally, topically, or transmucosally, *e.g.*, orally, nasally, or rectally, or transdermally. Parenteral administration includes, for example, intravenous injection, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

[0049] The compounds (*e.g.*, antipurinergic agents such as suramin and/or derivatives thereof) are suitable for oral, parenteral or intravenous administration. Alternatively, the

compound can be modified or otherwise altered so that it can effectively cross or be transported across the blood brain barrier. Many strategies known in the art are available for molecules crossing the blood-brain barrier, including but not limited to, increasing the hydrophobic nature of a molecule; introducing the molecule as a conjugate to a carrier, such as transferring, targeted to a receptor in the blood-brain barrier, or to docosahexaenoic acid and the like.

[0050] In another embodiment, a compound of the disclosure can be administered intracranially or intraventricularly. In another embodiment, osmotic disruption of the blood-brain barrier can be used to effect delivery of the compound to the brain (Nilayer *et al.*, *Proc. Natl. Acad. Sci.* USA 92:9829-9833 (1995)). In yet another embodiment, a compound of the disclosure can be administered in a liposome targeted to the blood-brain barrier. Administration of pharmaceutical agents in liposomes are known (see Langer, *Science* 249:1527-1533 (1990); Treat *et al.*, *Liposomes in the Therapy of infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. pp. 317-327 and 353-365 (1989).

[0051] In another embodiment, a therapeutic formulation comprising an antipurinergic agent can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 317-327 and 353-365 (1989)).

[0052] In another embodiment, a therapeutic formulation comprising a compound of the disclosure can be delivered in a controlled release system. For example, the antipurinergic agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald *et al.*, *Surgery* 88:507 (1980); Saudek *et al.*, *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Fla. (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and

Ball (eds.), Wiley: New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy *et al.*, *Science* 228:190 (1985); During *et al.*, *Ann. Neurol.* 25:351 (1989); Howard *et al.*, *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *e.g.*, the brain, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

[0053] In addition, any of the materials described herein can be administered to any part of the mammal's body including, without limitation, brain, spinal fluid, blood stream, lungs, nasal cavity, intestines, stomach, muscle tissues, skin, peritoneal cavity, and the like. Thus, a compound (*e.g.*, an antipurinergic agent) can be administered by intravenous, intraperitoneal, intramuscular, subcutaneous, extracranial, intrathecal, and intradermal injection, by oral administration, by inhalation, or by gradual perfusion over time. For example, an aerosol preparation can be given to a mammal by inhalation. It is noted that the duration of treatment with the materials described herein can be any length of time from as short as one day to as long as a lifetime (*e.g.*, many years). For example, a formulation comprising a compound of the disclosure can be administered once (or twice, three times, etc.) daily, weekly, monthly, or yearly.

[0054] Preparations for administration can include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include, without limitation, propylene glycol, polyethylene glycol, vegetable oils, and injectable organic esters. Aqueous carriers include, without limitation, water as well as alcohol, saline, and buffered solutions. Preservatives, flavorings, and other additives such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, and the like may also be present.

[0055] A compound of the disclosure is administered in a dose sufficient to provide a therapeutically effective amount to an individual to provide a beneficial effect (*e.g.*, to treat a symptom

of mitochondrial disorders, autism spectrum disorders or chronic fatigue syndrome) of a subject. A therapeutically effective dose of a compound of the disclosure can be determined empirically and depends on the type of treatment, the route of administration, and the size, weight, age and overall health of the patient, as is within the skill of one in the art such as a medical practitioner.

[0056] The amount of an antipurinergic compound (*e.g.*, suramin and/or derivatives thereof) disclosed herein which is administered as a unit dose will depend upon the type of pharmaceutical composition being administered, for example, a solution, a suspension, a gel, a film, an emulsion, a powder, or a sustained-release formulation. The quantity of formulation needed to deliver the desired dose will also depend on the concentration of the compound of the disclosure in the composition. Such determinations are within the skill of one in the art.

[0057] The therapeutic dosage of an antipurinergic compound disclosed herein in the pharmaceutical compositions used in the methods of the disclosure will depend on a number of factors such as the chemical composition and/or modification of the compound, its bioavailability by the chosen route of administration, its efficacy, the desired frequency of administration combined with the desired single dosage of the formulation and whether the compound is administered in combination with other active agent(s). Particularly, the dosage of a compound disclosed herein will be chosen to maximize cognitive functions of a subject. Pharmacological data can be obtained from animal models and clinical trials with normal human volunteers or patients by one with skill in the art.

[0058] As stated above, an effective amount of an antipurinergic compound (*e.g.*, suramin and/or derivative thereof) of the disclosure will depend on the form and composition being used in the method. For example, dosages used for administration can include, but are not limited to, an effective amount within the dosage range of about 1-100mg per dose, typically about 100mg to 1 g per day (*e.g.*, about 10-20 mg/kg).

[0059] Dosages can be administered in a single dose or in multiple doses, for example, dosages can be administered two,

three, four, up to ten times daily depending on the type of treatment as well as on individual susceptibility. Dosages can be administered in a sustained release formulation which may allow for a compound disclosed herein to be administered less frequently such as six times a week, five times a week, four times a week, three times a week, twice a week, or once a week, once a month, once every two months, three months, four months, five months or six months or more. Infrequent administration can be accomplished by sustained release formulations.

[0060] In some embodiments of the disclosure, a composition comprising an antipurinergic compound such as suramin and/or a derivative thereof may further comprise an additional active agent, wherein the compound and the additional active agent(s) are administered as a mixture, separately and simultaneously, or separately in any order. In some examples the composition comprising a compound disclosed herein is administered in combination with at least one additional active agent (*e.g.*, cysteamine and/or cystamine). In other examples, the composition comprising a compound of the disclosure is administered in combination with at least two additional active agents. In a particular embodiment, a composition comprising a compound disclosed herein can also be administered in combination with other classes of compounds, including, but not limited to, sepsis treatments, such as drotrecogin-α; steroidals, such as hydrocortisone; local or general anesthetics, such as ketamine; platelet aggregation inhibitors, such as clopidogrel; HMG-CoA reductase inhibitors (statins), such as atorvastatin; anticoagulants, such as heparin; thrombolytics, such as streptokinase; fibrates, such as clofibrate; bile acid sequestrants, such as colestipol; non-steroidal anti-inflammatory agents (NSAIDs), such as naproxen; cholesteryl ester transfer protein (CETP) inhibitors, such as anacetrapib; anti-bacterial agents, such as ampicillin; anti-fungal agents, such as amorolfine; norepinephrine reuptake inhibitors (NRIs), such as atomoxetine; dopamine reuptake inhibitors (DARIs), such as methylphenidate; sedatives, such as diazepham; norepinephrine-dopamine reuptake inhibitor (NDRIs), such as bupropion; serotonin-norepinephrine-

dopamine-reuptake-inhibitors (SNDRIs), such as venlafaxine; monoamine oxidase inhibitors, such as selegiline; hypothalamic phospholipids; endothelin converting enzyme (ECE) inhibitors, such as phosphoramidon; opioids, such as tramadol; thromboxane receptor antagonists, such as ifetroban; potassium channel openers; thrombin inhibitors, such as hirudin; hypothalamic phospholipids; growth factor inhibitors, such as modulators of PDGF activity; platelet activating factor (PAF) antagonists; anti-platelet agents, such as GPIIb/IIIa blockers (*e.g.*, abdximab, eptifibatide, and tirofiban), P2Y(AC) antagonists (*e.g.*, clopidogrel, ticlopidine and CS-747), and aspirin; low molecular weight heparins, such as enoxaparin; Factor VIIa Inhibitors and Factor Xa Inhibitors; renin inhibitors; neutral endopeptidase (NEP) inhibitors; vasopepsidase inhibitors (dual NEP-ACE inhibitors), such as omapatrilat and gemopatrilat; squalene synthetase inhibitors; fibrates; niacin; anti-atherosclerotic agents, such as ACAT inhibitors; MTP Inhibitors; calcium channel blockers, such as amlodipine besylate; potassium channel activators; alpha-muscarinic agents; beta-muscarinic agents, such as carvedilol and metoprolol; antiarrhythmic agents; diuretics, such as chlorothiazide, hydrochlorothiazide, flumethiazide, hydroflumethiazide, bendroflumethiazide, methylchlorothiazide, trichloromethiazide, polythiazide, benzothiazide, ethacrynic acid, tricrynafen, chlorthalidone, furosenilde, musolimine, bumetanide, triamterene, amiloride, and spironolactone; recombinant tPA, streptokinase, urokinase, prourokinase, and anisoylated plasminogen streptokinase activator complex (APSAC); anti-diabetic agents, such as biguanides (*e.g.* metformin), glucosidase inhibitors (*e.g.*, acarbose), insulins, meglitinides (*e.g.*, repaglinide), sulfonylureas (*e.g.*, glimepiride, glyburide, and glipizide), thiozolidinediones (*e.g.* troglitazone, rosiglitazone and pioglitazone), and PPAR-gamma agonists; mineralocorticoid receptor antagonists, such as spironolactone and eplerenone; growth hormone secretagogues; aP2 inhibitors; phosphodiesterase inhibitors, such as PDE III inhibitors (*e.g.*, cilostazol) and PDE V inhibitors (*e.g.*, sildenafil, tadalafil, vardenafil); protein tyrosine kinase inhibitors; antiinflammatories; antiproliferatives, such as methotrexate, FK506

(tacrolimus, Prograf), mycophenolate mofetil; chemotherapeutic agents; immunosuppressants; anticancer agents and cytotoxic agents (*e.g.*, alkylating agents, such as nitrogen mustards, alkyl sulfonates, nitrosoureas, ethylenimines, and triazenes); antimetabolites, such as folate antagonists, purine analogues, and pyridine analogues; antibiotics, such as anthracyclines, bleomycins, mitomycin, dactinomycin, and plicamycin; enzymes, such as L-asparaginase; farnesyl-protein transferase inhibitors; hormonal agents, such as glucocorticoids (*e.g.*, cortisone), estrogens/antiestrogens, androgens/antiandrogens, progestins, and luteinizing hormone-releasing hormone anatagonists, and octreotide acetate; microtubule-disruptor agents, such as ecteinascidins; microtubule-stabilizing agents, such as pacitaxel, docetaxel, and epothilones A-F; plant-derived products, such as vinca alkaloids, epipodophyllotoxins, and taxanes; and topoisomerase inhibitors; prenyl-protein transferase inhibitors; and cyclosporins; steroids, such as prednisone and dexamethasone; cytotoxic drugs, such as azathiprine and cyclophosphamide; TNF-alpha inhibitors, such as tenidap; anti-TNF antibodies or soluble TNF receptor, such as etanercept, rapamycin, and leflunomide; and cyclooxygenase-2 (COX-2) inhibitors, such as celecoxib and rofecoxib; and miscellaneous agents such as, hydroxyurea, procarbazine, mitotane, hexamethylmelamine, gold compounds, platinum coordination complexes, such as cisplatin, satraplatin, and carboplatin.

[0049] As mentioned above, the disclosure also provides a methods of diagnosing mitochondrial diseases and disorders including chronic fatigue syndrome and autism spectrum disorders using various metabolic signatures or metabolomics information.

[0050] Metabolomics has several advantages over genomics for the diagnosis of complex chronic disease and for growing interest in precision medicine. First, fewer than 2000 metabolites constitute the majority of the parent molecules in the blood that are used for cell-to-cell communication and metabolism, compared to 6 billion bases in the diploid human genome. Second, metabolites reflect the current functional state of the individual. Collective cellular chemistry represents the functional interaction of genes and environment. This is metabolism. In contrast, the genome

represents an admixture of ancestral genotypes that were selected for fitness in ancestral environments. The metabolic state of an individual at the time of illness is produced by both current conditions, age, and the aggregate history, timing, and magnitude of exposures to physical and emotional stress, trauma, diet, exercise, infections, and the microbiome recorded as metabolic memory. Analysis of metabolites may provide a more technically and bioinformatically tractable, physiologically relevant, chemically comprehensive, and cost-effective method of diagnosis of complex chronic diseases. In addition, because metabolomics provides direct small molecule information, the results can provide immediately actionable treatment information using readily available small molecule nutrients, cofactors, and life style interventions. The results presented herein demonstrates that chronic fatigue syndrome has an objectively identifiable chemical signature in both men and women and that targeted metabolomics can be used to uncover biological insights that may prove useful for both diagnosis, and personalized treatment.

[0051] The results presented herein also demonstrate that the metabolic features of chronic fatigue syndrome are consistent with a hypometabolic state. Sphingolipids, glycosphingolipids, phospholipids, purines, microbiome aromatic amino acid and branch chain amino acid metabolites, FAD, and lathosterol were decreased. The decreases in these metabolites correlated with disease severity as measured by Karnofsky scores (see **TABLES 1-4**).

Table 1. The Top 25 Diagnostic Metabolites in Chronic Fatigue Syndrome (Males).

No.	MRM Name	Pathway Name	VIP Score	P Value	FDR	Fold Change	Z Score	KPS* Correlation Spearman r	KPS* Correlation Spearman p
1	PC(18:0/16:0)	Phospholipid Metabolism	2.65	5.32E-05	0.023	0.78	-1.61	0.59	6.43E-05
2	GC(18:1/16:0)	Glycosphingolipid Metabolism	2.48	2.32E-04	0.025	0.59	-1.44	0.63	1.55E-05
3	Ceramide(d18:1/16:0)	Sphingolipid Metabolism	2.43	2.77E-04	0.025	0.69	-1.23	0.59	7.01E-05
4	THC 18:1/24:0	Glycosphingolipid Metabolism	2.39	3.74E-04	0.025	0.69	-1.22	0.55	2.12E-04
5	Ceramide(d18:1/24:2)	Sphingolipid Metabolism	2.51	1.61E-04	0.025	0.56	-1.32	0.54	3.10E-04
6	PI(38:4)	Phospholipid Metabolism	2.39	3.66E-04	0.025	0.78	-1.45	0.53	4.10E-04
7	DHC(18:1/16:0)	Glycosphingolipid Metabolism	2.38	4.04E-04	0.025	0.57	-1.26	0.53	4.72E-04
8	PA(16:0/18:0)	Phospholipid Metabolism	2.35	4.90E-04	0.026	0.75	-1.08	0.49	1.40E-03
9	1-Pyrroline-5-carboxylic acid	P5C, Arginine, Ornithine, Proline	2.26	8.32E-04	0.040	1.45	1.23	-0.53	4.91E-04
10	SM(d18:1/22:1 OH)	Sphingolipid Metabolism	2.20	1.24E-03	0.041	0.78	-1.31	0.53	4.01E-04
11	SM(d18:1/24:2 OH)	Sphingolipid Metabolism	2.21	1.15E-03	0.041	0.76	-1.19	0.48	2.84E-03
12	Ceramide(d18:1/16:1 OH)	Sphingolipid Metabolism	2.20	1.19E-03	0.041	0.71	-1.16	0.44	4.68E-03
13	SM(d18:1/22:0)	Sphingolipid Metabolism	2.20	1.22E-03	0.041	0.72	-0.99	0.43	5.29E-03
14	Ethanolamine	Phospholipid and Plasmalogen Metabolism	2.13	1.79E-03	0.056	0.81	-1.17	0.47	2.12E-03
15	FAD	Vitamin B2 (Riboflavin) Metabolism	2.05	2.83E-03	0.076	0.69	-0.85	0.49	1.29E-03
16	4-Hydroxyphenyllactic acid	Microbiome Metabolism	2.06	2.66E-03	0.076	0.73	-1.10	0.48	1.71E-03
17	Ceramide(d18:1/16:1)	Sphingolipid Metabolism	2.02	3.42E-03	0.085	0.78	-1.16	0.50	1.14E-03
18	Ceramide(d18:1/18:0)	Sphingolipid Metabolism	2.01	3.50E-03	0.085	0.61	-0.92	0.42	6.77E-03
19	SM(d18:1/16:0)	Sphingolipid Metabolism	1.91	5.66E-03	0.090	0.77	-1.01	0.54	3.65E-04
20	SM(d18:1/18:2 OH)	Sphingolipid Metabolism	1.92	5.55E-03	0.090	0.83	-1.02	0.44	4.85E-03
21	L-Serine	1-Carbon, Folate, Formate, Glycine, Serine	1.95	4.86E-03	0.090	1.27	1.25	-0.41	7.96E-03
22	Ceramide(d18:1/24:1)	Sphingolipid Metabolism	1.92	5.59E-03	0.090	0.71	-0.96	0.41	9.37E-03
23	Arginine	P5C, Arginine, Ornithine, Proline	1.91	5.71E-03	0.090	1.30	1.10	-0.40	1.10E-02
24	Methionine sulfoxide	SAM, SAH, Methionine, Cysteine, Glutathione	1.94	4.93E-03	0.090	1.47	0.94	-0.39	1.18E-02
25	Ceramide(d18:1/26:2)	Sphingolipid Metabolism	1.98	4.18E-03	0.090	0.66	-0.95	0.38	1.50E-02
						Decreased	0.72	-1.16	0.49	N = 21
						Increased	1.37	1.13	-0.43	N = 4

*KPS = Karnofsky performance scale
Metabolites that were negatively correlated with KPS scores and elevated in CFS are indicated in red

Table 2. The Top 25 Diagnostic Metabolites in Chronic Fatigue Syndrome (Females).

No.	MRM Name	Pathway Name	VIP Score	P Value	FDR	Fold Change	Z Score	KPS* Correlation Spearman r	KPS* Correlation Spearman p
1	Ceramide(d18:1/25:0)	Sphingolipid Metabolism	2.76	3.64E-05	0.009	0.55	-1.41	0.69	4.63E-07
2	THC 18:1/24:0	Glycosphingolipid Metabolism	2.72	4.83E-05	0.009	0.66	-1.48	0.66	8.49E-06
3	PC(16:0/16:0)	Phospholipid Metabolism	2.69	6.11E-05	0.009	0.79	-1.30	0.68	3.77E-06
4	Lathosterol	Cholesterol, Cortisol, Non-Gonadal Steroids	2.46	3.15E-04	0.028	0.67	-1.16	0.62	3.17E-04
5	Hydroxyproline	Vitamin C (Ascorbate) Metabolism/Collagen	2.44	3.73E-04	0.028	1.66	1.50	-0.59	5.62E-04
6	PI(16:0/16:0)	Phospholipid Metabolism	2.42	4.06E-04	0.028	0.40	-1.19	0.64	1.76E-04
7	Ceramide(d18:1/22:2)	Sphingolipid Metabolism	2.39	5.17E-04	0.031	0.59	-1.05	0.68	3.08E-06
8	Adenosine	Purine Metabolism	2.36	6.15E-04	0.032	0.49	-1.59	0.41	5.91E-03
9	Ceramide(d18:1/24:2)	Sphingolipid Metabolism	2.27	1.02E-03	0.044	0.55	-1.02	0.61	1.33E-05
10	THC 18:1/16:0	Glycosphingolipid Metabolism	2.26	1.10E-03	0.044	0.64	-1.22	0.49	7.85E-04
11	2-Octenoylcarnitine	Fatty Acid Oxidation and Synthesis	2.25	1.14E-03	0.044	0.55	-1.05	0.55	1.19E-04
12	GC(18:1/16:0)	Glycosphingolipid Metabolism	2.23	1.32E-03	0.046	0.66	-1.32	0.45	2.17E-03
13	Phenyllactic acid	Microbiome Metabolism	2.21	1.49E-03	0.048	0.85	-0.83	0.57	5.37E-05
14	Ceramide(d18:1/26:0)	Sphingolipid Metabolism	2.19	1.63E-03	0.048	0.66	-0.93	0.47	1.22E-03
15	Ceramide(d18:1/24:0)	Sphingolipid Metabolism	2.18	1.73E-03	0.048	0.65	-1.03	0.48	1.03E-03
16	DHC(18:1/16:0)	Glycosphingolipid Metabolism	2.16	1.95E-03	0.049	0.69	-1.23	0.44	2.68E-03
17	Ceramide(d18:1/26:2)	Sphingolipid Metabolism	2.16	1.96E-03	0.049	0.58	-0.88	0.54	1.33E-04
18	FAD	Vitamin B2 (Riboflavin) Metabolism	2.10	2.73E-03	0.064	0.73	-0.89	0.36	1.67E-02
19	1-Pyrroline-5-carboxylic acid	PSC, Arginine, Ornithine, Proline	2.08	2.96E-03	0.066	1.37	1.13	-0.35	2.05E-02
20	Ceramide(d18:1/16:0)	Sphingolipid Metabolism	2.03	3.86E-03	0.081	0.65	-0.90	0.47	1.21E-03
21	SM(d18:1/22:2)	Sphingolipid Metabolism	2.01	4.13E-03	0.081	0.74	-0.82	0.38	8.52E-02
22	Adenosine monophosphate	Purine Metabolism	2.01	4.21E-03	0.081	0.82	-0.82	0.40	7.16E-03
23	PC(18:1/22:5)	Phospholipid Metabolism	1.99	4.64E-03	0.085	1.33	0.87	-0.34	2.41E-02
24	PI(38:3)	Phospholipid Metabolism	1.97	5.07E-03	0.089	0.76	-0.93	0.41	5.24E-03
25	PC(22:6/P-18:0)	Plasmalogen Metabolism	1.96	5.37E-03	0.090	1.52	1.29	-0.42	4.08E-03
					Decreased	0.64	-1.10	0.49	N = 21
					Increased	1.47	1.20	-0.40	N = 4

*KPS = Karnofsky performance scale
Metabolites that were negatively correlated with KPS scores and elevated in CFS are indicated in red

Table 3. Diagnostic Metabolites #26-61 in Chronic Fatigue Syndrome (Males).

No.	MRM Name	Pathway Name	VIP Score	P Value	FDR	Fold Change	Z Score	KPS* Correlation Spearman r	KPS* Correlation Spearman p
26	Ceramide(d18:1/25:0)	Sphingolipid Metabolism	1.92	0.005	0.090	0.889	-0.803	0.362	0.022
27	Ceramide(d18:1/22:2)	Sphingolipid Metabolism	1.95	0.005	0.090	0.710	-0.883	0.358	0.023
28	Ceramide(d18:1/22:1)	Sphingolipid Metabolism	1.93	0.005	0.090	0.883	-0.946	0.351	0.026
29	Behenic acid	Very Long Chain Fatty Acid Oxidation	1.84	0.008	0.124	0.852	-0.718	0.279	0.081
30	Hydroxyisocaproic acid	Branch Chain Amino Acid Metabolism	1.83	0.010	0.139	0.756	-0.920	0.339	0.032
31	Uric acid	Purine Metabolism	1.79	0.010	0.142	0.886	-0.672	0.398	0.011
32	Pyroglutamic acid	GABA, Glutamate Metabolism	1.78	0.012	0.146	0.839	-1.432	0.487	0.001
33	SM(d18:1/24:0)	Sphingolipid Metabolism	1.75	0.012	0.146	0.801	-0.829	0.369	0.019
34	Lathosterol	Cholesterol, Cortisol, Non-Gonadal Steroid	1.78	0.011	0.146	0.825	-1.051	0.317	0.046
35	PC(16:0/20:4)	Phospholipid Metabolism	1.77	0.011	0.146	0.828	-1.039	0.295	0.064
36	PC(18:1/22:6)	Phospholipid Metabolism	1.76	0.012	0.146	1.283	0.924	-0.281	0.104
37	SM(d18:1/22:1)	Sphingolipid Metabolism	1.74	0.013	0.146	0.795	-0.918	0.408	0.009
38	Ceramide(d18:1/22:0)	Sphingolipid Metabolism	1.72	0.014	0.159	0.743	-0.785	0.395	0.014
39	SM(d18:1/16:0 OH)	Sphingolipid Metabolism	1.71	0.015	0.165	0.837	-1.012	0.415	0.008
40	Ceramide(d18:1/24:0)	Sphingolipid Metabolism	1.69	0.016	0.169	0.788	-0.822	0.403	0.010
41	SM(d18:1/20:2 OH)	Sphingolipid Metabolism	1.69	0.016	0.170	0.826	-0.974	0.299	0.061
42	PC(18:1/18:1)	Phospholipid Metabolism	1.67	0.017	0.174	0.828	-0.901	0.319	0.046
43	Ceramide(d18:1/16:2 OH)	Sphingolipid Metabolism	1.66	0.018	0.178	0.780	-0.886	0.377	0.016
44	PC(20:5/P-18:0)	Phospholipid Metabolism	1.65	0.019	0.183	1.444	0.902	-0.273	0.088
45	PI(38:3)	Phospholipid Metabolism	1.62	0.021	0.192	0.847	-0.763	0.374	0.017
46	L-Threonine	Amino Acid Metabolism	1.62	0.021	0.192	1.188	0.871	-0.368	0.030
47	2-Methylcitric acid	Isoleucine, Valine, Threonine, or Methionine	1.63	0.021	0.192	0.769	-1.177	0.362	0.022
48	SM(d18:1/22:0 OH)	Sphingolipid Metabolism	1.61	0.022	0.192	0.847	-0.756	0.341	0.032
49	24,25-Epoxycholesterol	Cholesterol, Cortisol, Non-Gonadal Steroid	1.62	0.022	0.192	0.782	-0.859	0.272	0.089
50	Cholesterol	Cholesterol, Cortisol, Non-Gonadal Steroid	1.60	0.023	0.200	0.882	-0.750	0.419	0.007
51	SM(d18:1/18:0)	Sphingolipid Metabolism	1.59	0.024	0.205	0.816	-0.681	0.369	0.011
52	Ceramide(d18:1/24:0 OH)	Sphingolipid Metabolism	1.58	0.026	0.212	0.708	-0.685	0.336	0.034
53	SM(d18:1/22:2)	Sphingolipid Metabolism	1.57	0.026	0.212	0.776	-0.835	0.285	0.074
54	2-Hydroxy-3-methylbutyrate	Branch Chain Amino Acid Metabolism	1.56	0.027	0.212	0.761	-0.710	0.226	0.162
55	Ceramide(d18:1/18:0 OH)	Sphingolipid Metabolism	1.55	0.028	0.214	0.750	-0.683	0.370	0.019
56	Deoxyguanosine	Purine Metabolism	1.55	0.028	0.214	0.769	-0.755	0.257	0.110
57	Gamma-Aminobutyric acid	GABA, Glutamate Metabolism	1.55	0.028	0.214	1.221	0.705	-0.168	0.244
58	Tiglylcarnitine	Branch Chain Amino Acid Metabolism	1.51	0.033	0.230	0.750	-0.725	0.435	0.005
59	SM(d18:1/18:1 OH)	Sphingolipid Metabolism	1.51	0.032	0.230	0.867	-0.926	0.417	0.007
60	Ceramide(d18:1/18:1 OH)	Sphingolipid Metabolism	1.52	0.031	0.230	0.814	-0.795	0.257	0.109
61	SM(d18:1/25:0)	Sphingolipid Metabolism	1.51	0.032	0.230	0.827	-0.625	0.208	0.197
					Decreased	0.789	-0.852	0.348	N = 32
					Increased	1.284	0.851	-0.272	N = 4

*KPS = Karnofsky performance scale
Metabolites that were negatively correlated with KPS scores and were elevated in CFS are indicated in red.

Table 4. Diagnostic Metabolites #26-61 in Chronic Fatigue Syndrome (Females)

No.	MRM Name	Pathway Name	VIP Score	P Value	FDR	Fold Change	Z Score	KPS* Correlation Spearman r	KPS* Correlation Spearman p
26	PC(36:0)	Phospholipid Metabolism	1.93	0.006	0.103	1.274	1.142	-0.375	0.012
27	Chenodeoxycholic acid	Bile Salt Metabolism	1.90	0.007	0.107	0.504	-0.828	0.403	0.007
28	Ceramide(d18:1/20:0)	Sphingolipid Metabolism	1.90	0.007	0.107	0.654	-0.853	0.437	0.003
29	PC(18:1/22:6)	Phospholipid Metabolism	1.88	0.008	0.113	1.373	1.151	-0.392	0.009
30	Ceramide(d18:1/22:1 OH)	Sphingolipid Metabolism	1.86	0.009	0.116	0.637	-0.741	0.480	0.001
31	Ceramide(d18:1/18:2 OH)	Sphingolipid Metabolism	1.86	0.009	0.116	0.662	-0.785	0.450	0.002
32	Ceramide(d18:1/22:0)	Sphingolipid Metabolism	1.83	0.010	0.129	0.667	-0.801	0.445	0.002
33	Ceramide(d18:1/18:0)	Sphingolipid Metabolism	1.82	0.010	0.129	0.567	-0.730	0.415	0.005
34	Ceramide(d18:1/16:1 OH)	Sphingolipid Metabolism	1.82	0.010	0.129	0.704	-0.809	0.429	0.004
35	Ceramide(d18:1/24:2 OH)	Sphingolipid Metabolism	1.79	0.012	0.136	0.644	-0.766	0.457	0.002
36	SM(d18:1/16:0)	Sphingolipid Metabolism	1.79	0.012	0.136	0.748	-0.890	0.274	0.072
37	Ceramide(d18:1/24:1)	Sphingolipid Metabolism	1.77	0.013	0.144	0.685	-0.813	0.500	0.001
38	PI(34:1)	Phospholipid Metabolism	1.75	0.014	0.152	0.765	-0.792	0.362	0.016
39	PI(36:0)	Phospholipid Metabolism	1.72	0.016	0.171	0.742	-0.781	0.369	0.014
40	SM(d18:1/20:1)	Sphingolipid Metabolism	1.71	0.016	0.171	0.775	-0.843	0.415	0.005
41	Adipoylcarnitine	Fatty Acid Oxidation and Synthesis	1.71	0.017	0.172	1.401	1.338	-0.380	0.016
42	2-Arachidonylglycerol	Endocannabinoid Metabolism	1.69	0.018	0.174	0.681	-0.749	0.428	0.004
43	THC 18:1/18:0	Glycosphingolipid Metabolism	1.69	0.018	0.174	0.756	-0.906	0.346	0.021
44	Ceramide(d18:1/22:1)	Sphingolipid Metabolism	1.68	0.019	0.180	0.680	-0.712	0.414	0.005
45	PI(34:0)	Phospholipid Metabolism	1.66	0.020	0.183	0.757	-0.714	0.380	0.011
46	Ceramide(d18:1/26:1 OH)	Sphingolipid Metabolism	1.66	0.020	0.183	0.655	-0.717	0.469	0.001
47	PI(38:4)	Phospholipid Metabolism	1.65	0.021	0.185	0.812	-0.665	0.351	0.020
48	Hydroxyisocaproic acid	Branch chain amino acids	1.64	0.022	0.193	0.720	-0.618	0.381	0.011
49	PC(16:0/22:6)	Phospholipid Metabolism	1.61	0.025	0.211	1.249	0.711	-0.306	0.043
50	PC(30:0)	Phospholipid Metabolism	1.60	0.025	0.211	0.840	-0.729	0.358	0.017
51	Cobalamin	Vitamin B12 (Cobalamin) Metabolism	1.60	0.026	0.211	0.803	-1.062	0.157	0.308
52	Ceramide(d18:1/20:1 OH)	Sphingolipid Metabolism	1.59	0.026	0.213	0.659	-0.603	0.447	0.002
53	dAMP	Purine Metabolism	1.59	0.027	0.214	0.902	-0.865	0.326	0.031
54	Arginine	P5C, Arginine, Ornithine, Proline	1.56	0.030	0.233	1.206	0.821	-0.131	0.397
55	PC(38:5)	Phospholipid Metabolism	1.54	0.031	0.233	1.229	0.710	-0.298	0.051
56	Gluconic acid	Microbiome Metabolism	1.54	0.032	0.233	1.261	0.894	-0.257	0.092
57	PG(32:2)	Phospholipid Metabolism	1.54	0.032	0.233	0.492	-0.565	0.364	0.015
58	Vitamin K2	Microbiome Metabolism	1.53	0.033	0.233	1.168	0.683	-0.225	0.145
59	PC(16:0/18:2)	Phospholipid Metabolism	1.53	0.033	0.233	0.922	-0.599	0.344	0.022
60	Glucosamine 6-phosphate	Amino sugars	1.52	0.034	0.233	1.307	0.688	-0.285	0.060
61	PI(36:1)	Phospholipid Metabolism	1.52	0.034	0.233	0.773	-0.691	0.329	0.029
					Decreased	0.686	-0.737	0.376	N = 27
					Increased	1.263	0.792	-0.263	N = 9

*KPS = Karnofsky performance scale
Metabolites that were negatively correlated with KPS scores and were elevated in CFS are indicated in red.

[0052] Research has been performed on the hypometabolic phenotype in various biologic systems, including dauer, diapause, hibernation, estivation, torpor, ischemic preconditioning, ER stress, the unfolded protein response, autophagy, and caloric restriction. Dauer, which means persistence or long-lived in German, is an example of one well-studied system. The developmental stage of dauer is a hypometabolic state capable of living efficiently by altering a number of basic mitochondrial functions, fuel preferences, behavior, and physical features. Dauer is comprised of an evolutionarily conserved and synergistic suite of metabolic and structural changes that are triggered by exposure to adverse environmental conditions. Entry into dauer confers a survival advantage in harsh conditions. When the dauer response is blocked by certain mutations (dauer defectives), animals are short-lived when exposed to environmental stress. These mutations show that the latent ability to enter into a hypometabolic state during times of environmental threat is adaptive, even though it comes at the cost of decreasing the optimal functional capacity. Similar to dauer, chronic fatigue syndrome appears to represent a hypometabolic survival state that is triggered by environmental stress. The metabolic features of chronic fatigue syndrome and dauer correspond to the same pathways that characterize the acute cell danger response and metabolic syndrome, but are regulated in the opposite direction. For example, cholesterol, phospholipids, and uric acid are often elevated in the acute cell danger response and metabolic syndrome, but these metabolites were decreased in chronic fatigue syndrome patients. A prediction based on these findings is that patients with chronic fatigue syndrome would be more resistant to the constellation of hypertension, dyslipidemia, central obesity, and insulin resistance that increase all-cause mortality associated with metabolic syndrome, but at the cost of significant long-term disability, pain, and suffering.

