



(51) International Patent Classification:

C12Q 1/6883 (2018.01) A61P 25/00 (2006.01)

A61K 39/395 (2006.01) A61P 25/14 (2006.01)

(21) International Application Number:

PCT/US2024/036262

(22) International Filing Date:

28 June 2024 (28.06.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/511,638 30 June 2023 (30.06.2023) US

63/554,913 16 February 2024 (16.02.2024) US

(71) Applicant: UNIVERSITY OF MASSACHUSETTS

[US/US]; 50 Washington Street, Westborough, Massachusetts 01581 (US).

(72) Inventors: RICHTER, Joel D.; c/o University of Massa-

chusetts, 50 Washington Street, Westborough, Massachusetts 01581 (US). SHAH, Sneha; c/o University of Massachusetts, 50 Washington Street, Westborough, Massachusetts 01581 (US). SPINELLI, Jessica; c/o University of Massachusetts, 50 Washington Street, Westborough, Massachusetts 01581 (US).

(74) Agent: SANDERS, Deirdre et al.; Hamilton, Brook, Smith

& Reynolds, P.C., 55 Old Bedford Rd., Suite 200, Lincoln, Massachusetts 01773 (US).

(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ,

RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: BIOMARKERS AND METHODS RELATED TO FRAGILE X SYNDROME

(57) Abstract: Provided herein, in various embodiments, are methods of treating fragile X syndrome (FXS), comprising determining in a sample from the subject: a presence, an absence, or a level of an FXS-associated metabolite; an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite; a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites, or a combination of the foregoing. Also provided herein, in various embodiments, are methods of diagnosing, prognosing, subclassifying and/or predicting a treatment outcome of FXS in a subject.



BIOMARKERS AND METHODS RELATED TO FRAGILE X SYNDROME

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/554,913, filed on February 16, 2024, which claims the benefit of U.S. Provisional Patent Application No. 63/511,638, filed on June 30, 2023, each of which is incorporated by reference herein in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL IN XML

[0002] This application incorporates by reference the Sequence Listing contained in the following eXtensible Markup Language (XML) file being submitted concurrently herewith: File name: 54391032-002_SL.xml; created April 1, 2024, 41,224 Bytes in size.

GOVERNMENT SUPPORT

[0003] This invention was made with government support under GM135087 and GM046779 from National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0004] Fragile X syndrome (FXS) is an autism spectrum disorder that is the most frequently inherited form of intellectual impairment. FXS afflicts 1 in approximately 4,000 boys and 1 in approximately 7,000 girls. In addition to intellectual impairment, children with FXS present a range of symptoms including speech and developmental delays, perseveration, hyperactivity, aggression, and epilepsy, among other maladies. FXS is caused by a CGG triplet repeat expansion in a single gene, *fragile X messenger ribonucleoprotein 1 (FMR1)*, which resides on the X chromosome. When the CGG triplet expands to 200 repeats or more, the *FMR1* gene is methylated and thereby transcriptionally inactivated. The loss of the *FMR1* gene product, the protein fragile X messenger ribonucleoprotein (FMRP), is the cause of the disorder.

[0005] There is a critical need to develop methods and biomarkers to supplement treatment of fragile X-associated disorders such as fragile X syndrome (FXS).

SUMMARY

[0006] The present disclosure relates to methods and biomarkers to treat, and to supplement treatment of, fragile X-associated disorders such as fragile X syndrome (FXS).

[0007] The present disclosure relates to biomarkers, for example, metabolites that are differentially present in FXS subjects. Experiments conducted during the course of development of embodiments of the present disclosure identified a series of metabolites as being differentially present in samples of FXS subjects (*e.g.*, versus one or more control samples and/or reference levels). Accordingly, the present disclosure generally relates to biomarkers (*e.g.*, metabolites) and methods that are useful for diagnosing, making prognoses, and/or treating a subject, *e.g.*, a subject having, or at risk of having, a fragile X-associated disorder.

[0008] Provided herein, among other things, are methods of treating fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing; and

administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product.

[0009] The disclosure also provides, among other things, methods of predicting a treatment outcome of, diagnosing, prognosing, or subclassifying fragile X syndrome (FXS) in a subject, the methods comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0011] The foregoing will be apparent from the following more particular description of example embodiments, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments.

[0012] **FIGs. 1A-1B.** Metabolic changes observed in FXS patient-derived post-mortem cortex (brain) samples. **FIG. 1A.** Volcano plot depicting the changes in polar metabolite levels between post-mortem cortex tissue samples from six FXS males and six age-matched typically-developing (TD) males captured using liquid chromatography-mass spectrometry (LC-MS). The horizontal dotted line represents a significance threshold of a p-value equal to 0.05 ($-\log_{10}(0.05) = 1.3$). The left arm of the plot, where $\log_2$ of the fold change is less than zero, represents a decrease in levels of metabolites (18 metabolites reached statistical significance) in the FXS samples compared to TD samples. The right arm of the plot, where $\log_2$ of the fold change is greater than zero, represents an increase in levels of metabolites (four metabolites reached statistical significance) in the FXS samples compared to TD samples. *See also Table 1.* **FIG. 1B.** Ratios of glutathione (GSH) to glutathione disulfide (GSSG) levels compared between the FXS and TD samples used in FIG. 1A, reflecting increased oxidative stress in the FXS cortex samples.

[0013] **FIGs. 2A-2B.** Metabolic changes observed in FXS patient-derived PBMC samples. **FIG. 2A.** Volcano plot depicting the changes in polar metabolite levels between PBMC samples from six FXS males and five age-matched TD males captured using LC-MS. The horizontal dotted line represents a significance threshold of a p-value equal to 0.05 ($-\log_{10}(0.05) = 1.3$). The left arm of the plot, where $\log_2$ of the fold change is less than zero, represents a decrease in levels of metabolites (24 metabolites reached statistical significance) in the FXS samples compared to TD samples. *See also Table 2.* **FIG. 2B.** Ratios of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) levels compared between the FXS and TD samples used in FIG. 2A, reflecting altered mitochondrial metabolism in the FXS PBMC samples.

[0014] **FIGs. 3A-3C.** Common metabolic changes observed in FXS patient-derived PBMC and post-mortem cortex (brain) samples. **FIG. 3A.** Venn diagram depicting the polar metabolite levels significantly altered in FXS males compared to TD males in PBMC samples, cortex samples, and both sample types. The significance threshold of a p-value equal to 0.05 was used. The left circle shows the number of polar metabolites with significantly changed levels in PBMC samples from FXS males compared to TD males; the right circle shows the equivalent comparison in cortex samples. The overlapping region of the two circles and its accompanying list show the number and identity of polar metabolites with significantly changed levels in both PBMC and cortex samples from FXS males compared to TD males. **FIG. 3B.** Plots depicting levels of amino acids from the overlapping region of FIG. 3A that are reduced in cortex samples from FXS males compared to TD males. **FIG. 3C.** Plots depicting the levels of amino acids from the overlapping region of FIG. 3A that are reduced in PBMC samples from FXS males compared to TD males.

[0015] **FIG. 4.** Metabolic changes observed in FXS patient-derived lymphoblastoid cell lines (LCLs). Volcano plot depicting the changes in polar metabolite levels in two human male FXS LCLs (GM05131 and GM07072) compared to two TD LCLs (GM07174 and GM06890) captured using LC-MS. The horizontal dotted line represents a significance threshold of a p-value equal to 0.05 ($-\log_{10}(0.05) = 1.3$). The left arm of the plot, where $\log_2$ of the fold change is less than zero, represents a decrease in levels of metabolites (21 metabolites reached statistical significance) in the FXS samples compared to TD samples. *See also Table 4.*

[0016] **FIGs. 5A-5D.** A. Schematic of *FMRI* RNA isoforms in FXS. ASO against *FMRI-217* RNA used in FXS2 cells (160nM for 48hrs). B, C. qPCR data for *FMRI-217* RNA and *FMRI* RNA levels respectively (**** $p < 0.0001$, ** $p < 0.01$). D. Western blot data for FMRP and GAPDH proteins in TD and FXS2 cells (with vehicle or ASO).

[0017] **FIGs. 6A-6D.** Volcano plot illustrating $\log_2FC$ in FXS compared to TD samples (N=6 each). Dotted lines indicate cutoffs at $\log_2FC=0$ and $-\log(p>1.3)$. Amino acids and their derivatives are represented by green and red dots respectively in cerebellum (A) and cortex tissues (B). C. Box plots display the levels of leucine and lysine in TD and FXS samples (* $p < 0.05$, ** $p < 0.01$). D. Venn diagram shows the overlapping differential metabolites (amino acids and their derivatives highlighted in green and red respectively).

[0018] **FIGs. 7A-E.** Changes in metabolites may correlate with FMRP expression. **FIG. 7A.** Western blots to measure the FMRP levels in FXS1, FXS2 and TD lines compared to GAPDH. Below is a representation of FMRP levels in the cell lines. **FIG. 7B.** PCA plot demonstrating the clustering of the FXS2 line (partial FMRP) with the TD as against the FXS1 cell line (no FMRP). **FIG. 7, C, D and E.** Volcano plot depicting significantly altered metabolites (amino acids in green and their derivatives in red) in FXS2 vs TD (FIG. 7C.), FXS1 vs TD (FIG. 7D.) and FXS2 vs FXS1 (FIG. 7E., note the increase in amino acid levels). **FIG. 7F.** Examples of amino acid changes in the FXS lines compared to TD. (* $p < 0.05$, ** $p < 0.01$).

[0019] **FIGs. 8A-8B.** iPSC derived forebrain neuron cultures. **FIG 8A.** The diagram depicts the conversion of LCLs from FXS patients into iPSCs, their subsequent differentiation into neuronal lineage, and the generation of mature forebrain neurons. **FIG. 8B.** Representative images of cells in culture aligned to the different stages in **FIG 8A.** (panel A).

[0020] **FIG. 9.** Model proposing the influence of dysregulated proteostasis and/or mitochondrial function on free amino acids levels in FXS. FXS LCLs will be treated with various inhibitors such as MG-132 or Bortezomib against proteasome, 3-methyladenine (3-MA) against autophagosome, Rapamycin to block mTORC1 activation and dexpropamethazine (DEX) to block proton leak in the mitochondria will be used. LC-MS assay will be used to analyze the effect of each inhibitor on the metabolome. To assess the effect of reduced free amino acids on tRNA charging, the i-TRAP qPCR method will be used to quantify ratios of individual uncharged to charged tRNAs.

[0021] **FIG. 10.** Model proposing restoration of FMRP based rescue of metabolic deficits in FXS. Using ASOs to restore FMRP in FXS cells, whether the metabolite expression specifically amino acids are restored to those in TD cells will be tested.