[0053] As identified herein, all of the metabolic abnormalities in chronic fatigue syndrome were either directly regulated by redox, or the availability of NADPH. About 60% of NADPH is produced by the pentose phosphate pathway under baseline conditions. The other 40% is produced by the combined flux through 5 $NADP^+$ dependent

enzymes: 1) Malic enzyme (ME), 2) Isocitrate dehydrogenase (IDH), 3) Glutamate dehydrogenase (GDH), 4) Nicotinamide nucleotide transhydrogenase (NNT), and 5) Methylene tetrahydrofolate dehydrogenase (MTHFD2L). Each of these enzymes has at least one mitochondrial isoform and is known to be upregulated under conditions of environmental or developmental stress. It has recently been shown that mitochondrial MTHFD2L is responsible for producing 20-40% of cellular NADPH by the oxidation of methylene tetrahydrofolic acid to 10-formyl tetrahydrofolate. These data show that folates are not only important in methylation reactions, but also in regulating intracellular redox and NADPH levels (see **FIG. 8**). A number of single nucleotide polymorphisms (SNPs) have been identified in the MTHFD2L gene that correlate with the cell danger response and IL1β production triggered by smallpox vaccination. Mitochondrial pools of NADPH are in continuous communication with NADH levels through the enzyme nicotinamide nucleotide transhydrogenase (NNT). Therefore, NADPH acts as a global barometer of cellular fuel status by interrogating both mitochondrial electron (NADH) consumption, and the availability of cytoplasmic reducing equivalents as NADPH. When mitochondrial electron transport decreases for any reason, fewer molecules of oxygen are converted to water (H_2O) by cytochrome c oxidase. If capillary delivery of oxygen to the cell is unchanged, the concentration of dissolved oxygen rises in the cell like water in a bowl in response to instantaneous decreases in mitochondrial oxygen consumption. This activates scores of enzymes that are kinetically regulated by the availability of dissolved oxygen and can act as oxygen sensors. Some of these include NADPH oxidases like Nox4 that make hydrogen peroxide (H_2O_2) from the excess diatomic oxygen (O_2) to initiate the oxidative shielding response. When reduced (NADPH) and total (NADPH plus $NADP^+$) pools are low, sterol, fatty acid, protein, and nucleotide synthesis fall to baseline survival levels. When NADPH levels are higher, metabolism is shifted from persistence to normal cell function and growth, anabolic pathways are stimulated, biomass is created, and carbons and electrons are stored as biopolymers for cell growth and repair in the form of lipids, protein, glycogen, glycans, and nucleic acids.

[0054] It is important to emphasize that NADPH is neither the problem nor the solution by itself. It is a messenger and cofactor. NADPH cannot work without the availability of hundreds of carbon skeletons of intermediary metabolism needed to carry out the message—the signal that fuel stores are either replete or limiting and metabolism must be adjusted accordingly. Specifically, NADPH cannot be simply added as a nutritional supplement to produce the tidal change in metabolism needed to shift the dauer state of chronic fatigue syndrome to normal health. Incremental improvements in NADPH production could theoretically be supported by interventions directed at folate, B12, glycine, and serine pools, and B6 metabolism (see **FIG. 8**), however the safety and efficacy of these manipulations have not yet been tested in a rigorously designed clinical trial. Ultimately, effective treatments for chronic fatigue syndrome are likely to be achieved by careful attention to nutrition, metabolism, triggers, stressors, and physical activity as an integrated system, combined with a systems biological understanding of the triggers of the cell danger response, and dauer entry and exit.

[0055] Chronic fatigue syndrome has a chemical signature that can be identified using targeted plasma metabolomics. Receiver operator characteristic (ROC) curve analysis showed a diagnostic accuracy that exceeded 90%. The pattern and directionality of these changes showed that chronic fatigue syndrome is a conserved, hypometabolic response to environmental stress similar to dauer. Only about 25% of the metabolite disturbances found in each person were needed for the diagnosis of CFS. About 75% of the metabolite abnormalities were unique to the individual and useful in guiding personalized treatment. The finding of an objective chemical signature in chronic fatigue syndrome helps to remove diagnostic uncertainty, will help clinicians monitor individualized responses to treatment, and will facilitate multi-center clinical trials.

[0056] In some embodiments, small molecule metabolite profile(s) or metabolomic profile(s) from a test subject or patient is/are compared to that/those of a control small molecule or control metabolomic profile. In some embodiments, detected amounts of metabolites are compared to normal or control amounts, such as

amounts detected performing similar methods on a normal or control sample. A normal or control sample in some aspects is one obtained from a subject who does not have, or is known not to have developed, *e.g.*, subsequent to obtaining the sample, the disease or disorder being assessed, or having a relatively low risk for the same. Such comparisons can be made by individuals, *e.g.*, visually, or can be made using software designed to make such comparisons, *e.g.*, a software program may provide a secondary output which provides useful information to a user. For example, a software program can be used to confirm a profile or can be used to provide a readout when a comparison between profiles is not possible with a "naked eye". The selection of an appropriate software program, *e.g.*, a pattern recognition software program, is within the ordinary skill of the art. An example of such a program is Pirouette® by InfoMetrix®.

[0057] Also as used herein, the term "test metabolite" is intended to indicate a substance the concentration of which in a biological sample is to be measured; the test metabolite is a substance that is a by-product of or corresponds to a specific end product or intermediate of metabolism.

[0058] The collection of metabolomic data, including small molecule metabolite profiles and metabolic profiles, can be through, for example, a single technique or a combination of techniques for separating and/or identifying small molecules known in the art. Small molecule metabolites can be detected in a variety of ways known to one of skill in the art, including the refractive index spectroscopy (RI), ultra-violet spectroscopy (UV), fluorescence analysis, radiochemical analysis, near-infrared spectroscopy (near-IR), nuclear magnetic resonance spectroscopy (NMR), light scattering analysis (LS), mass spectrometry, pyrolysis mass spectrometry, nephelometry, dispersive Raman spectroscopy, gas chromatography, gas chromatography combined with mass spectrometry, high pressure liquid chromatography (HPLC), liquid chromatography combined with mass spectrometry, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) combined with mass spectrometry, ion spray spectroscopy combined with mass spectrometry, capillary electrophoresis, NMR and IR detection.

[0059] A biological sample obtained from a subject can be prepared for use in one or more of the foregoing identification/detection methods. The biological sample, can be divided for multiple parallel measurements and/or can be enriched for a particularly type of small molecule metabolite(s). For example, different fractionation procedures can be used to enrich the fractions for small molecules. For example, small molecules obtained can be passed over several fractionation columns. The fractionation columns will employ a variety of detectors used in tandem or parallel to generate the small molecule metabolite profile.

[0060] For example, to generate a small molecule metabolite profile of water soluble molecules, the biological sample will be fractionated on HPLC columns with a water soluble array. The water soluble small molecule metabolites can then be detected using fluorescence or UV detectors to generate the small molecule metabolite profiles. For detecting non water soluble molecules, hydrophobic columns can also be used to generate small molecule metabolite profiles. In addition, gas chromatography combined with mass spectroscopy, liquid chromatography combined with mass spectroscopy, MALDI combined with mass spectroscopy, ion spray spectroscopy combined with mass spectroscopy, capillary electrophoresis, NMR and IR detection are among the many other combinations of separation and detection tools can be used to generate small molecule metabolite profiles.

[0061] Provided are methods to diagnose and/or provide predictive and/or risk information about chronic fatigue syndrome (CFS) and/or mitochondrial related disease by analyzing metabolites found in easily obtained biospecimens (*e.g.*, blood, urine). In one embodiment, the methods of the disclosure allows clinicians to stratify subjects according to the risk of CFS or the occurrence of CFS. In one embodiment, the methods use high performance liquid chromatography (HPLC) chromatography, tandem Mass Spectrometry (LC-MS/MS), and analytical statistical techniques to identify and analyze metabolomic profiles.

[0062] The methods of the disclosure can utilize the measurement of a thousand or more metabolites (*e.g.*, up to 2500 or more) or fewer than 2500 (*e.g.*, 15-30, 30-60, 60-100, 100-200, 200-500, 500-

1000, 1000-1500, 1500-2000, 2000-2500 and any number there between 15 and 2500). While several hundred small molecule metabolites can be measured, in practice 30 or fewer small molecule metabolites may be sufficient for diagnostic and prognostic purposes. Furthermore, the small molecule metabolites being measured can include more than one metabolite from a particular metabolic pathway. Thus, for example, 30 or fewer small molecule metabolites may be representative of 15 or fewer metabolic pathways (*e.g.*, more than one metabolite is from the same catabolic or anabolic pathway). Analysis of these metabolites may be performed using HPLC and Mass Spectrometry or with techniques other than HPLC and/or Mass Spectrometry.

[0063] For example, small molecule metabolites are collected and subjected to chemical extraction. Internal isotopically labeled standards can be added to the sample and injected into an HPLC-Mass Spectrometer. Small molecule metabolites are separated and then measured via mass spectrometry. Subjects having or at risk of having CFS (or other mitochondrial disease or disorder to be analyzed) have a distinct set of metabolites (*e.g.*, a "CFS small molecule metabolite profile") that are indicative of a CFS metabolomic profile that distinguish them from healthy controls.

[0064] In some embodiments, the small molecule metabolites are collected, processed to non-naturally occurring analytes (*e.g.*, mass fragments), the analytes processed to determine their identities and the data plotted in 2D or 3D coordinates and compared to a control small molecule metabolite profile or a control metabolomics profile, which can be plotted on the same coordinate system (*e.g.*, a mass spectroscopy plot, an HPLC plot or the like). This plot can then be output to a user or medical technician for analysis.

[0065] For example, the method of the disclosure includes obtaining a small molecule metabolite profile from a test subject, identifying small molecule analytes that are over produced or under produced (including presence and absence) generating a metabolomics profile which is indicative of the activity of the various metabolic pathways associated with the small molecule metabolites and comparing metabolomics profiles of the test subject/patients to

a standard, normal control metabolomics profile. In one embodiment, an over or under production of a metabolite compared to a control by at least 2 standard deviations is indicative of an aberrant metabolic pathway. In another embodiment, a difference in the amount of metabolite by 10% or more (*e.g.*, 10%-100% or more) compared to a control value is indicative of an aberrant metabolic pathway. The method thus involves identifying the small molecules which are present in aberrant amounts in the test small molecule metabolite profile. The small molecules present in aberrant amounts are indicative of a diseased or dysfunctional metabolic pathway.

[0066] An "aberrant amount" includes any level, amount, or concentration of a small molecule metabolite, which is different from the level of the small molecule of a standard sample by at least 1 standard deviation (typically 2 standard deviations are used). The aberrant amount can be higher or lower than the control amount.

[0067] The method of the disclosure includes measuring a plurality of pathways and metabolites. **Tables 2-4**, provides an exemplary list of such pathways and an exemplary number of metabolites that can be measure in each pathway.

[0068] In certain embodiments, the methods disclosed herein can be used to characterize CFS subjects based upon metabolomics profiles. In some embodiments, a method comprises obtaining a sample from a subject (*e.g.*, blood, urine, tissue); preparing the sample (*e.g.*, extracting, enriching, and the like) metabolites, which can include the addition of internal standards; performing a technique to quantitate metabolites in the sample (*e.g.*, HPLC, Mass spectroscopy, LC-MS/MS, and the like); identifying aberrant quantities of metabolites; and generating heat maps, biochemical pathway visualization or other data output for analysis. The resulting data output in some aspects is then compared to a "normal" or "control" data. Using a CFS metabolomics profile, 5 to 61 metabolites were determined in one study to be useful in characterizing a CFS subject (see, *e.g.*, **Tables 11** and **12**). In a further embodiment, the number of metabolites that can be used to characterize a CFS subject is selected from 7, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or a range between any two of the

foregoing numbers. It should be noted herein, that CFS metabolomics profiles can be further differentiated based upon the sex of the subject, wherein the optimal metabolomics profile that is indicative of the risk that a male subject has CFS is not necessarily likewise optimal for a female subject, or vice versa. Moreover, based upon the results presented in this disclosure, it is clear that there is difference between metabolomics profiles for CFS between male and female subjects.

[0069] In some embodiments, the disclosure provides methods of using metabolomics profile information to study the effectiveness of a therapy or intervention for CFS, such as the administration of antipurinergic compounds like suramin and/or a derivative thereof to a subject. For example, by obtaining and comparing the metabolomics profiles, amounts of metabolites, and/or alterations in pathways, from a subject having CFS and a control population, certain aberrant small molecule metabolites can be identified and their corresponding metabolic pathways identified. A therapy (*e.g.*, antipurinergic compounds) can then be administered or provided to a subject having CFS and a small molecule metabolite profile and metabolomics profile obtain from the subject during or after therapy. The small molecule and metabolomics profiles from the subject are analyzed with particular attention to any previously identified aberrant measurement from the disease state. A change in the small molecule metabolite or metabolomics profile of the treated subject that is more consistent with a normal control profile would be indicative of an effective therapy. By "more consistent" means that the aberrant values or pathway are trending towards or are within a desired range considered "normal" for the population.

[0070] Based upon the genetic model disclosed herein, use of antipurinergic therapy (APT) (*e.g.*, administration of suramin) would be expected to reset metabolism and facilitate "healing" in chronic fatigue syndrome subject. The usefulness of the antipurinergic therapy is ultimately traceable to mitochondria which is regulated by purinergic signaling. Treatment with antipurinergic compounds is expected to turn off the cell danger response (CDR) which is controlled by purinergic signaling, which

has been identified herein as a component of chronic fatigue syndrome. The primary pharmacologic mechanism of action of antipurinergic compounds, like suramin, is as a competitive antagonist of extracellular ATP and other nucleotides, acting at purinergic receptors. For example, antipurinergic agents that block P2X/P2Y signaling or extracellular ATP, ADP, and/or P1-receptor (Adenosine) signaling by extracellular adenosine or AMP. Examples of antipurinergic compounds that can be used in the methods disclosed herein, include but are not limited to, P2Y inhibitors like 2-methylthioladenosine monophosphate, A3P5PS, AMPαS, diadenosine tetraphosphate, AR-C66096, AR-C67085MX, AR-C69931MX, AR-C118925XX, C1330-7, Cangrelor, Clopidrogel, Elinogrel, IP51, MRS-2179, MRS-2211, MRS-2279, MRS-2395, MRS-2500, MRS-2578, NF-157, NF-340, 2,2'-pyridylisatogen tosylate, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid, prasugrel, PSB-0739, RB-2, regrelor, suramin, ticagrelor, and ticlopidine.

[0071] The following examples are intended to illustrate but not limit the disclosure. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

Example 1

[0072] **Sample Collection.** Venous blood was collected between the hours of 8 am and 5 pm, at least 3 hours after the last meal, into lithium-heparin vacutainer tubes (BD #367884). Plasma was separated by centrifugation at 900g x 10 minutes at room temperature within one hour of collection. The resulting fresh lithium-heparin plasma was transferred to labeled 1.2 mL or 2.0 mL externally threaded, cryotubes with a minimum headspace air gap for storage at -80 °C for analysis.

[0073] **Metabolomics.** Samples were analyzed on an AB SCIEX QTRAP 5500 triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization (ESI) source, Shimadzu LC-20A UHPLC system, and a PAL CTC autosampler. Typically, 90 µL of plasma was thawed on ice and transferred to a 1.7 mL Eppendorf tube. Five (5.0) µL of a cocktail containing 25-35 commercial stable isotope internal standards, and 5.0 µL of 57 stable isotope internal standards that

were custom-synthesized in *E. coli* and *S. cerevisiae* by metabolic labeling with ^{13}C-glucose and ^{13}C-bicarbonate, were added, mixed, and incubated for 10 min at 20 °C to permit small molecules and vitamins in the internal standards to associate with plasma binding proteins. Macromolecules (protein, DNA, RNA, glycans, etc.) were precipitated by extraction with 4 volumes (400 µL) of cold (-20 °C), acetonitrile:methanol (50:50) (LCMS grade, Cat# LC015-2.5 and GC230-4, Burdick & Jackson, Honeywell), vortexed vigorously, and incubated on crushed ice for 10 min, then removed by centrifugation at 16,000g x 10 min at 4°C. The supernatants containing the extracted metabolites and internal standards in the resulting 40:40:20 solvent mix of acetonitrile:methanol:water were transferred to labeled cryotubes and stored at -80 °C for LC-MS/MS analysis.

[0074] LC-MS/MS analysis was performed by scheduled multiple reaction monitoring (sMRM) under Analyst v1.6.2 software control in both negative and positive mode with rapid polarity switching (50 ms). Nitrogen was used for curtain gas (set to 30), collision gas (set to high), ion source gas 1 and 2 (set to 35). The source temperature was 500 °C. Spray voltage was set to -4500 V in negative mode and 5500 V in positive mode. The values for Q1 and Q3 mass-to-charge ratios (m/*z*), declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were determined and optimized for each MRM for each metabolite. Ten microliters of extract was injected by PAL CTC autosampler via a 10 µL stainless steel loop into a 250 mm × 2.0 mm, 4 µm polymer based NH_2 HPLC column (Asahipak NH2P-40 2E, Showa Denko America, Inc., NY) held at 25 °C for chromatographic separation. The mobile phase was solvent A: 95% water with 20 mM $(NH_4)_2CO_3$ (Sigma, Fluka Cat# 74415-250G-F), 5% acetonitrile, and 38 mM NH_4OH (Sigma, Fluka Cat# 17837-100ML), final pH 9.75; solvent B: 100% acetonitrile. Separation was achieved using the following gradient: 0-3.5 min: 95%B, 3.6-8 min: 85% B, 8.1-13 min: 75% B, 13.5-35 min: 0% B, 36-46 min: 95% B, 46.1 min: end. The flow rate was 200 µL/min. Pump pressures ranged from 920-2600 psi over the course of the gradient. All the samples were kept at 4 °C during analysis. The chromatographic peaks were identified using

MultiQuant (v3, AB Sciex), confirmed by manual inspection, and the peak areas integrated.

[0075] Metabolomics Quality Control. Two levels of reproducibility were routinely quantified before passing metabolomic results for data analysis. In the first level, the variability introduced by pipetting of stable-isotope labeled internal standards and sample extraction efficiency was quantified. The foregoing was termed work-flow quality control. Starting from a standardized lot of pooled human plasma, it was divided this into 3 samples. Stable isotope-labeled internal standards (SIL-ISDs) were added to each sample and extracted. Each extract was then injected 3 times each day for 3 days. Measurements of 27 stable isotope-labeled standards and 50 endogenous metabolites were monitored daily for process control. Reproducibility was quantified by calculating the within day, and within plus between day Pearson correlations, and median of the relative standard deviations (RSD) (**see Table 5**).

Table 5. Metabolomics Quality Control: Work-Flow Reproducibility.

Daily QC Metabolites	Within Day Pearson r	Within + Between Day Pearson r	Within Day Median RSD	Within + Between Day Median RSD
27 Stable Isotope (^{13}C, ^{15}N, ^{2}H)-Labeled Internal Standards	0.999	0.987	6.2%	9.1%
50 Endogenous Metabolites in Standard Pooled Human Plasma	0.998	0.981	8.4%	10.7%

[0076] In the second level of quality control the metabolite areas under the curve (AUCs) of 420 to 460 targeted molecules were quantified by scheduled Multiple Reaction Monitoring (sMRM) using at least 2 mass transitions per molecule, in 2 representative plasma samples injected in triplicate on 3 days (**see Table 6**).

Table 6. Metabolomics Quality Control: Reproducibility of Metabolite Quantification

Representative Plasma Samples	Within Day Pearson r	Within + Between Day Pearson r	Within Day Median RSD	Within + Between Day Median RSD
458 Metabolites in Sample 1	0.998	0.983	10.2%	12.1%
458 Metabolites in Sample 2	0.997	0.981	11.3%	12.5%

[0077] Metabolic Pathway Visualization in Cytoscape. A rendering of mammalian intermediary metabolism in Cytoscape v 3.1.1 (http:]]---[[//www.cytoscape.org/) was constructed. Pathways represented in

the network for Chronic Fatigue Syndrome included the 20 metabolic pathways associated with the cell danger response(36) that were dysregulated. Nodes in the Cytoscape network represent metabolites within the pathways and have been colored according to the Z-score. The Z-score was computed as the arithmetic difference between the mean concentration of each metabolite in Chronic Fatigue and controls, divided by the standard deviation in the controls. Node colors were arranged on a red-green color scale with green representing ≤-2.00 Z-score, red representing ≥+2.00 Z-score, and with a zero (0) Z-score represented as white. The sum of the VIP scores from metabolites with VIP scores >1.5 for each metabolic pathway was displayed percentage of total biochemical impact next to the pathway name.

[0078] Demographics. 84 subjects in the study were recruited from 51 zip codes around the US and Canada. Eighty subjects were from California. All chronic fatigue syndrome subjects met the 2015 diagnostic criteria published by the Institute of Medicine (IOM), and the Canadian working group. Although the Institute of Medicine has suggested the use of the new name, "systemic exertion intolerance disease" (SEID), chronic fatigue syndrome (CFS) will be referred herein. The average age of men with CFS in this study was 53 (+/- 2.8) (see **TABLE 7**). The average age of the women with CFS was 52 (+/- 2.5). The average age of onset was 30 (+/- 2.6) years for the men and 33 (+/- 2.3) years for the women. The average duration of illness was 21 (+/- 3.0) years for men and 17 (+/- 2.3) years for women. The Karnofsky quality of life performance score for men was 62 (+/- 3.2), and 54 (+/- 3.3) for women (see **Table 7**).

TABLE 7. DEMOGRAPHICS

	Males			Females		
Parameters	Chronic Fatigue Mean (SEM) [Range]	Controls Mean (SEM) [Range]	p-Value	Chronic Fatigue Mean (SEM) [Range]	Controls Mean (SEM) [Range]	p-Value
Subject Number (N = 84)	22	18		23	21	
Age (years)	53 (2.8) [21-67]	53 (3.5) [23-69]	ns	52 (2.5) [20-67]	48 (2.8) [25-69]	ns
Age of Onset (years)	30 (2.6) [13-54]	n/a	n/a	33 (2.3) [7-52]	n/a	n/a
Duration of Illness (years)	21 (3.0) [3-49]	n/a	n/a	17 (2.3) [2-40]	n/a	n/a
Karnofsky Performance Score	62 (3.2) [30-90]	100 (0) [100]	$4x10^{-13}$	54 (3.3) [30-90]	100 (0.5) [90-100]	$3x10^{-16}$
Number of Medications	4.1 (0.9) [0-16]	0.2 (0.2) [0-3]	0.0005	4.6 (0.9) [0-20]	0.3 (0.1) [0-2]	$6x10^{-6}$
Years of Education[a]	16 (0.8) [8-21]	18 (0.9) [10-25]	ns	16 (0.6) [9-21]	16 (0.8) [11-25]	ns
BMI	25.0 (0.7) [17-31]	26.7 (0.8) [21-34]	ns	24.6 (1.2) [18-44]	23.8 (0.8) [19-32]	ns
Ethnicity						
White/Non-Hispanic	22	16	ns	22	19	ns
Hispanic	0	2		0	1	
Asian	0	0		0	1	
Native American	0	0		1	0	
African American	0	0		0	0	

[a]Years of education: HS=12; AA=14; Bachelor's=16; Master's=18; JD=19; MD, DO, or ND=20; PhD=21; MD-PhD=25

[0079] Fatty Acid and Endocannabinoid Metabolism was Disturbed in Females. Plasma adipoylcarnitine is a six-carbon dicarboxylic acid (C_6DC) carnitine ester that was increased in females with chronic fatigue syndrome, but not in males (see **TABLES 8-9**).

Table 8. Biochemical Pathway Abnormalities in Chronic Fatigue Syndrome (Male)

No.	Pathway Name	Measured Metabolites in the Pathway (N)	Expected Pathway Proportion (P = N/431)	Expected Hits in Sample of 61 (P * 61)	Observed Hits in the Top 61 Metabolites	Fold Enrichment (Obs/Exp)	Impact (Sum VIP Score)	Fraction of Impact Explained (% of 114.7)	Increased	Decrea
1	Sphingolipids	72	0.167	10.2	30	2.9	55.7	49%	0	30
2	Phospholipids	76	0.176	10.8	9	0.8	18.0	16%	2	7
3	P5C, Arginine, Ornithine, Proline	6	0.014	0.8	4	4.7	7.5	7%	3	1
4	Glycosphingolipids	13	0.030	1.8	3	1.6	7.2	6%	0	3
5	Cholesterol, Cortisol, Non-Gonadal Steroids	15	0.035	2.1	3	1.4	5.0	4%	0	3
6	Branch Chain Amino Acids	10	0.023	1.4	3	2.1	4.9	4%	0	3
7	Purines	19	0.044	2.7	2	0.7	3.3	3%	0	2
8	Microbiome Metabolism	20	0.046	2.8	1	0.4	2.1	2%	0	1
9	Vitamin B2 (Riboflavin)	2	0.005	0.3	1	3.5	2.1	2%	0	1
10	Serine, 1-Carbon Metabolism	5	0.012	0.7	1	1.4	1.9	2%	1	0
11	SAM, SAH, Methionine, Cysteine, Glutathione	13	0.030	1.8	1	0.5	1.9	2%	1	0
12	Very Long Chain Fatty Acid Oxidation	2	0.005	0.3	1	3.5	1.8	2%	0	1
13	Propiogenic Amino Acids	2	0.005	0.3	1	3.5	1.6	1%	0	1
14	Threonine Metabolism	4	0.009	0.6	1	1.8	1.6	1%	1	0
								Subtotal	8	53
								Total	61	

Table 9. Biochemical Pathway Abnormalities in Chronic Fatigue Syndrome (Female)

No.	Pathway Name	Measured Metabolites in the Pathway (N)	Expected Pathway Proportion (P = N/421)	Expected Hits in Sample of 61 (P * 61)	Observed Hits in the Top 61 Metabolites	Fold Enrichment (Obs/Exp)	Impact (Sum VIP Score)	Fraction of Impact Explained (% of 117.3)	Increased	Decrea
1	Sphingolipids	71	0.169	10.29	21	2.0	41.1	35%	0	21
2	Phospholipids	77	0.183	11.16	17	1.5	31.0	26%	6	11
3	Glycosphingolipids	12	0.029	1.74	5	2.9	11.1	9%	0	5
4	Purines	20	0.048	2.90	3	1.0	6.0	5%	0	3
5	Microbiome Metabolism	21	0.050	3.04	3	1.0	5.3	5%	2	1
6	Fatty Acid Oxidation and Synthesis	36	0.086	5.22	2	0.4	4.0	3%	1	1
7	P5C, Arginine, Ornithine, Proline	6	0.014	0.87	2	2.3	3.6	3%	2	0
8	Cholesterol, Cortisol, Non-Gonadal Steroids	16	0.038	2.32	1	0.4	2.5	2%	0	1
9	Collagen/Hydroxyproline Metabolism	2	0.005	0.29	1	3.5	2.4	2%	1	0
10	Vitamin B2 (Riboflavin)	2	0.005	0.29	1	3.5	2.1	2%	0	1
11	Bile Salt Metabolism	7	0.017	1.01	1	1.0	1.9	2%	0	1
12	Endocannabinoids	2	0.005	0.29	1	3.5	1.7	1%	0	1
13	Branch Chain Amino Acids	10	0.024	1.45	1	0.7	1.6	1%	0	1
14	Vitamin B12 (Cobalamin) Metabolism	2	0.005	0.29	1	3.5	1.6	1%	0	1
15	Amino-Sugar, Galactose, & Non-Glucose	4	0.010	0.58	1	1.7	1.5	1%	1	0
								Subtotal	13	48
								Total	61	

[0080] Elevations in adipoylcarnitine are sensitive indicator of decrease in riboflavin dependent mitochondrial beta oxidation of fatty acid, and fasting. Fasting, or decreased intracellular allocation of fatty acids for oxidation in mitochondria results in the induction of the peroxisomal enzyme known as the NAD^+ activated, L-bifunctional enzyme (enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase, EHHADH). This enzyme is required for the synthesis of medium chain dicarboxylic acids like adipic acid. Spurious elevations of adipoylcarnitine can be seen as the result of high volume consumption of adipic acid-containing gelatin. Plasma 2-arachidinoyl glycerol (2AG) was decreased in females with CFS, but not in males (see **TABLES 8-9**). 2AG is a natural cannabinoid agonist of CB_1 signaling. Inhibition of CB_1 receptors is known to inhibit fatty acid oxidation and increase plasma levels of adipoyl carnitine. The observed decrease in 2AG might contribute to decreased oxidation of adipic acid and increased plasma adipoylcarnitine.

[0081] Plasma 2-octenoylcarnitine is an eight-carbon mono-unsaturated fatty acid (C_8:1) carnitine ester that was decreased in females with chronic fatigue syndrome, but not in males (see **TABLES 8-9**). Elevations of octenoylcarnitine are seen in calorie excess conditions such as obesity and metabolic syndrome. Decreased octenoic acid is also known to inhibit the susceptibility to Herpes virus infections and to increase cell membrane stiffness. Octenoic acid is produced as an intermediate of mitochondrial fatty acid and lipoic acid synthesis. It is the substrate for mitochondrial enoyl thioester reductase (ETR), a family of enzymes that requires NADPH to reduce the double bond to octanoic acid, which is then used for lipoic acid synthesis. Decreased levels of this C_8:1 acylcarnitine are consistent with decreased mitochondrial fatty acid synthesis, increased oxidation, increased renal secretion, or a combination of the three. All other acylcarnitine species measured (C_2-C_{24}) were normal in both males and females. No abnormalities of fatty acid oxidation were found in males with chronic fatigue syndrome.

[0082] **Serine and Threonine were Increased in Males.** Plasma serine and threonine levels were increased in males with chronic fatigue syndrome, but not in females (see **TABLES 8-9**). The

intracellular ratio of serine to glycine is a key regulator of 1-carbon, nucleotide, and folate metabolism. L-Serine is also an essential precursor of sphingolipid, phosphatidylserine, D-serine, and *de novo* cysteine and glutathioine synthesis. Both serine and threonine are gluconeogenic amino acids that can be used to make glucose during fasting or stress. Acute stress or infection results in a decrease in both plasma serine and threonine levels. An increase in these amino acids is consistent with increased synthesis, decreased utilization, decreased renal excretion, or a combination of all three in males with chronic fatigue syndrome. With regards to decreased utilization, the sharply reduced levels of sphingolipids that require serine for synthesis, would contribute to a serine sparing.

[0083] Sphingolipids and Glycosphingolipids. Increased sphingolipids are a recently recognized hallmark of obesity, metabolic syndrome, insulin resistance, and a risk factor in Alzheimer dementia. These abnormalities are improved by exercise, and caloric restriction. Effective methods to increase abnormally low sphingolipids have not yet been developed.

[0084] Cholesterol Synthesis through the Lathosterol Pathway was Decreased. The final conversion of desmosterol to cholesterol is catalyzed by the FAD-, and NADPH-requiring enzyme, 24-dehydrocholesterol reductase (DHCR24). When DHCR24 is downregulated, desmosterol accumulates in cell membranes, but does not change significantly in plasma. Membrane accumulation of desmosterol inhibits endocytosis and pinocytotic import through caveolae, and inhibiting the production of sphingolipid-rich and cholesterol-rich lipid rafts needed for cell-cell signaling. This would serve as a cell defense mechanism that inhibits the uptake of certain intracellular bacterial pathogens as Coxiella (Q fever) and Borrelia (Lyme disease).

[0085] Data Analysis. Metabolomic data were log-transformed, scaled by control standard deviations, and analyzed by multivariate partial least squares discriminant analysis (PLSDA), principal components analysis (PCA), t test, univariate ANOVA with pairwise comparisons and *post hoc* correction for multiple hypothesis testing using Fisher's least significant difference method in

MetaboAnalyst, or the false discovery rate (FDR) method of Benjamini and Hochberg. Metabolites with variable importance in projection (VIP) scores determined by PLSDA that were greater than 1.5 were considered significant. Metabolite correlations with Karnofsky performance scores were calculated by Pearson parametric and Spearman non-parametric methods implemented in Stata (Stata/SE12.1, StataCorp, College Station, TX), Prism (Prism 6, GraphPad Software, La Jolla, CA), or R. Significant metabolites were grouped into pathways and their VIP scores summed to determine the rank-ordered significance of each biochemical pathway. Sets of 5-15 metabolites were selected manually from the top 60 significant metabolites as candidate diagnostic classifiers using two multivariate methods: random forests (RF), and linear support vector machine (SVM) implemented in MetabolAnalyst. The diagnostic performance of the selected classifiers was then visualized and quantified by area under the receiver operator characteristic (AUROC) curve analysis. Classifier robustness was estimated by repeated double cross validation (rdCV), and permutation testing 1000 times in MetaboAnalyst. Confidence intervals for the ROC curves were calculated by bootstrap resampling. Sensitivity, specificity, accuracy, positive predictive value, negative predictive value, and number of misclassifications were estimated by conventional 2 x 2 contingency table analysis and p values calculated by Fisher's exact test in Prism.

[0086] Chronic Fatigue Syndrome Patients had an Average of 40 Metabolic Abnormalities. The mean number of metabolites falling outside of the 95% confidence limits (beyond 2 standard deviations from the mean) in females with chronic fatigue patients was 39 (+/- 3.5) (mean +/- SEM), and 41 (+/- 4.4) in males (See **FIG 3A-B**). This was significantly more than the number found among age and sex-matched controls, which was 16 (+/- 1.8) ($p < 2 \times 10^{-6}$; see **FIG 3A-B**). The mean and 95% confidence interval for the number of abnormalities expected by chance in the controls was calculated from the binomial distribution of 420 measured metabolites with a mean chance of abnormality equal to the p value of 0.05 for each test. The total number of abnormalities expected in controls was 21, with a range of 13-32 (95% CI = 13-32; 420 x 0.05 = 21). The

metabolites that were altered most in chronic fatigue syndrome were ranked by multivariate analysis (see **FIG. 4A-B**). Individual metabolite abnormalities were visualized according to a conventional biochemical pathway wall chart-style organization created in Cytoscape (see **FIG. 5A-B**). Metabolites that were decreased were colored green and those that were increased in chronic fatigue were colored red.

[0087] Mass Spectrometry and Metabolomics Quality Control. Data from a representative quality control series are reported in **Tables 5-6.** The within-day Pearson correlation of 27 internal stable-isotope labeled standards and 50 endogenous unlabeled metabolites was 0.999 and 0.998, respectively (see **Table 5**). The total variation associated with replicate sample processing was found to be 9-11% (see **Table 5**).

[0088] The reproducibility of 420-460 targeted metabolites was quantified by running two representative plasma samples in triplicate on three sequential days. A total of 458 metabolite AUCs in two plasma samples were quantified in the series illustrated. The Pearson correlation r values were 0.983 in one sample and 0.981 in another. The median of the relative standard deviations (RSD) from 458 metabolites measured in triplicate on 3 days was 12.1%-12.5% (see **Table 6**).

[0089] A Homogeneous Metabolic Response to Heterogeneous Triggers. Although the current study was not designed to examine the role of different triggering events, some basic data were collected. Possible triggering events fell broadly into 5 groups: biological (viral, bacterial, fungal/mold, and parasitic infections), chemical exposures, physical trauma, psychological trauma, and unknown. The specific biological and chemical exposures, and the precise nature of the physical and psychological traumas were diverse, numbering more than a dozen in just this small sample. Several patients had multiple triggers that converged in the same year. Although biological triggers were most common, no single infectious agent or other stressor was statistically more prevalent, and comprehensive testing for biological exposures in the control group was beyond the scope of this study.

[0090] Despite the heterogeneity of triggers, the cellular response to these environmental stressors in patients who developed CFS was homogeneous and statistically robust. These data supported the notion that it is the unified cellular response, and not the specific trigger, that lies at the root of the metabolic features of chronic fatigue syndrome.

[0091] Metabolomics Revealed a Chemical Signature of Chronic Fatigue Syndrome. Multivariate analysis was used to identify the pattern of chemical abnormalities in chronic fatigue syndrome compared to healthy controls. In the 3-dimensional plot of the results (see **FIG. 2A-B**), it was found that both males and females with chronic fatigue had a chemical signature that was distinct from healthy controls. The relative pathway impact and statistical significance were visualized in **FIG. 2C-D**. The nine biochemical pathway disturbances that were common to both males and females with chronic fatigue syndrome were visualized in a Venn diagram (see **FIG. 2E**). Eleven pathways were represented by metabolite disturbances that showed a degree of sex specificity. The biochemical pathways and metabolites that were altered in chronic fatigue syndrome were then ranked and tabulated (see **Tables 8-9**; see also **FIGs. 3A-B** and **4A-B**), and visualized by Cytoscape pathway analysis (see **FIGs. 5A-B**). The dominant finding from the pathway analysis was that sphingolipid abnormalities constituted close to 50% of all the metabolic disturbances associated with chronic fatigue syndrome in both males and females. Phospholipid abnormalities constituted 16% of the metabolic disturbances in males and 26% in females (see **Tables 8-9**).

[0092] Metabolites Correlated with the Clinical Severity of Chronic Fatigue Syndrome. Next was examined how each of the top 25 metabolite abnormalities were related to clinical functional status by Spearman correlation analysis. Each of these metabolites was found to have false discovery rates (FDR) of less than 10% (see **Tables 1-2**). A list of the top 61 metabolites appears in **Tables 3-4**. Twenty-one of the top 25 (84%) discriminating metabolites were low. These findings were consistent with the notion that chronic fatigue syndrome is a coordinated hypometabolic state.

[0093] Sphingolipids, Chronic Fatigue, and Dauer. All sphingolipids are synthesized from the amino acid serine and palmitoyl-CoA. Ceramides are produced rapidly from sphingomyelins by the inducible enzyme acid sphingomyelinase (ASMase) and redox regulated neutral sphingomyelinase (nSMase). Ceramides, and gangliosides made from ceramides, are important components of cell membrane patches and microdomains called lipid rafts that mobilize and aggregate during cell activation or infection. Sphingolipid rafts are enriched in cholesterol, and are crucial for cell-cell signaling, and reactive oxygen species (ROS) production for oxidative shielding and defense. Ceramide-, ganglioside-, and cholesterol-enriched cell membrane rafts are needed to recruit protein receptor and effector subunits during cell activation, cardiovascular and renal disease, and bacterial and protozoal infections. Increased membrane expression and release of sphingolipids is a universal response to acute infection, but can also be hijacked as a vehicle for virus entry and spread, and can result in autoimmune responses to gangliosides, and to lipid raft-associated receptors like CD46. Down-regulation of sphingolipids in chronic fatigue syndrome is consistent with a compensatory response to environmental stress, inflammation, or infection. Down-regulation of sphingolipid synthesis in general, and ceramide synthesis specifically, decreases the risk of apoptosis in response to new viral or bacterial infections, and is a feature of dauer. Mechanistic studies of the path by which the hypometabolic state is normally exited after viral illness, or in model systems after dauer provide insights that are useful in treating patients with chronic fatigue syndrome.

[0094] Sphingolipids and Glycosphingolipids were Decreased. The largest disturbances in the chemical signature of chronic fatigue syndrome were produced by widespread decrease in plasma sphingo- and glycosphingolipids (see **FIG. 2C-D**, see **Tables 8-9**). Thirty molecular species of sphingolipids were decreased in males, and 21 were decreased in females. Sphingolipid and glycosphingolipid abnormalities explained 55% of the metabolic impact in males, and 44% in females (**Tables 8-9**). Measured glycosphingolipids included glucosyl- (GC), dihexosyl- (DHC), and trihexosyl (THC) ceramides.

In males, over 50% (16/30) of the sphingolipids that were decreased were ceramides, and 47% (14/30) were sphingomyelin species. In females, 86% (18/21) were ceramides and 14% (3/21) were sphingomyelins in females (see **Tables 1-4**). In general, females with chronic fatigue retained more sphingomyelin species in the normal range than males. The low sphingolipid profile in chronic fatigue syndrome appears to be an adaptive response that is opposite to the increased sphingolipids observed in metabolic syndrome and the acute cell danger response, and ultimately may represent a fundamental response to oppose the spread of persistent viral and intracellular bacterial infections.

[0095] Phospholipids were Decreased. Several plasma phosphatidylcholine (PC) phospholipids were decreased in both males and females with chronic fatigue syndrome (see **Tables 8-9**). In contrast, it was found that a very specific molecular species of phospholipid, PC(18:1/22:6), containing the essential omega 3 fatty acid docosahexaenoic acid (DHA, C22:6) and oleic acid (C18:1) was increased. This pattern is opposite to that seen in response to acute infection and the cell danger response and metabolic syndrome.

[0096] Purines were Decreased. Plasma uric acid was decreased in males with chronic fatigue syndrome (see **Table 8**). Uric acid is the end product of purine metabolism and an important antioxidant molecule. Plasma adenosine was decreased in females (see **Table 9**). Plasma adenosine is produced from ATP and ADP released from cell surface ectonucleotidases, and by S-adenosylhomocysteine hydrolase (SAHH), during acute infection, inflammation, or stress. The decrease in plasma purines in chronic fatigue syndrome is consistent with decreased synthesis and/or turnover (flux) of ATP and GTP, and decreased reserve capacity caused in part by a generalized decrease in the ability to restore high-energy phosphate stores after exertion.

[0097] Aromatic Amino Acid Metabolites from the Microbiome were Decreased. Plasma 4-hydroxyphenyllactic acid (HPLA) was decreased in males with chronic fatigue syndrome (see **Table 8**). Plasma phenyllactic acid (PLA) was decreased in females (see **Table 9**). HPLA is a microbiome metabolite of tyrosine. PLA is a microbiome

metabolite of phenylalanine. This pattern is also opposite of what is found during acute inflammation, and infection.

[0098] Flavin Adenine Dinucleotide (FAD) was Decreased. Plasma FAD was decreased in both males and females with chronic fatigue syndrome (see **Tables 8-9**). FAD is synthesized from riboflavin (vitamin B2) and ATP. The GI absorption, distribution, and transporter-mediated uptake of FAD are carefully regulated during health and disease. FAD is mobilized from tissues, increased in the plasma, and renal secretion is increased under conditions of stress or infection. FAD is an important cofactor for fatty acid oxidation and sterol synthesis, is required for activation and oxidation of vitamin B6 (pyridoxine), lipoic acid metabolism (E3 subunit) needed for pyruvate, alpha-ketoglutarate, and branched chain amino acid oxidation, vitamin A activation, 5-methyltetrahydrofolic acid synthesis, niacin and NAD synthesis, and glutathione reduction. Functional deficiency of riboflavin can be produced by dietary and environmental factors. Severe riboflavin deficiency can present with a plasma acyl-carnitine pattern similar to multiple acyl-CoA dehydrogenase deficiency (MADD), also known as glutaric aciduria type II (GAII). GAII-like acyl-carnitine abnormalities did not appear in chronic fatigue syndrome patients.

[0099] Cholesterol and Bile Acid Synthesis through the Lathosterol Pathway were Decreased. Plasma lathosterol was decreased in both males and females with chronic fatigue syndrome (see **Tables 8-9**). Total plasma cholesterol, desmosterol, cortisol, and aldosterone were normal in both males and females with chronic fatigue syndrome. Two pathways are used in mammalian cells to synthesize cholesterol. These are the Kandutsch-Russell (K-R) pathway through lathosterol, and the Bloch pathway through desmosterol. The K-R pathway is preferred for cholesterol synthesis in the brain, heart, skeletal muscle, and skin, making up as much as 80% of cholesterol synthesis in these tissues under baseline conditions. The Bloch pathway is normally used preferentially in certain metabolic stress-response tissues like the gonads, spleen, adrenal glands, kidney, and adipose tissue. Under baseline conditions of health, the liver uses a nearly-equal blend of Bloch and K-R pathways. The data are consistent with increased flux through the desmosterol

pathway to maintain normal cellular levels of cholesterol. The desmosterol pathway corresponds to the stress-inducible arm of *de novo* cholesterol and sterol synthesis.

[00100] Plasma chenodeoxycholic acid (CDCA) was decreased in females (see **Table 9**). CDCA is a primary bile acid made from cholesterol. Decreased cholesterol flux can result in decreased substrate for bile acid synthesis needed for normal fat digestion and microbiome signaling. The absence of adequate bile acid delivery can lead to a loss in intestinal mucosal integrity and leaky gut via a cascade of events stemming in part from disrupted farnesoid X receptor signaling.

[00101] Pyrroline-5-Carboxylate and Arginine were Increased. Pyrroline-5-carboxylic acid (P5C) was increased in both males and females with chronic fatigue syndrome (see **Tables 8-9**). P5C production is a well-studied response to stress in plants, and mammals. P5C can be produced by the stress-induced oxidation of proline and hydroxyproline from collagen turnover via the enzyme proline oxidase, or from glutamate oxidation via pyrroline-5-carboxylate synthase (P5CS). P5C is converted to glutamate semialdehyde (GSA) non-enzymatically, then to ornithine under stress conditions. This reaction is catalyzed by what is often considered the reverse reaction of the mitochondrial enzyme, ornithine amino transferase (OAT). Hydroxyproline was increased in females with chronic fatigue (see **Table 9**). Hydroxyproline is converted to proline, then to P5C and GSA, which is then used as the precursor for arginine synthesis from ornithine in the epithelium of the small intestine under conditions of decreased calorie or protein intake. Another metabolic fate of hydroxyproline is glyoxylate, which can be transaminated in mitochondria to produce glycine, and metabolized in peroxisomes to oxalate and peroxide for cell defense, innate and antiviral immunity.

[00102] Plasma arginine levels were also increased in chronic fatigue males and females. Arginine is both a source of urea by arginase in the urea cycle, but more importantly, it is an activator of *N*-acetylglutamate (NAG) synthesis. NAG is the obligate activator of carbamoyl phosphate synthetase I (CPS-I).

CPS-I is required for the introduction of ammonia into the urea cycle via the synthesis of citrulline from ornithine and carbamoylphosphate by ornithine transcarbamoylase (OTC). Citrulline, ornithine, proline, glutamine, and glutamate levels were all normal. Under stress conditions, proline from collagen breakdown is shunted to arginine synthesis to spare nitrogen from other amino acids, and limit wasting during periods of decreased calorie and or protein intake. Increased arginine might theoretically be used for nitric oxide (NO) synthesis and contribute to vascular headaches or migraines, however the linkage between arginine and migraine is complex, and this usage would run counter to the nitrogen sparing use of arginine needed during times of environmental stress. Another metabolic fate of arginine is the NO inhibitor, asymmetric dimethylarginine (ADMA). CFS patients did not have an increase in plasma ADMA. Increased arginine is associated with a decreased risk of infection after operative stress and is used to synthesize the antimicrobial molecule agmatine under conditions of active infection.

[00103] Branch Chain Amino Acid Metabolic Intermediates were Decreased. 2-Hydoxyisocaproic acid (HICA) is derived from alpha ketoisocaproic acid, the transamination product of leucine. HICA was decreased in both males and females with chronic fatigue syndrome. This is consistent with decreased gut absorption, increased renal excretion, increased mitochondrial oxidation, or a combination of the three. HICA has antibacterial and antifungal activity.

[00104] Diagnostic vs Personalized Metabolic Disturbances. All of the metabolite abnormalities were classified in each patient as either being one of the abnormalities that defined chronic fatigue syndrome patients as a group (see **Tables 1-4**, and **8-9**), or as abnormalities that differed from controls, but did not contribute to the CFS diagnosis. Chronic fatigue syndrome patients had an average of 10 (+/-1.0) metabolite abnormalities that contributed to the CFS diagnosis, and 30 (+/-2.0) metabolites that were abnormal, but non-contributory for purposes of CFS diagnosis (see **FIG. 6A-B**). This means that 75% of the chemical abnormalities identified by metabolomic analysis were personalized, and 25% provided diagnostic

group information. The foregoing suggests that symptom improvements can be achieved more reliably by addressing the personalized abnormalities, than by assuming a chemical abnormality without actual measurement.

[00105] Assessment of Metabolomics as a Diagnostic Test in Chronic Fatigue Syndrome. After identifying over 60 metabolites that differed between chronic fatigue syndrome and controls in both males and females (see **Tables 1-4**), smaller sets of analytes were investigated for chronic fatigue syndrome diagnosis. Samples of 5-15 of the top 60 metabolites were manually selected to broadly interrogate several of the discriminating biochemical pathways (see Tables 8-9) in males and females. The performance of each classifier set of metabolites was then tested by area under the receiver operator characteristic (AUROC) curve analysis. It was found that the exact specification of metabolites in the classifier was flexible. Using both forward selection and backward elimination method, once a set of 5-15 analytes was found, the addition or removal of one or a few analytes had little effect on the overall quality of the classifier. In males, it was found a set of 8 analytes performed well (see **FIG. 7A**). In females, it was found a set of 13 analytes performed well (see **FIG. 7B**). It was further found that a single-analyte classification method performed surprisingly well in this small sample of 84 subjects (see **Table 10**).

Table 10. Diagnostic Accuracy of Targeted Plasma Metabolomics in Chronic Fatigue Syndrome.

Sex	Classifiers	AUROC[c]	95% CI	rdCV[e] Accuracy	Permutation[f] p Value	2x2[g] Accuracy	2x2 Sensitivity	2x2 Specificity
Males	8-Analyte Example[a]	0.94	0.84 -1.0	0.84	0.001	0.90	0.91	0.89
	1-Analyte Example[b]	0.71	0.50 - 0.88	0.62	0.009	0.72	0.73	0.72
Females	13-Analyte Example[c]	0.96	0.87 -1.0	0.90	0.001	0.93	0.91	0.95
	1-Analyte Example[d]	0.68	0.42 - 0.86	0.58	0.009	0.68	0.70	0.67

[a]8:analytes in males: phosphatidyl choline PC(16:0/16:0), glucosylceramide GC(18:1/16:0), 1-pyrroline-5-carboxylate (P5C),

flavin adenine dinucleotide (FAD), pyroglutamic acid (also known as 5-oxoproline), 2-hydroxyisocaproic acid (HICA), L-serine, and lathosterol.
[b]1-analyte: phosphatidyl choline PC(16:0/16:0)
[c]13-analytes in females: trihexosylceramide THC(18:1/24:0), phosphatidyl choline PC(16:0/16:0), hydroxyproline, ceramide(d18:1/22:2), lathosterol, adenosine, phosphatidylinositol PI(16:0/16:0), flavin adenine dinucleotide (FAD), 2-octenoylcarnitine, phosphatidyl choline plasmalogen PC(22:6/P18:0), phosphatidyl choline PC(18:1/22:6), 1-pyrroline-5-carboxylate (P5C), and chenodeoxycholic acid (CDCA).
[d]AUROC = area under the receiver operator curve reflects the overall accuracy of diagnosis using these analytes.
[e]rdCV = repeated random sub-sample (2/3 in, 1/3 out) double cross validation.
[f]Permutation p values represent the probability that the random forest classification of cases and controls using the specified analytes could be obtained by chance.
[g]Values calculated by standard 2x2 contingency table analysis
N = 18 control males and 22 CFS males, and N = 21 control females and 23 CFS females.

[00106] However, single-biomarkers are biologically implausible as a diagnostic test for complex diseases like chronic fatigue syndrome, and are likely to perform poorly in larger populations. By using classifiers constructed from 5 or more metabolites, natural biological variation is more readily accommodated and diagnostic accuracy is more robust. A principal components analysis (PCA) was also performed to identify orthogonal components of the metabolomic signature (see **Tables 11-12**; and see **FIG. 9A-B**). PCA was found to be less robust than PLSDA and random forest analysis in identifying diagnostically useful metabolites in independent clinical settings.

Table 11. Principal Components Analysis (Males). Top 10 Metabolites.

No.	PC1 Metabolite	PC1-12.4%	PC2 Metabolite	PC2-8.2%
1	Ceramide(d18:1/18:0)	0.11255	L-Kynurenine	0.12502
2	Ceramide(d18:1/16:0)	0.10299	Methylcysteine	0.11606
3	BMP(18:1/16:0)	0.099692	L-Lysine	0.11594
4	Ceramide(d18:1/24:0)	0.099161	Ureidopropionic acid	0.11379
5	SM(d18:1/20:2)	0.099053	L-Tyrosine	0.1135
6	Ceramide(d18:1/16:1 OH)	0.09877	1-Methylhistidine	0.10692
7	Ceramide(d18:1/24:2)	0.098766	Ornithine	0.10628
8	Ceramide(d18:1/20:1)	0.098707	L-Glutamine	0.10504
9	Ceramide(d18:1/22:0)	0.098347	Imidazoleacetic acid	0.10467
10	Ceramide(d18:1/24:0 OH)	0.097983	Kynurenic acid	0.089947

Table 12. Principal Components Analysis (Females). Top 10 Metabolites.

No.	PC1 Metabolite	PC1-12%	PC2 Metabolite	PC2-8.9%
1	N-Acetyl-L-aspartic acid	0.071768	Oleic acid	0.09896
2	Arachidonyl carnitine	0.069874	Linoleic acid	0.098395
3	Hexose Monophosphate Pool	0.058809	12-HETE	0.090984
4	3, 5-Tetradecadiencarnitine	0.058589	L-Acetylcarnitine	0.090299
5	Glycine	0.057178	Glyoxylic acid	0.088276
6	Oleoylcarnitine	0.05587	3-Hydroxyhexadecenoylcarnitine	0.080783
7	2-Aminoisobutyric acid	0.055047	Arachidonic Acid	0.078768
8	N-acetylserine	0.053443	PS(36:2)	0.076863
9	O-acetylserine	0.052228	PS(18:0/20:4)	0.076464
10	3-Hydroxy-cis-5-tetradecenoylcarnitine	0.052165	4-Hydroxybenzoic acid	0.075434

[00107] Metabolic Similarities Between CFS and Dauer. Many of the pathways and metabolites that were abnormal in chronic fatigue syndrome are also known to be features of dauer, a well-studied, long-lived survival and persistence state triggered by environmental stress (**Table 13**). Interestingly, it was found that the direction of CFS abnormalities was opposite to metabolic syndrome, and opposite to the metabolic response to infection, inflammation, or environmental stress that has been called the cell danger response. For example, cholesterol, phospholipid, sphingolipid, and purine metabolism are all decreased in CFS and dauer, but are increased in metabolic syndrome and the stereotyped cell danger response (see **Table 13**). These facts suggest that chronic fatigue syndrome is an evolutionarily conserved, genetically regulated, hypometabolic state similar to dauer that permits survival and persistence under conditions of environmental stress, but at the cost of severely curtailed function and quality of life.

Table 13. Metabolic Similarities and Contrasts Between CFS and Dauer, Cell Danger and Metabolic Syndrome.

Plasma Metabolites	Chronic Fatigue Syndrome	Dauer	Cell Danger Response(7)	Metabolic Syndrome
Sphingolipids	Decreased (M+F)[a]	Decreased(62)	Increased(63)	Increased(64)
Glycosphingolipids	Decreased (M+F)	Decreased(62)	Increased(63)	Increased(65)
Phospholipids (most species)	Decreased (M+F)	Decreased(66)	Increased(67)	Increased(68)
PC(18:1/22:6)—Oleoyl/DHA phospholipids	Increased (M+F)	No data	Decreased(67)	Decreased(13)
Cholesterol, Sterol Synthesis	Decreased (M+F)	Decreased(69)	Increased(70)	Increased(71)
Purines	Decreased (M+F)	Decreased(72)	Increased(73)	Increased(74)
Uric Acid	Decreased (M)	N/A[b]	Increased(75)	Increased(76)
Pyrroline-5-Carboxylate/Arginine	Increased (M+F)	No data	Decreased(77)	No data
FAD/Riboflavin	Decreased (M+F)	Decreased(72)	Increased(28)	No data

[a]M+F = males and females. M = males only. F = females only
[b]N/A: the end products of purine metabolism in worms are glyoxylate and ammonia, not uric acid.

Example 2

[00108] Study Design and Participants. An investigator-initiated, phase I/II, double blind, placebo-controlled, randomized clinical trial was performed to examine the safety and activity of single-dose suramin or placebo in 10 children with autism spectrum disorders (ASD). All children met DSM-5 diagnostic criteria for autism spectrum disorder, and received confirmatory testing by Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2) examination. Inclusion criteria were males, ages 4-17 years, living in the San Diego, California region, with a confirmed diagnosis of ASD. Exclusion criteria included children who weighed less than the 5th percentile for age, took prescription medications, or had laboratory evidence of liver, kidney, heart, or adrenal abnormalities. Children living more than a 90-minute drive from the testing sites were excluded to eliminate the possibility of aberrant behaviors resulting from extended car travel. Children with known syndromic forms of ASD caused by DNA mutation or chromosomal copy number variation (CNV) were excluded in this first study. Families were asked not to change their children's therapy (*e.g.*, supplements, speech, and behavioral therapies) or diet throughout the study period.

[00109] Standardized Testing and Questionnaires. Two observational examinations were performed by a clinician at 3 time points: baseline (56 ± 8 days; mean ± SEM; before the infusion), 2-days post-infusion, and 6-weeks post-infusion. The two examiner-based metrics were the Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2), with video and audio files recorded on 3 cameras, and the Expressive One Word Picture Vocabulary Testing (EOWPVT). Both of these observational metrics were administered by a trained and certified examiner using approved test materials. Three standardized questionnaires were completed by parents at 3 time points: baseline, 7-days post-infusion, and 6-weeks post-infusion. The three standardized questionnaires completed by parents were the 58-question Aberrant Behavior Checklist (ABC), the 75-item Autism Treatment Evaluation Checklist (ATEC), and the 33-item repetitive behavior questionnaire (RBQ). Parents were asked to complete these three instruments with reference to how their child behaved in the

previous 7 days. At the end of the six weeks, a 24-question Clinical Global Impression (CGI) questionnaire was used. In addition, parents were asked to list the 3 top behaviors or symptoms that they observed to be most changed over the previous 6-weeks. To minimize the misinterpretation of natural day-to-day variations in symptoms, parents were asked to mark a symptom as changed in the 6-week CGI only if it had lasted for at least 1 week.

[00110] Storyboards and Social Stories. A graphic artist was commissioned to prepare a storyboard of each step of the procedure. The panel contents and color schemes were reviewed, and revisions recommended, by a 16-year old artist with Asperger syndrome to optimize the informational value and minimize any sensory issues. Next, the developmental neuropsychologist created social stories to accompany each panel of the storyboard.

[00111] Phone Interviews, Parent Reports, and Clinical Observations. Scripted phone interviews were conducted daily for the first week, then weekly until the completion of the study for each child 6-weeks after the infusion. Parents also kept study journals throughout the six weeks to document their observations. These scripted and narrative observations were used to permit discovery of any changes in ASD, behavior, or constitutional symptoms such as sleep and appetite, or any adverse or unanticipated events. The parent reports also provided insight regarding the timing and pattern of the responses after the infusion that were not predicted prior to the study, and were not adequately captured by the scheduled observations.

[00112] *Daily Calls.* Parents were contacted by phone on days 1-7 after the infusion to ensure close follow-up and to provide the opportunity for parents to report any positive or negative observations.

[00113] *Weekly Calls.* Parents were called weekly on days 14, 21, 28, and 35 after the infusion to ensure close follow-up and to provide the opportunity for parents to report any positive or negative observations.

[00114] *Clinical Global Impression (CGI).* A 24-question Clinical Global Impression (CGI) instrument was developed to assess the core

symptoms of autism spectrum disorders and some of the most common comorbid features. The CGI instrument scoring system was the traditional 7-point, CGI-Improvement scale. In this scale, the historian gives a score of 0 if the symptom "was never a problem", a 1 for "very much improved", a 4 for "no change", and a 7 for "very much worse". In addition to the 24 structured questions, the parents were asked to write in the top 3 symptoms or behaviors that were most changed over the 6 weeks since the suramin infusion. This hybrid design of structured and open-ended responses permitted the capture of a large number of clinical outcomes associated with single-dose suramin treatment.

[00115] Randomization and Masking. 20 males with ASD were screened. 16 met entry criteria. 10 participants could be matched by age, non-verbal IQ, and ADOS scores into 5 pairs. The randomization sequence was generated electronically by the biostatistical team. Subjects within each pair were allocated to receive suramin or saline according to the prospectively determined randomization sequence. The randomization sequence was concealed from the clinical team and implemented by the investigational pharmacy, which prepared drug and placebo for infusion. The design was double-blind. The mask was not broken until all subjects had completed the study and all clinical data had been collected.

[00116] Diagnostic and Outcome Procedures. Examiner-based outcomes (ADOS and EOWPVT) were assessed at 2-days and 6-weeks after the infusion. Parent-based outcomes (ABC, ATEC, CGI, and RBQ) were assessed at 7-days and 6-weeks after the infusion. To minimize the effects of natural behavioral variability, the parents were instructed to mark a behavior as changed only if it was persistently changed for at least 1 week. Storyboards and accompanying social stories were created to illustrate each step of the study for parents to review with each child before the study.

[00117] The diagnosis of each of the enrolled participants was confirmed by ADOS-2 comparison scores of ≥ 7. Non-verbal IQ was tested by Leiter-3 examination. The primary behavioral outcomes were ADOS scores and language assessed by standardized vocabulary testing. Expressive vocabulary was assessed by Expressive One Word Picture Vocabulary Test (EOWPVT). Primary outcomes were measured

at baseline, and 2-days and 6-weeks after infusion. Secondary outcomes were the Aberrant Behavior Checklist (ABC), Autism Treatment Evaluation Checklist (ATEC), Clinical Global Impression of Improvement (CGI), and Repetitive Behavior Questionnaire (RBQ). Secondary outcomes were measured at baseline, and 7-days and 6-weeks after infusion.

[00118] Protocol Deviations. The original protocol was designed to collect electroencephalography (EEG), heart rate variability (HRV), balance, gait, fine motor, and sensory motor data as secondary outcomes. However, the wide range in ages and abilities, small subject numbers, and task compliance difficulties made collection of these data incomplete and insufficiently powered to draw any conclusions. In addition, it was found that major language advances were in the form of new speech fluency and new interest in speech and social communication, and not in new vocabulary. Peabody Picture Vocabulary testing (PPVT) did not capture this new interest in communication. These data were incomplete and insufficiently powered for analysis.

[00119] Drug and Placebo Administration. Suramin was provided as the hexasodium salt (MW 1429.2 g/mol) in 1 gram lyophilized vials by Bayer Pharmaceuticals, Inc. A 1-gram vial was reconstituted in 10 mL of sterile water for infusion to prepare a 10% (100 mg/mL) solution. Height and weight were recorded, vital signs and capillary oxygen saturation (pulse oximetry) measured, physical and neurological examinations were conducted, and urine and blood for safety monitoring, pharmacology, and metabolomics was collected before the infusion. Each child then received a 50 mg test dose (0.5 mL of a freshly reconstituted 10% solution) of suramin in 5 mL of saline, or 5 mL of saline only given by slow intravenous (IV) push over 3 minutes, followed by a 10 mL flush of saline. One hour after the test dose, vital signs were repeated and a single infusion of either suramin (20 mg/kg, minus the 50 mg test dose, in 50 mL, up to a maximum of 1 gram) or saline (50 mL IV) was given over 30 minutes, followed by a 10 mL flush of saline. One hour after completion of the infusion, vital signs and the physical and neurological examinations were repeated, blood was collected for safety monitoring and pharmacology, and the family discharged to

home. A typical infusion visit lasted about 4 hours from start to finish.

[00120] Safety and Adverse Event Monitoring. Blood and urine for safety and toxicity monitoring were collected immediately before the infusion, 1 hour after the infusion, 2 days after, and 45 days after the infusion. Vital signs and anthropomorphic measurements were also collected. Safety surveillance included 18 vital sign and anthropometric features, 19 complete blood count (CBC) parameters, 20 blood chemistry measures, 3 thyroid and cortisol measures, and 5 lipid measures at the 5 time points. 24 urinalysis features were measured at 4 times: baseline, pre-infusion, 2-days post-infusion, and 45-days post-infusion.

[00121] Blood and urine samples were collected for safety and toxicity monitoring at 5 times throughout the study: at baseline (32 ± 6 days before the infusion; mean ± SEM), immediately before the infusion, 1 hour after the infusion, 2 days after, and 45 days after the infusion. Unexpected and adverse events were recorded as they occurred and graded in severity according to the National Cancer Institute Common Terminology Criteria for Adverse Events v4.03 (CTCAE) scale. Additional pharmaco-vigilance monitoring included daily scripted phone calls in the first week, then 4 weekly calls until the exit examinations at 6 weeks. Each child received a formal neurological examination by a board-certified pediatric neurologist at baseline and at the end of the study. An independent data safety monitoring board (DSMB) reviewed the data and IRB communications for the study.

[00122] Pharmacokinetics. Plasma samples were collected for suramin pharmacokinetics (PK) before the infusion, at 1 hour, 2 days, and 45 days post-infusion. Suramin concentrations were measured by high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS). The small number of PK samples per subject prevented a standard, non-compartmental analysis in individual subjects. The suramin drug concentrations were analyzed using a population PK approach with post-hoc empiric Bayesian estimate of PK parameters in individual subjects. The PK data were fit to a two-compartment model using the computer program NONMEM (ICON, Dublin, Ireland). PK parameters were scaled allometrically

with volume terms scaled to linear body weight (kg1.0) and clearance terms scaled to weight (kg0.75). Scaled adult suramin parameters of compartmental volumes of distribution and clearance were used as initial parameter estimates and between subject variability only estimated for clearance (CL) and the peripheral volume of distribution (Vd).

[00123] Pharmacometabolomics. Targeted, broad-spectrum, plasma metabolomic analysis, covering 63 biochemical pathways, was performed by LC-MS/MS as described with minor modifications. Four hundred thirty-one (431) of 610 targeted metabolites were measurable in plasma.

[00124] Sample Size Calculation and Statistical Analysis. This was a pilot study designed to obtain activity data and effect size estimates upon which future sample size calculations could be based. No data on suramin in autism were available for sample size calculations prior to this study. Each child was used as his own control to examine before and after treatment effects in a paired t-test design for the analysis of the ADOS, EOWPVT, ABC, ATEC, RBQ, and blood and urine safety data. Paired, non-parametric analysis was by Wilcoxon signed-rank sum test. Categorical data, such as the presence or absence of adverse events or historical symptoms was analyzed by Fisher's exact test. Two-way ANOVA (treatment x time), with Sidak *post hoc* correction, was used to analyze the 6-week summaries captured by the ADOS, CGI, and blood and urine safety analysis. Cohen's d—calculated as the mean difference of the paired, within-subject scores before and after treatment, divided by the standard deviation of the differences—was used as an estimate of effect size. Metabolomic data were log-transformed, scaled by control standard deviations, and analyzed by multivariate partial least squares discriminant analysis (PLSDA), with pairwise comparisons and *post hoc* correction for multiple hypothesis testing using Fisher's least significant difference method in MetaboAnalyst, or the false discovery rate (FDR) method of Benjamini and Hochberg. Metabolites with variable importance in projection (VIP) scores determined by PLSDA that were greater than 1.5 were considered significant. Methods were implemented in Stata (Stata/SE12.1, StataCorp, College Station, TX), Prism (Prism 6,

GraphPad Software, La Jolla, CA), or R. Significant metabolites were grouped into pathways and their VIP scores summed to determine the rank-ordered significance of each biochemical pathway.

[00125] Verification of Data Completeness and Transcription Accuracy. Standardized questionnaire responses and the ADOS-2 and EOWPVT scores (5,490 cells of data) were compiled in spreadsheets from the original hard copy forms and from the electronic medical records. A total of 87 cells (1.6%) of the 5,490 outcome scores were either left blank, asked about a symptom that did not apply, or were missing. One participant missed the 6-week ADOS and EOWPVT evaluations because of scheduling difficulties. His 2-day results were used as an estimate of his 6-week scores. ADOS scores remained significant when this subject was dropped from the analysis (See **FIG. 16T**). EOWPVT results were also unchanged (See **FIG. 16X**). The 4,210 cells of laboratory and vital sign data were also collected and reviewed. When specific cells of data were found to be missing, they were manually confirmed by inspection of the original questionnaire, laboratory results, and clinical data sheets. A random generator program was written that randomly selected 5% of the data. These randomly selected cells of data that were then manually checked for transcription accuracy by reviewing the hard copy responses and Red Cap electronic medical records.

[00126] Study Participant Disposition and Characteristics. FIG. 9 illustrates the CONSORT flow diagram for patient recruitment, allocation, and analysis in the SAT-1 study. The two treatment groups were well matched (**Table 14**).