[0022] **FIGs. 11A-C.** **FIG. 11A.** Lymphoblastoid cell lines (LCLs) from a typically developing (TD) male individual, a Fragile X Syndrome male (FXS) expressing some FMRP (FXS2), and a FXS male expressing no FMRP (FXS1) were analyzed for FMRP and GAPDH (as a loading control) on western blots in triplicate. **FIG. 11B.** Polar metabolites from the cell lines noted in **FIG. 11A.** (panel A) were assessed by LC/MS. A Principal Component Analysis (PCA) shows tight clustering of the metabolites based on the genotype. **FIG. 11C.** Relative amounts of leucine and methionine in the LCLs from the 3 genotypes noted in **FIG. 11A.**

- [0023] **FIGs. 12A-12B. FIG. 12A.** The culture media from the LCLs noted in **FIG. 11** were analyzed for metabolites by LC/MS. Shown are relative amounts of leucine and methionine.
- FIG. 12B.** PCA plot for metabolites in the culture media from the LCL lines denoted in **FIG. 11**.
- [0024] **FIG. 13.** LC/MS analysis of the metabolome comparing FXS1 to TD LCLs. The volcano plot at left is the cell pellets and at right is the culture media. The metabolites above the dashed line are statistically different between the genotypes. The vertical line separates metabolites that are decreased (to the left) or increased (to the right) when comparing FXS1 to TD. *See also Table 5 (cell pellet) and Table 6 (culture media).*
- [0025] **FIG. 14.** LC/MS analysis of the metabolome comparing FXS2 to TD LCLs. The volcano plot at left is the cell pellets and at right is the culture media. The metabolites above the dashed line are statistically different between the genotypes. The vertical line separates metabolites that are decreased (to the left) or increased (to the right) when comparing FXS2 to TD. *See also Table 7 (cell pellet) and Table 8 (culture media).*
- [0026] **FIG. 15.** LC/MS analysis of the metabolome comparing FXS2 to FXS1 LCLs. The volcano plot at left is the cell pellets and at right is the culture media. The metabolites above the dashed line are statistically different between the genotypes. The vertical line separates metabolites that are decreased (to the left) or increased (to the right) when comparing FXS2 to FXS1. *See also Table 9 (cell pellet) and Table 10 (culture media).*
- [0027] **FIG. 16A.** Relative amounts of $^{13}\text{C}_5$ citrate in wild-type and FXS2 cells.
- [0028] **FIG. 16B.** Amount of extracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamate with respect to the amount of extracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamine.
- [0029] **FIG. 16C.** Amount of intracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamate with respect to the amount of intracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamine.
- [0030] **FIG. 16D.** Relative amounts of extracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamate in wild-type and FXS2 cells.
- [0031] **FIG. 16E.** Relative amounts of intracellular $^{13}\text{C}_5, ^{15}\text{N}_2$ glutamate in wild-type and FXS2 cells.

DETAILED DESCRIPTION

- [0032] A description of example embodiments follows.

[0033] Several aspects of the invention are described below, with reference to examples for illustrative purposes only. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or practiced with other methods, protocols, reagents, cell lines and animals. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts, steps or events are required to implement a methodology in accordance with the present invention. Many of the techniques and procedures described, or referenced herein, are well understood and commonly employed using conventional methodology by those skilled in the art.

Definitions

[0034] Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this disclosure pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. It will be further understood that terms, such as those defined in commonly-used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and/or as otherwise defined herein.

[0035] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0036] When introducing elements disclosed herein, the articles “a,” “an,” “the,” and “said” are intended to mean that there are one or more of the elements. Further, the one or more elements may be the same or different. For example, unless the context clearly indicates otherwise, “an FXS-associated metabolite” includes a single metabolite, and two or more metabolites. In some embodiments, a first metabolite has a reduced level in a sample from a subject, and a second metabolite has an increased level in the sample from a subject.

[0037] Throughout this specification and the claims which follow, unless the context requires otherwise, the term “comprise,” and variations such as “comprises” and “comprising,” will be understood to imply the inclusion of, *e.g.*, a stated integer or step or group of integers or steps, but not the exclusion of any other integer or step or group of integer or step. When used herein, the term “comprising” can be substituted with the term “containing” or “including.”

[0038] As used herein, the term “consisting of” excludes any element, step, or ingredient not specified in the claim element. When used herein, the term “consisting essentially of” does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[0039] Any of the terms “comprising,” “containing,” “including,” and “having,” whenever used herein in the context of an aspect or embodiment disclosed herein, can in some embodiments, be replaced with the term “consisting of,” or “consisting essentially of” to vary scopes disclosed herein.

[0040] As used herein, the conjunctive term “and/or” between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by “and/or,” a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and, therefore, satisfy the requirement of the term “and/or” as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and, therefore, satisfy the requirement of the term “and/or.”

[0041] It should be understood that for all numerical bounds describing some parameter in this application, such as “about,” “at least,” “less than,” “fewer than,” and “more than,” the description also necessarily encompasses any range bounded by the recited values. Accordingly, for example, the description “at least 1, 2, 3, 4, or 5” also describes, *inter alia*, the ranges 1-2, 1-3, 1-4, 1-5, 2-3, 2-4, 2-5, 3-4, 3-5, and 4-5, *et cetera*.

[0042] When a list is presented, unless stated otherwise, it is to be understood that each individual element of that list, and every combination of that list, is a separate embodiment. For

example, a list of embodiments presented as “A, B, or C” is to be interpreted as including the embodiments, “A,” “B,” “C,” “A or B,” “A or C,” “B or C,” or “A, B, or C.”

[0043] As used herein, the term “about” means within an acceptable error range for a particular value, as determined by one of ordinary skill in the art. Typically, an acceptable error range for a particular value depends, at least in part, on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, “about” can mean within an acceptable standard deviation, per the practice in the art. Alternatively, “about” can mean a range of $\pm 20\%$, *e.g.*, $\pm 10\%$, $\pm 5\%$ or $\pm 1\%$ of a given value. It is to be understood that the term “about” can precede any particular value specified herein, except for particular values used in the Exemplification. When “about” precedes a range, as in “about 55-200 CGG repeats,” the term “about” should be read as applying to both given values of the range, such that “about 55-200 CGG repeats” means about 55 CGG repeats to about 200 CGG repeats.

[0044] The term “subject” refers to a mammalian subject, preferably human, diagnosed with or suspected of having a fragile X-associated disorder (*e.g.*, FXS).

[0045] As used herein, the term “metabolite” refers to an intermediate or product resulting from metabolism. As used herein, the term “metabolism” refers to chemical changes occurring within an organism. In some embodiments, a metabolite results from anabolism. In some embodiments, a metabolite results from catabolism.

[0046] Compounds described herein include those described generally, and are further illustrated by the classes, subclasses, and species disclosed herein. As used herein, the following definitions shall apply unless otherwise indicated. For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed. Additionally, general principles of organic chemistry are described in “Organic Chemistry”, Thomas Sorrell, University Science Books, Sausalito: 1999, and “March’s Advanced Organic Chemistry”, 5th Ed., Ed.: Smith, M.B. and March, J., John Wiley & Sons, New York: 2001, the relevant contents of which are incorporated herein by reference.

[0047] Unless specified otherwise within this specification, the nomenclature used in this specification generally follows the examples and rules stated in Nomenclature of Organic Chemistry, Sections A, B, C, D, E, F, and H, Pergamon Press, Oxford, 1979, which is

incorporated by reference herein for its chemical structure names and rules on naming chemical structures. Optionally, a name of a compound may be generated using a chemical naming program (*e.g.*, CHEMDRAW®, version 17.0.0.206, PerkinElmer Informatics, Inc.).

[0048] As used herein, the term “sample” refers to any sample that can be from or derived from a subject (*e.g.*, a mammalian subject such as a human subject). The methods disclosed herein can be performed using a variety of possible sample types. For example, a bodily fluid, a single cell or cell lysate, a population of cells, a cell culture, and/or a tissue.

[0049] The phrase “pharmaceutically acceptable” means that the substance or composition the phrase modifies is, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, and the like, and are commensurate with a reasonable benefit/risk ratio.

[0050] As used herein, the term “pharmaceutically acceptable salt” refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of mammals without undue toxicity, irritation, allergic response, and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge *et al.*, describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 1977, 66, 1-19, the relevant teachings of which are incorporated herein by reference in their entirety. Pharmaceutically acceptable salts of the agents/compounds described herein include salts derived from suitable inorganic and organic acids, and suitable inorganic and organic bases.

[0051] Examples of salts derived from suitable acids include salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid, or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art, such as ion exchange. Other pharmaceutically acceptable salts derived from suitable acids include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, cinnamate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, glutarate, glycolate, hemisulfate, heptanoate, hexanoate, hydroiodide, hydroxybenzoate, 2-hydroxy-ethanesulfonate, hydroxymaleate, lactobionate, lactate, laurate,

lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 2-phenoxybenzoate, phenylacetate, 3-phenylpropionate, phosphate, pivalate, propionate, pyruvate, salicylate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like.

[0052] Either the mono-, di- or tri-acid salts can be formed, and such salts can exist in either a hydrated, solvated or substantially anhydrous form.

[0053] Salts derived from appropriate bases include salts derived from inorganic bases, such as alkali metal, alkaline earth metal, and ammonium bases, and salts derived from aliphatic, alicyclic or aromatic organic amines, such as methylamine, trimethylamine and picoline, or $N^+((C_1-C_4)alkyl)_4$ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, barium and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxyl, sulfate, phosphate, nitrate, lower alkyl sulfonate and aryl sulfonate.

[0054] “Pharmaceutically acceptable carrier” refers to a non-toxic carrier or excipient that does not destroy the pharmacological activity of the agent with which it is formulated and is nontoxic when administered in doses sufficient to deliver a therapeutic amount of the agent. Pharmaceutically acceptable carriers that may be used in the compositions described herein include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

[0055] “Treating” or “treatment,” as used herein, refers to taking steps to deliver a therapy to a subject, such as a mammal, in need thereof (*e.g.*, as by administering to a mammal one or more therapeutic agents). “Treating” or “treatment” includes inhibiting the disease or condition (*e.g.*,

as by slowing or stopping its progression or causing regression of the disease or condition) and relieving the symptoms resulting from the disease or condition. The term “treating,” or “treatment” refers to the medical management of a subject with the intent to improve, ameliorate, stabilize (*i.e.*, not worsen), prevent, or cure a disease, pathological condition, or disorder—such as the particular indications exemplified herein. This term includes active treatment (treatment directed to improve the disease, pathological condition, or disorder), causal treatment (treatment directed to the cause of the associated disease, pathological condition, or disorder), palliative treatment (treatment designed for the relief of symptoms), preventative treatment (treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder); and supportive treatment (treatment employed to supplement another therapy). Treatment also includes diminishment of the extent of the disease or condition; preventing spread of the disease or condition; delay or slowing the progress of the disease or condition; amelioration or palliation of the disease or condition; and remission (whether partial or total), whether detectable or undetectable. “Ameliorating” or “palliating” a disease or condition means that the extent and/or undesirable clinical manifestations of the disease, disorder, or condition are lessened and/or time course of the progression is slowed or lengthened, as compared to the extent or time course in the absence of treatment. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder, as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented. In some embodiments, treatment includes change or restoration of expression, *e.g.*, endogenous FMRP expression. In some embodiments, treatment includes change or restoration of FMRP level(s), *e.g.*, to ameliorate metabolic deficit(s) in FXS.