TABLE 14 Group characteristics

Parameter	Suramin Group Mean ± SD (Range) or Number	Placebo Group Mean ± SD (Range) or Number	P value[1]
Number	5	5	n/a
Age (years)	8.9 ± 3.3 (5.7-13.6)	9.2 ± 3.8 (6.2-14.7)	0.88
Leiter IQ	82 ± 7.8 (75-92)	79 ± 8.8 (66-87)	0.69
ADOS Score	8.6 ± 0.9 (8-10)	9.4 ± 1.3 (7-10)	0.30
Weight (kg)	32 ± 14 (23-55)	40 ± 23 (24-80)	0.53
Weight Percentile	64 ± 16 (42-84)	78 ± 30 (25-98)	0.40
Height (cm)	136 ± 23 (118-174)	137 ± 28 (113-180)	0.92
BSA* (m^2)	1.09 ± 0.32 (0.87-1.63)	1.21 ± 0.46 (0.87-1.99)	0.64
Body Mass Index (kg/m^2)	16.8 ± 1.1 (15.5-18.1)	19.9 ± 3.1 (16.2-24.7)	0.07
Head Circumference (cm)	54.3 ± 2.8 (51.5-57.5)	54.5 ± 2.3 (51.5-57)	0.90
HC Percentile	75 ± 30 (35-99)	75 ± 27 (42-97)	0.97
Age at ASD diagnosis (yrs)	3.2 ± 0.5 (2.5-3.75)	2.7 ± 0.3 (2.5-3.0)	0.10
Paternal age at birth (yrs)	37 ± 3.2 (35-41)	43 ± 12 (33-64)	0.62
Maternal age at birth (yrs)	35 ± 2.8 (32-38)	41 ± 6 (33-47)	0.053
Sibling with ASD	0	1	0.99
History of GI issues—current	0	1	0.99
Maintains a gluten-free diet	0	1	0.99
IVF conception	1	0	0.99
C-section delivery	1	1	0.99
History of premature birth	0	1	0.99
History of epilepsy[2]—current	0	0	0.99
History of developmental regression(s)	3	2	0.99
History of asthma—current	0	0	0.99
ASD symptom improvement with fever	2	1	0.99

*Mosteller method. **Abbreviations:** BSA: body surface area; HC: head circumference; GI: gastrointestinal; IVF: in vitro fertilization; ASD: autism spectrum disorder. [1]Student's *t*-test for continuous data; Fisher's exact test for categorical data. [2]Patients taking prescription drugs were excluded from the study. This included anticonvulsant medications.

The mean age was 9.1 years (range = 5-14). The mean non-verbal Leiter IQ was 80 (range = 66-92). The mean Autism Diagnostic Observation Schedule, 2nd edition (ADOS-2) comparison score was 9.0 (range = 7-10).

[00127] Safety Monitoring and Adverse Events. Extensive monitoring revealed no serious toxicities (CTCAE grades 3-5). Neurologic examinations showed there was no peripheral neuropathy (**Table 15**).

TABLE 15 Summary of adverse or unanticipated events

No.	Events	Suramin (N=5)	CTCAE[a] Grade	Placebo (N=5)	CTCAE[a] Grade	P Value[b]
1	Asymptomatic rash	5	1	0	-	0.0079
2	Uncomplicated URI[c]	2	1	2	1	0.99
3	Headache	1	1	0	-	0.99
4	Emesis x 1	1[e]	1	1[e]	1	0.99
5	Hyperactivity	2[e]	1	1	1	0.99
6	Hypoglycemia[f]	1	2	1	2	0.99
7	Leukocytosis	0	-	1[g]	1	0.99
8	Enuresis	1[h]	1	0	-	0.99
9	Peripheral neuropathy	0	-	0	-	0.99
	Total:	13	-	6	-	0.12
	Total, excluding rash:	8	-	6	-	0.77

[a]CTCAE: Common terminology criteria for adverse events v4.03. Mild to moderate = Grade 1-2; Serious = Grade 3-5. [b]Fisher's exact test. [c]URI: upper respiratory tract infection, common cold. Infusions occurred October-February. [e]In 7-year old after pizza and slushee consumption after playing youth league basketball. [f]In a 6-year old after a car ride. [g]In a 5 and 14-year old intermixed with periods of calm focus in first week (the 14-year old) or first 3 weeks (the 5-year old). [f]6-weeks after the infusion, after several days of a cold and fasting before lunch. Hypoglycemia was asymptomatic and corrected after a normal lunch. [g]Leukocytosis (12.2k WBC) occurred on the day of the saline infusion and preceded a URI. [h]In a 7-year old briefly for a few days while sick with a cold. None of the events required medical intervention. No serious adverse events (SAEs) occurred in this study.

Analysis of free cortisol, hemoglobin, white blood cell count (WBC), platelets, liver transaminases, creatinine, and urine protein showed no differences in children who received suramin and placebo (see **FIG. 11**). The 5 children who received suramin developed a self-limited, evanescent, asymptomatic, fine macular, patchy, morbilliform rash over 1%-20% of their body (see **FIG. 11H-I**). This peaked 1 day after the infusion and disappeared spontaneously in 2-4 days. The mean number of AEs per participant was 1.9 (1.2 in the placebo group and 2.6 in the suramin group; 1.6 in the suramin group for a non-rash AE; RR = 1.3; 95% CI = 0.5-3.4; p = 0.77; see **Table 15**). No serious adverse events (SAEs) occurred in this study. An independent data and safety monitoring board (DSMB) reviewed this information, as well as the clinical safety and toxicity data and IRB communications from the study and found no safety concerns.

[00128] Pharmacokinetics. Pharmacokinetic analysis showed that at 1 hour after intravenous infusion of 20 mg/kg (558 ± 41 mg/m2; mean ± sd; and **Table 16**), the suramin concentration was 104 ± 11.6 μM (see **FIG. 12A**).

Table 16 Single-dose suramin pharmacokinetics

Pair Block	ID	Age (yrs)	Height (m)	Weight (kg)	BSA* (m^2)	20 mg/kg Dose (mg)	Dose (mg/m^2)	1-Hour Plasma Conc (μM)	2-Day Plasma Conc (μM)	45-Day Plasma Conc (μM)	Plasma Half-Life (days)
1	001	11	1.395	34.4	1.15	680	591	101.2	13.2	0.96	12.6
2	007	5	1.169	22.9	0.87	460	529	87.9	11.9	1.67	14.7
3	014	14	1.74	54.7	1.63	1000	613	110.9	10.6	1.04	14.9
4	012	6	1.18	23.1	0.87	460	529	118.6	13.8	2.28	16.5
5	005	7	1.271	28.1	0.95	500	526	101.8	10.6	1.76	15.0
						Mean:	558	104.1	12.0	1.54	14.7
						sd:	41	11.6	1.5	0.6	1.4

*Mosteller method

[00129] The distribution phase half-life was 7.4 ± 0.55 hours. The suramin levels rapidly fell below 100 μM and into the target range before day 2 in all subjects, with an average plasma level of suramin of 12.0 ± 1.5 μM on day 2 (see **FIG. 12B**, and **Table 16**). Target concentrations of 1.5-15 μM were maintained between 2 days and 6 weeks following the dose (see **FIG. 12**). The steady-state volume of distribution was 0.83 ± 0.014 L/kg (22.7 ± 2.6 L/m^2). The clearance was 1.95 ± 0.21 mL/hr/kg (0.056 ± 0.011 $L/hr/m^2$). The terminal elimination phase half-life (t1/2) was 14.7 ± 1.4 days (see **FIG. 12C-D**). These data are the first in the published literature on the pharmacokinetics of suramin in a pediatric population.

[00130] Additional pharmacokinetic results are illustrated in **Table 16.** Although no behavioral outcomes were significant at 2 days after infusion, it was found that 28 biochemical pathways were changed by suramin 2-days after the infusion (see **Table 18**). Twenty-two of these (79%) remained changed at the 6-week time point (See **Table 19**). The rank order of metabolites most changed at day 2, and their associated metabolic pathway is illustrated in **FIG. 15.** The full list of 61 metabolites on day 2 and 48 metabolites at 6-weeks that were significantly changed by suramin appears in **Tables 19-20.** A wallchart-style biochemical pathway map was created in Cytoscape to illustrate the organization of metabolites that were increased and decreased by suramin treatment (See **FIG. 15**).

[00131] Pharmacometabolomics. Targeted, broad-spectrum, plasma metabolomic analysis of 610 metabolites from 63 biochemical pathways was performed by high performance liquid chromatography

and tandem mass spectrometry (LC-MS/MS) as described with minor modifications. 431 metabolites were above the lower limit of quantitation (LLOQ) in this study. Venous blood was collected between the hours of 8 am and 5 pm, at least 3 hours after the last meal, into lithium-heparin vacutainer tubes (BD #367884). Plasma was separated by centrifugation at 900g x 10 minutes at room temperature within one hour of collection. The resulting fresh lithium-heparin plasma was transferred to labeled 1.2 mL or 2.0 mL externally threaded, cryotubes with a minimum headspace air gap for storage at -80 °C for analysis. Samples were analyzed on an AB SCIEX QTRAP 5500 triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization (ESI) source, Shimadzu LC-20A UHPLC system, and a PAL CTC autosampler. Typically, 90 µl of plasma was thawed on ice and transferred to a 1.7 mL Eppendorf tube. Five (5.0) µL of a cocktail containing 25-35 commercial stable isotope internal standards, and 5.0 µL of 57 stable isotope internal standards that were custom-synthesized in *E. coli NCM3722, Caenorhabditis elegans N2,* and *Komagataella phaffii* (ATCC 76273; formerly known as *Pichia pastoris*) by metabolic labeling with ^{13}C-glucose and ^{13}C-bicarbonate, were added, mixed, and incubated for 10 min at 20 °C to permit small molecules and vitamins in the internal standards to associate with plasma binding proteins. Macromolecules (protein, DNA, RNA, glycans, etc.) were precipitated by extraction with 4 volumes (400 µL) of cold (-20 °C), acetonitrile:methanol (50:50) (LCMS grade, Cat# LC015-2.5 and GC230-4, Burdick & Jackson, Honeywell), vortexed vigorously, and incubated on crushed ice for 10 min, then removed by centrifugation at 16,000g x 10 min at 4°C. The supernatants containing the extracted metabolites and internal standards in the resulting 40:40:20 solvent mix of acetonitrile:methanol:water were transferred to labeled cryotubes and stored at -80 °C for LC-MS/MS analysis.

[00132] LC-MS/MS analysis was performed by scheduled multiple reaction monitoring (sMRM) under Analyst v1.6.2 software control in both negative and positive mode with rapid polarity switching (50 ms). Nitrogen was used for curtain gas (set to 30), collision gas (set to high), ion source gas 1 and 2 (set to 35). The source

temperature was 500 °C. Spray voltage was set to -4500 V in negative mode and 5500 V in positive mode. The values for Q1 and Q3 mass-to-charge ratios (m/z), de-clustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were determined and optimized for each MRM for each metabolite. Ten microliters of extract was injected by PAL CTC autosampler via a 10 µl stainless steel loop into a 250 mm × 2.0 mm, 4µm polymer based NH2 HPLC column (Asahipak NH2P-40 2E, Showa Denko America, Inc., NY) held at 25 °C for chromatographic separation. The mobile phase was solvent A: 95% water with 20 mM $(NH_4)_2CO_3$ (Sigma, Fluka Cat# 74415-250G-F), 5% acetonitrile, and 38 mM NH_4OH (Sigma, Fluka Cat# 17837-100ML), final pH 9.75; solvent B: 100% acetonitrile. Separation was achieved using the following gradient: 0-3.5 min: 95%B, 3.6-8 min: 85% B, 8.1-13 min: 75% B, 13.5-35 min: 0% B, 36-46 min: 95% B, 46.1 min: end. The flow rate was 200 µL/min. Pump pressures ranged from 920-2600 psi over the course of the gradient. All the samples were kept at 4 °C during analysis. The chromatographic peaks were identified using MultiQuant (v3.0, Sciex), confirmed by manual inspection, and the peak areas integrated.

[00133] The small number of subjects in this trial precluded conventional treatment group analysis because of high false discovery rates associated with measuring 431 metabolites in groups with just 5 subjects. However, by using each child as their own control in a paired analysis of pre-infusion and post-infusion results, the pharmacometabolomic effects of suramin could be characterized (see **FIG. 12**).

[00134] Targeted plasma metabolomics was performed immediately before infusion, at 2 days, and 6 weeks after the infusion. The rank order of the top 35 of 48 significant metabolites 6-weeks after suramin treatment is illustrated in **FIG. 13A**. The rank order after 2-days is illustrated in **FIG. 14**. Consistent with our previously published work using mouse models, the metabolic effects of suramin resulted in a decrease of the cell danger response and restored more normal metabolism (**Table 17**).

TABLE 17 Suramin pharmacometabolomics: biochemical pathways changed at 6-weeks

No.	Pathway Name	Measured Metabolites in the Pathway (N)	Expected Pathway Proportion (P = N/429)	Expected Hits in Sample of 48 (P * 48)	Observed Hits in the Top 48 Metabolite s	Fold Enrichment (Obs/Exp)	Impact (Sum VIP Score)	Fraction of Impact (VIP Score) Explained (% of 94.6)	Increased	Decreased
1	Purine Metabolism	28	0.061	2.9	5	1.7	10.2	11%	3	2
2	SAM, SAH, Methionine, Cysteine, Glutathione	15	0.035	1.7	5	3.0	9.5	10%	5	0
3	Microbiome Metabolism	18	0.042	2.0	4	2.0	8.4	9%	4	0
4	Branch Chain Amino Acid Metabolism	12	0.028	1.3	4	3.0	7.4	8%	4	0
5	Bile Acid Metabolism	6	0.014	0.7	3	4.5	5.7	6%	3	0
6	Fatty Acid Oxidation and Synthesis	37	0.086	4.1	3	0.7	5.0	5%	0	3
7	Amino Acid Metabolism (not otherwise covered)	4	0.009	0.4	2	4.5	4.3	5%	2	0
8	Krebs Cycle	9	0.021	1.0	2	2.0	4.3	5%	2	0
9	Pyrimidine Metabolism	9	0.021	1.0	2	2.0	4.2	4%	2	0
10	Sphingomyelin Metabolism	38	0.084	4.0	2	0.5	4.1	4%	2	0
11	1-Carbon, Folate, Formate, Glycine, Serine Metabolism	5	0.012	0.6	2	3.6	4.0	4%	2	0
12	GABA, Glutamate, Arginine, Ornithine, Proline	8	0.014	0.7	2	3.0	3.9	4%	2	0
13	Tyrosine and Phenylalanine Metabolism	3	0.007	0.3	2	6.0	3.7	4%	2	0
14	Cholesterol, Cortisol, Non-Gonadal Steroid Metabolism	16	0.037	1.8	2	1.1	3.5	4%	2	0
15	Gamma-Glutamyl and other Dipeptides	2	0.005	0.2	1	4.5	2.4	2%	1	0
16	Histidine, Histamine, Carnosine Metabolism	4	0.009	0.4	1	2.2	2.3	2%	1	0
17	Nitric Oxide, Superoxide, Peroxide Metabolism	2	0.005	0.2	1	4.5	2.2	2%	1	0
18	Tryptophan, Kynurenine, Serotonin, Melatonin	6	0.014	0.7	1	1.5	2.1	2%	1	0
19	Glycolysis and Gluconeogenesis Metabolism	7	0.016	0.8	1	1.3	2.1	2%	1	0
20	Vitamin C (Ascorbate) Metabolism	2	0.005	0.2	1	4.5	2.0	2%	1	0
21	Amino-Sugar, Galactose, & Non-Glucose Metabolism	5	0.012	0.6	1	1.8	1.9	2%	1	0
22	Phospholipid Metabolism	73	0.170	8.2	1	0.1	1.6	2%	0	1
								Subtotal	42	6
								Total	48	

[00135] Purine metabolism was the single most changed pathway. Suramin increased healthy purines such as AICAR, which is an activator of the master metabolic regulator AMP-dependent protein kinase (AMPK). 1-Methyl-adenine (1-MA) was also increased. 1-MA is derived from 1-methyl-adenosine, a recently recognized marker of new protein synthesis and cell growth. Suramin decreased other purines in the plasma such as cAMP and dGDP (see **FIG. 13A, Tables 18-20**). Improvements in 1-carbon, folate, methionine, and cysteine metabolism were also found (**Table 21**, and **FIG. 15**). **FIG. 13B** illustrates the similarities found in the pharmacometabolomic response to suramin in MIA13 and Fragile X mouse models and in children with ASD in this study. Twenty-one of the 28 pathways (75%) changed in ASD were also changed by suramin treatment in the mouse models of ASD (see **FIG. 13B**).

Table 18 Suramin pharmacometabolomics. Pathways changed at 2-days

No.	Pathway Name	Measured Metabolites in the Pathway (N)	Expected Pathway Proportion (P = N/431)	Expected Hits in Sample of 61 (P * 61)	Observed Hits in the Top 61 Metabolites	Fold Enrichment (Obs/Exp)	Impact (Sum VIP Score)	Fraction of Impact (VIP Score) Explained (% of 115.7)	Increased	Decreased
1	Purine Metabolism	26	0.060	3.7	9	2.4	17.6	15%	4	5
2	Bile Salt Metabolism	6	0.014	0.8	4	4.7	11.9	10%	4	0
3	Microbiome Metabolism	18	0.042	2.5	4	1.6	9.3	8%	4	0
4	Branch Chain Amino Acid Metabolism	12	0.028	1.7	4	2.4	7.3	6%	4	0
5	Eicosanoid and Resolvin Metabolism	13	0.030	1.8	4	2.2	7.1	6%	0	4
6	Phospholipid Metabolism	74	0.172	10.5	3	0.3	5.7	5%	0	3
7	SAM, SAH, Methionine, Cysteine, Glutathione	15	0.035	2.1	3	1.4	5.6	5%	2	1
8	GABA, Glutamate, Arginine, Ornithine	6	0.014	0.8	3	3.5	4.7	4%	3	0
9	Pyrimidine Metabolism	9	0.021	1.3	2	1.6	4.3	4%	1	1
10	Glycolysis and Gluconeogenesis Metabolism	7	0.016	1.0	2	2.0	4.3	4%	2	0
11	Gamma-Glutamyl and other Dipeptides	2	0.005	0.3	2	7.1	3.8	3%	2	0
12	Sphingomyelin Metabolism	36	0.084	5.1	2	0.4	3.6	3%	0	2
13	Biogenic and Neurotransmitter Metabolism	9	0.021	1.3	2	1.6	3.3	3%	0	2
14	Krebs Cycle	9	0.021	1.3	2	1.6	3.3	3%	2	0
15	Vitamin D (Calciferol) Metabolism	3	0.007	0.4	1	2.4	3.1	3%	0	1
16	Cardiolipin Metabolism	7	0.016	1.0	2	2.0	3.1	3%	2	0
17	Glycosphingolipid Metabolism	12	0.028	1.7	1	0.6	2.1	2%	1	0
18	Taurine, Hypotaurine Metabolism	2	0.005	0.3	1	3.5	2.0	2%	0	1
19	Nitric Oxide, Superoxide, Peroxide	2	0.005	0.3	1	3.5	1.9	2%	0	1
20	Histidine, Histamine, Carnosine Metabolism	4	0.009	0.6	1	1.8	1.8	2%	1	0
21	Tyrosine and Phenylalanine Metabolism	3	0.007	0.4	1	2.4	1.8	2%	1	0
22	Fatty Acid Oxidation and Synthesis	37	0.086	5.2	1	0.2	1.8	2%	0	1
23	Cholesterol, Cortisol, Non-Gonadal Steroid	16	0.037	2.3	1	0.4	1.8	2%	1	0
24	Amino Acid Metabolism	4	0.009	0.6	1	1.8	1.8	1%	1	0
25	Endocannabinoid Metabolism	4	0.009	0.6	1	1.8	1.7	1%	0	1
26	Amino-Sugar, Galactose, & Non-Glucose	5	0.012	0.7	1	1.4	1.6	1%	1	0
27	Tryptophan, Kynurenine, Serotonin	6	0.014	0.8	1	1.2	1.6	1%	1	0
28	Ceramide Metabolism	34	0.079	4.8	1	0.2	1.5	1%	1	0
								Subtotals	38	23
								Totals	61	

Table 19 Suramin pharmacometabolomics. Metabolites changed at 2-days

No.	Metabolite	Pathway Name	VIP Score	Z Score	Z Abs	Linear Ratio	Best p-value
1	Chenodeoxyglycocholic acid	Bile Salt Metabolism	3.371	1.620	1.620	1.787	0.159
2	1,25-Dihydroxyvitamin D3	Vitamin D (Calciferol) Metabolism	3.134	-1.447	1.447	0.371	0.311
3	Glycocholic acid	Bile Salt Metabolism	3.090	2.020	2.020	2.344	0.103
4	Taurodeoxycholic acid Peak	Bile Salt Metabolism	3.048	1.326	1.326	1.614	0.068
5	2-Keto-L-gluconate	Microbiome Metabolism	2.994	2.386	2.386	1.364	0.444
6	Taurocholic acid	Bile Salt Metabolism	2.813	1.102	1.102	2.183	0.002
7	2,3-Diphosphoglyceric acid	Glycolysis and Gluconeogenesis Metabolism	2.600	0.990	0.990	1.188	0.322
8	Cytidine	Pyrimidine Metabolism	2.556	2.083	2.083	1.489	0.118
9	p-Hydroxyphenylacetic acid	Microbiome Metabolism	2.548	1.464	1.464	1.192	0.149
10	11(S)-HETE	Eicosanoid and Resolvin Metabolism	2.400	-0.875	0.875	0.748	0.085
11	Hypoxanthine	Purine Metabolism	2.287	-1.000	1.000	0.743	0.108
12	Deoxyguanosine diphosphate	Purine Metabolism	2.264	-1.276	1.276	0.859	0.821
13	Glycylproline	Gamma-Glutamyl and other Dipeptides	2.209	1.212	1.212	1.773	0.919
14	Allantoin	Purine Metabolism	2.193	0.926	0.926	1.663	0.171
15	L-Isoleucine	Branch Chain Amino Acid Metabolism	2.136	0.813	0.813	1.094	0.041
16	GC(18:1/22:0)	Glycosphingolipid Metabolism	2.123	1.037	1.037	1.299	0.448
17	Cysteamine	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	2.072	1.398	1.398	1.107	0.180
18	LysoPC(14:0)	Phospholipid Metabolism	2.067	-0.808	0.808	0.777	0.176
19	Taurine	Taurine, Hypotaurine Metabolism	2.043	-0.942	0.942	0.736	0.644
20	1-Methyladenine	Purine Metabolism	2.032	1.327	1.327	1.631	0.038
21	SM(d18:1/20:1)	Sphingomyelin Metabolism	2.031	-1.230	1.230	0.745	0.288
22	PA(18:0/18:1)	Phospholipid Metabolism	1.992	-0.813	0.813	0.793	0.419
23	Cyclic adenosine monophosphate	Purine Metabolism	1.949	-0.681	0.681	0.825	0.668
24	Ascorbic acid	Nitric Oxide, Superoxide, Peroxide Metabolism	1.929	-2.024	2.024	0.914	0.828
25	Shikimate-3-phosphate	Microbiome Metabolism	1.898	1.033	1.033	1.047	0.709
26	Indoxyl sulfate	Microbiome Metabolism	1.858	0.702	0.702	1.280	0.059
27	1-Methylhistidine	Histidine, Histamine, Carnosine Metabolism	1.848	0.899	0.899	1.143	0.731
28	Purine	Purine Metabolism	1.847	1.137	1.137	1.208	0.131
29	L-Phenylalanine	Tyrosine and Phenylalanine Metabolism	1.839	0.897	0.897	1.364	0.718
30	Malonic acid	Fatty Acid Oxidation and Synthesis	1.833	-0.825	0.825	0.904	0.904
31	Methionine sulfoxide	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	1.817	1.738	1.738	1.331	0.085
32	L-Valine	Branch Chain Amino Acid Metabolism	1.808	0.749	0.749	1.163	0.783
33	7A,25-Dihydroxycholesterol	Cholesterol, Cortisol, Non-Gonadal Steroid Metabolism	1.807	1.014	1.014	1.382	0.744
34	Orotic acid	Pyrimidine Metabolism	1.787	-0.812	0.812	0.870	0.734
35	AICAR	Purine Metabolism	1.787	1.310	1.310	1.308	0.720
36	Isovalerylglycine	Branch Chain Amino Acid Metabolism	1.783	0.832	0.832	1.301	0.590
37	Alanine	Amino Acid Metabolism (not otherwise covered)	1.776	1.068	1.068	1.183	0.234
38	Xanthosine	Purine Metabolism	1.764	-1.116	1.116	0.811	0.902
39	Anandamide	Endocannabinoid Metabolism	1.713	-0.709	0.709	0.484	0.132
40	Cisaconitic acid	Krebs Cycle	1.704	1.229	1.229	1.121	0.475
41	Cysteine-S-sulfate	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	1.682	-0.644	0.644	0.869	0.862
42	PG(16:0/18:0)	Phospholipid Metabolism	1.664	-0.687	0.687	0.348	0.303
43	Dopamine	Bioamines and Neurotransmitter Metabolism	1.653	-0.642	0.642	0.877	0.182
44	Glycerol 3-phosphate	Glycolysis and Gluconeogenesis Metabolism	1.651	1.131	1.131	1.187	0.418
45	5-HETE	Eicosanoid and Resolvin Metabolism	1.646	-0.671	0.671	0.866	0.714
46	Myoinositol	Amino-Sugar, Galactose, & Non-Glucose Metabolism	1.645	0.783	0.783	1.206	0.188
47	L-Glutamic acid	Bioamines and Neurotransmitter Metabolism	1.641	-0.619	0.619	0.797	0.458
48	Gamma-Aminobutyric acid	GABA, Glutamate, Arginine, Ornithine, Proline Metabolism	1.628	1.101	1.101	1.068	0.991
49	L-Kynurenine	Tryptophan, Kynurenine, Serotonin, Melatonin Metabolism	1.617	0.623	0.623	1.099	0.238
50	Citric acid	Krebs Cycle	1.590	0.759	0.759	1.142	0.537
51	SM(d18:1/20:0)	Sphingomyelin Metabolism	1.576	-0.770	0.770	0.712	0.775
52	Gamma-glutamyl-Alanine	Gamma-Glutamyl and other Dipeptides	1.573	0.896	0.896	1.284	0.942
53	Tiglylglycine	Branch Chain Amino Acid Metabolism	1.562	0.627	0.627	1.141	0.971
54	L-Proline	GABA, Glutamate, Arginine, Ornithine, Proline Metabolism	1.548	0.603	0.603	1.135	0.598
55	CL(18:1/18:2/18:2/18:2)	Cardiolipin Metabolism	1.538	0.506	0.506	1.131	0.378
56	CL(18:2/18:2/18:2/18:1)	Cardiolipin Metabolism	1.523	0.474	0.474	1.102	0.072
57	11,12-Epoxyeicosatrienoic acid	Eicosanoid and Resolvin Metabolism	1.523	-0.634	0.634	0.828	0.131
58	Ceramide(d18:1/18:2)	Ceramide Metabolism	1.521	0.510	0.510	1.187	0.348
59	Guanosine	Purine Metabolism	1.519	-0.766	0.766	0.702	0.177
60	Prostaglandin J2	Eicosanoid and Resolvin Metabolism	1.509	-0.601	0.601	0.849	0.972
61	N-Acetylglutamic acid	GABA, Glutamate, Arginine, Ornithine, Proline Metabolism	1.503	0.613	0.613	1.120	0.283

Table 20 Suramin pharmacometabolomics. Metabolites changed at 6-weeks

No.	Metabolite	Pathway Name	MP Score	T Score	Z Abs	Linear Ratio	Test p-value
1	Deoxycytidine	Microbiome Metabolism	2.606	2.365	2.138	1.239	0.023
2	SM(d18:1/26:0 OH)	Sphingomyelin Metabolism	2.822	2.002	2.002	1.671	0.023
3	Glycine	1-Carbon, Folate, Formate, Glycine, Serine Metabolism	2.823	1.891	1.891	1.292	0.009
4	1-Methyladenine	Purine Metabolism	2.459	2.259	2.259	2.287	0.016
5	Alanine	Amino Acid Metabolism (not otherwise covered)	2.456	1.987	1.987	1.312	0.019
6	Cytidine	Pyrimidine Metabolism	2.442	2.552	2.552	1.932	0.010
7	Citric acid	Krebs Cycle	2.410	1.772	1.772	1.363	0.009
8	1-Pyrroline-5-carboxylic acid	GABA, Glutamate, Arginine, Ornithine, Proline Metabolism	2.358	1.922	1.922	1.298	0.008
9	Gamma-glutamylalanine	Gamma-Glutamyl and other Dipeptides	2.353	1.725	1.725	1.644	0.045
10	Histamine	Histidine, Histamine, Carnosine Metabolism	2.279	1.813	1.813	1.319	0.043
11	p-Hydroxyphenylacetic acid	Microbiome Metabolism	2.263	2.226	2.226	1.306	0.031
12	Alanine	Nitric Oxide, Superoxide, Peroxide Metabolism	2.151	2.558	2.558	1.123	0.013
13	Methionine sulfoxide	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	2.104	2.053	2.053	1.409	0.028
14	Kynurenine	Tryptophan, Kynurenine, Serotonin, Melatonin Metabolism	2.099	1.731	1.731	1.203	0.145
15	Glycerol 3-phosphate	Glycolysis and Gluconeogenesis Metabolism	2.081	1.731	1.731	1.264	0.006
16	Cystathionine	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	2.060	2.007	2.007	1.157	0.023
17	Chenodeoxyglycocholic acid	Bile Salt Metabolism	2.052	1.650	1.650	1.858	0.072
18	Hydroxyproline	Vitamin C (Ascorbate) Metabolism	2.039	3.009	3.009	1.210	0.118
19	2-Hydroxyisovaleric acid	Branch Chain Amino Acid Metabolism	1.888	1.246	1.246	1.236	0.043
20	Purine	Purine Metabolism	1.880	1.650	1.650	1.307	0.127
21	Cyclic adenosine monophosphate	Purine Metabolism	1.862	-1.544	1.544	0.701	0.007
22	Glycocholic acid	Bile Salt Metabolism	1.856	1.648	1.648	2.278	0.119
23	4-Hydroxyphenyllactic acid	Microbiome Metabolism	1.845	1.172	1.172	1.254	0.019
24	Deoxyguanosine diphosphate	Purine Metabolism	1.819	-1.583	1.583	0.884	0.062
25	Hexose Disaccharide (Hex)	Amino Sugar, Galactose, & Non-Glucose Metabolism	1.811	1.220	1.220	1.121	0.140
26	S-Adenosylhomocysteine	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	1.804	0.871	0.871	1.417	0.071
27	Isovalerylglycine	Branch Chain Amino Acid Metabolism	1.888	0.901	0.901	2.027	0.091
28	Allantoin	Purine Metabolism	1.882	1.068	1.068	1.798	0.191
29	Tigylglycine	Branch Chain Amino Acid Metabolism	1.878	1.310	1.310	1.302	0.101
30	Hippuric acid	Tyrosine and Phenylalanine Metabolism	1.875	1.381	1.381	1.245	0.143
31	Oxaloacetic acid	Krebs Cycle	1.862	0.928	0.928	1.278	0.143
32	Methodesol	Cholesterol, Cortisol, Non-Gonadal Steroid Metabolism	1.844	1.079	1.079	1.284	0.206
33	L-Asparagine	Amino Acid Metabolism (not otherwise covered)	1.824	1.381	1.381	1.360	0.209
34	Cinnamoylglycine	Tyrosine and Phenylalanine Metabolism	1.790	2.218	2.218	1.190	0.204
35	Octanoylcarnitine	Fatty Acid Oxidation and Synthesis	1.780	-1.451	1.451	0.703	0.059
36	L-Cystine	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	1.774	1.660	1.660	1.190	0.050
37	Uridine	Pyrimidine Metabolism	1.764	0.928	0.928	1.244	0.013
38	Mevalonic acid	Cholesterol, Cortisol, Non-Gonadal Steroid Metabolism	1.673	1.636	1.636	1.388	0.082
39	Chenodeoxycholic acid	Bile Salt Metabolism	1.670	1.575	1.575	2.088	0.031
40	Guanidinoacetic acid	SAM, SAH, Methionine, Cysteine, Glutathione Metabolism	1.644	1.284	1.284	1.217	0.161
41	3-Hydroxyisobutyric acid	Branch Chain Amino Acid Metabolism	1.622	0.997	0.997	1.308	0.078
42	Decanoylcarnitine	Fatty Acid Oxidation and Synthesis	1.617	-1.157	1.157	0.644	0.087
43	3-Hydroxy-cis-5-tetradecenoylcarnitine	Fatty Acid Oxidation and Synthesis	1.612	-1.096	1.096	0.734	0.099
44	Hippuric acid	Microbiome Metabolism	1.599	0.885	0.885	1.602	0.275
45	PE (18:0/18:0)	Phospholipid Metabolism	1.595	-1.331	1.331	0.844	0.070
46	L-Proline	GABA, Glutamate, Arginine, Ornithine, Proline Metabolism	1.546	0.749	0.749	1.196	0.114
47	SM(d18:1/18:1)	Sphingomyelin Metabolism	1.508	0.730	0.730	1.424	0.084
48	L-Serine	1-Carbon, Folate, Formate, Glycine, Serine Metabolism	1.505	0.954	0.954	1.192	0.275

TABLE 21 Outcomes

Outcome			Suramin						Placebo					
Instrument	Factor or behavior	Time after treatment (days)	Difference from baseline (mean ± sd)	95% CI	d[1]	N	P[2]	P[3]	Difference from baseline (mean ± sd)	95% CI	d[1]	N	P[2]	P[3]
Primary Outcomes														
ADOS-2	Comparison	45	-1.6 ± 0.55	-2.3 to -0.9	2.9	5	0.0028	0.038	-0.4 ± 0.55	-1.1 to +0.28	0.7	5	0.18	0.16
	Raw	45	-4.6 ± 1.9	-7.0 to -2.2	2.4	5	0.0062	0.039	-0.4 ± 1.8	-2.7 to +1.9	0.22	5	0.65	0.58
	Social	45	-3.2 ± 1.9	-5.6 to -0.8	1.7	5	0.020	0.043	0.0 ± 1.7	-2.2 to +2.2	0	5	0.99	0.71
	Restr/Rep	45	-1.4 ± 0.89	-2.5 to -0.29	1.6	5	0.025	0.059	-0.4 ± 2.1	-3.0 to +2.2	0.19	5	0.69	0.58
EOWPVT	Vocabulary	45	-4.2 ± 8.3	-14.5 to +6.1	-0.51	5	0.32	0.50	+2.0 ± 4.6	-3.8 to +7.8	0.43	5	0.39	0.50
Secondary Outcomes														
ABC	Stereotypy	7	-3.6 ± 2.1	-6.2 to -1.0	1.7	5	0.018	0.043	+0.4 ± 1.9	-2.0 to +2.8	-0.21	5	0.67	0.68
	Stereotypy	45	-4.0 ± 2.3	-6.9 to -1.1	1.7	5	0.019	0.042	+1.0 ± 4.3	-4.3 to +6.3	-0.23	5	0.63	0.69
ATEC	Total	7	-10 ± 7.7	-20 to -0.46	1.3	5	0.044	0.043	+7.2 ± 14	-10 to +25	-0.51	5	0.32	0.35
	Language	7	-2.2 ± 1.5	-4.0 to -0.36	1.4	5	0.021	0.059	0.0 ± 4.1	-5.0 to +5.0	0	5	0.99	0.89
	Sociability	7	-3.6 ± 2.6	-6.8 to -0.36	1.4	5	0.025	0.063	-0.8 ± 2.8	-4.3 to +2.6	0.29	5	0.55	0.58
	Language	45	-2.0 ± 1.4	-2.7 to -0.49	1.4	5	0.034	0.059	-0.2 ± 2.9	-3.8 to +3.4	0.07	5	0.88	0.79
CGI	Overall ASD	45	-1.8 ± 1.04	-3.4 to -0.15	1.7	5	0.05	n/a	0.0 ± 0.34	-0.55 to +0.55	0	5	0.99	n/a
	E. Language	45	-2.0 ± 1.04	-3.6 to -0.35	1.9	5	0.01	n/a	0.0 ± 0.34	-0.55 to +0.55	0	5	0.99	n/a
	Social Inter.	45	-2.0 ± 1.04	-3.6 to -0.35	1.9	5	0.01	n/a	0.0 ± 0.34	-0.55 to +0.55	0	5	0.99	n/a
RBQ	Total	45	-3.2 ± 5.8	-10.4 to +4.0	0.55	5	0.28	0.22	-0.8 ± 3.3	-4.9 to 3.3	0.24	5	0.62	0.47

[1]A positive Cohen's d reflects improvement, and a negative d reflects a decrease by convention. Cohen's d is likely an overestimate of the actual treatment effect based on the large mean differences and small standard deviations found before and after treatment in this small study. [2]P value from parametric paired *t*-test analysis. [3]P value from non-parametric paired Wilcoxon signed-rank sum analysis. **Abbreviations.** ADOS-2: autism diagnostic observation schedule, 2nd edition. EOWPVT: expressive one-word picture vocabulary test. ABC: aberrant behavior checklist. ATEC: autism treatment evaluation checklist. CGI: clinical global impression survey. RBQ: repetitive behavior questionnaire. Restr/Rep: restricted or repetitive behaviors. Overall ASD Sx: overall ASD symptoms. E. Language: expressive language. Social Inter.: social interaction. **Analysis.** ADOS, EOWPVT, ABC, ATEC, and RBQ scores were analyzed by paired analysis before and after treatment using each subject as their own control. CGI was analyzed by 2-way ANOVA (symptom x time before and after treatment) with post hoc correction. Non-parametric p values were not calculated (n/a). **Interpretation.** ADOS, ABC, ATEC, CGI, and RBQ are severity scores; negative differences from baseline reflect decreased severity, i.e., improvement. EOWPVT is a performance score; negative differences reflect a decrease.

[00136] Suramin Quantitation. Suramin concentrations were measured by LC-MS/MS as previously described with modifications. Plasma suramin samples were collected at 1 hour, 2 days and 42 days post-infusion. Heparinized plasma, 90 µl was used. Ten (10) µL of 50 µM stock of trypan blue was added to achieve an internal standard concentration of 5 µM. This was incubated at room temperature for 10 min to permit metabolite interaction with binding proteins, then extracted with 4 volumes (400 µL) of pre-chilled methanol-acetonitrile (50:50) to produce a final concentration of 40:40:20 (methanol:acetonitrile:H_2O), and precipitated on ice for 10 minutes. The samples were deproteinated and macromolecules removed by precipitation on crushed ice for 10 min. The mixture was centrifuged at 16,000g for 10 min at 4 °C and the supernatant was transferred to a new tube and kept at -80 °C for further LC-MS/MS analysis.

[00137] Suramin was analyzed on an AB SCIEX QTRAP 5500 triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization (ESI) source, Shimadzu LC-20A UHPLC system, and a PAL CTC autosampler. Ten microliters of extract were injected onto a Kinetix F5 column (100 × 2.1 mm, 2.6 µm; Phenomenex, CA) held at 30 °C for chromatographic separation. The mobile phase A was water with 20 mM ammonium acetate (NH_4OAC) (pH 7) and mobile phase B was methanol with 20 mM NH_4OAC (pH 7). Elution was performed using the following gradient: 0-1.5 min-0% B, 1.6-3 min-15% B, 3.1-7 min-60% B, 7.1-13 min-100% B, 14 min-0% B, 18 min-0% B, 18.1 minute-end. The flow rate was 400 µl/min. All the samples were kept at 4 °C during analysis. Suramin and trypan blue were detected using MRM scanning mode with the dwell time of 180 ms. MRM transitions for the doubly-charged form of suramin were 647.0 m/z for the (Q1) precursor and 382.0 m/z for the (Q3) product. MRM transitions for trypan blue were 435.2 (Q1) and 185.0 (Q3). Absolute concentrations of suramin were determined using a standard curve prepared in plasma to account for matrix effects, and the peak area ratio of suramin to the internal standard trypan blue. The declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were -104, -9.5, -32 and -16.9, and

-144.58, -7, -57.8 and -20.94, for suramin and trypan blue, respectively. The ESI source parameters were set as follows: source temperature 500 °C; curtain gas 30; ion source gas 1, 35; ion source gas 2 35; spray voltage -4500 V. Analyst v1.6 was used for data acquisition and analysis.

[00138] Outcomes. The primary outcome measures were ADOS-2 and expressive one-word picture vocabulary (EOWPVT) scores (**Table 21**). Parents reported that after suramin treatment, the rate of language, social, behavioral, and developmental improvements continued to increase for 3 weeks, then gradually decreased toward baseline over the next 3 weeks. The blood levels of suramin at 3 weeks were estimated to be 4.2 ± 0.5 μM using our PK model. ADOS-2 comparison scores at 6-weeks improved by an average of -1.6 ± 0.55 points (mean ± SD; n = 5; 95% CI = -2.3 to -0.9; Cohen's d = 2.9; p = 0.0028) in the suramin treatment group and did not change in the saline group. We calculated p values by both parametric and non-parametric methods (**Table 21**). The mean ADOS comparison score in the suramin-treated group was 8.6 ± 0.4 at baseline and 7.0 ± 0.3 at 6-weeks. Two-way ANOVA of ADOS scores of suramin and placebo groups measured at baseline and at 6-weeks were also significant (treatment x time interaction $F(1,8) = 12.0$; $p = 0.0085$; and **FIG. 16A**). ADOS scores were not changed in the saline treated group (**Table 21**). EOWPVT scores did not change (**Table 21**). Several secondary outcome measures also showed improvements. These included improvements in ABC, ATEC, and CGI scores (**Table 21**). The Repetitive Behavior Questionnaire (RBQ) scores did not capture a change.

[00139] ADOS comparison scores were improved in the suramin treatment group at 6-weeks (see **FIG. 16A-B**) but were unchanged in the saline group (See **FIG. 16A**, and **16F**). ADOS scores at 2-days after treatment were not changed (See **FIG. 16E**). EOWPVT scores were not changed (See **FIG. 16I**). Secondary outcomes included Aberrant Behavior Checklist (ABC), Autism Treatment Evaluation Checklist (ATEC), the Clinical Global Impression (CGI), and the Repetitive Behavior Questionnaire (RBQ). Suramin treatment was associated with improvements in the ABC, ATEC, and CGI, but not in the RBQ

(See **FIG. 16**). Three of 24 symptoms covered in the CGI were significant (See **FIG. 16aa**). Parents were also asked to specify the three top, most-changed behaviors as an unstructured component of the CGI at 6-weeks after the infusion. Five symptoms were named that achieved statistically significant results. These were social communication and play, speech and language, calm and focus, stims or stereotypies, and coping skills (See **FIG. 16bb**).

[00140] Discussion. The aim of the SAT-1 trial was to test the safety, pharmacokinetics, and pharmacodynamics of low-dose suramin in children with ASD. A self-limited rash was seen, but no serious adverse events occurred. Pharmacometabolomic analysis showed that the pathways changed by suramin treatment in ASD were previously known mediators of the cell danger response (CDR) and that purine metabolism was changed most. Seventy-five percent of the pathways changed by suramin in children with ASD were also changed by suramin in mouse models.

[00141] Safety. Suramin has been used safely for nearly a century to treat both children and adults with African sleeping sickness. Although side effects occurred occasionally, toxicity was rare and could be further minimized by attention to patient nutritional status, proper dose, administration procedures, and measured blood levels of suramin. In this study, a single, 20 mg/kg dose of suramin was administered intravenously to help restore normal purinergic signaling and metabolism. This dose produced blood levels of 1.5-15 μM for 6 weeks. Previous uses of suramin have never studied the side effect profile in this low-dose range. The side effect profile of high-dose suramin (150-270 μM) is known from cancer chemotherapy studies. The side effect profile from medium-dose suramin (50-100 μM) is known from African sleeping sickness studies. However, the side effect profile of low-dose suramin (5-15 μM) used for antipurinergic therapy (APT) in autism is unknown. Low-dose suramin was found to be safe in 5 children, ages 5-14 years, in this study.

[00142] The rash caused by suramin in this study was not raised and did not itch. It was not urticarial. The children did not appear to notice it. Any residual rash was covered by clothing and not

visible on exposed skin at the 2-day evaluation. Parents were instructed not to discuss it with the neuropsychology team to decrease the chance of examiner bias. Video camera records of the ADOS testing confirmed the absence of any visible rash. The rash was a known risk of suramin treatment.

[00143] Study Considerations. Parents reported that the rate of new behavioral and developmental improvements continued to increase for 3 weeks after the single dose of suramin, as blood levels of suramin fell from 12 to 4 μM, then gradually decreased toward baseline over the next 3 weeks, as blood levels fell further from 4 to 1.5 μM. This pattern of response suggested a threshold effect that could not have been predicted on the basis of what was known about suramin before this study, and outcomes were not measured at 3 weeks.

[00144] Psychopharmacology. Suramin has objective central nervous system (CNS) effects in animal models and children with autism despite being unable to penetrate the blood brain barrier. Suramin also has a number of peripheral effects on innate immunity, metabolism, pain, gut, autonomic, inflammatory, and other pathways regulated by purinergic signaling that may contribute to the beneficial effects observed. Previous studies have shown that suramin is taken up into the CNS at the level of the brainstem, although not appreciably into the cerebrum or cerebellum. There are 8 circumventricular organs (CVOs) in the brain that contain neurons that lack a blood brain barrier. The area postrema in the brainstem is one of these CVOs that monitors the chemistry of the blood and transduces this information to higher centers in the brain for neuroendocrine, affective, cognitive, and behavioral integration. Rather than being a disadvantage, the peripheral actions and indirect CNS effects of suramin may have certain advantages by minimizing the risk of CNS toxicity. While new antipurinergic drugs (APDs) may soon be developed that can pass the blood brain barrier, this appears not to be required to produce the behavioral effects of suramin in ASD.

[00145] The SAT-1 trial examined the effects of low-dose suramin or placebo in 10 children with autism spectrum disorder. No safety

concerns were found. A two-compartment pharmacokinetic model permitted accurate forecasting of plasma drug levels from 1 hour to 6-weeks after the infusion. Metabolomic studies confirmed the importance of the cell danger response (CDR)8 and purinergic signaling. A single, 20 mg/kg intravenous dose of suramin was associated with improved scores for language, social interaction, and decreased restricted or repetitive behaviors measured by ADOS, ABC, ATEC, and CGI scores. None of these improvements occurred in the 5 children who received placebo.

EXAMPLE 3

[00146] The following results were collected from a cohort of about 80 mice. These animals were exposed *in utero* to either saline (Controls = 40), or a simulated viral infection by injection of the double strand RNA (PolyIC) (ASD = 40) into pregnant dams on gestational day E12.5. Equal numbers of males and females were used in each experimental group. Half of the controls (N= 20) and half of the PolyIC-exposed animals (N = 20) were treated at 8-weeks of age after the onset of ASD-like behaviors. Treatment consisted of weekly injections of either Saline or antipurinergic therapy with suramin. The 4 experimental groups of animals (N = 20 per group; 10 males and 10 females each) were then comprehensively evaluated beginning at 12 weeks of age. **Table 22** provides a list of the tests that have been completed to date.

Table 22. Summary of Antipurinergic Therapy Results in the Poly(IC) Mouse Model of Autism Spectrum Disorders

Feature	Abnormality in Males	Response to Antipurinergic Therapy
Social Preference	Decreased	Normalized ($p<0.05$)
Sensorimotor Coordination (Rotarod)	Decreased	Normalized ($p<0.001$)
Basal Body Temperature	Decreased	Normalized ($p<0.001$)
Oxygen Consumption During Sleep	Unchanged*	Increased ($p<0.001$)
Plasma Immunoglobulins	Unchanged*	Increased ($p<0.05$)
Plasma Corticosterone	Unchanged*	Increased ($p<0.03$)
Synaptosomal Structure by Electron Microscopy	Fragile and malformed post-synaptic densities; Accumulation of electron dense material	Normalized
Cerebral Mitochondrial Respiratory Chain Complex I Activity	Increased	Normalized ($p<0.02$)
Cerebral Mitochondrial Respiratory Chain Complex IV Activity	Increased	Normalized ($p<0.02$)
Synaptosomal Purinergic Receptor (P2Y1) Expression	Decreased	Normalized ($p<0.02$)
Synaptosomal Purinergic Receptor (P2Y2) Expression	Decreased	Normalized ($p<0.02$)
Synaptosomal Purinergic Receptor (P2X7) Expression	Decreased	Normalized ($p<0.02$)
Synaptosomal ERK1/2 Phosphorylation	Decreased	Normalized ($p<0.001$)
Synaptosomal CAMKII Phosphorylation	Decreased	Normalized ($p<0.001$)
Synaptosomal FMRP Expression	Decreased	Normalized ($p<0.02$)
Synaptosomal Nictotinic Acetylcholine Receptor subunit α7 Expression	Unchanged*	Increased ($p<0.001$)
Cerebellar Vermis Lobule VII Purkinje Cell Number	Decreased	Preserved ($p<0.05$)

[00147] Social Approach. A classic 3-chamber Crawley social interaction paradigm was used with automated and hand scoring of the number and duration of social encounters of an experimental mouse with a novel mouse placed under an inverted, wire cup. It was found that antipurinergic therapy with suramin significantly increased the social encounter duration of male ASD mice (see **FIG. 17**). No effect was found in the females (data not shown).

[00148] Neuromuscular Coordination—Rotarod. Purkinje cell abnormalities in the cerebellum are a consistent feature of the brain abnormalities in the MIA mouse model of autism. Because of the critical role of cerebellar processing in whole-body neuromuscular coordination, a rotarod test was used to detect

difference between treatment groups. In this first cohort of ASD mice, a decrease in rotarod latency to fall in the Poly(IC)-exposed animals was not shown. However, antipurinergic therapy with suramin showed a trend toward improved rotarod performance in the females (n = 10) (see **FIG. 18**). ASD males had normal rotarod performance compared to controls and no effect of suramin (data not shown).

[00149] Hyperactivity. It was found that hyperactivity, measured by center entries in a standard open field test, was sexually dimorphic in this cohort of ASD mice. Male ASD mice showed an increased number (see **FIG. 19**) of center entries. This increase was returned to control levels by antipurinergic therapy with suramin. Females did not show hyperactivity and did not show a suramin effect (data not shown).

[00150] Self-Calming and Autonomic Balance. Perhaps the most fundamental change in autism is the inability to self-calm after an excitatory stimulus. These stimuli may come in the form of an attempt at social engagement by a parent, a touch, a sound, bright lights, or even a particular taste of food. A classic paradigm of stress-induced hyperthermia (SIH) was used to test the hypothesis that a standardized stress will produce an exaggerated response in ASD mice. It was found that the ASD mice had an exaggerated SIH response and that this was normalized by antipurinergic therapy with suramin (see **FIG. 20**). This effect was observed in both males and females. All of these measures were performed in 4- month old animals after 1 month of antipurinergic or saline treatment.

[00151] It was next asked, "Is the increased SIH response in our ASD mice a function of an increased stress temperature, a decreased basal temperature, or both?" SIH is measured by taking the difference between a basal body temperature taken at time 0 and a post-stress temperature taken at time 10 minutes (see **FIG. 21A**). Interestingly, it was noted that the increased SIH in the maternal immune activation model of autism was primarily the result of a decrease in the baseline body temperature of the mice (See **FIG. 21A**). Antipurinergic therapy restored normal core temperatures (see **FIG. 21B**).

[00152] Basal Body Temperature and Purinergic Signaling. Next, the focus was on the baseline core temperatures of the control mice. It was found that there were no sex differences between males and females (data not shown). When we analyzed the core temperatures of all animals, we found that animals that were exposed to Poly(IC) in utero had a 0.4 °C lower temperature than saline controls (see **FIG. 22A**).

[00153] Since in **Figures 22A** and **22B** it was found that antipurinergic therapy with suramin returned the basal body temperature of ASD mice to normal, next the effect of a direct purinergic stimulus with ATP on body temperature was tested. An experiment was performed in an unrelated cohort of animals to test the effect of an ip injection of ATP on body temperature. It was found that direct injection ip of ATP produced an average temperature drop of 1.8 °C compared to controls (see **FIG. 22B**).

[00154] A Single Dose of Suramin Corrects the Short-term Memory Defect in the Poly(IC) Mouse. Five month old, ASD-like animals displayed a short-term memory defect that was quantified using the T-maze paradigm. Control C57BL/6 mice will recall the immediately previous T-maze arm exploration and will spontaneously alternate into right and left arms 75% of the time (see **FIG. 23;** Sal-Sal). This alternation falls to random choice (50%) in ASD-like animals (see **FIG. 23;** PIC-Sal). A single ip dose of suramin given to animals that have been symptomatic and untreated for 5 months of age causes complete correction of this short-term memory deficit (see **FIG. 23;** PIC-Sur).

[00155] A Single Dose of Suramin Corrected the Social Abnormalities in the Poly(IC) Mouse. Six month old, ASD-like animals had a 30% sustained decrease in their social interactions measured in the classical 3-chamber social approach chamber. When given a single dose of Suramin, their social behavior was completely restored to normal. This is unprecedented in the history of autism animal model research that a single dose of a drug can normalize the classical social inhibitions of autism-like behavior. The striking effectiveness of single-dose therapy implies the presence of a neurochemical switch that controls autism-like behavior and

learning disabilities.

[00156] It was shown that antipurinergic therapy is a promising new treatment for autism in a mouse model. In addition the first steps have taken in the exploration of completely new way of understanding the root cause and pathogenetic mechanisms of many of the different forms of human autism. It is clear that there are over a dozen monogenic forms of human autism, and scores of ecogenetic forms that result from a combination of environmental and genetic factors. Yet it is believed that abnormal purinergic signaling is a common denominator of many of these different forms of autism.

[00157] In the first experiments, antipurinergic therapy was sometimes more effective in males, as in the social approach paradigm and hyperactivity (see **FIG. 17** and **FIG. 19**). In some cases, it was more effective in females, as in correcting the coordination abnormalities (see **FIG. 18**). In many cases, antipurinergic therapy with suramin was equally effective in both males and females, as seen in the SIH and in the restoration of normal core body temperatures (see **FIG. 20** and **FIG. 21**).

[00158] As a serendipitous discovery made in the process of these studies, it was found that direct purinergic stimulation by ip injection of ATP caused a rapid decrease in core body temperature (see **FIG. 22B**). This helped explain why the ASD mice in the study had a significantly decreased basal body temperature (see **FIG. 22A**). A unifying hypothesis that explains all of these observations is that the maternal immune activation model of autism in particular, and potentially all human autism in general, is fundamentally the result of dysregulated purinergic signaling. Antipurinergic therapy holds great promise in treating this fundamental defect in autism.

[00159] Antipurinergic therapy (APT) is also effective in adult animals, 6 months of age (see **FIG. 23** and **FIG. 24**). In these animals, a single dose of suramin corrected behavioral and learning abnormalities that were present since shortly after birth. The results suggest that APT is capable of triggering a previously unknown, neurochemical switch that lies at the cause of

abnormalities that were formerly thought to be a fixed feature of disease. Based on these discoveries, and the long reach of the Cell Danger Theory of Autism and Related Spectrum Disorders, it is believed that APT will be effective for at least treating the following: autism, chronic fatigue syndrome, fibromyalgia, obsessive compulsive disorder, generalized anxiety disorder, schizophrenia, bipolar depression, subacute therapy for traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), chronic traumatic encephalopathy (CTE), disabling Attention Deficit Hyperactivity Disorder (ADHD, ADD), acute therapy of lupus encephalitis, and early Alzheimer Disease.

[00160] A number of embodiments have been described herein. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of this disclosure. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating mitochondrial disease or disorder comprising, administering an effective amount of an antipurinergic agent to a subject suffering from a mitochondrial disease or disorder.

2. The method of claim 1, wherein the mitochondrial disease or disorder is selected from the group consisting of autism spectrum disorders, chronic fatigue syndrome, Friedreich's ataxia, Leber's hereditary optic neuropathy (LHON), myoclonic epilepsy and ragged-red fibers (MERRF), mitochondrial encephalomyopathy, mitochondrial neurogastrointestinal encephalopathy (MNGIE), lactic acidosis, stroke-like syndrome (MELAS), Kearns-Sayre syndrome, Pearson marrow syndrome, neuropathy ataxia and retinitis pigmentosa (NARP), Polymerase gamma (POLG), Alpers-Huttenlocher syndrome, ataxia neuropathy spectrum (ANS) disorders, mitochondrial DNA depletion syndromes, mitochondrial DNA depletion syndromes resulting from gene defects and subacute necrotizing encephalopathy (Leigh's Syndrome).

3. The method of claim 1 or 2, wherein the antipurinergic agent is suramin or a derivative thereof.

4. The method of claim 3, wherein the antipurinergic agent is administered in combination with a second agent.

5. The method of claim 4, wherein the second agent comprises cysteamine or derivative thereof, cystamine or derivative thereof and/or triacetyluridine (TAU) or derivative thereof.

6. The method of claim 1, wherein the subject has decreased thiol levels compared to a subject that does not have a mitochondrial disease or disorder.

7. The method of claim 1, wherein the administering results in improvement in mitochondrial activity markers compared to levels before administration of the antipurinergic agent.

8. The method of claim 7, wherein the mitochondrial activity marker is selected from the group consisting free thiol levels, glutathione (GSH), reduced glutathione (GSSH), total glutathione, advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), lactic acid, pyruvic acid, lactate/pyruvate ratios, phosphocreatine, NADH levels, NADPH levels, NAD levels, NADP levels, ATP, anaerobic threshold, reduced coenzyme Q, oxidized coenzyme Q; total coenzyme Q, oxidized cytochrome C, reduced cytochrome C, oxidized cytochrome C/reduced cytochrome C ratio, acetoacetate, β-hydroxy butyrate, acetoacetate/β-hydroxy butyrate ratio, 8-hydroxy-2'-deoxyguanosine (8-OHdG), levels of reactive oxygen species, levels of oxygen consumption (VO2), levels of carbon dioxide output (VCO2), and respiratory quotient (VCO2/VO2).

9. The method of claim 3, wherein the administering results in increased thiol levels compared to levels before administration of the antipurinergic agent.

10. The method of claim 1, wherein the subject is a child or adolescent.

11. The method of claim 1, wherein the subject has a cognitive disorder selected from the group consisting of aphasia, delirium, dementia, amnesia, executive dysfunction and cerebrovascular disease.

12. The method of claim 11, wherein the dementia is selected from the group consisting of Alzheimer's disease, cortical dementia, and subcortical dementia.

13. The method of claim 1, wherein the subject has an autism spectrum disorder.

14. The method of claim 13, wherein the subject has a disorder selected from the group consisting of attention-deficit hyperactivity disorder, autism, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder and Asperger's disorder.

15. The method of claim 1, wherein the antipurinergic agent blocks P2X/P2Y signaling of extracellular ATP, ADP, and P1-receptor (adenosine) signaling of extracellular adenosine or AMP.

16. The method of claim 3, wherein the suramin is administered at dose so as to maintain blood levels of 4-20 μM of suramin.

17. The method of claim 16, wherein the suramin is administered at intervals of 3 to 8 weeks.

18. The method of claim 16 or 17, wherein the suramin is administered for 2 to 12 months.

19. The method of claim 1, wherein the method comprises administering the antipurinergic agent and at least one additional active agent, and wherein the at least one additional active agent is used to treat a mental, developmental and/or cognitive disorder.

20. The method of claim 19, wherein the at least one additional active agent is an antipsychotic agent, a stimulant, and/or antidementia agent.

21. The method of claim 2, wherein the mitochondrial disease or disorder is chronic fatigue syndrome.

23. A method to determine a subject’s risk of having or developing chronic fatigue syndrome (CFS) comprising:

detecting an amount of each of a plurality of metabolites in a biological sample obtained from the subject, the plurality of metabolites comprising at least seven metabolites, each of the at least seven metabolites being in a metabolic pathway selected from the group of pathways consisting of: a sphingolipid metabolic pathway, a phospholipid metabolic pathway, a glycosphingolipid metabolic pathway, a purine metabolic pathway, a microbiome metabolic pathway, a cholesterol metabolic pathway, a vitamin B2 metabolic pathway, a pyrroline-5-carboxylic acid metabolic pathway, an arginine metabolic pathway, a proline metabolic pathway, and a branch chain amino acid pathway;

determining the presence or absence of an alteration in the metabolic pathways of the subject based upon comparing the amounts of the detected metabolites of the subject versus the amounts of the metabolites detected from a control population that does not have CFS; and

indicating that the subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the subject are altered in comparison to the control population; and

treating the subject according to claim 1.

24. The method of claim 23, wherein the subject is a male subject, and the method further comprises:

detecting an amount of each of a plurality of metabolites in a biological sample obtained from the male subject, the plurality of metabolites comprising at least three metabolites, each of the at least 3 metabolites being in a metabolic pathway selected from the group of pathways consisting of: a serine/1-carbon metabolic pathway, a S-adenosyl methionine pathway, a *S*-adenosylhomocysteine metabolic pathway, a methionine metabolic pathway, a very long chain fatty acid oxidation metabolic pathway, a propiogenic ammino acid metabolic pathway, and a threonine metabolic pathway;

determining the presence or absence of an alteration in the metabolic pathways of the male subject based upon comparing the amounts of the detected metabolites from the male subject versus

the amounts of the metabolites detected from a control population of male subjects that do not have CFS; and

indicating that the male subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the male subject are altered in comparison to the control population of male subjects.

25. The method of claim 24, wherein the metabolites that are detected are phosphatidyl choline PC(16:0/16:0), glucosylceramide GC(18:1/16:0), 1-pyrroline-5-carboxylate (P5C), flavin adenine dinucleotide (FAD), pyroglutamic acid, 2-hydroxyisoccaproic acid, L-serine, and lathosterol.

26. The method of claim 23, wherein the subject is a female subject, and the method further comprises:

detecting an amount of each of a plurality of metabolites in a biological sample obtained from the female subject, the plurality of metabolites comprising at least three metabolites, each of the at least 3 metabolites being in a metabolic pathway selected from the group of pathways consisting of: a fatty acid oxidation metabolic pathway, a vitamin C/collagen metabolic pathway, a bile acid metabolic pathway; an endocannabinoid metabolic pathway, a vitamin B12 metabolic pathway; and an amino sugar metabolic pathway;

determining the presence or absence of an alteration in the metabolic pathways of the female subject based upon comparing the amounts of the detected metabolites from the female subject versus the amounts of the metabolites detected from a control population of female subjects that do not have CFS; and

indicating that the female subject has or is at risk of developing CFS based upon the determining that the metabolic pathways in the female subject are altered in comparison to the control population of female subjects.

27. The method of claim 26, wherein the metabolites that are detected are trihexosylceramide THC(18:1/24:0), phosphatidyl

choline PC(16:0/16:0), hydroxyproline, ceramide(d18:1/22:2),lathosterol, adenosine, PI(16:0/16:0), flavin adenine dinucleotide (FAD), 2-octenoylcarnitine, phosphatidyl choline plasmalogen PC(22:6/P18:0), phosphatidyl choline PC(18:1/22:6), 1-pyrroline-5-carboxylate, and chenodeoxycholic acid.

28. The method of claim 23, wherein the metabolites that are detected are selected from group consisting of PC(16:0/16:0), ceramide(d18:1/24:2), GC(18:1/16:0), ceramide (d18:1/16:0), THC(d18:1/24:0), PI(38:4), DHC(18:1/16:0), PA(16:0/16:0), 1-pyrroline-5-carboxylic acid, SM(d18:1/24:2), ceramide(d18:1/16:1OH), SM(d18:1/22:0), SM(d18:1/23:0), ethanolamine, 4-hydroxyphenyllactic acid, FAD, ceramide(d18:1/16:1), ceramide(d18:1/18:0), ceramide(d18:1/26:2), ceramide(d18:1/22:2), L-serine, methionine sulfoxide, ceramide(d18:1/22:1), ceramide(d18:1/25:0), SM(d18:1/18:2OH), ceramide(d18:1/24:1), arginine, SM(d18:1/16:0), behenic acid, hydroxyisocaproic acid, uric acid, lathosterol, PC(16:0/20:4), PC(18:1/22:6), and SM(d18:1/24:0).

29. The method of claim 23, wherein the metabolites that detected are selected from the group consisting of ceramide(d18:1/25:0), THC(d18:1/24:0), PC(16:0/16:0), lathosterol, hydroxyproline, PI(16:0/16:0), ceramide(d18:1/22:2), adenosine, ceramide(d18:1/24:2), THC(d18:1/16:0), 2-octenoylcarnitine, GC(18:1/16:0), phenyllactic acid, ceramide(d18:1/26:0), ceramide (d18:1/24:0), DHC(d18:1/16:0), ceramide(d18:1/26:2), FAD, 1-pyrroline-5-carboxylic acid, ceramide(d18:1/16:0), SM(d18:1/22:2), adenosine monophosphate, PC(40:6), PI(38:3), PC(22:6/P-18:0), PC(36:0), PC(22:6/P-18:0), PC(36:0), chenodeoxycholic acid, ceramide(d18:1/20:0), PC(18:1/22:6), ceramide(d18:1/23:0), ceramide(d18:1/18:2OH), ceramide(d18:1/22:0), ceramide(d18:1/18:0), ceramide(d18:1/16:1OH), and ceramide(d18:1/24:2OH).

30. The method of claim 23, wherein the at least 7 metabolites comprise a metabolite in each of at least 7 of the group of metabolic pathways.

31. The method of claim 23, wherein an alteration in a metabolic pathway is determined by detecting a reduced or elevated amount of a metabolite from the subject in comparison to the control population.

32. The method of claim 23, wherein the metabolites from the subject are converted to a non-naturally occurring by-product that is analyzed.

33. The method of claim 32, wherein the non-naturally occurring by-product is a mass fragment.

34. The method of claim 23, wherein the metabolites are detected by using one or more of the following: HPLC, TLC, electrochemical analysis, mass spectroscopy, refractive index spectroscopy (RI), Ultra-Violet spectroscopy (UV), fluorescent analysis, gas chromatography (GC), radiochemical analysis, Near-InfraRed spectroscopy (Near-IR), Nuclear Magnetic Resonance spectroscopy (NMR), and Light Scattering analysis (LS).

35. The method of claim 23, wherein the biological sample is selected from the group consisting of cells, cellular organelles, interstitial fluid, blood, blood-derived samples, cerebral spinal fluid, and saliva.