[0056] As used herein, a “polynucleotide” is defined as a plurality of nucleotides and/or nucleotide analogs linked together in a single molecule. In some embodiments, a polynucleotide disclosed herein comprises deoxyribonucleotides. In some embodiments, the polynucleotide comprises ribonucleotides. Non-limiting examples of polynucleotides include single-, double- or multi-stranded DNA or RNA, DNA-RNA hybrids (*e.g.*, each “T” position may be independently substituted by a “U” or vice versa), or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide

bases. The backbone of the polynucleotide can comprise sugars and phosphate groups, modified or substituted sugar or phosphate groups, a polymer of synthetic subunits such as phosphoramidates, or a combination thereof.

[0057] As used herein, the term “nucleotide analog” or “altered nucleotide” or “modified nucleotide” refers to a non-standard nucleotide, including non-naturally occurring ribonucleotides or deoxyribonucleotides. A nucleotide analog may be modified at any position so as to alter certain chemical properties of the nucleotide yet retain the ability to perform its intended function. Non-limiting examples of positions of the nucleotide which may be derivatized include the 5 position, *e.g.*, 5-(2-amino)propyl uridine, 5-bromo uridine, 5-propyne uridine, and 5-propenyl uridine; the 6 position, *e.g.*, 6-(2-amino)propyl uridine; the 8-position for adenosine and/or guanosines, *e.g.*, 8-bromo guanosine, 8-chloro guanosine, and 8-fluoroguanosine. Nucleotide analogs also include deaza nucleotides, *e.g.*, 7-deaza-adenosine; O- and N-modified (*e.g.*, alkylated or N6-methyl adenosine) nucleotides.

[0058] As used herein, the term “complementary” refers to sequence complementarity between two different polynucleotides or between two regions of the same polynucleotide. A first region of a polynucleotide is complementary to a second region of the same or a different polynucleotide if, when the two regions are arranged in an anti-parallel fashion, at least one nucleotide residue of the first region is capable of base pairing (*i.e.*, hydrogen bonding) with a residue of the second region, thus forming a hydrogen-bonded duplex.

[0059] As used herein, the term “sequence identity” refers to the extent to which two nucleotide sequences have the same residues at the same positions when the sequences are aligned to achieve a maximal level of identity, expressed as a percentage. For sequence alignment and comparison, typically one sequence is designated as a reference sequence, to which test sequences are compared. Sequence identity between reference and test sequences is expressed as a percentage of positions across the entire length of the reference sequence where the reference and test sequences share the same nucleotide or amino acid upon alignment of the reference and test sequences to achieve a maximal level of identity. As an example, two sequences are considered to have 70% sequence identity when, upon alignment to achieve a maximal level of identity, the test sequence has the same nucleotide residue at 70% of the same positions over the entire length of the reference sequence.

[0060] Alignment of sequences for comparison to achieve maximal levels of identity can be readily performed by a person of ordinary skill in the art using an appropriate alignment method or algorithm. In some instances, alignment can include introduced gaps to provide for the maximal level of identity. Examples include the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), and visual inspection (see generally Ausubel *et al.*, *Current Protocols in Molecular Biology*).

[0061] When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequent coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. A commonly used tool for determining percent sequence identity is Protein Basic Local Alignment Search Tool (BLASTP) available through National Center for Biotechnology Information, National Library of Medicine, of the United States National Institutes of Health. (Altschul *et al.*, 1990).

[0062] As used herein, the term “polypeptide” refers to a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation). A polypeptide can comprise any suitable L-and/or D-amino acid, for example, common α -amino acids (*e.g.*, alanine, glycine, valine), non- α -amino acids (*e.g.*, β -alanine, 4-aminobutyric acid, 6-aminocaproic acid, sarcosine, statine), and unusual amino acids (*e.g.*, citrulline, homocitrulline, homoserine, norleucine, norvaline, ornithine). The amino, carboxyl, and/or other functional groups on a polypeptide can be free (*e.g.*, unmodified) or protected with a suitable protecting group. Suitable protecting groups for amino and carboxyl groups, and methods for adding or removing protecting groups are known in the art and are disclosed in, for example, Green and Wuts, “*Protecting Groups in Organic Synthesis*,” John Wiley and Sons, 1991. The functional groups of a polypeptide can also be derivatized (*e.g.*, alkylated) or labeled (*e.g.*, with a detectable label, such as a fluorogen or a hapten) using

methods known in the art. A polypeptide can comprise one or more modifications (*e.g.*, amino acid linkers, acylation, acetylation, amidation, methylation, terminal modifiers (*e.g.*, cyclizing modifications), *N*-methyl- α -amino group substitution), if desired. In addition, a polypeptide can be an analog of a known and/or naturally-occurring peptide, for example, a peptide analog having conservative amino acid residue substitution(s).

[0063] As used herein, the term “antibody mimetic” refers to polypeptides capable of mimicking an antibody’s ability to bind an antigen, but structurally differ from native antibody structures. Examples of antibody mimetics include, but not limited to, Adnectins, Affibodies, Affilins, Affimers, Affitins, Alphabodies, Anticalins, Avimers, DARPinS, Fynomers, Kunitz domain peptides, monobodies, nanobodies, nanoCLAMPs, and Versabodies.

[0064] A “pharmaceutical composition” refers to a formulation of one or more therapeutic agents and a medium generally accepted in the art for delivery of a biologically active agent to subjects, *e.g.*, humans. In some embodiments, a pharmaceutical composition may include one or more pharmaceutically acceptable excipients, diluents, or carriers. In some embodiments, a pharmaceutical composition suitable for use in methods disclosed herein further comprises one or more pharmaceutically acceptable carriers.

[0065] “Pharmaceutically acceptable carrier, diluent, or excipient” includes any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals.

[0066] “Pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative. In some embodiments, the carrier may be a diluent, adjuvant, excipient, or vehicle with which the agent (*e.g.*, polynucleotide) is administered. Such vehicles may be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. For example, 0.4% saline and 0.3% glycine can be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (*e.g.*, filtration). The compositions may

contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating, and coloring agents, *etc.* The concentration of the agent in such pharmaceutical formulation may vary widely, *i.e.*, from less than about 0.5%, to at least about 1%, or to as much as 15% or 20%, 25%, 30%, 35%, 40%, 45% or 50% by weight. The concentration will be selected primarily based on required dose, fluid volumes, viscosities, *etc.*, according to the mode of administration. Suitable vehicles and formulations, inclusive of other human proteins, *e.g.*, human serum albumin, are described, for example, in Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing: 691-1092 (*e.g.*, pages 958-89).

[0067] Non-limiting examples of pharmaceutically acceptable carriers are solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible, such as salts, buffers, antioxidants, saccharides, aqueous or non-aqueous carriers, preservatives, wetting agents, surfactants or emulsifying agents, or combinations thereof.

[0068] Non-limiting examples of buffers are acetic acid, citric acid, formic acid, succinic acid, phosphoric acid, carbonic acid, malic acid, aspartic acid, histidine, boric acid, Tris buffers, HEPPSO, and HEPES.

[0069] Non-limiting examples of antioxidants are ascorbic acid, methionine, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite, lecithin, citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, and tartaric acid.

[0070] Non-limiting examples of amino acids are histidine, isoleucine, methionine, glycine, arginine, lysine, L-leucine, tri-leucine, alanine, glutamic acid, L-threonine, and 2-phenylamine.

[0071] Non-limiting examples of surfactants are polysorbates (*e.g.*, polysorbate-20 or polysorbate-80); polyoxamers (*e.g.*, poloxamer 188); Triton; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (*e.g.*, lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-aurate; and the MONAQUA™ series (Mona Industries, Inc.,

Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.*, PLURONICS™, PF68, *etc.*).

[0072] Non-limiting examples of preservatives are phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride, alkylparaben (methyl, ethyl, propyl, butyl, and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate, and thimerosal, or mixtures thereof.

[0073] Non-limiting examples of saccharides are monosaccharides, disaccharides, trisaccharides, polysaccharides, sugar alcohols, reducing sugars, nonreducing sugars such as glucose, sucrose, trehalose, lactose, fructose, maltose, dextran, glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, mannitol, melibiose, melezitose, raffinose, mannatriose, stachyose, maltose, lactulose, maltulose, glucitol, maltitol, lactitol, or iso-maltulose.

[0074] Non-limiting examples of salts are acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous, and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids, and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium, and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine, and the like. In some embodiments, the salt is sodium chloride (NaCl).

[0075] Agents (*e.g.*, polynucleotides) described herein may be prepared in accordance with standard procedures and are administered at dosages that are selected to reduce, prevent, or eliminate, or to slow or halt progression of, a condition being treated (see, *e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, and Goodman and Gilman's The Pharmaceutical Basis of Therapeutics, McGraw-Hill, New York, N.Y., the contents of which are incorporated herein by reference, for a general description of methods for administering various agents for human therapy).

[0076] “Administering” or “administration,” as used herein, refers to providing a compound, composition, or pharmaceutically acceptable salt thereof described herein to a subject in need of treatment or prevention. Administering can be performed, for example, once, a plurality of times, and/or over one or more extended periods. Administration includes both direct administration (including self-administration), and indirect administration (including an act of prescribing a drug or directing a subject to consume an agent). For example, as used herein, one (*e.g.*, a physician) who instructs a subject (*e.g.*, a human patient) to self-administer an agent (*e.g.*, a drug), or to have an agent administered by another and/or who provides a patient with a prescription for a drug is administering an agent to a subject.

[0077] “A therapeutically effective amount” or “an effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic or biological result (*e.g.*, treatment, healing, inhibition or amelioration of physiological response or condition, *etc.*). A therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of a therapeutic or a combination of therapeutics to elicit a desired response in the individual.

[0078] Non-limiting examples of desired therapeutic or biological results include improvement of behavior and/or cognition (*e.g.*, clinical global impression-improvement (CGI-I) scale, pediatric anxiety rating scale (PARS) total score, visual analogue scale (VAS), anxiety, depression, and mood scale (ADAMS), aberrant behavior checklist (ABC), receptive language, attention, vigilance), improvement of mitochondrial function (*e.g.*, mitochondrial membrane potential, mass, ATP production, and/or Ca^{2+} transfer), mitigation of mitochondrial dysregulation (*e.g.*, mitochondrial ER stress (*e.g.*, GRP78 level and/or CHOP level) and/or Bax/Bcl-2 ratio), or any combination of the foregoing.

[0079] As used herein, the term “treat,” “treating” or “treatment” refers to therapeutic treatment wherein the objective is to slow down (lessen) an undesired physiological change or disease, such as the development or progression of the fragile X-associated disorder (*e.g.*, FXS), or to provide a beneficial or desired clinical outcome during treatment. Beneficial or desired clinical outcomes include alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, whether detectable or undetectable. Non-limiting examples of

symptoms include speech and motor development symptoms; cognitive disabilities, including learning and intellectual disabilities, hyperactivity, short attention span, anxiety, sensitivity to sensory stimulation, sleep problems, and seizures; recurrent ear infections, and mitral valve prolapse.

[0080] The term “poor” or “poorer” refers to greater degree of fragile X-associated disorder (*e.g.*, FXS) symptoms, increased extent of disease, decreased (*i.e.*, worsening) state of disease, increased or enhanced state of disease progression, deterioration or worsening of the disease state, whether detectable or undetectable.

[0081] “Mass Spectrometry” (MS) is a technique for measuring and analyzing molecules to produce a mass spectrum that serves as a “molecular fingerprint.” MS involves fragmenting a target molecule (*e.g.*, a metabolite), then analyzing the fragments, based on their mass/charge ratios. A “mass spectrum” is a plot of data produced by a mass spectrometer. In some embodiments, a mass spectrum contains m/z values on x-axis and intensity values on y-axis. The term “ m/z ” or “mass-to-charge” ratio refers to the dimensionless quantity formed by dividing the mass number of an ion by its charge number. In liquid chromatography-mass spectrometry-based (LC-MS) metabolomics, a group of ions may originate from the same metabolite, and one metabolite can be represented by multiple peaks in LC-MS data with distinct m/z values but, at similar retention times, due to the presence of adducts (*e.g.*, H^+ , Na^+ or K^+). A “peak” is a point on a mass spectrum with a relatively high y-value.

Methods of Treating Fragile X-Associated Disorders

[0082] Treatments for fragile X syndrome (and other autism spectrum disorders), which are mostly based on animal models, have met with very limited success in human clinical trials (Hagerman et al., *Nature Rev Disease Primers* 3:17065 (2017); Berry-Kravis *et al.*, *Nature Rev Drug Disc.* 17:280-299 (2018)). There is no widely available therapy that shows even modest efficacy for FXS. Moreover, there are no established molecular readouts with statistical rigor of any FXS treatment.

[0083] Provided herein, among other methods, are methods of treating fragile X-associated disorders.

[0084] In some embodiments, methods of treating fragile X syndrome (FXS) in a subject disclosed herein comprise determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[0085] In some embodiments, methods of treating fragile X syndrome (FXS) in a subject disclosed herein comprise determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing; and

administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product.

[0086] In some embodiments, the agent is an antisense oligonucleotide.

[0087] In some embodiments, a therapeutically effective amount of the agent decreases an aberrant *FMR1* transcript, a protein encoded by the aberrant *FMR1* transcript, or both.

[0088] In some embodiments, an aberrant *FMR1* gene product comprises *FMR1*-217.

[0089] For example, if the ratio between levels of two FXS-associated metabolites in a sample from the subject is determined to be 1:3 and suppose the reference ratio of the two FXS-associated metabolites is 1:5, the subject may be administered a therapeutically effective amount of an agent that that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product. In another example, after administering a therapeutically effective amount of an agent, if the ratio between levels of two FXS-associated metabolites in a sample from the subject is determined to be 1:2 a month later, the treatment regimen for the subject may be modified.

[0090] In some embodiments, a method disclosed herein comprises determining a presence, absence, amount, or alteration of at least one FXS-associated metabolite (e.g., an FXS-associated metabolite), or an alteration of an FXS-associated ratio of two metabolites, in a sample from the

subject. In some embodiments, a method further comprises treating FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, or the alteration of the FXS-associated ratio, in the sample.

Fragile X-Associated Disorders

[0091] Fragile X-associated disorders are caused by mutation of the *fragile X messenger ribonucleoprotein 1* (*FMRI*, previously known as *fragile X mental retardation 1*) gene, located in the q27.3 locus of the X chromosome. The expansion of the trinucleotide CGG repeat above the normal range (greater than 54 repeats) in the non-coding region of the *FMRI* gene has been associated with the development of fragile X-associated disorders. For example, in those carrying the premutation, the trinucleotide CGG repeat can range from 55-200 CGG repeats. In some embodiments, a fragile X-associated disorder is linked to greater than 77 CGG repeats in *FMRI*, e.g., greater than 98 CGG repeats in *FMRI*. In some embodiments, a fragile X-associated disorder is linked to at least 140 CGG repeats in *FMRI*. In some embodiments, a fragile X-associated disorder is linked to at least 201 CGG repeats in *FMRI*.

[0092] Non-limiting examples of fragile X-associated disorders include fragile X-associated tremor/ataxia syndrome (FXTAS), fragile X-associated primary ovarian insufficiency (FXPOI), fragile X-associated neuropsychiatric disorders (FXAND), and fragile X syndrome (FXS). In some embodiments, a fragile X-associated disorder is fragile X syndrome (FXS), fragile X-associated primary ovarian insufficiency (FXPOI), or fragile X-associated tremor/ataxia syndrome (FXTAS), or a combination thereof. In some embodiments, a fragile X-associated disorder is FXS.

Subjects

[0093] In some embodiments, a subject is a mammal. As used herein, the term “subject” includes humans, domestic animals, such as laboratory animals (e.g., dogs, monkeys, pigs, rats, mice, *etc.*), household pets (e.g., cats, dogs, rabbits, *etc.*), livestock (e.g., pigs, cattle, sheep, goats, horses, *etc.*), and non-domestic animals. In some embodiments, a subject is a human. In some embodiments, a subject (e.g., human) is male. In some embodiments a subject (e.g., human) is female.

[0094] In some embodiments, a subject (*e.g.*, a human) has, or is predisposed to have, a fragile X-associated disorder (*e.g.*, FXS). In some embodiments, a subject (*e.g.*, a human) has a fragile X-associated disorder (*e.g.*, FXS). In some embodiments, a subject (*e.g.*, a human) is predisposed to have a fragile X-associated disorder (*e.g.*, FXS).

[0095] In some embodiments, a subject is diagnosed with a fragile X-associated disorder (*e.g.*, FXS). In some embodiments, a subject is suspected of having a fragile X-associated disorder (*e.g.*, FXS).

[0096] In some embodiments, a subject has one or more of the physical and/or medical features associated with a fragile X-associated disorder (*e.g.*, FXS). Non-limiting examples of physical features associated with FXS include a long face, prominent ears and chin, arched palate, large testicles at puberty, low muscle tone, flat feet, and hyperextensible joints. Non-limiting examples of medical or behavioral features associated with FXS include sleep problems, seizures, recurrent ear infections, mitral valve prolapse, behaviors of hyperactivity, short attention span, hand biting or hand flapping, poor eye contact and social skills, shyness, anxiety, autism, epilepsy, aggression, delayed speech and/or motor development, repetitive speech, sensitivity to sensory stimulation (including a hypersensitivity to being touched, to light or to sound), or any combination thereof. In some embodiments, a subject is a female with an IQ score of less than 115, 110, 105, 100, 95, or 90. In some embodiments, a subject is a male with an IQ score of less than 60, 55, 50, or 45.

[0097] In some embodiments, a subject has one or more of the following: irregular menses, fertility problem, elevated FSH (follicle-stimulating hormone) level, premature ovarian failure, primary ovarian insufficiency, and vasomotor symptoms (*e.g.*, “hot flash”). In some embodiments, a subject has one or more of the following: intention tremor, parkinsonism, ataxia, memory loss, white matter lesion involving middle cerebellar peduncles, and cognitive decline.

[0098] In some embodiments, a subject has at least 55 CGG repeats in the 5' untranslated region of an *FMRI* gene, for example, having at least 77, at least 78, at least 98, at least 99, at least 140, or at least 201 CGG repeats in the 5' untranslated region of the *FMRI* gene. In some embodiments, a subject has between about 55-200 CGG repeats in the 5' untranslated region of an *FMRI* gene. In some embodiments, a subject has at least 200 CGG repeats in the 5'

untranslated region of an *FMRI* gene. In some embodiments, a subject has at least 201 CGG repeats in the 5' untranslated region of an *FMRI* gene.

[0099] In some embodiments, a subject (*e.g.*, a human) has a CGG repeat expansion that is at least partially methylated. In some embodiments, a subject (*e.g.*, a human) has a CGG repeat expansion that is partially methylated. In some embodiments, a subject has a CGG repeat expansion that is fully methylated.

[00100] In some embodiments, a subject is a premutation carrier for FXS. In some embodiments, a subject has FXS.

Aberrant *FMRI* Gene Products

[00101] In some embodiments, a subject (*e.g.*, a human) expresses or has an elevated expression of an aberrant *FMRI* gene product.

[00102] A *FMRI* gene encodes a fragile X messenger ribonucleoprotein (FMRP, previously known as fragile X mental retardation protein). In some embodiments, an *FMRI* gene described herein is a human *FMRI* gene (*e.g.*, corresponding to GenBank reference number NC_000023.11), a mouse *FMRI* gene (*e.g.*, NC_000086.8), a rat *FMRI* gene (*e.g.*, NC_051356.1), a golden hamster *FMRI* gene (*e.g.*, NW_024429188.1), a Chinese hamster *FMRI* gene (*e.g.*, NW_003614110.1), a dog *FMRI* gene (*e.g.*, NC_051843.1), a pig *FMRI* gene (*e.g.*, NC_046383.1), or a monkey *FMRI* gene (*e.g.*, NC_041774.1). In some embodiments, the *FMRI* gene is a human *FMRI* gene. The human *FMRI* gene (Ensembl: ENSG00000102081.16) is located within chromosome band Xq27.3 between base pairs 147,911,919 and 147,951,125 (the numberings referring to Genome Reference Consortium Human Build 38 (GRCh38)).

[00103] In some embodiments, an aberrant *FMRI* gene product (*e.g.*, an aberrant *FMRI* transcript and/or its protein product) contributes to pathology of a fragile X-associated disorder (*e.g.*, FXS). In some embodiments, an aberrant *FMRI* transcript contributes to pathology of a fragile X-associated disorder. In some embodiments, a protein encoded by an aberrant *FMRI* transcript contributes to pathology of a fragile X-associated disorder. In some embodiments, an aberrant *FMRI* transcript and its protein product contribute to pathology of a fragile X-associated disorder.

[00104] In some embodiments, an aberrant *FMRI* gene product is produced from a CGG expansion-dependent mis-splicing of an *FMRI* transcript.