36. The method of claim 23, wherein the biological sample is a fluid sample.

37. The method of claim 36, wherein the fluid sample is a serum sample.

38. The method of claim 36, wherein the fluid sample is a urine sample.

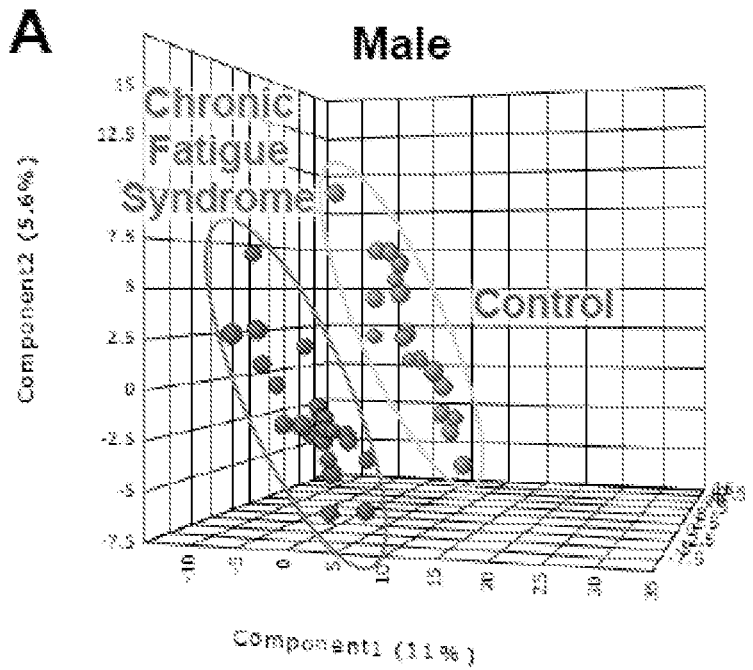

FIG. 1A

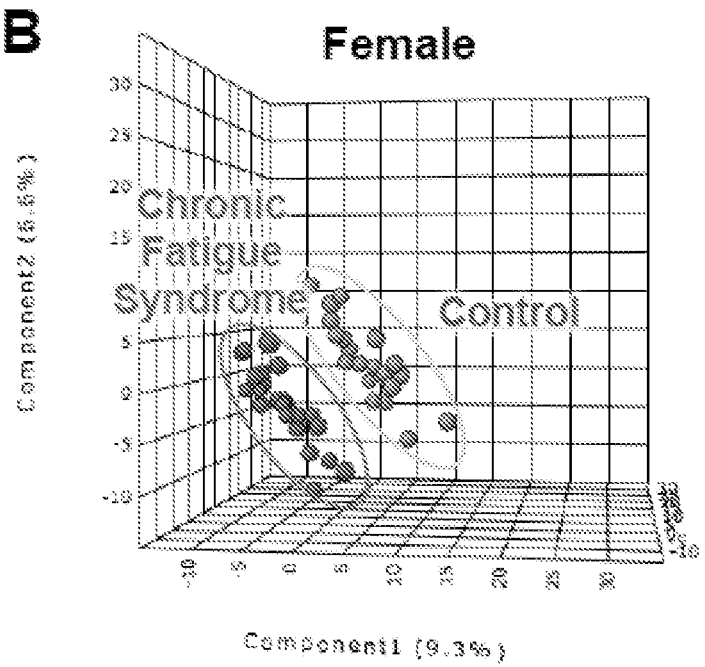

FIG. 1B

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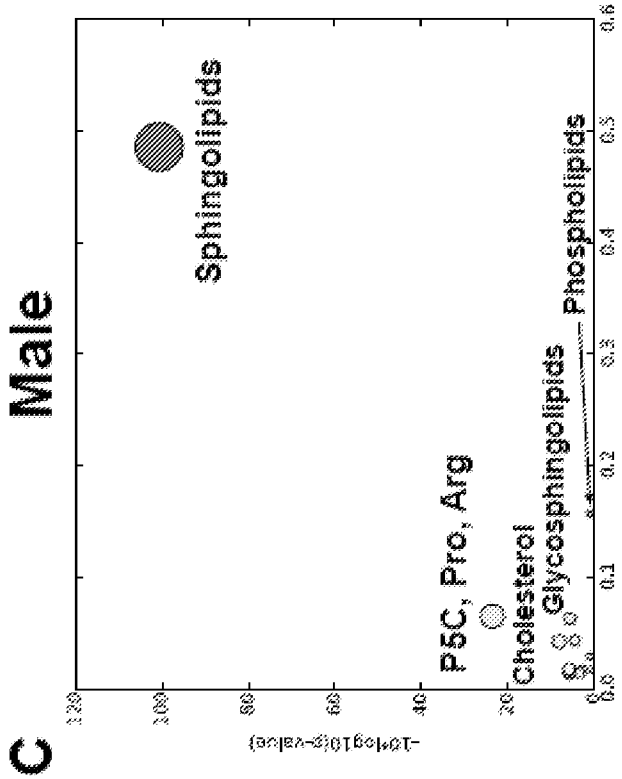
C
Male
Sphingolipids
P5C, Pro, Arg
Cholesterol
Glycosphingolipids
Phospholipids
Fractional Impact
-10*log10(p-value)

FIG. 1C

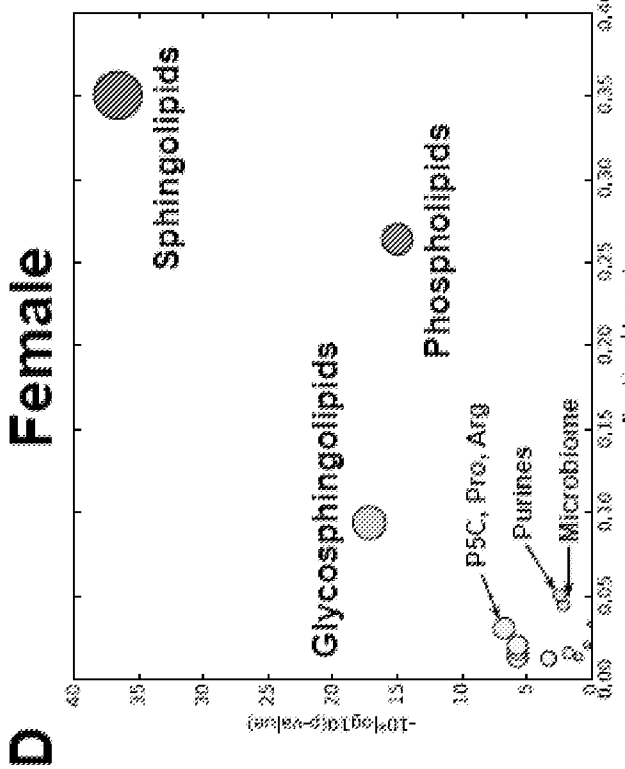
D
Female
Sphingolipids
Glycosphingolipids
Phospholipids
P5C, Pro, Arg
Purines
Microbiome
Fractional Impact
-10*log10(p-value)

FIG. 1D

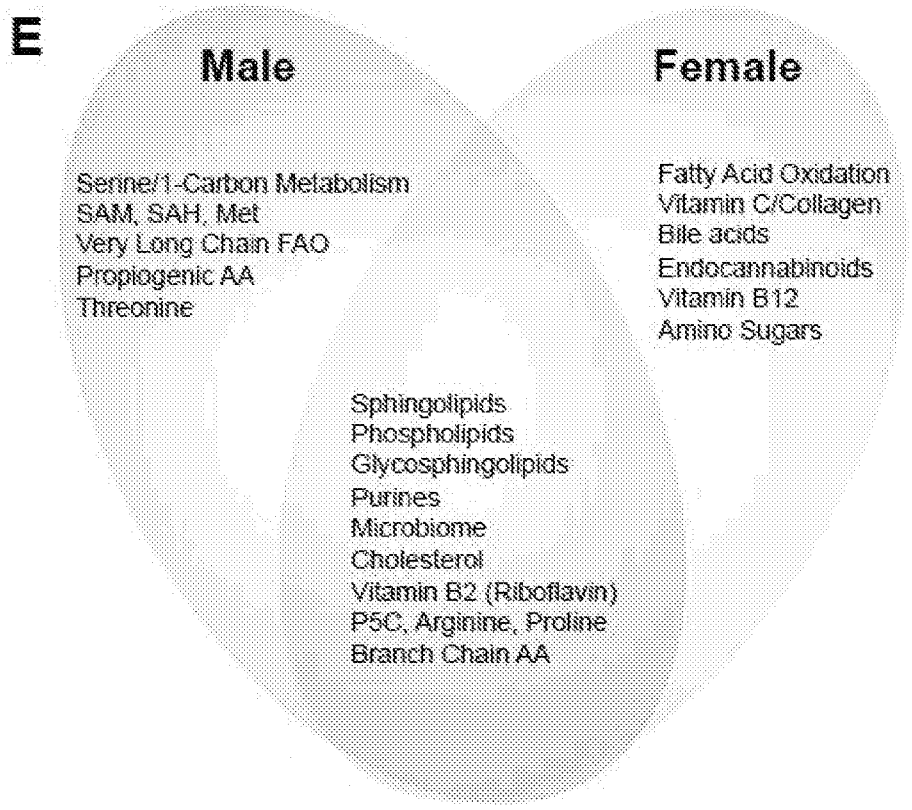
E
Male
Female
Serine/1-Carbon Metabolism
SAM, SAH, Met
Very Long Chain FAO
Propiogenic AA
Threonine
Fatty Acid Oxidation
Vitamin C/Collagen
Bile acids
Endocannabinoids
Vitamin B12
Amino Sugars
Sphingolipids
Phospholipids
Glycosphingolipids
Purines
Microbiome
Cholesterol
Vitamin B2 (Riboflavin)
P5C, Arginine, Proline
Branch Chain AA

FIG. 1E

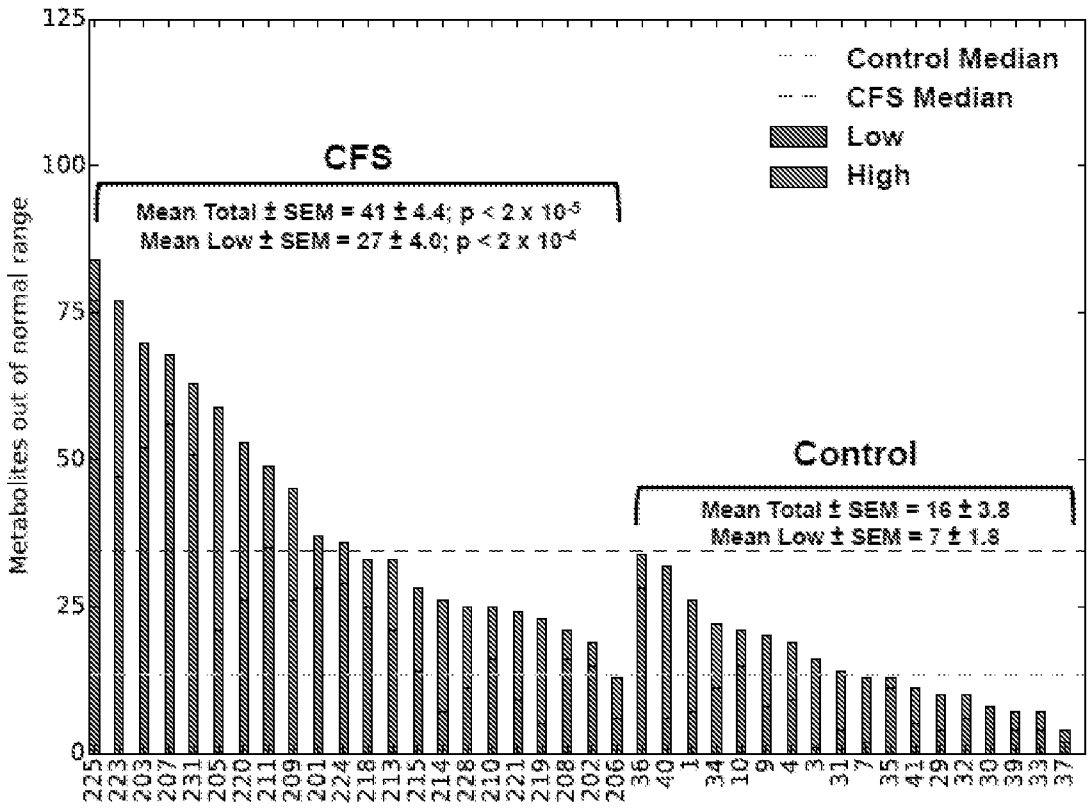
Control Median
CFS Median
Low
High
CFS
Mean Total ± SEM = 41 ± 4.4; p < 2 x 10-5
Mean Low ± SEM = 27 ± 4.0; p < 2 x 10-4
Control
Mean Total ± SEM = 16 ± 3.8
Mean Low ± SEM = 7 ± 1.8
Metabolites out of normal range
125
100
75
50
25
0
225 223 203 207 231 205 220 211 209 201 224 218 213 215 214 228 210 221 219 208 202 206 36 40 1 34 10 9 4 3 31 7 35 41 29 32 30 39 33 37

FIG 2A

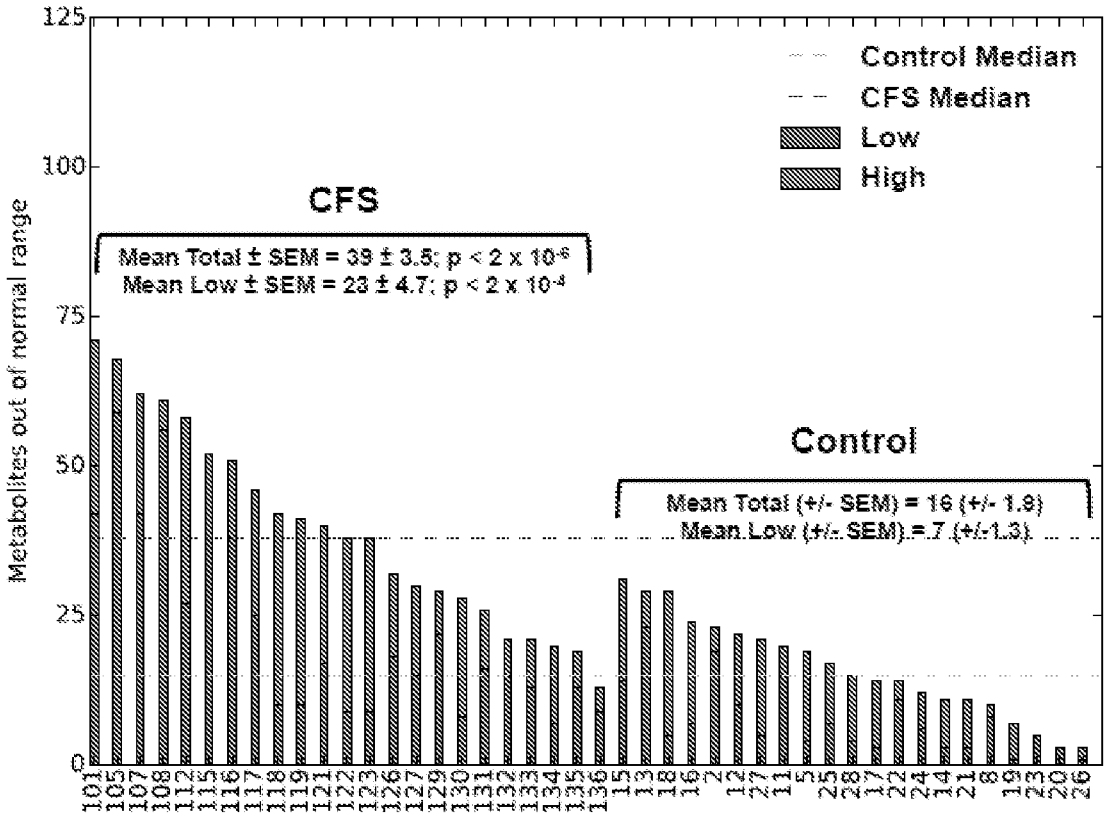
Control Median
CFS Median
Low
High
CFS
Mean Total ± SEM = 39 ± 3.5; p < 2 x 10-6
Mean Low ± SEM = 23 ± 4.7; p < 2 x 10-4
Control
Mean Total (+/- SEM) = 16 (+/- 1.9)
Mean Low (+/- SEM) = 7 (+/-1.3)
Metabolites out of normal range
125
100
75
50
25
0
101 105 107 108 112 115 116 117 118 119 121 122 123 126 127 129 130 131 132 133 134 135 136 15 13 18 16 2 12 27 11 5 25 28 17 22 24 14 21 8 19 23 20 26

FIG. 2B

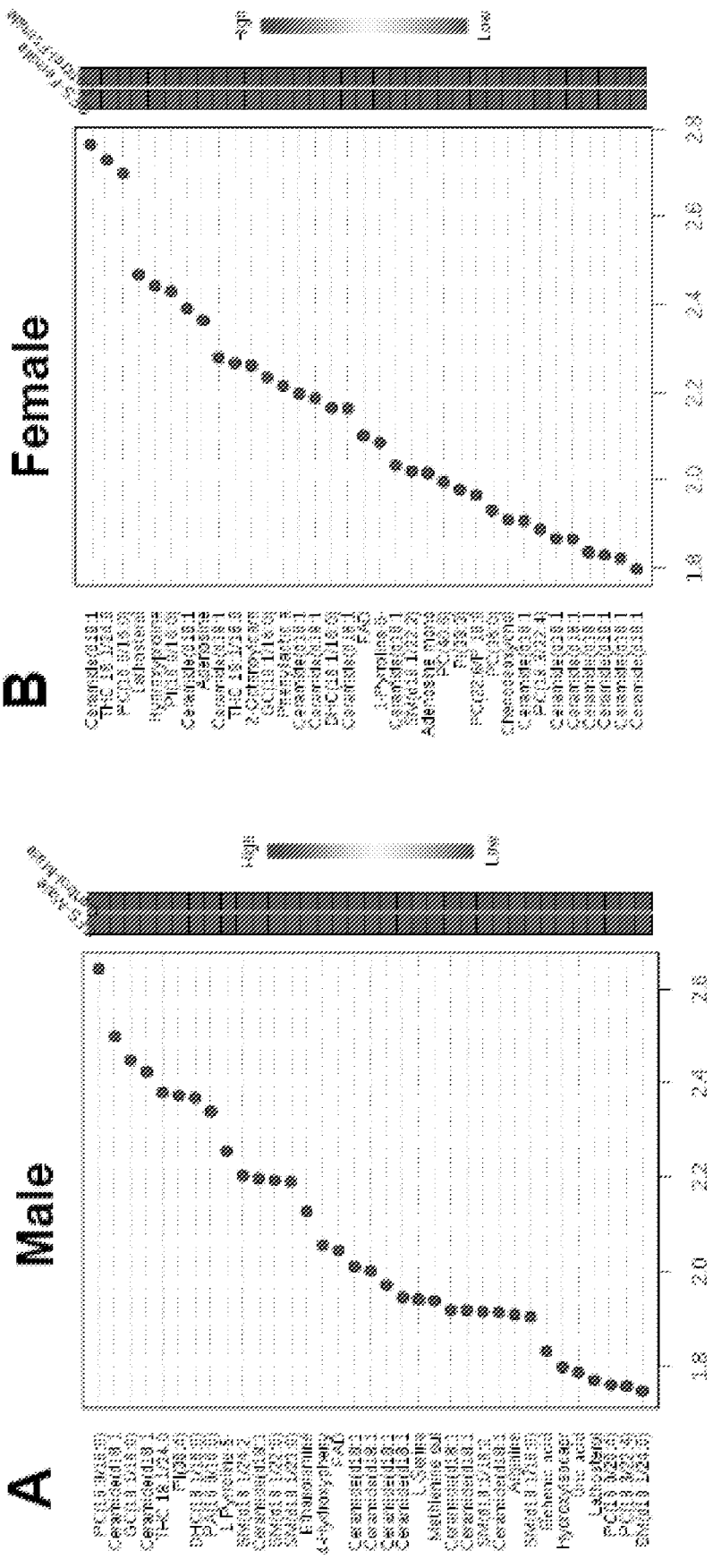

FIG. 3A

FIG. 3B

113

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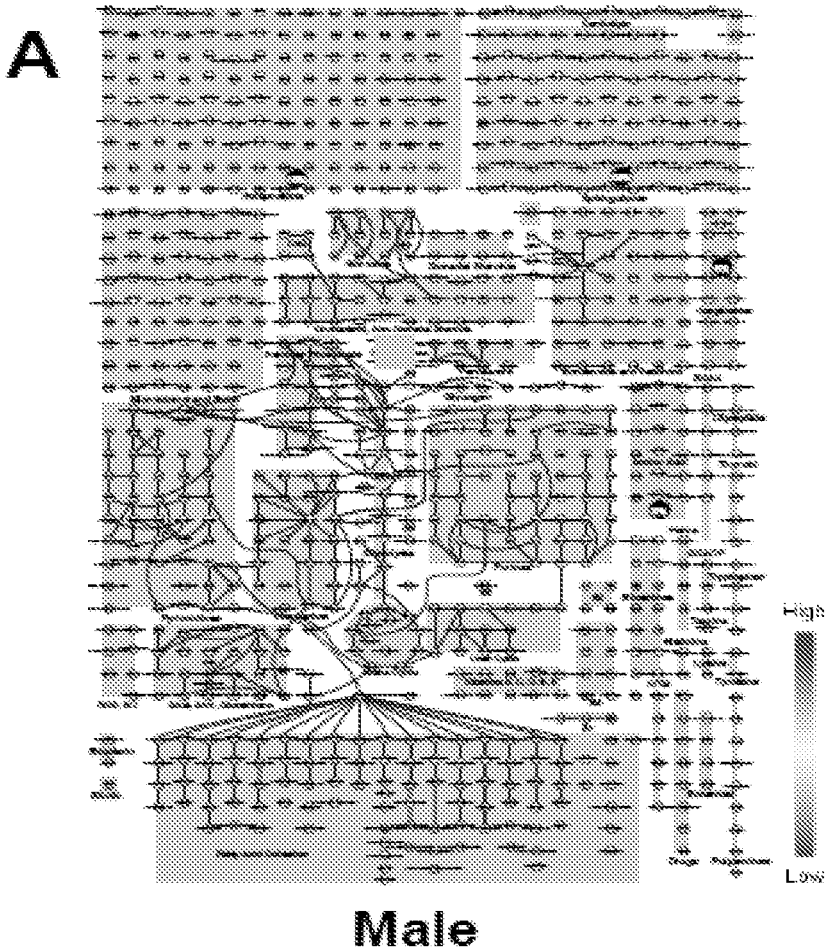

FIG. 4A

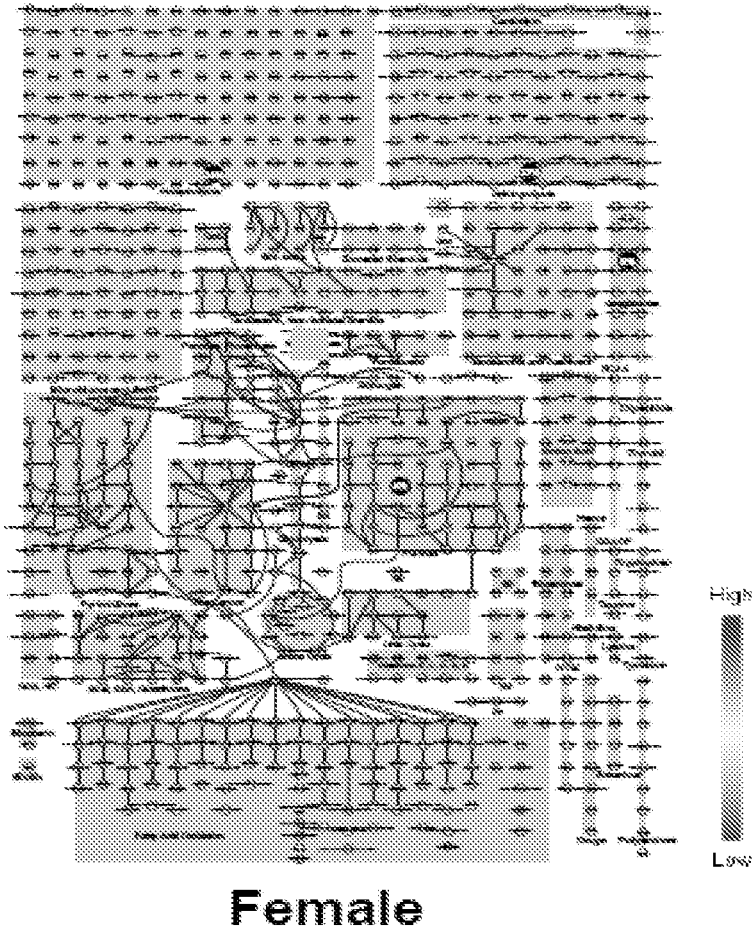

FIG. 4B

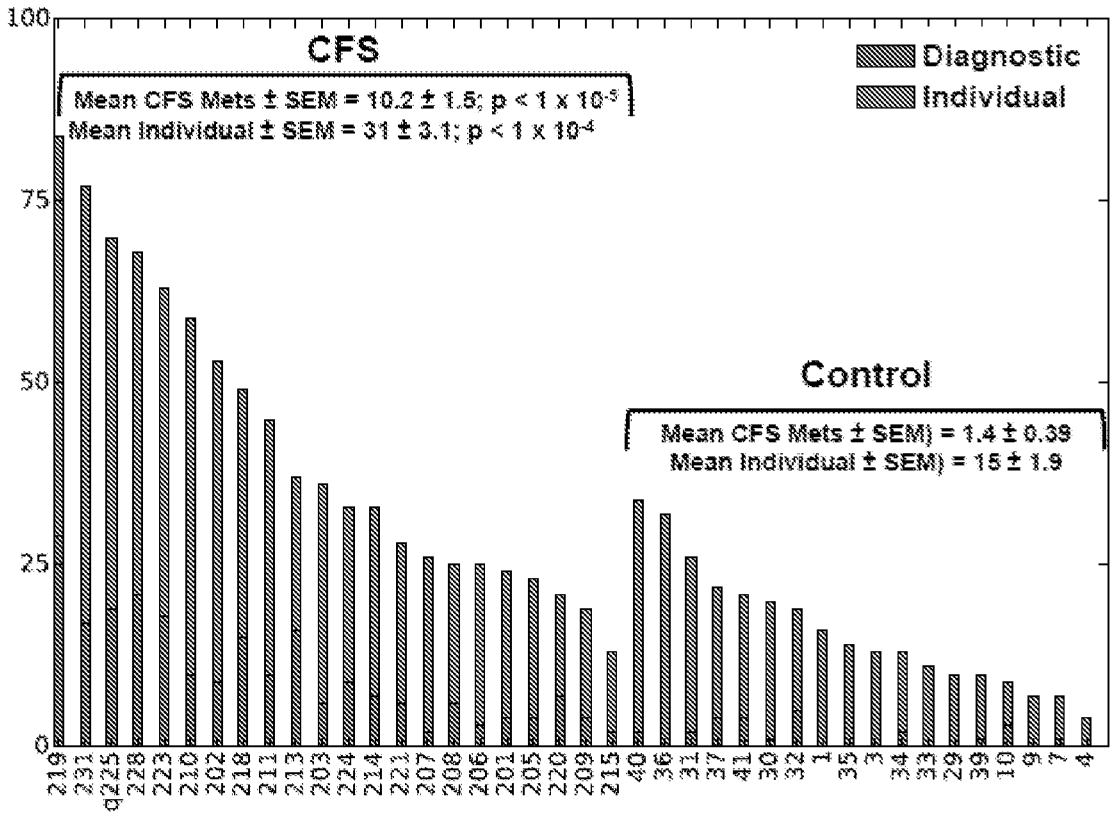
CFS
Diagnostic
Individual
Mean CFS Mets ± SEM = 10.2 ± 1.5; p < 1 x 10-5
Mean Individual ± SEM = 31 ± 3.1; p < 1 x 10-4
Control
Mean CFS Mets ± SEM) = 1.4 ± 0.39
Mean Individual ± SEM) = 15 ± 1.9
100
75
50
25
0
219 231 Q225 228 223 210 202 218 211 213 203 224 214 221 207 208 206 201 205 220 209 215 40 36 31 37 41 30 32 1 35 3 34 33 29 39 10 9 7 4

FIG. 5A

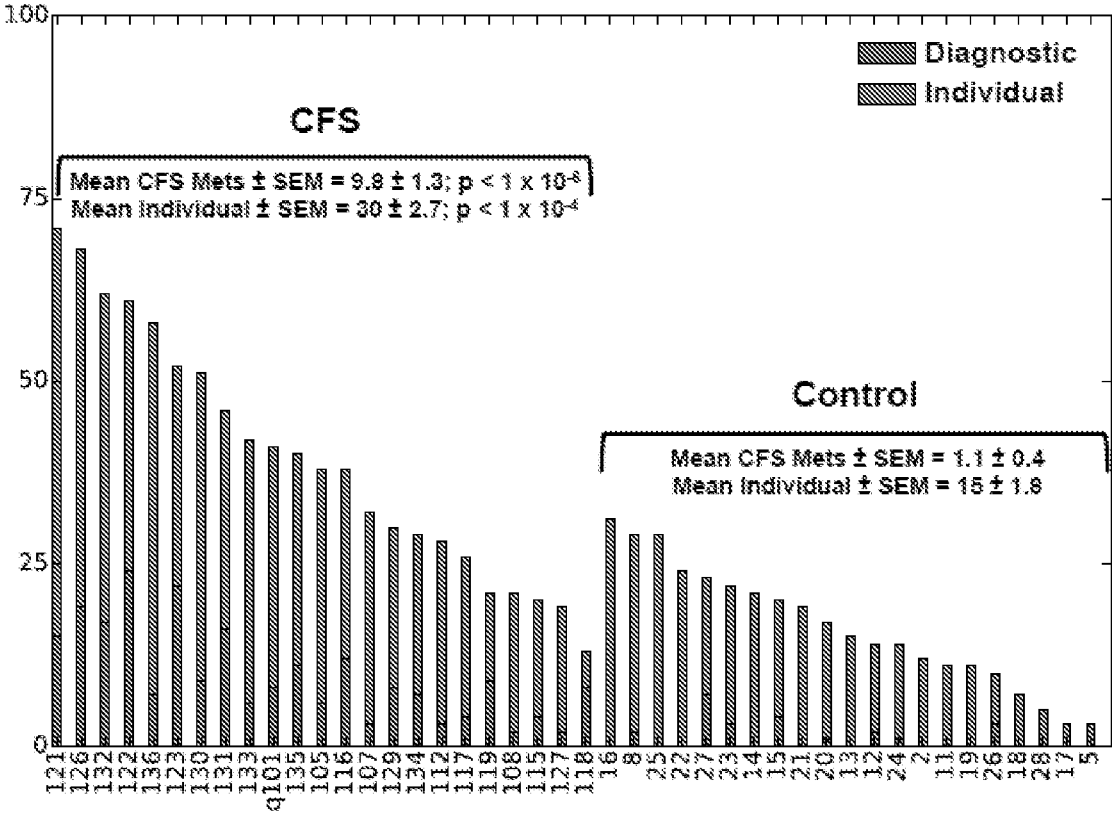
100
75
50
25
0
Diagnostic
Individual
CFS
Mean CFS Mets ± SEM = 9.8 ± 1.3; p < 1 x 10-6
Mean Individual ± SEM = 30 ± 2.7; p < 1 x 10-4
Control
Mean CFS Mets ± SEM = 1.1 ± 0.4
Mean Individual ± SEM = 15 ± 1.8
121 126 132 122 136 123 130 131 133 q101 135 105 116 107 129 134 112 117 119 108 115 127 118 16 8 25 22 27 23 24 15 21 20 13 12 24 2 11 19 26 18 28 17 5

FIG. 5B

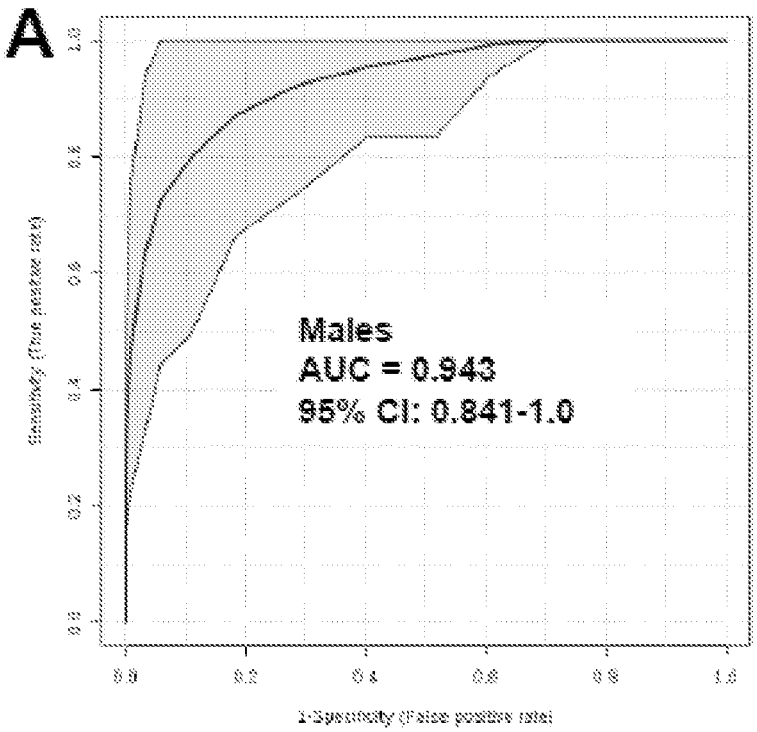
A
Males
AUC = 0.943
95% CI: 0.841-1.0
1-Specificity (False positive rate)

FIG. 6A

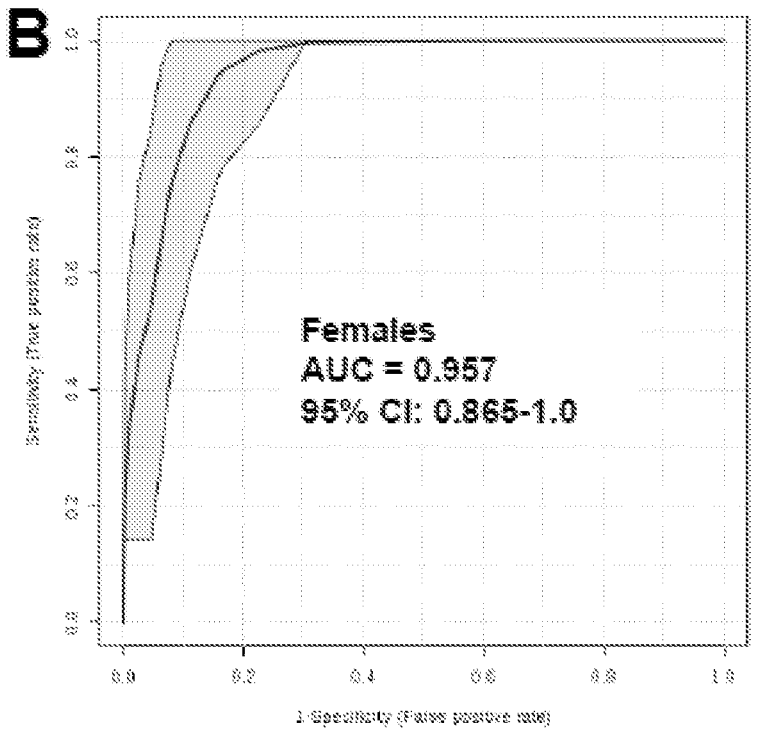
B
Females
AUC = 0.957
95% CI: 0.865-1.0
1-Specificity (False positive rate)