[00105] In some embodiments, an aberrant *FMRI* transcript is *FMRI*-217. In humans, *FMRI*-217, also referred to as “isoform 12” or “iso12,” is a transcript corresponding to A0A087X1M7 (ENST00000621447.1, 1,832 nucleotides). *FMRI*-217 has 2 exons, and the splicing between Exon 1 of *FMRI*-217 (between base pairs 147,912,123 and 147,912,230, SEQ ID NO:1) and Exon 2 of *FMRI*-217 (between base pairs 147,912,728 and 147,914,451, SEQ ID NO:2) is considered aberrant *FMRI* RNA splicing. *FMRI*-217 is detected in a subpopulation of subjects with fragile X-associated disorder, including a subpopulation of FXS patients, and a subpopulation of premutation carriers for FXS.

[00106] CGCCCGCAGCCACCTCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGAGCTGGTGGTGGGAAGTGCGGGGCTCCAATGGCGCTTTCTACAAG (SEQ ID NO:1).

[00107] CATTGGGACTTCGGAGAGCTCCACTGTTCTGGGCGAGGGCTGTGAAGAAA
GAGTAGTAAGAAGCGGTAGTCGGCACCAAATCACAAATGGCAACTGATTTTTAGTGG
CTTCTCTTTGTGGATTTCGGAGGAGATTTTAGATCCAAAAGTTTCAGGAAGACCCTA
ACATGGCCCAGCAGTGCATTGAAGAAGTTGATCATCGTGAATATTCGCGTCCCCCTT
TTTGTTAAACGGGGTAAATTCAGGAATGCACATGCTTCAGCGTCTAAAACCATTAGC
AGCGCTGCTACTTAAAAATTGTGTGTGTGTGTTTAAGTTTCCAAAGACCTAAATATA
TGCCATGAACTTCAGGTAATTAAGTGAAGATATATTACTAGGGCATTTTTTTTTT
TAACTGAGCGAAAATATTTTTGTGCCCTAAGAACTTGACCACATTTCTTTGAATTT
GTGGTGTTCAGTGGACTGAATTGTTGAGGCTTTATATAGGCATTCATGGGTTTACT
GTGCTTTTTAAAGTTACACCATTGCAGATCAACTAACACCTTTCAGTTTTAAAGGA
AGATTTACAAATTTGATGTAGCAGTAGTGCGTTTGTGTTGGTATGTAGGTGCTGTATAA
ATTCATCTATAAATTCTCATTTCCTTTTGAATGTCTATAACCTCTTCAATAATATCCC
ACCTTACTACAGTATTTTGGCAATAGAAGGTGCGTGTGGAAGGAAGGCTGGAAAAT
AGCTATTAGCAGTGTCCAACACAATTCTTAAATGTATTGTAGAATGGCTTGAATGTT
TCAGACAGGACACGTTTGGCTATAGGAAAATAACAATTGACTTTATTCTGTGTTTA
CCAATTTTATGAAGACATTTGGAGATCAGTATATTTCATAAATGAGTAAAGTATGTA
AACTGTTCCATACTTTGAGCACAAAGATAAAGCCTTTTGCTGTAAAAGGAGGCAAA
AGGTAACCCCGCGTTTATGTTCTTAACAGTCTCATGAATATGAAATTGTTTCAGTTGA
CTCTGCAGTCAAAATTTTAATTCATTGATTTTATTGATCCATAATTTCTTCTGGTGA

GTTTGCGTAGAATCGTTCACGGTCCTAGATTAGTGGTTTTGGTCACTAGATTTCTGGC
 ACTAATAACTATAATACATATACATATATATGTGTGAGTAACGGCTAATGGTTAGGC
 AAGATTTTGATTGACCTGTGATATAAACTTAGATTGGATGCCACTAAAGTTTGCTTA
 TCACAGAGGGCAAGTAGCACATTATGGCCTTGAAGTACTTATTGTTCTCTCCAGCA
 ACTTATGATTTGCTCCAGTGATTTTGCTTGCACTGACTGGAATATAAGAAATGCC
 TTCTATTTTTGCTATTAATTCCCTCCTTTTTTGTGTTTTGTGTAACGAAGTTGTTTAA
 CTTGAAGGTGAATGAAGAATAGGTTGGTTGCCCTTAGTTCCCTGAGGAGAAATGTT
 AATACTTGAACAAGTGTGTGTCAGACAAATTGCTGTTATGTTTATTTAATTAAGTTTG
 ATTTCTAAGAAAATCTCAAATGGTCTGCACTGATGGAAGAACAGTTTCTGTAACAAA
 AAAGCTTGAAATTTTTATATGACTTATAATACTGCTGTGAGTTTTAAAAGTAAAGCA
 AAAGTAACTGAGTTGCTTGCTCCAGTGGGATGGACAGGAAAGATGTGAAATAAAAA
 CCAATGAAAAATGAA (SEQ ID NO:2).

[00108] *FMRI*-217 encodes a 31-amino acid protein (SEQ ID NO:3).

[00109] MEELVVEVRGSNGAFYKHWDFGELHCSGRGL (SEQ ID NO:3).

[00110] Additional information on *FMRI*-217 and its protein product can be found at the web address below, the contents of which are incorporated herein by reference in their entirety:
useast.ensembl.org/Homo_sapiens/Transcript/Summary?db=core;g=ENSG00000102081;r=X:147911951-147951125;t=ENST00000621447.

[00111] In some embodiments, a subject (*e.g.*, a human) does not express and/or does not have an elevated expression of an aberrant *FMRI* gene product, for example, compared to typically developing subjects. In some embodiments, a subject does not express an aberrant *FMRI* gene product. In some embodiments, a subject does not have an elevated expression of an aberrant *FMRI* gene product.

Normal *FMRI* Gene Products

[00112] In some embodiments, a subject (*e.g.*, a human) expresses a normal *FMRI* gene product (*i.e.*, a *FMRI* gene product expressed in typically developing subjects). Non-limiting examples of “normal” human *FMRI* gene products include:

a transcript corresponding to Q06787 (*FMRI*-205, ENST00000370475.9, 4,441 nucleotides), and its protein product (a 632-amino acid protein (NP_002015.1)),

a transcript corresponding to NM_001185075.2 (4,170 nucleotides), and its protein product (a 537-amino acid protein (NP_001172004.1)),

a transcript corresponding to NM_001185076.2 (4,378 nucleotides), and its protein product (a 611-amino acid protein (NP_001172005.1)),

a transcript corresponding to NM_001185082.2 (4,303 nucleotides), and its protein product (a 586-amino acid protein (NP_001172011.1)),

a transcript corresponding to NM_001185081.2 (4,107 nucleotides), and its protein product (a 516-amino acid protein (NP_001172010.1)),

a transcript corresponding to Q06787-9 (*FMRI-201*, ENST00000218200.12, 4,333 nucleotides), and its protein product (a 611-amino acid protein),

a transcript corresponding to Q06787-8 (*FMRI-208*, ENST00000440235.6, 4,271 nucleotides), and its protein product (a 586-amino acid protein),

a transcript corresponding to X5D907 (*FMRI-223*, ENST00000687593.1, 4,159 nucleotides), and its protein product (a 594-amino acid protein),

a transcript corresponding to Q06787-10 (*FMRI-204*, ENST00000370471.7, 4,125 nucleotides), and its protein product (a 537-amino acid protein),

a transcript corresponding to G3V0J0 (*FMRI-207*, ENST00000439526.6, 3,699 nucleotides), and its protein product (a 592-amino acid protein),

a transcript corresponding to A8MQB8 (*FMRI-206*, ENST00000370477.5, 3,437 nucleotides), and its protein product (a 582-amino acid protein),

a transcript corresponding to A0A087WY29 (*FMRI-212*, ENST00000495717.6, 2,874 nucleotides), and its protein product (a 561-amino acid protein),

a transcript corresponding to A0A087WXI3 (*FMRI-214*, ENST00000616382.5, 2,799 nucleotides), and its protein product (a 536-amino acid protein), and

a transcript corresponding to R9WNI0 ("*FMRI-218*", ENST00000621453.5, 1,827 nucleotides), and its protein product (a 548-amino acid protein).

[00113] In some embodiments, a normal *FMRI* transcript is Q06787 (*FMRI-205*, ENST00000370475.9, 4,441 nucleotides, produces a 632-amino acid protein (NP_002015.1)). *FMRI-205*, also referred to as "isoform 1" or "iso1," is produced in typical developing subjects (subjects, e.g., humans who do not have FXS) and a subpopulation of FXS subjects. *FMRI-205*

has 17 exons, and the splicing between Exon 1 of *FMRI*-205 (between base pairs 147,911,919 and 147,912,230, SEQ ID NO:4) and Exon 2 of *FMRI*-205 (between base pairs 147,921,933 and 147,921,985, SEQ ID NO:5) is considered normal *FMRI* RNA splicing. Additional information on *FMRI*-205 and its protein product can be found at the web address below, the contents of which are incorporated herein by reference in their entirety:

useast.ensembl.org/Homo_sapiens/Transcript/Summary?db=core;g=ENSG00000102081;r=X:147911951-147951125;t=ENST00000370475.

[00114] CTCAGTCAGGCGCTCAGCTCCGTTTCGGTTTCACTTCCGGTGGAGGGCCGC
CTCTGAGCGGGCGGCGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCG
CCGCTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG
GAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCTGGGCCTCGAGCGCCCGCAGCCC
ACCTCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGAGCT
GGTGGTGGAAAGTGCGGGGCTCCAATGGCGCTTTCTACAAG (SEQ ID NO:4).

[00115] GCATTTGTAAAGGATGTTTCATGAAGATTCAATAACAGTTGCATTTGAAAA
CAA (SEQ ID NO:5).

[00116] In some embodiments, a subject has a decreased level of isoform 1 of *FMRI*. In some embodiments, a subject has a decreased level of isoform 1 of *FMRI* and an increased level of isoform 12 of *FMRI*.

Age

[00117] In some embodiments, a subject (*e.g.*, a human) is a child (*e.g.*, birth to 17 years of age). In some embodiments, a subject (*e.g.*, a human) is an adult (18-64 years of age). In some embodiments, a subject (*e.g.*, a human) is an older adult (65 years of age or older).

[00118] In some embodiments, a subject (*e.g.*, a human) is at least about 1 month of age, for example, at least about: 2, 5, 10, 12, 18, 24, 30, 36, 42, or 48 months of age. In some embodiments, a subject is at least about 18 months of age. In some embodiments, a subject is at least about 24 months of age. In some embodiments, a subject is at least about 42 months of age.

[00119] In some embodiments, a subject (*e.g.*, a human) is at least about 1 year of age, for example, at least about: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 years of age. In some embodiments, a subject is at least about: 2, 3, 5, 6, 7, 8, 10, 12, 13,

14, 15, 18, 30, or 50 years of age. In some embodiments, a subject is at least 14 years of age. In some embodiments, a subject is at least 18 years of age.

[00120] In some embodiments, a subject (*e.g.*, a human) is about 90 years of age or younger, for example, about: 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 29, 25, 24, 23, 22, 20, 18, 17, 16, 15, 13, 11, 10, 7, 6 years of age or younger. In some embodiments, a subject is about 68 months of age or younger.

[00121] In some embodiments, a subject (*e.g.*, a human) is about 0-100 years of age, for example, about: 1-100, 1-85, 1-80, 1-65, 1-60, 1-55, 1-50, 1-45, 1-40, 1-35, 1-30, 1-29, 1-24, 1-23, 1-22, 1-20, 1-18, 1-17, 1-16, 1-13, 1-12, 1-11, 1-10, 1-8, 1-7, 1-6, 2-100, 2-85, 2-80, 2-65, 2-60, 2-55, 2-50, 2-45, 2-40, 2-35, 2-30, 2-29, 2-24, 2-23, 2-22, 2-20, 2-18, 2-17, 2-16, 2-13, 2-12, 2-11, 2-10, 2-8, 2-7, 2-6, 3-100, 3-85, 3-80, 3-65, 3-60, 3-55, 3-50, 3-45, 3-40, 3-35, 3-30, 3-29, 3-24, 3-23, 3-22, 3-20, 3-18, 3-17, 3-16, 3-13, 3-12, 3-11, 3-10, 3-8, 3-7, 3-6, 4-100, 4-85, 4-80, 4-65, 4-60, 4-55, 4-50, 4-45, 4-40, 4-35, 4-30, 4-29, 4-24, 4-23, 4-22, 4-20, 4-18, 4-17, 4-16, 4-13, 4-12, 4-11, 4-10, 4-8, 4-7, 4-6, 5-100, 5-85, 5-80, 5-65, 5-60, 5-55, 5-50, 5-45, 5-40, 5-35, 5-30, 5-29, 5-24, 5-23, 5-22, 5-20, 5-18, 5-17, 5-16, 5-13, 5-12, 5-11, 5-10, 5-8, 5-7, 5-6, 6-100, 6-85, 6-80, 6-65, 6-60, 6-55, 6-50, 6-45, 6-40, 6-35, 6-30, 6-29, 6-24, 6-23, 6-22, 6-20, 6-18, 6-17, 6-16, 6-13, 6-12, 6-11, 6-10, 6-8, 6-7, 7-100, 7-85, 7-80, 7-65, 7-60, 7-55, 7-50, 7-45, 7-40, 7-35, 7-30, 7-29, 7-24, 7-23, 7-22, 7-20, 7-18, 7-17, 7-16, 7-13, 7-12, 7-11, 7-10, 7-8, 8-100, 8-85, 8-80, 8-65, 8-60, 8-55, 8-50, 8-45, 8-40, 8-35, 8-30, 8-29, 8-24, 8-23, 8-22, 8-20, 8-18, 8-17, 8-16, 8-13, 8-12, 8-11, 8-10, 10-100, 10-85, 10-80, 10-65, 10-60, 10-55, 10-50, 10-45, 10-40, 10-35, 10-30, 10-29, 10-24, 10-23, 10-22, 10-20, 10-18, 10-17, 10-16, 10-13, 10-12, 10-11, 12-100, 12-85, 12-80, 12-65, 12-60, 12-55, 12-50, 12-45, 12-40, 12-35, 12-30, 12-29, 12-24, 12-23, 12-22, 12-20, 12-18, 12-17, 12-16, 12-13, 13-100, 13-85, 13-80, 13-65, 13-60, 13-55, 13-50, 13-45, 13-40, 13-35, 13-30, 13-29, 13-24, 13-23, 13-22, 13-20, 13-18, 13-17, 13-16, 14-100, 14-85, 14-80, 14-65, 14-60, 14-55, 14-50, 14-45, 14-40, 14-35, 14-30, 14-29, 14-24, 14-23, 14-22, 14-20, 14-18, 14-17, 14-16, 15-100, 15-85, 15-80, 15-65, 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, 15-29, 15-24, 15-23, 15-22, 15-20, 15-18, 15-17, 15-16, 18-100, 18-85, 18-80, 18-65, 18-60, 18-55, 18-50, 18-45, 18-40, 18-35, 18-30, 18-29, 18-24, 18-23, 18-22, 18-20, 20-100, 20-85, 20-80, 20-65, 20-60, 20-55, 20-50, 20-45, 20-40, 20-35, 20-30, 20-29, 20-24, 20-23, 20-22, 30-100, 30-85, 30-80, 30-65, 30-60, 30-55, 30-50, 30-45, 30-40, 30-35, 40-100, 40-85, 40-80, 40-65, 40-60,

40-55, 40-50, 40-45, 50-100, 50-85, 50-80, 50-65, 50-60, or 50-55 years of age.

[00122] In some embodiments, a subject is about: 1-7, 2-16, 3-10, 3-11, 3-17, 3-18, 3-23, 5-11, 5-13, 5-23, 5-35, 5-50, 6-18, 6-40, 7-16, 8-18, 8-45, 10-17, 10-23, 10-45, 12-18, 12-29, 12-40, 12-45, 12-50, 13-22, 13-29, 13-35, 13-40, 14-50, 14-100, 15-55, 18-24, 18-45, 18-50, 18-60, 18-65, 18-100, 30-80, or 50-85 years of age. In some embodiments, a subject is about 24-68 months of age. In some embodiments, a subject is about 42 months to 16 years of age. In some embodiments, a subject is 12 years or older, and less than 18 years of age.

Samples

[00123] In some embodiments, a sample is a sample from or derived from a human subject. In some embodiments, a sample comprises a single cell or cell lysate, a population of cells, a cell culture, a tissue, or a bodily fluid.

[00124] In some embodiments, a sample comprises a bodily fluid sample, a hair sample (*e.g.*, from hair follicles), a nasal sample (*e.g.*, nasal swab), a buccal sample (*e.g.*, buccal swab), or a skin sample.

[00125] In some embodiments, a sample comprises a bodily fluid sample. Non-limiting examples of bodily fluids include cerebrospinal fluid (CSF), blood (*e.g.*, whole blood and derivatives and fractions of blood, such as plasma or serum), bone marrow aspirates, extracted galls, gingival crevicular fluid (GCF), milk, prostate fluid, pus, saliva (including whole saliva, individual gland secretions, oral rinse), skin scrapes, sputum, surface washings, tears (liquid secreted by lacrimal glands), and urine. In some embodiments, a bodily fluid comprises CSF, blood, saliva, sputum, tears, urine or semen, or a combination thereof. In some embodiments, a bodily fluid comprises CSF, blood, serum, plasma, or urine, or a combination thereof. In some embodiments, a bodily fluid comprises CSF.

[00126] In some embodiments, a sample comprises a bodily fluid selected from cerebrospinal fluid (CSF), blood, serum, plasma, or urine. In some embodiments, a sample comprises peripheral blood mononuclear cells (PBMCs).

[00127] In some embodiments, a sample comprises a tissue sample. In some embodiments, a sample comprises a brain sample. In some embodiments, a sample comprises a non-brain sample. In some embodiments, a sample comprises a non-neural sample.

[00128] In some embodiments, a sample comprises a cell sample. In some embodiments, a

sample comprises a blood cell (*e.g.*, peripheral blood mononuclear cell (PBMC) or white blood cell), a fetal cell (*e.g.*, circulating fetal cell), a blastomere, a trophectoderm cell, a stem cell (*e.g.*, induced pluripotent stem cell (iPSC) or derived stem cell), a fibroblast (*e.g.*, a dermal derived fibroblast cell or lung-derived fibroblast cell), a modified fibroblast, a leukocyte, a pluripotent cell, or a cultured cell, or a combination thereof. In some embodiments, a sample comprises a PBMC. In some embodiments, a sample comprises a white blood cell. In some embodiments, a sample comprises transformed lymphoblastoid B-cells (*e.g.*, human lymphoblastoid cell line).

[00129] In some embodiments, a sample comprises a brain sample (*e.g.*, a human brain sample), a CSF sample (*e.g.*, a human CSF sample), a PBMC sample (*e.g.*, a human PBMC sample), or transformed lymphoblastoid B-cells (*e.g.*, transformed human lymphoblastoid B-cells), or a combination thereof. In some embodiments, a sample comprises a brain sample, a PBMC sample, or a peripheral blood lymphocyte sample, or a combination thereof.

[00130] In some embodiments, a method further comprises enriching (*e.g.*, isolating) a cellular compartment or organelle from a sample. In some embodiments, a method further comprises enriching a cellular compartment (*e.g.*, cytoplasm) from a sample. In some embodiments, a method further comprises enriching an organelle from a sample. Non-limiting examples of organelles include mitochondria, nuclei, Golgi apparatus, endoplasmic reticulum (ER), and ribosomes. In some embodiments, a method further comprises enriching mitochondria from a sample.

[00131] In some embodiments, a sample is substantially free of macromolecules, *e.g.*, molecules having molecular weights of greater than about 1,500 Daltons, for example, greater than about: 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, or 10,000 Daltons.

FXS-Associated Metabolites

[00132] In some embodiments, a method comprises determining, in a sample from a subject, a presence, an absence, or a level of an FXS-associated metabolite.

[00133] In some embodiments, a method comprises determining, in a sample from a subject, an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite.

[00134] In some embodiments, a method comprises determining, in a sample from a subject, a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites.

[00135] In some embodiments, a method comprises determining a presence, absence, amount, and/or alteration of at least one FXS-associated metabolite (*e.g.*, an FXS-associated metabolite).

[00136] In some embodiments, an FXS-associated metabolite is present in aberrant amount in a FXS subject, is potentially involved in FXS initiation, progression, and/or prediction, or both. In some embodiments, an FXS-associated metabolite is present in aberrant amount in a FXS subject. In some embodiments, an FXS-associated metabolite is potentially involved in FXS initiation, progression, and/or prediction.

[00137] In some embodiments, a method comprises determining a presence of at least one FXS-associated metabolite. In some embodiments, a method comprises determining an absence of at least one FXS-associated metabolite. In some embodiments, a method comprises determining an amount of at least one FXS-associated metabolite. In some embodiments, a method comprises determining an alteration of at least one FXS-associated metabolite.

[00138] In other embodiments, an FXS-associated metabolite is absent in a sample (*e.g.*, a CSF sample) from a subject (*e.g.*, a human), but present in a control sample and/or a reference.

[00139] In other embodiments, an FXS-associated metabolite is present in a sample (*e.g.*, a CSF sample) from a subject (*e.g.*, a human), but absent in a control sample and/or a reference.