FIG. 6B

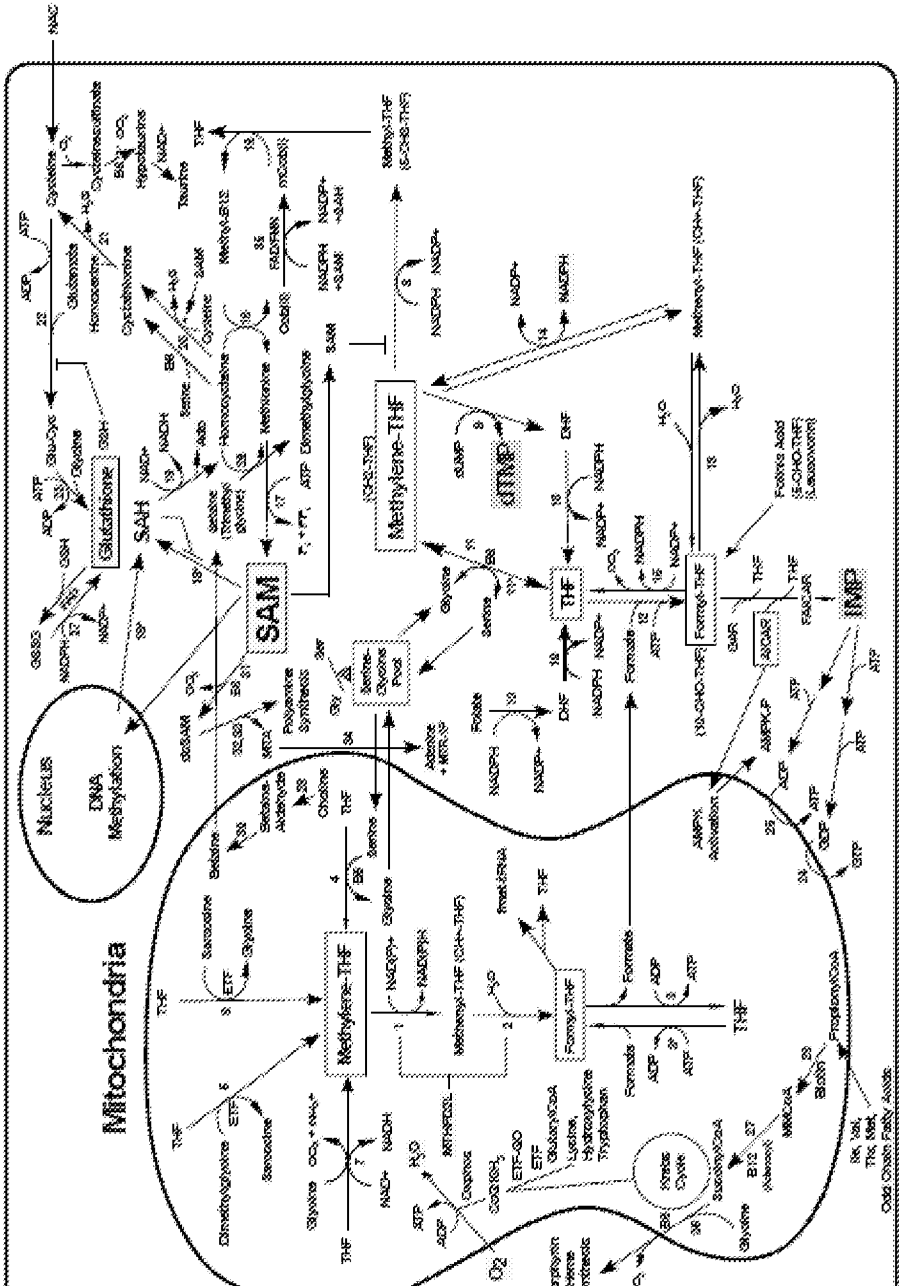
Mitochondria
Nucleus
DNA Methylation
SAM
SAH
Glutathione
Methylene-THF
THF
Formyl-THF
IMP
dTMP

FIG. 7

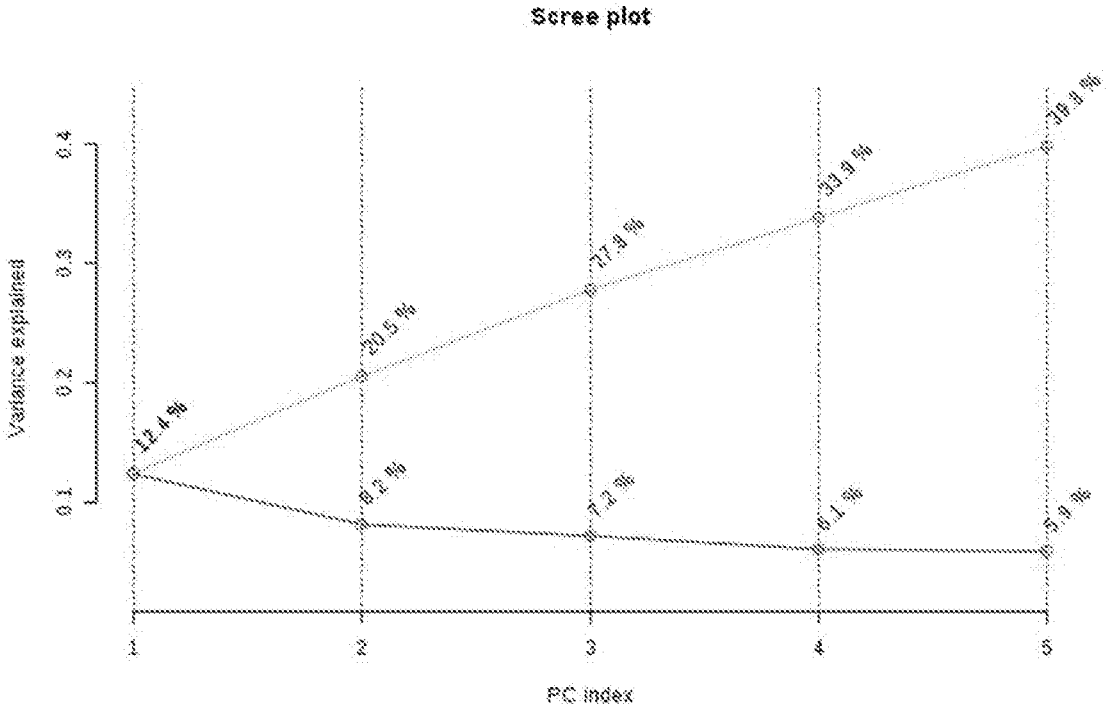
Scree plot
Variance explained
0.1
0.2
0.3
0.4
12.4 %
20.5 %
27.9 %
33.9 %
38.8 %
8.2 %
7.3 %
6.1 %
5.0 %
1
2
3
4
5
PC index

FIG. 8A

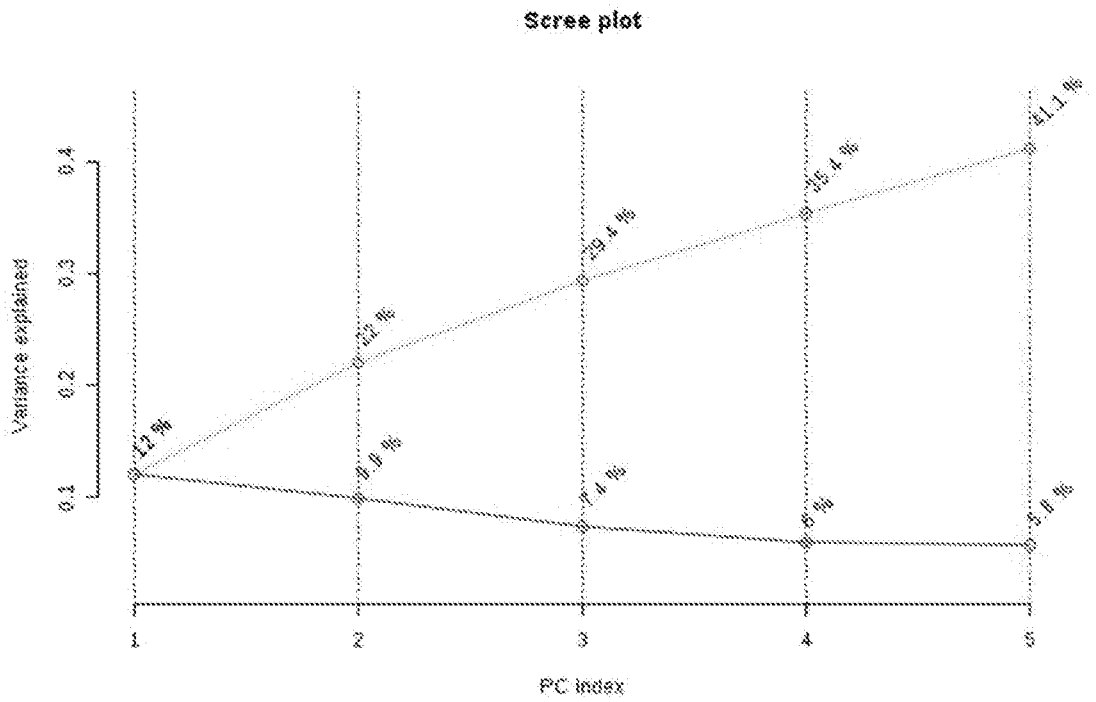
Scree plot
Variance explained
0.1
0.2
0.3
0.4
42 %
22 %
8.0 %
4.4 %
1
2
3
4
5
PC Index

FIG. 8B

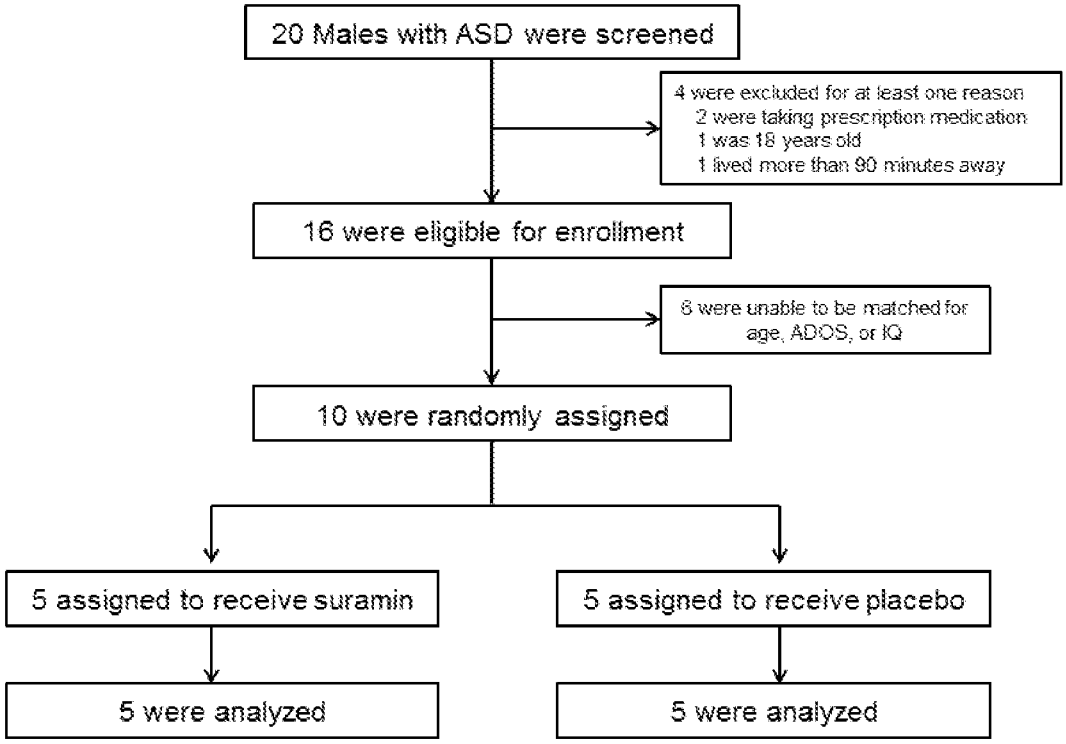
20 Males with ASD were screened
4 were excluded for at least one reason
2 were taking prescription medication
1 was 18 years old
1 lived more than 90 minutes away
16 were eligible for enrollment
6 were unable to be matched for age, ADOS, or IQ
10 were randomly assigned
5 assigned to receive suramin
5 assigned to receive placebo
5 were analyzed
5 were analyzed

FIG. 9

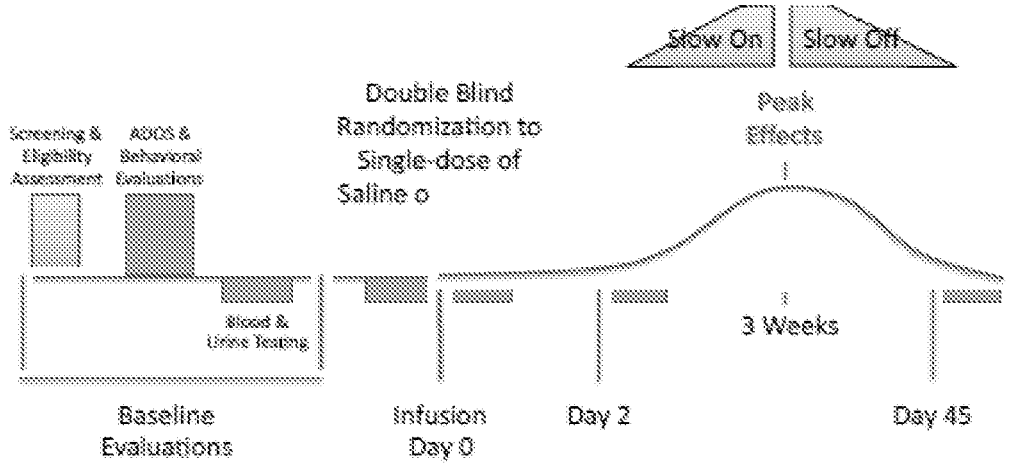
Slow On
Slow Off
Double Blind
Randomization to
Single-dose of
Saline o
Peak
Effects
3 Weeks
Baseline
Evaluations
Infusion
Day 0
Day 2
Day 45

FIG. 10

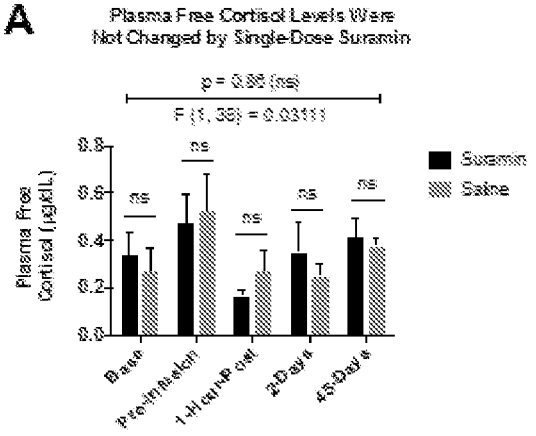

FIG. 11A

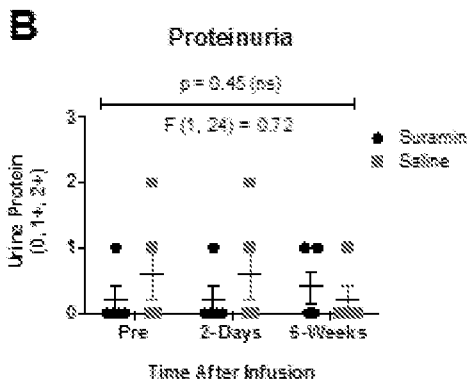

FIG. 11B

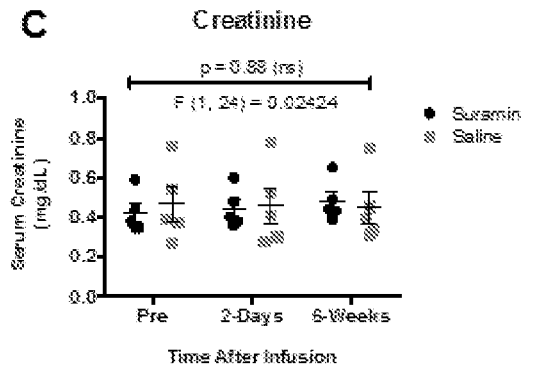

FIG. 11C

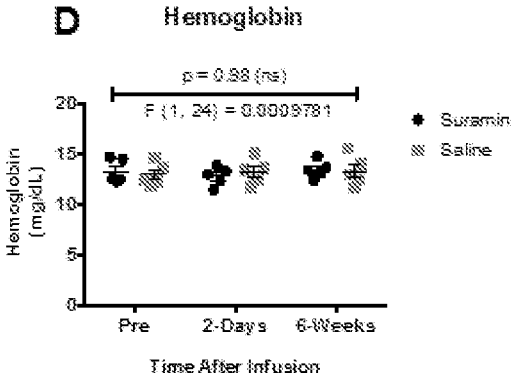

FIG. 11D

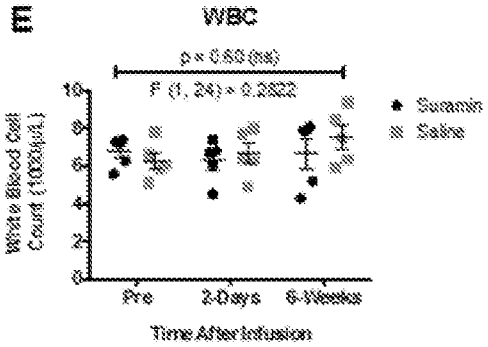

FIG. 11E

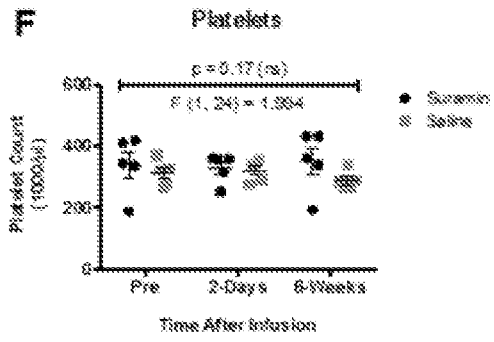

FIG. 11F

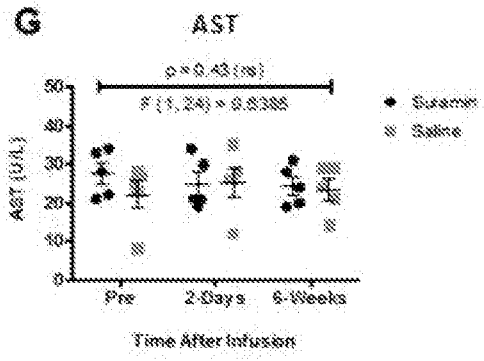

FIG. 11G

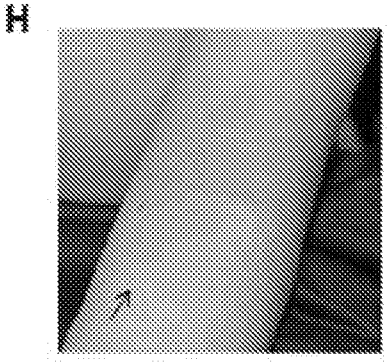

FIG. 11H

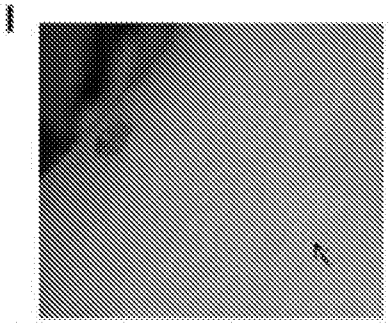

FIG. 11I

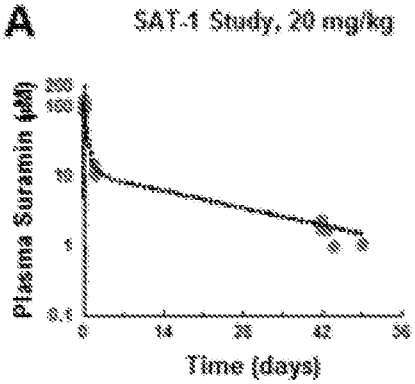

FIG. 12A

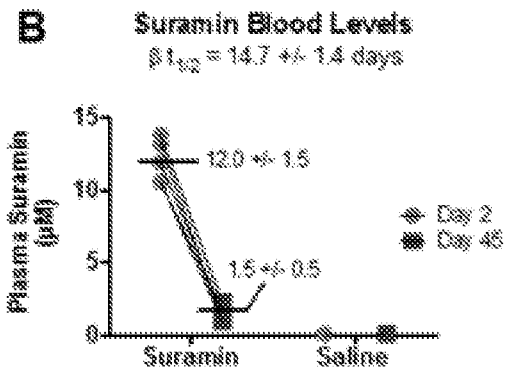

FIG. 12B

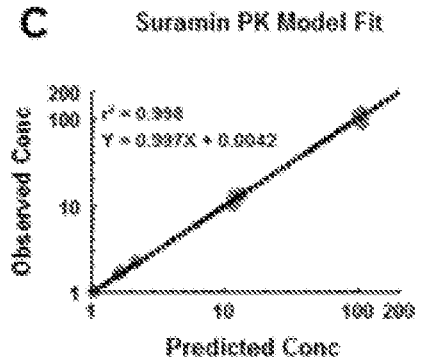

FIG. 12C

D

Parameter	Symbol	Mean ± sd
Beta elimination half-life	$\beta t_{1/2}$	14.7 ± 1.4 days
Alpha elimination half-life	$\alpha t_{1/2}$	7.41 ± 0.55 hours
Clearance	Cl	1.95 ± 0.21 ml/hr/kg
Steady state volume of distribution	Vd_{ss}	0.83 ± 0.014 L/kg

FIG. 12D

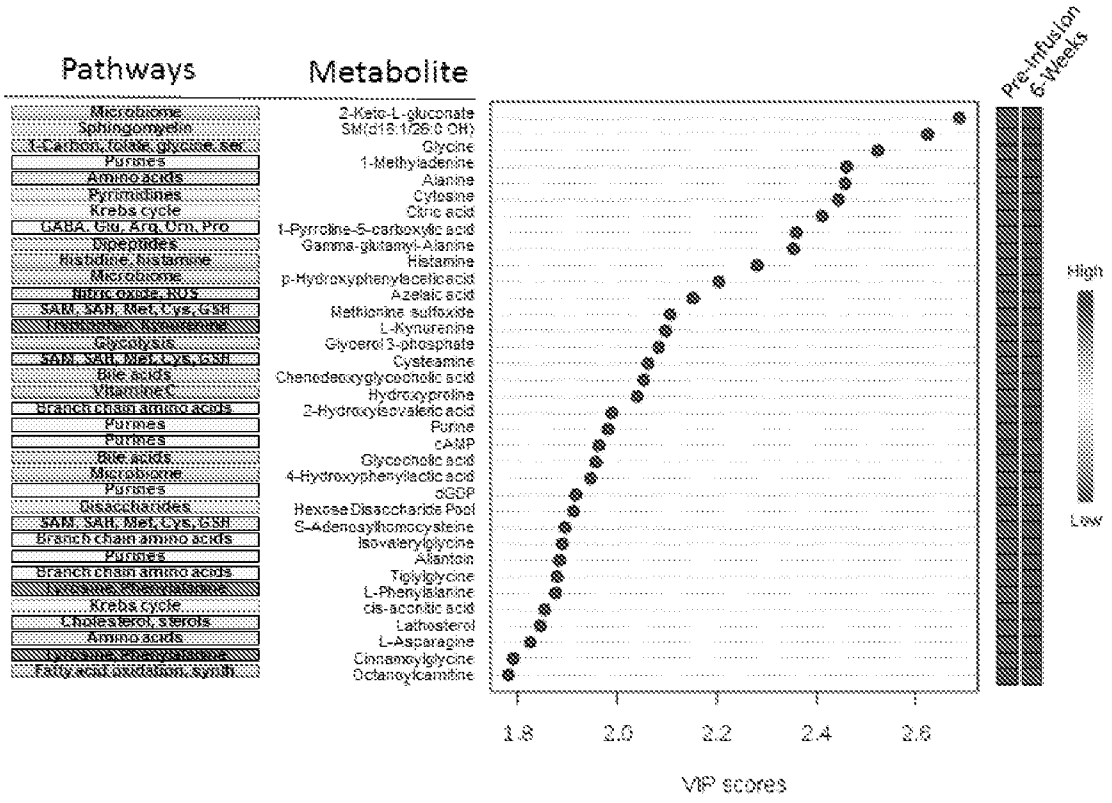
Pathways
Metabolite
Microbiome
Sphingomyelin
1-Carbon, folate, glycine, ser
Purines
Amino acids
Pyrimidines
Krebs cycle
GABA, Glu, Arg, Orn, Pro
Dipeptides
Histidine, histamine
Microbiome
Nitric oxide, ROS
SAM, SAH, Met, Cys, GSH
Tryptophan, Kynurenine
Glycolysis
SAM, SAH, Met, Cys, GSH
Bile acids
Vitamin C
Branch chain amino acids
Purines
Purines
Bile acids
Microbiome
Purines
Disaccharides
SAM, SAH, Met, Cys, GSH
Branch chain amino acids
Purines
Branch chain amino acids
Tyrosine, Phenylalanine
Krebs cycle
Cholesterol, sterols
Amino acids
Tyrosine, Phenylalanine
Fatty acid oxidation, synth
2-Keto-L-gluconate
SM(d18:1/26:0 OH)
Glycine
1-Methyladenine
Alanine
Cytosine
Citric acid
1-Pyrroline-5-carboxylic acid
Gamma-glutamyl-Alanine
Histamine
p-Hydroxyphenylacetic acid
Azelaic acid
Methionine sulfoxide
L-Kynurenine
Glycerol 3-phosphate
Cysteamine
Chenodeoxyglycocholic acid
Hydroxyproline
2-Hydroxyisovaleric acid
Purine
cAMP
Glycocholic acid
4-Hydroxyphenyllactic acid
dGDP
Hexose Disaccharide Pool
S-Adenosylhomocysteine
Isovalerylglycine
Allantoin
Tigylglycine
L-Phenylalanine
cis-aconitic acid
Lathosterol
L-Asparagine
Cinnamoylglycine
Octanoylcarnitine
Pre-Infusion
6-Weeks
High
Low
1.8
2.0
2.2
2.4
2.6
VIP scores

FIG. 13A

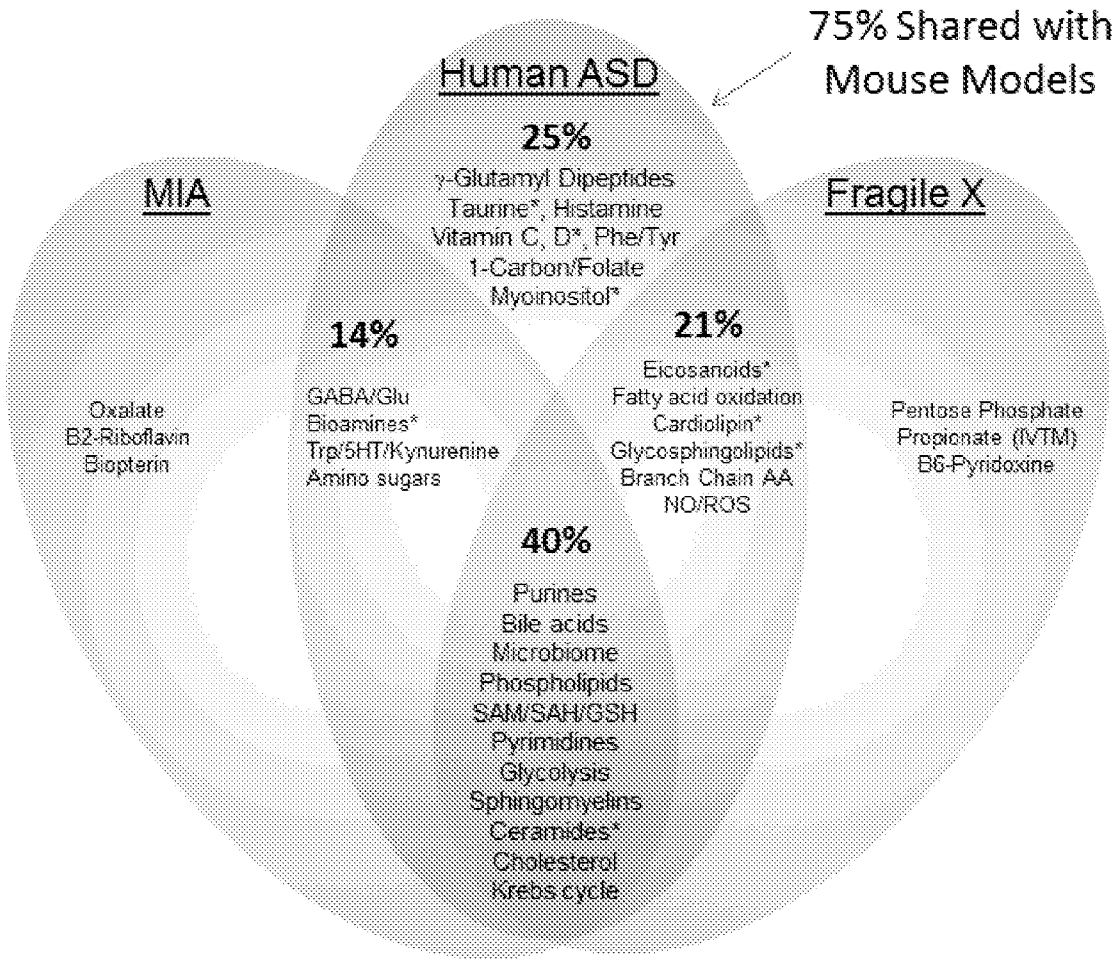
75% Shared with Mouse Models
Human ASD
25%
γ-Glutamyl Dipeptides
Taurine*, Histamine
Vitamin C, D*, Phe/Tyr
1-Carbon/Folate
Myoinositol*
MIA
Oxalate
B2-Riboflavin
Biopterin
14%
GABA/Glu
Bioamines*
Trp/5HT/Kynurenine
Amino sugars
21%
Eicosanoids*
Fatty acid oxidation
Cardiolipin*
Glycosphingolipids*
Branch Chain AA
NO/ROS
Fragile X
Pentose Phosphate
Propionate (IVTM)
B6-Pyridoxine
40%
Purines
Bile acids
Microbiome
Phospholipids
SAM/SAH/GSH
Pyrimidines
Glycolysis
Sphingomyelins
Ceramides*
Cholesterol
Krebs cycle

FIG. 13B

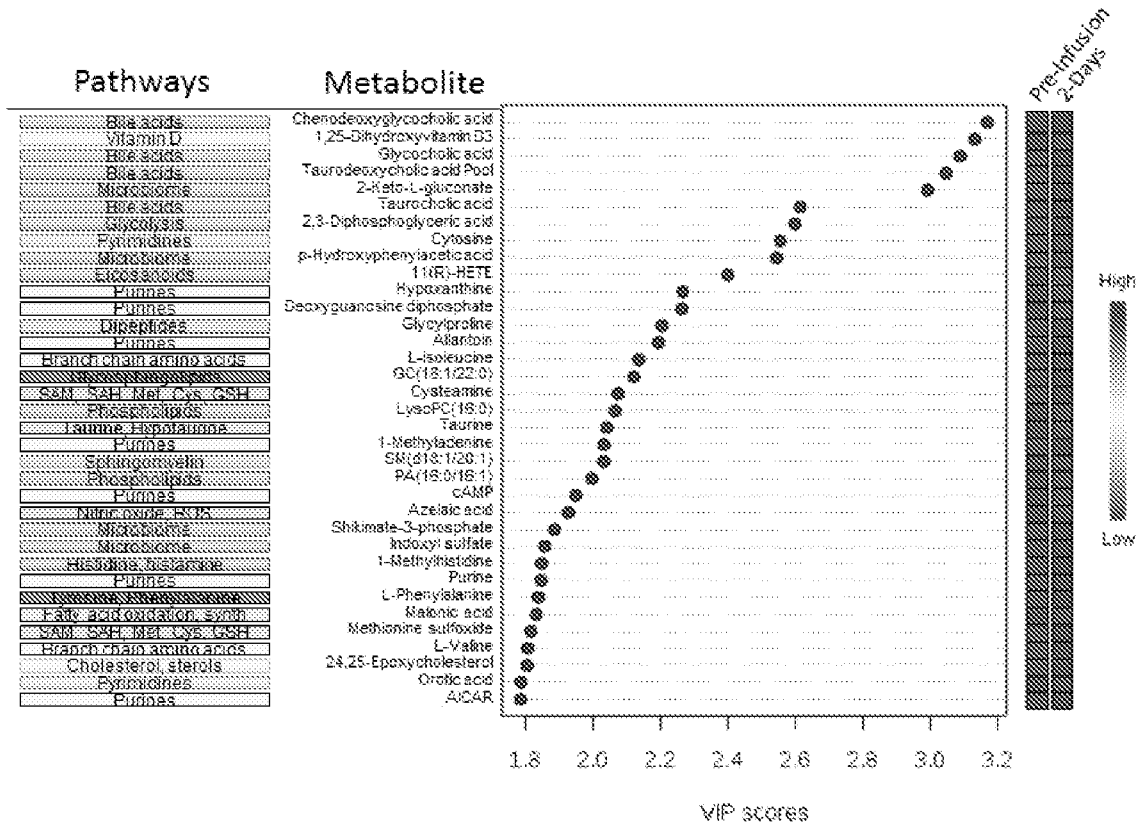
Pathways
Metabolite
Pre-Infusion
2-Days
High
Low
Bile acids
Vitamin D
Bile acids
Bile acids
Microbiome
Bile acids
Glycolysis
Pyrimidines
Microbiome
Eicosanoids
Purines
Purines
Dipeptides
Purines
Branch chain amino acids
Sphingomyelin
SAM, SAH, Met, Cys, GSH
Phospholipids
Taurine, Histamine
Purines
Sphingomyelin
Phospholipids
Purines
Nitric oxide, ROS
Microbiome
Microbiome
Histidine, histamine
Purines
Urea cycle, Arg, Pro, NO
Fatty acid oxidation, synth
SAM, SAH, Met, Cys, GSH
Branch chain amino acids
Cholesterol, sterols
Pyrimidines
Purines
Chenodeoxyglycocholic acid
1,25-Dihydroxyvitamin D3
Glycocholic acid
Taurodeoxycholic acid Pool
2-Keto-L-gluconate
Taurocholic acid
2,3-Diphosphoglyceric acid
Cytosine
p-Hydroxyphenylacetic acid
11(R)-HETE
Hypoxanthine
Deoxyguanosine diphosphate
Glycylproline
Allantoin
L-isoleucine
GC(18:1/22:6)
Cysteamine
LysoPC(16:0)
Taurine
1-Methyladenine
SM(d18:1/26:1)
PA(18:0/18:1)
cAMP
Azelaic acid
Shikimate-3-phosphate
Indoxyl sulfate
1-Methylhistidine
Purine
L-Phenylalanine
Malonic acid
Methionine sulfoxide
L-Valine
24,25-Epoxycholesterol
Orotic acid
AICAR
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
VIP scores