[00140] In some embodiments, at least one metabolite has an altered level in a sample (*e.g.*, a CSF sample) from a subject (*e.g.*, a human), relative to a control sample and/or a reference (*e.g.*, reference level). In some embodiments, at least two (*e.g.*, at least three, four, five, six, seven, eight, nine, or ten) metabolites have altered levels in a sample (*e.g.*, a CSF sample) from a subject (*e.g.*, a human), relative to a control sample and/or a reference.

[00141] In some embodiments, a control sample comprises a sample from one or more age-matched typically-developing individuals (*e.g.*, two or more age-matched typically-developing individuals), a baseline sample from a subject (*e.g.*, prior to the onset of the disease state), or a sample derived from healthy cells (*e.g.*, not affected by the disease state and from a similar origin), or any combination thereof. In some embodiments, a control sample is a sample from one or more age-matched typically-developing individuals. In some embodiments, a control

sample is a baseline sample from a subject. In some embodiments, a control sample is a sample derived from healthy cells.

[00142] In some embodiments, the reference level is based on:

a sample from an age-matched typically-developing individual, or
a baseline sample from the subject.

[00143] In some embodiments, a level of at least one metabolite is compared to a level of a reference (*e.g.*, standard). In some embodiments, a reference (*e.g.*, reference level) is a theoretical value obtained from a population, *e.g.*, a general population (*e.g.*, from the same tissue, organ, bodily fluid, cell, organelle, and/or cellular compartment as a FXS sample). In some embodiments, a reference is a theoretical value obtained from one or more FXS subjects who were treated successfully.

[00144] In some embodiments, the term “metabolite” (*e.g.*, an FXS-associated metabolite) excludes molecules having molecular weights over 2,000 Daltons. In some embodiments, the term “metabolite” excludes molecules having molecular weights over 1,500 Daltons. In some embodiments, the term “metabolite” excludes molecules having molecular weights over 1,000 Daltons.

[00145] In some embodiments, an FXS-associated metabolite comprises a signaling molecule. In some embodiments, an FXS-associated metabolite comprises an amino acid, fatty acid, nucleotide, sugar, or an intermediate of a cellular process, or a combination thereof.

[00146] In some embodiments, at least one metabolite (*e.g.*, an FXS-associated metabolite) having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises n-acetylneuraminate, D-alanine, L-alanine arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, L-carnitine, carnosine, citrulline, creatine, cystine, cytosine, fumarate, alpha-D-glucose, glucuronate, oxidized glutathione (GSSG), reduced glutathione (GSH), glycerol, glycine, guanine, 3-methyl-L-histidine, homoserine, hypoxanthine, indoleacetaldehyde, isoleucine, kynurenate, leucine, lysine, malate, methionine, methyl acetoacetate, methylmalonate, mevalonate, n-formyl-L-methionine, nicotinamide, phenylalanine, proline, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, D-sedoheptulose, serine, succinate, threonine, allothreonine, tryptophan, tyramine, tyrosine, uracil, urate, valine, norvaline, xanthine, phosphoenolpyruvate, orotate, guanosine, trehalose, or a combination thereof.

[00147] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises n-acetylneuraminate, D-alanine, L-alanine arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, L-carnitine, carnosine, citrulline, creatine, cystine, cytosine, fumarate, alpha-D-glucose, glucuronate, oxidized glutathione (GSSG), reduced glutathione (GSH), glycerol, glycine, guanine, 3-methyl-L-histidine, homoserine, hypoxanthine, indoleacetaldehyde, isoleucine, kynurenate, leucine, lysine, malate, methionine, methyl acetoacetate, methylmalonate, mevalonate, n-formyl-L-methionine, nicotinamide, phenylalanine, proline, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, D-sedoheptulose, serine, succinate, threonine, allothreonine, tryptophan, tyramine, tyrosine, uracil, urate, valine, norvaline, or xanthine, or a combination thereof.

[00148] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises:

- a) lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, GSH, phosphoenolpyruvate, GSSG, orotate, or guanosine, or a combination thereof,
- b) argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, azelate, trehalose, or D-sedoheptulose, or a combination thereof, or
- c) valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof,

or a combination thereof.

[00149] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises lysine, proline, glycine,

leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, GSH, phosphoenolpyruvate, GSSG, orotate, or guanosine, or a combination thereof.

[00150] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, azelate, trehalose, or D-sedoheptulose, or a combination thereof.

[00151] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminic acid, asparagine, or L-alanine, or a combination thereof.

[00152] In some embodiments, at least one metabolite having an altered level in a sample from a subject, relative to a control sample and/or a reference, comprises arginine, leucine, lysine, or D-sedoheptulose, or a combination thereof.

[00153] In some embodiments, at least one metabolite has a reduced level in a sample from a subject, relative to a control sample and/or a reference. In some embodiments, the FXS-associated metabolite has a reduced level in the sample from the subject, relative to a reference level of the same metabolite.

[00154] In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises:

- a) lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or GSH, or a combination thereof,
- b) argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine,

glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof, or

- c) valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof,

or a combination thereof.

[00155] In some embodiments, a FXS-associated metabolite comprises n-acetylneuraminate, D-alanine, L-alanine arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, L-carnitine, carnosine, citrulline, creatine, cystine, cytosine, fumarate, alpha-D-glucose, glucuronate, oxidized glutathione (GSSG), reduced glutathione (GSH), glycerol, glycine, guanine, 3-methyl-L-histidine, homoserine, hypoxanthine, indoleacetaldehyde, isoleucine, kynurenate, leucine, lysine, malate, methionine, methyl acetoacetate, methylmalonate, mevalonate, n-formyl-L-methionine, nicotinamide, phenylalanine, proline, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, D-sedoheptulose, serine, succinate, threonine, allothreonine, tryptophan, tyramine, tyrosine, uracil, urate, valine, norvaline, xanthine, phosphoenolpyruvate, orotate, guanosine, trehalose, or a combination thereof.

[00156] In some embodiments, a FXS-associated metabolite comprises:

- a) lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or reduced glutathione (GSH), or a combination thereof,
- b) argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof, or
- c) valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-

D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof,

or a combination thereof.

[00157] In some embodiments, a FXS-associated metabolite comprises arginine, leucine, or lysine, or a combination thereof.

[00158] In some embodiments, a FXS-associated metabolite comprises:

- a) phosphoenolpyruvate, oxidized glutathione disulfide (GSSG), orotate, or guanosine, or a combination thereof, or
- b) trehalose, or D-sedoheptulose, or both,

or a combination thereof.

[00159] In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or GSH, or a combination thereof.

[00160] In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof.

[00161] In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof.

[00162] In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises arginine, leucine,

lysine, or D-sedoheptulose, or a combination thereof. In some embodiments, at least one metabolite having a reduced level in a sample from a subject, relative to a control sample and/or a reference, comprises arginine, leucine, or lysine, or a combination thereof.

[00163] In some embodiments, the alteration of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite, is a $\log_2$ fold reduction of from about -30 to about 0 (*e.g.*, from about -20 to about 0, from about -20 to about -0.90, from about -10 to about 0, from about -10 to about -0.90, from about -9 to about 0, from about -8 to about 0, from about -7 to about 0, from about -6 to about 0, from about -5 to about 0, from about -4 to about 0, from about -3 to about 0, from about -2 to about 0, from about -1 to about 0, from about -0.5 to about 0, etc.) or a $\log_2$ fold increase of from about 0 to about 30 (*e.g.*, from about 0 to about 20, from about 0.90 to about 20, from about 0 to about 10, from about 0.90 to about 10, from about 0 to about 9, from about 0 to about 8, from about 0 to about 7, from about 0 to about 6, from about 0 to about 5, from about 0 to about 4, from about 0 to about 3, from about 0 to about 2, from about 0 to about 1, from about 0 to about 0.5, etc.). In some embodiments, the alteration of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite, is a $\log_2$ fold reduction of from about -10 to about 0 or a $\log_2$ fold increase of from about 0 to about 10.

[00164] In some embodiments, the alteration of a ratio between levels of two FXS-associated metabolites relative to a reference ratio of the two FXS-associated metabolites is a decrease of at least about 10% (*e.g.*, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500% etc.). For example, the ratio between levels of two FXS-associated metabolites is smaller than a reference ratio of the same two metabolites by at least about 10%. In some embodiments, the alteration of a ratio between levels of two FXS-associated metabolites relative to a reference ratio of the two FXS-associated metabolites is a decrease of at least about 20%.

[00165] In some embodiments, the alteration of a ratio between levels of two FXS-associated metabolites relative to a reference ratio of the two FXS-associated metabolites is a decrease of from about 10% to about 500% (*e.g.*, about 10% to about 300%, about 10% to about 200%, about 10% about 100%, about 10% to about 80%, about 20% to about 80%, about 30% to about 70%, about 40% to about 70%, etc.). In some embodiments, the alteration of a ratio between levels of two FXS-associated metabolites relative to a reference ratio of the two FXS-associated metabolites is a decrease of from about 20% to about 80%.

[00166] In some embodiments, the alteration of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite, is a $\log_2$ fold reduction of equal to or less than about -2.0 (*e.g.*, -1.9, -1.8, -1.7, -1.6, -1.5, -1.4, -1.3, -1.2, -1.1, -1.0, -0.95, -0.90, -0.85, -0.80, -0.75, -0.70, -0.65, -0.60, -0.55, -0.50, -0.45, -0.40, -0.35, -0.30, -0.25, -0.20, -0.15, -0.10, etc.) or a $\log_2$ fold increase of equal to or greater than about 0.10 (*e.g.*, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, etc). In some embodiments, the alteration of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite, is a $\log_2$ fold reduction of equal to or less than about -0.50 or a $\log_2$ fold increase of equal to or greater than about 0.50.

[00167] In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq(-0.10)$ in a sample from a subject, relative to a control sample and/or a reference, for example, $\leq(-0.15)$, $\leq(-0.20)$, $\leq(-0.25)$, $\leq(-0.30)$, $\leq(-0.35)$, $\leq(-0.40)$, $\leq(-0.45)$, $\leq(-0.50)$, $\leq(-0.55)$, $\leq(-0.60)$, $\leq(-0.65)$, $\leq(-0.70)$, $\leq(-0.75)$, $\leq(-0.80)$, $\leq(-0.85)$, $\leq(-0.90)$, $\leq(-0.95)$, $\leq(-1.0)$, $\leq(-1.1)$, $\leq(-1.2)$, $\leq(-1.3)$, $\leq(-1.4)$, $\leq(-1.5)$, $\leq(-1.6)$, $\leq(-1.7)$, $\leq(-1.8)$, $\leq(-1.9)$, or $\leq(-2.0)$, relative to a control sample and/or a reference.

[00168] In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq(-0.20)$ in a sample from a subject, relative to a control sample and/or a reference. In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq(-0.50)$ in a sample from a subject, relative to a control sample and/or a reference. In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq(-0.90)$ in a sample from a subject, relative to a control sample and/or a reference.