FIG. 14

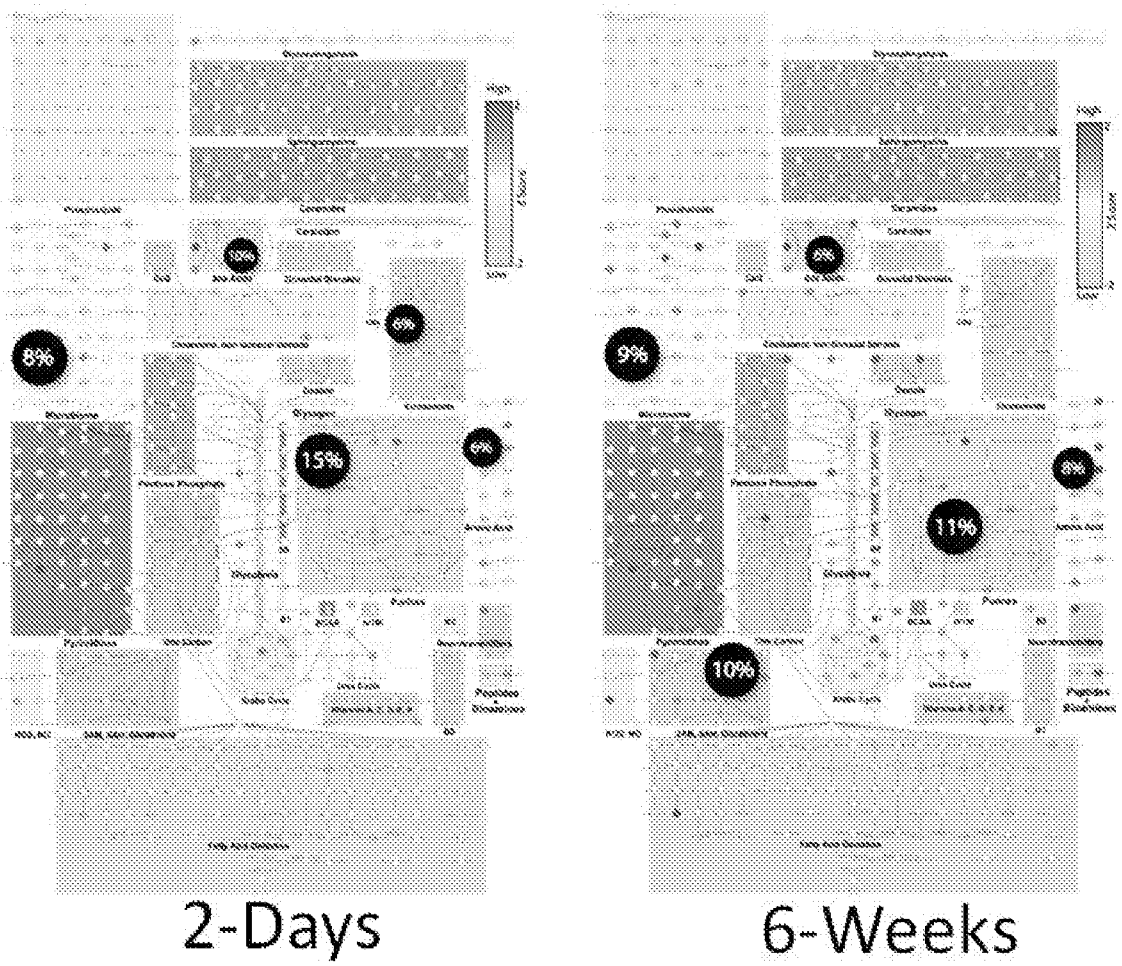

FIG. 15

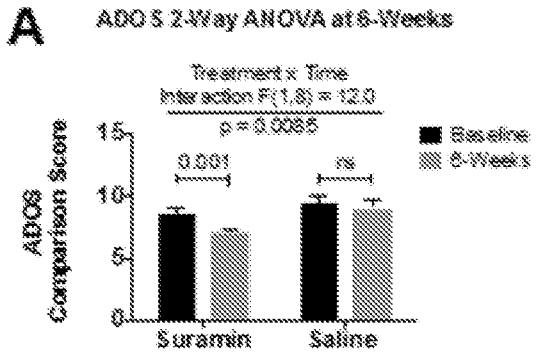

FIG. 16A

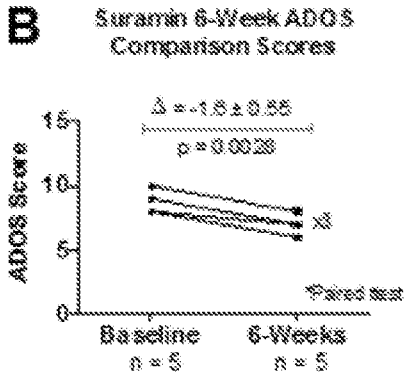

FIG. 16B

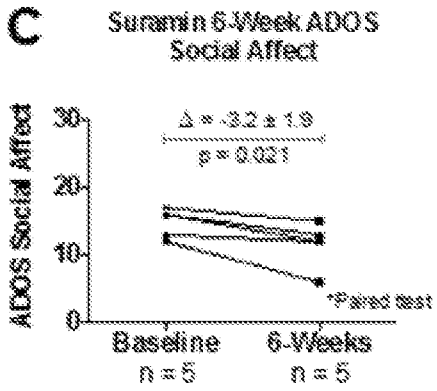

FIG. 16C

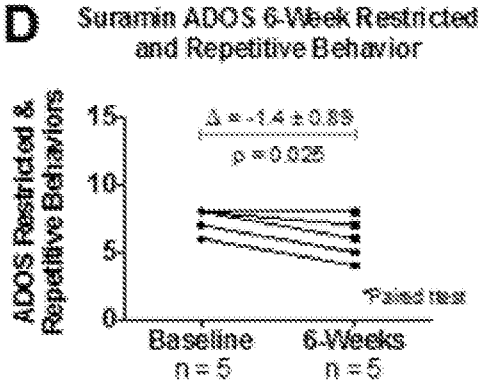

FIG. 16D

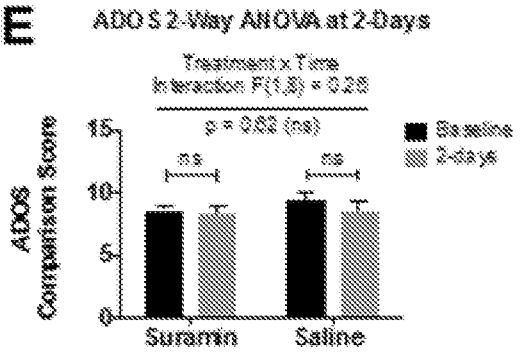

FIG. 16E

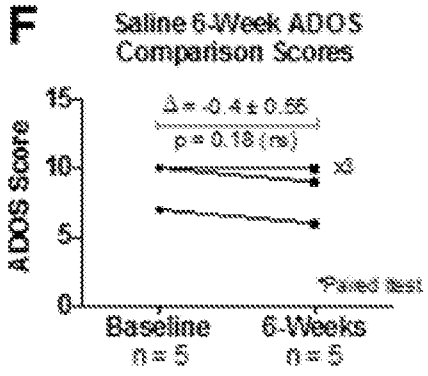

FIG. 16F

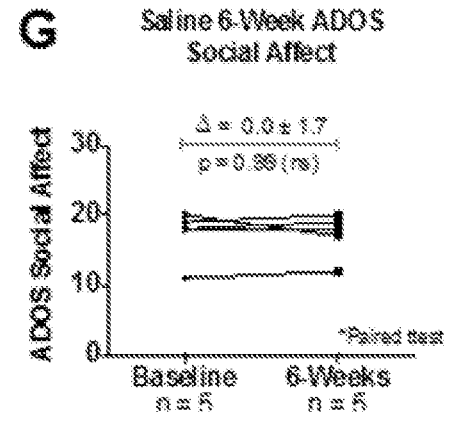

FIG. 16G

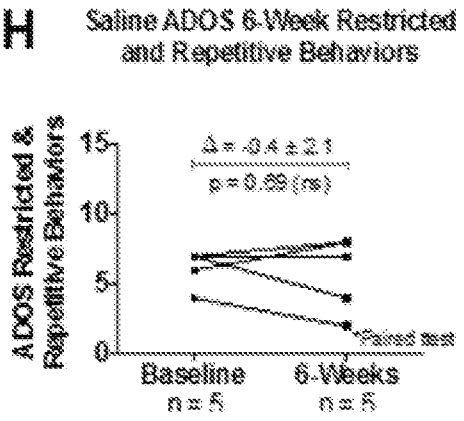

FIG. 16H

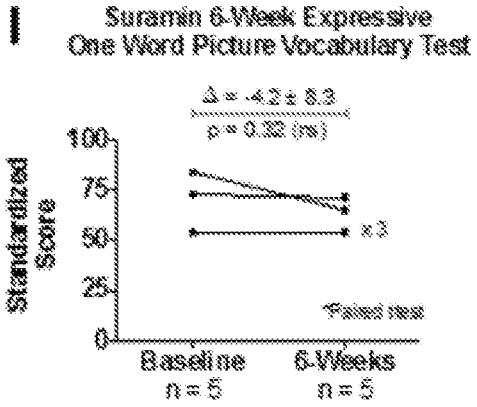

FIG. 16I

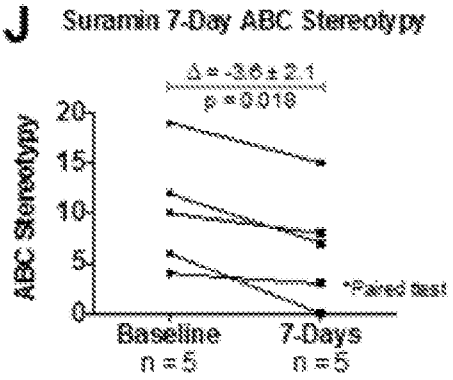

FIG. 16J

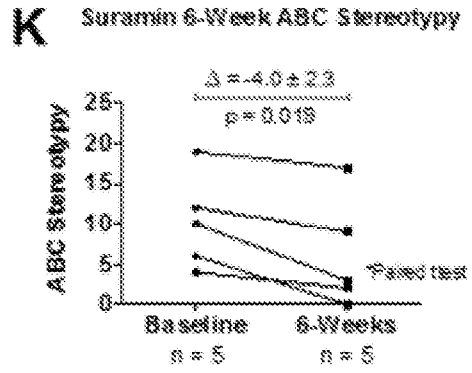

FIG. 16K

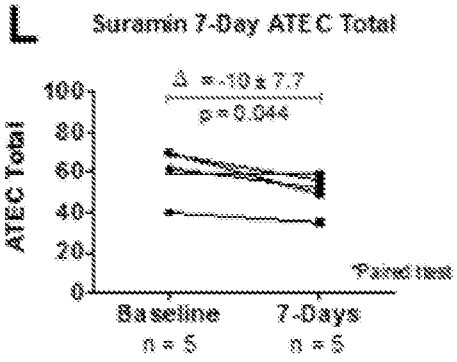

FIG. 16L

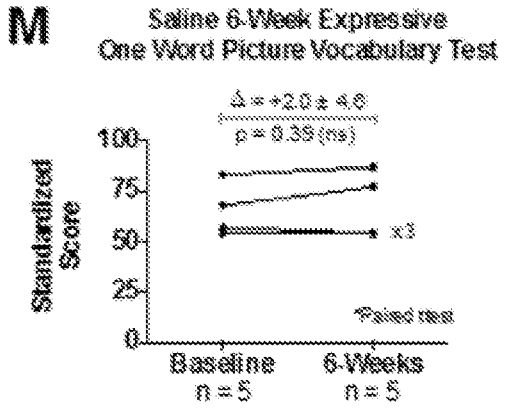

FIG. 16M

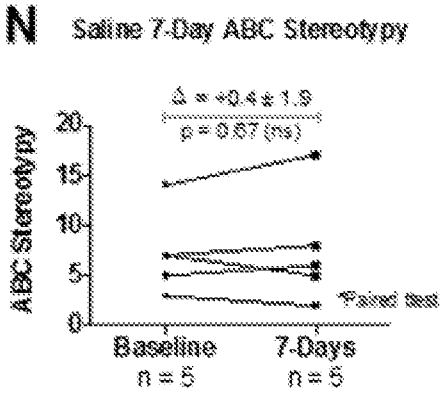

FIG. 16N

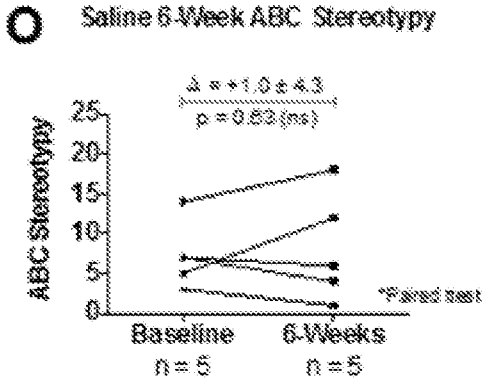

FIG. 16O

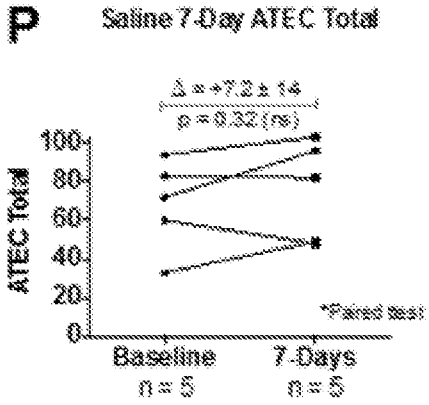

FIG. 16P

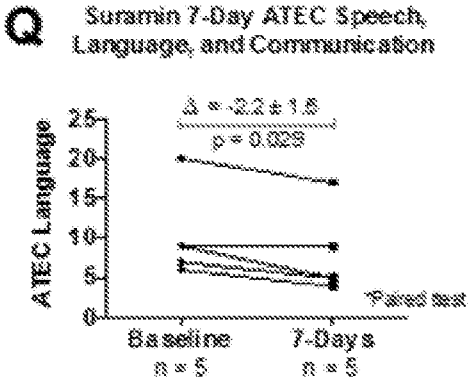

FIG. 16Q

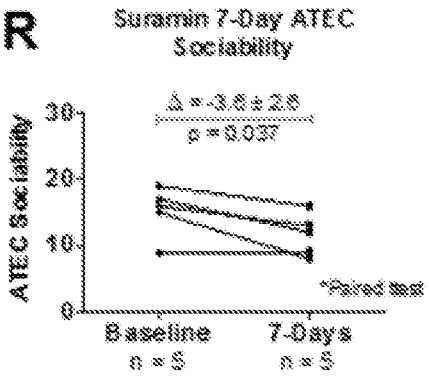

FIG. 16R

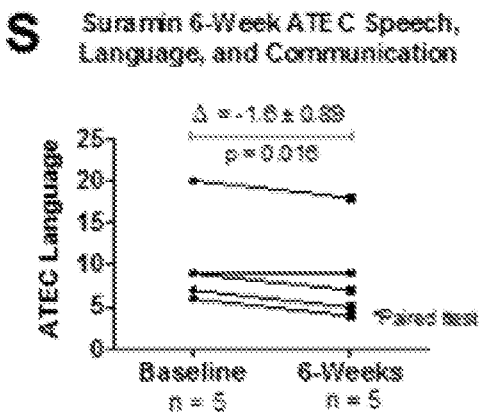

FIG. 16S

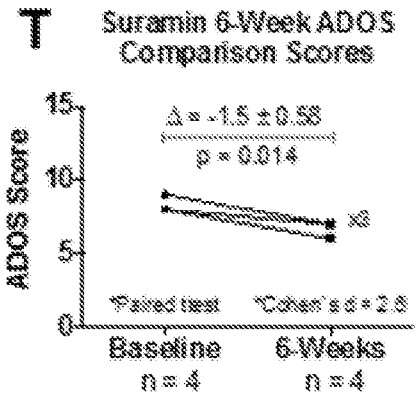

FIG. 16T

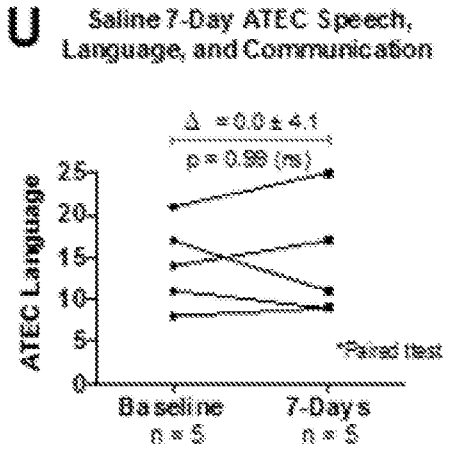

FIG. 16U

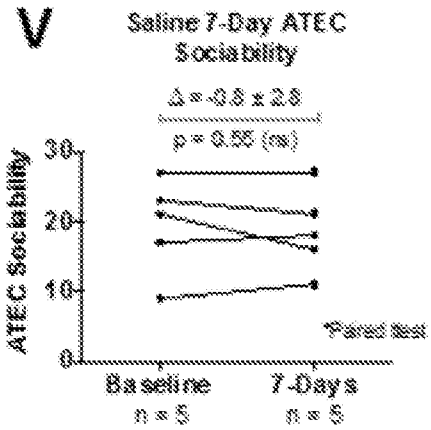

FIG. 16V

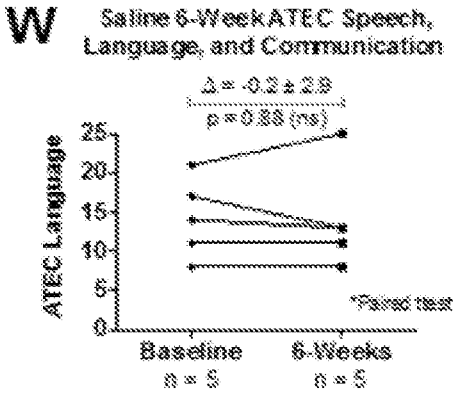

FIG. 16W

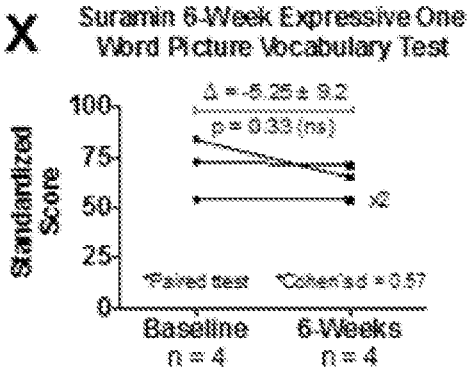

FIG. 16X

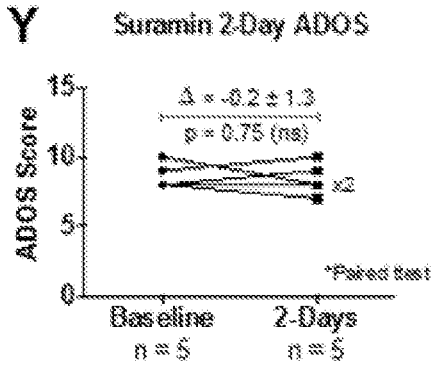

FIG. 16Y

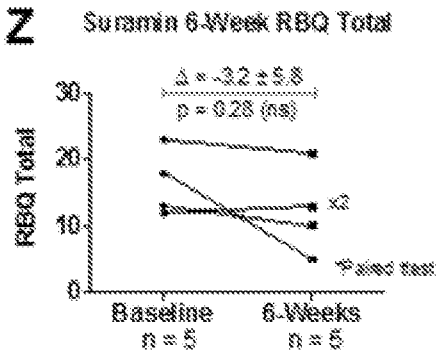

FIG. 16Z

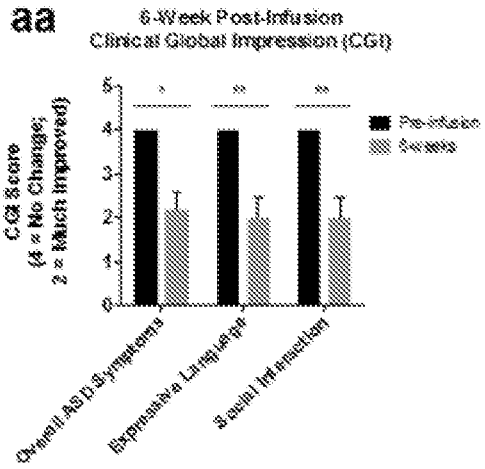

FIG. 16aa

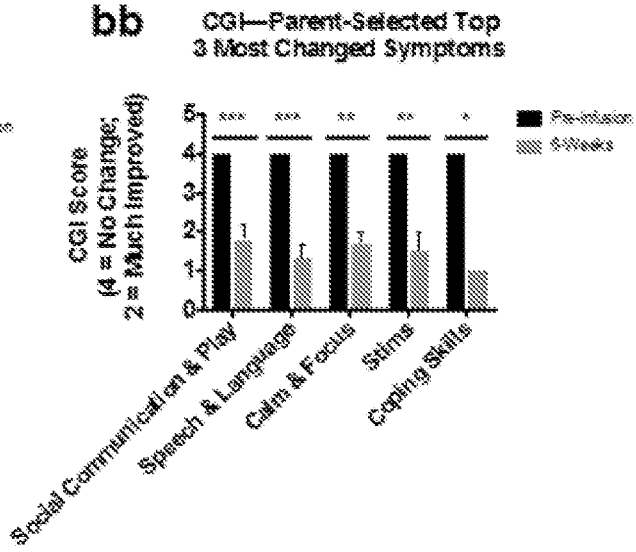

FIG. 16bb

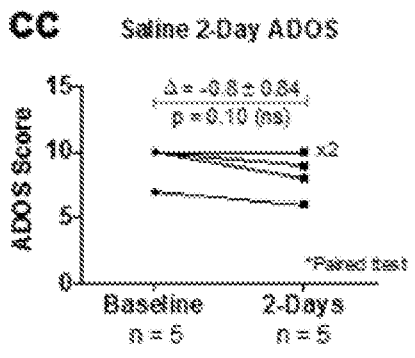

FIG. 16cc

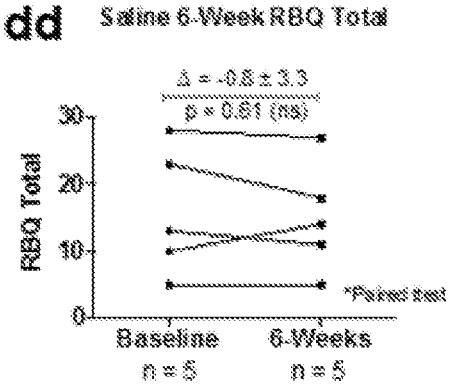

FIG. 16dd

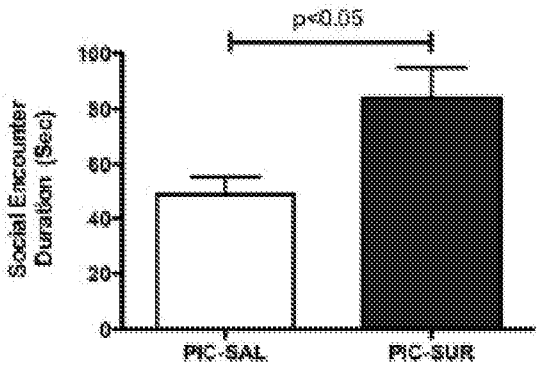

FIG. 17

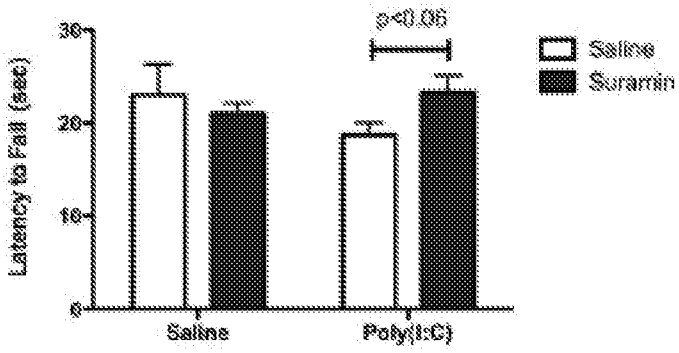

FIG. 18

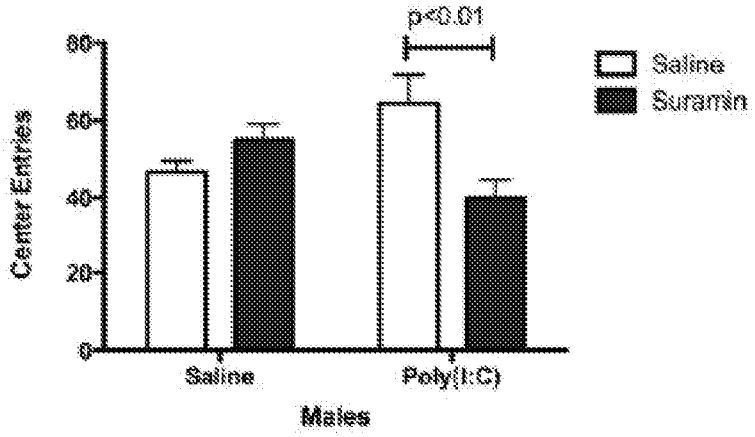

FIG. 19

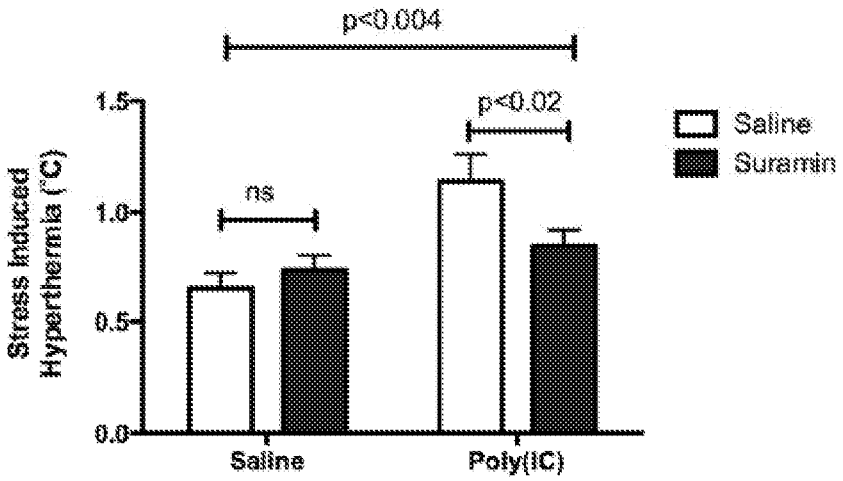

FIG. 20

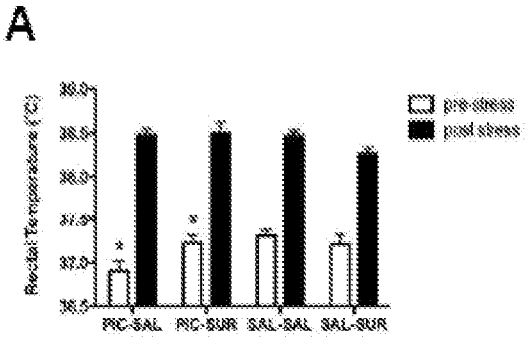

FIG. 21A

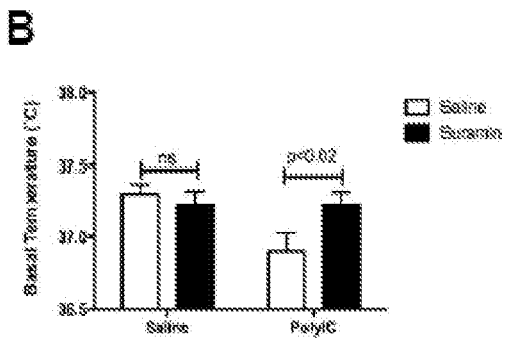

FIG. 21B

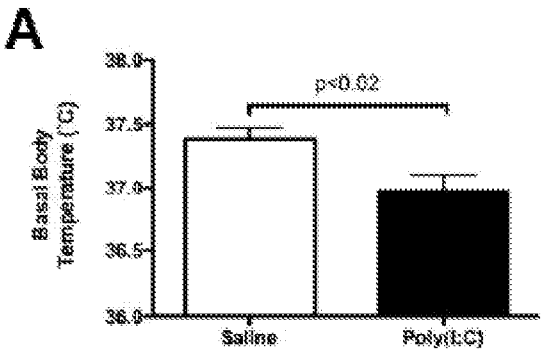

FIG. 22A

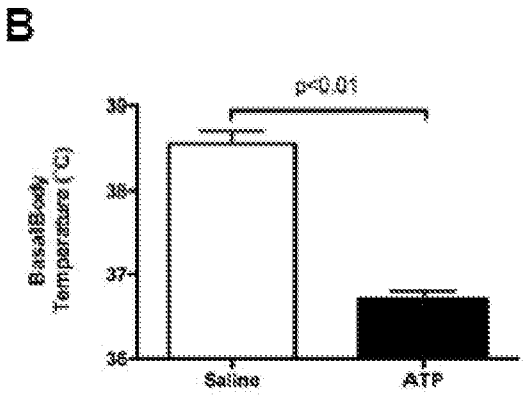

FIG. 22B

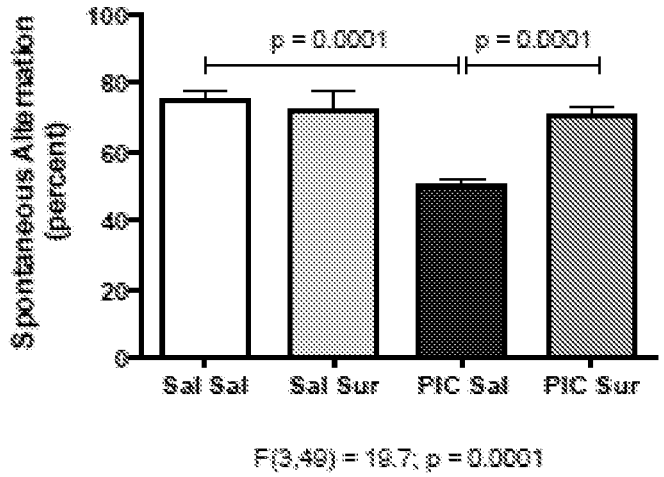
Spontaneous Alternation (percent)
100
80
60
40
20
0
p = 0.0001
p = 0.0001
Sal Sal
Sal Sur
PIC Sal
PIC Sur
F(3,49) = 19.7; p = 0.0001

FIG. 23

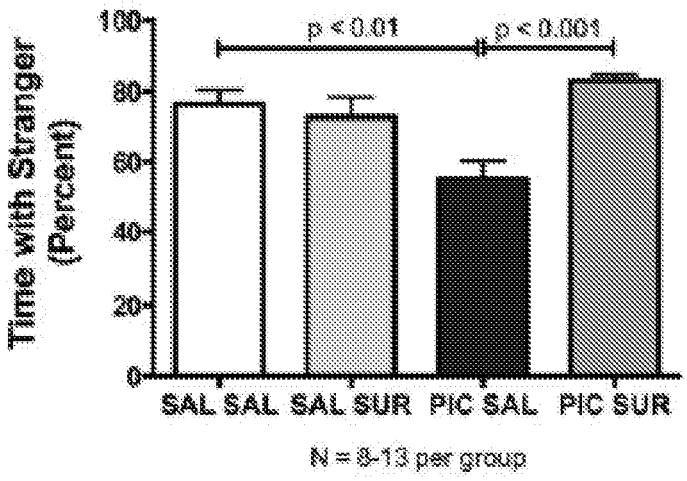
Time with Stranger (Percent)
100
80
60
40
20
0
p < 0.01
p < 0.001
SAL SAL
SAL SUR
PIC SAL
PIC SUR
N = 8-13 per group

FIG. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/41932

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/185, G01N 33/68 (2017.01)
CPC - G01N 2800/50, G01N 2800/301, A61K 31/185, G01N 2570/00

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)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2013/0267441 A1 (Momeni et al.) 10 October 2013 (10.10.2013); abstract; para [0007], [0037], [0061], [0115], [0150]	1-3, 7, 10-17, 21 --- 4-6, 8-9, 19-20, 23-38
Y	US 2015/0125526 A1 (Raptor Pharmaceuticals Inc.) 07 May 2015 (07.05.2015); abstract; para [0009], [0016], [0018], [0049], [0053]	4-6, 8-9, 19-20
Y	WO 2015/027116 A1 (The Regents Of The University Of California) 26 February 2015 (26.02.2015); para [0005], [0007]-[0009], [0011]-[0013], [0054], [0062], [00170], para [00201]	23-38
A	Wikipedia, Purinergic signalling, 28 May 2016; pg 2, table; Retrieved on 7 September 2017, from <https://en.wikipedia.org/wiki/Purinergic_signalling>	15
A	Hamidpour et al., Antipurinergic Therapy with Suramin as a Treatment for Autism Spectrum Disorder, 29 March 2016, Journal of Biomedical Sciences, vol 5, pages 1-7; abstract	15
A	Myhill et al., Chronic fatigue syndrome and mitochondrial dysfunction, 15 January 2009, Int J Clin Exp Med, vol 2, pages 1-16; pg 12, conclusion	21
A	US 2013/0237454 A1 (Schutzer) 12 September 2013 (12.09.2013); abstract	23

☐ 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	Date of mailing of the international search report
07 September 2017	06 OCT 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 17/41932

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims 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. ☒ Claims Nos.: 18
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:

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 claims 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 claims Nos.:

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.