[00169] In some embodiments, level of at least one FXS-associated metabolite is at least about 5% lower in a sample from a subject, relative to a control sample and/or a reference. For example, level of at least one FXS-associated metabolite is at least about: 10%, 12%, 15%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% lower in a sample from a subject, relative to a control sample and/or a reference.

[00170] In some embodiments, reduction of a level of at least one FXS-associated metabolite in a sample from a subject, relative to a control sample and/or a reference, is statistically significant. In some embodiments, p-value is less than 0.05. In some embodiments, p-value is less than 0.01. In some embodiments, p-value is less than 0.001.

[00171] In some embodiments, determining: an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite; or a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites, comprises determining the statistical significance of the alteration based on a significance threshold.

[00172] In some embodiments, the significance threshold is a p-value equal to or less than about 0.05. In some embodiments, p-value is less than 0.01. In some embodiments, p-value is less than 0.001.

[00173] In some embodiments, at least one metabolite has an increased level in a sample from a subject, relative to a control sample and/or a reference.

[00174] In some embodiments, at least one metabolite having an increased level in a sample from a subject, relative to a control sample and/or a reference, comprises:

- a) phosphoenolpyruvate, GSSG, orotate, or guanosine, or a combination thereof, or
 - b) trehalose, or D-sedoheptulose, or both,
- or a combination thereof.

[00175] In some embodiments, at least one metabolite having an increased level in a sample from a subject, relative to a control sample and/or a reference, comprises phosphoenolpyruvate, GSSG, orotate, or guanosine, or a combination thereof.

[00176] In some embodiments, at least one metabolite having an increased level in a sample from a subject, relative to a control sample and/or a reference, comprises trehalose, or D-sedoheptulose, or both.

[00177] In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.10 in a sample from a subject, relative to a control sample and/or a reference, for example, ≥ 0.15 , ≥ 0.20 , ≥ 0.25 , ≥ 0.30 , ≥ 0.35 , ≥ 0.40 , ≥ 0.45 , ≥ 0.50 , ≥ 0.55 , ≥ 0.60 , ≥ 0.65 , ≥ 0.70 , ≥ 0.75 , ≥ 0.80 , ≥ 0.85 , ≥ 0.90 , ≥ 0.95 , ≥ 1.0 , ≥ 1.1 , ≥ 1.2 , ≥ 1.3 , ≥ 1.4 , ≥ 1.5 , ≥ 1.6 , ≥ 1.7 , ≥ 1.8 , ≥ 1.9 , or ≥ 2.0 , relative to a control sample and/or a reference.

[00178] In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.20 in a sample from a subject, relative to a control sample and/or a reference. In some embodiments, at least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.50 in a sample from a subject, relative to a control sample and/or a reference. In some embodiments, at

least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.90 in a sample from a subject, relative to a control sample and/or a reference.

[00179] In some embodiments, level of at least one FXS-associated metabolite is at least about 5% higher in a sample from a subject, relative to a control sample and/or a reference. For example, level of at least one FXS-associated metabolite is at least about: 10%, 12%, 15%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, or 200%, higher in a sample from a subject, relative to a control sample and/or a reference.

[00180] In some embodiments, increase of a level of at least one FXS-associated metabolite in a sample from a subject, relative to a control sample and/or a reference, is statistically significant. In some embodiments, p-value is less than 0.05. In some embodiments, p-value is less than 0.01. In some embodiments, p-value is less than 0.001.

[00181] In some embodiments, a first metabolite and a second metabolite have an altered ratio in a sample from a subject (*e.g.*, a human), relative to a control sample and/or a reference.

[00182] In some embodiments, an altered ratio is indicative of increased oxidative stress in a sample from a subject. In some embodiments, a first metabolite is reduced glutathione (GSH), and a second metabolite is oxidized glutathione disulfide (GSSG).

[00183] In some embodiments, an altered ratio is indicative of suppressed mitochondrial metabolism in a sample from a subject. In some embodiments, a first metabolite is adenosine triphosphate (ATP), and a second metabolite is adenosine diphosphate (ADP).

[00184] In some embodiments, the ratio between the levels of a pair of FXS-associated metabolites is the ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG) or the ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). In some embodiments, the ratio between the levels of a pair of FXS-associated metabolites is the ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG). In some embodiments, the ratio between the levels of a pair of FXS-associated metabolites is the ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP).

[00185] In some embodiments, a first metabolite and a second metabolite have a reduced ratio in a sample from a subject (*e.g.*, a human), relative to a control sample and/or a reference.

[00186] In some embodiments, a reduced ratio is indicative of increased oxidative stress in a sample from a subject. In some embodiments, a first metabolite is reduced glutathione (GSH), and a second metabolite is oxidized glutathione disulfide (GSSG).

[00187] In some embodiments, a reduced ratio is indicative of suppressed mitochondrial metabolism in a sample from a subject. In some embodiments, a first metabolite is adenosine triphosphate (ATP), and a second metabolite is adenosine diphosphate (ADP).

[00188] In some embodiments, the methods and teachings disclosed herein can be used to assess efficacy of treatment (*e.g.*, therapeutics such as ASO therapeutics), *e.g.*, in the CSF. In some embodiments, the method comprises assessing the metabolome of a subject, *e.g.*, for restoration of biology in the brain, following ASO treatment, *e.g.*, ASO treatment comprising intra-thecal injection. In some embodiments, the method comprises assessing the metabolome in a sample (*e.g.*, CSF) of a subject with FSX, before and after treatment, *e.g.*, ASO treatment.

FXS-Associated Metabolites in the Brain

[00189] In some embodiments, at least one metabolite has an altered level in a brain sample from a subject (*e.g.*, a human), relative to a control brain sample. In some embodiments, at least one metabolite having an altered level in a brain sample from a subject, relative to a control brain sample, comprises arginine, L-carnitine, cystine, GSSG, GSH, glycine, guanine, guanosine, homoserine, isoleucine, leucine, lysine, methionine, orotate, phenylalanine, phosphoenolpyruvate, proline, D-sedoheptulose, serine, threonine, tryptophan, tyrosine, or urate, or a combination thereof.

[00190] In some embodiments, at least one metabolite has a reduced level in a brain sample from a subject (*e.g.*, a human), relative to a control brain sample. In some embodiments, at least one metabolite having a reduced level in a brain sample from a subject, relative to a control brain sample, comprises lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or GSH, or a combination thereof.

[00191] In some embodiments, at least one metabolite has an increased level in a brain sample from a subject, relative to a control brain sample. In some embodiments, at least one metabolite having an increased level in a brain sample from a subject, relative to a control brain sample comprises phosphoenolpyruvate, GSSG, orotate, or guanosine, or a combination thereof.

[00192] In some embodiments, a first metabolite and a second metabolite have an altered ratio in a brain sample from a subject (*e.g.*, a human), relative to a control brain sample. In some embodiments, an altered ratio is indicative of increased oxidative stress in a brain sample from a subject. In some embodiments, a first metabolite is reduced glutathione (GSH), and a second metabolite is oxidized glutathione disulfide (GSSG).

[00193] In some embodiments, a first metabolite and a second metabolite have a reduced ratio in a brain sample from a subject (*e.g.*, a human), relative to a control brain sample. In some embodiments, a reduced ratio is indicative of increased oxidative stress in a brain sample from a subject. In some embodiments, a first metabolite is reduced glutathione (GSH), and a second metabolite is oxidized glutathione disulfide (GSSG).

FXS-Associated Metabolites in Peripheral Blood Mononuclear Cell (PBMC) Samples

[00194] In some embodiments, at least one metabolite has an altered level in a PBMC sample from a subject (*e.g.*, a human), relative to a control PBMC sample. In some embodiments, at least one metabolite having an altered level in a PBMC sample from a subject, relative to a control PBMC sample, comprises methyl acetoacetate, arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, carnosine, cytosine, fumarate, glucuronate, glycerol, indoleacetaldehyde, kynurenate, leucine, lysine, 3-methyl-L-histidine, malate, n-formyl-L-methionine, mevalonate, D-sedoheptulose, trehalose, tyramine, uracil, or xanthine, or a combination thereof.

[00195] In some embodiments, at least one metabolite has a reduced level in a PBMC sample from a subject (*e.g.*, a human), relative to a control PBMC sample. In some embodiments, at least one metabolite having a reduced level in a PBMC sample from a subject, relative to a control PBMC sample, comprises argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof.

[00196] In some embodiments, at least one metabolite has an increased level in a PBMC sample from a subject (*e.g.*, a human), relative to a control PBMC sample. In some embodiments, at least one metabolite having an increased level in a PBMC sample from a subject, relative to a control PBMC sample, comprises trehalose, or D-sedoheptulose, or both.

[00197] In some embodiments, a first metabolite and a second metabolite have an altered ratio in a PBMC sample from a subject (*e.g.*, a human), relative to a control PBMC sample.

[00198] In some embodiments, an altered ratio is indicative of suppressed mitochondrial metabolism in a PBMC sample from a subject. In some embodiments, a first metabolite is adenosine triphosphate (ATP), and a second metabolite is adenosine diphosphate (ADP).

[00199] In some embodiments, a first metabolite and a second metabolite have a reduced ratio in a PBMC sample from a subject (*e.g.*, a human), relative to a control sample and/or a reference. In some embodiments, a reduced ratio is indicative of suppressed mitochondrial metabolism in a PBMC sample from a subject. In some embodiments, a first metabolite is adenosine triphosphate (ATP), and a second metabolite is adenosine diphosphate (ADP).

FXS-Associated Metabolites in Peripheral Blood Lymphocyte Samples

[00200] In some embodiments, at least one metabolite has an altered level in a peripheral blood lymphocyte sample from a subject (*e.g.*, a human), relative to a control peripheral blood lymphocyte sample. In some embodiments, at least one metabolite having an altered level in a peripheral blood lymphocyte sample from a subject, relative to a control peripheral blood lymphocyte sample, comprises valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof.

[00201] In some embodiments, at least one metabolite has a reduced level in a peripheral blood lymphocyte sample from a subject (*e.g.*, a human), relative to a control peripheral blood lymphocyte sample. In some embodiments, at least one metabolite having a reduced level in a peripheral blood lymphocyte sample from a subject, relative to a control peripheral blood lymphocyte sample, comprises valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate,

alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof.

Metabolites Detection & Quantification

[00202] A single technique or a combination of techniques known in the art may be used for separating, identifying, and/or quantifying metabolites. Non-limiting examples of techniques include mass spectroscopy, nuclear magnetic resonance spectroscopy (NMR), refractive index spectroscopy (RI), ultra-violet spectroscopy (UV), near-infrared spectroscopy (Near-IR), electrochemical analysis, fluorescent analysis, radiochemical analysis, and light scattering analysis (LS).

[00203] In some embodiments, determining a presence, absence, amount, or alteration of a FXS-associated metabolite, or an alteration of a FXS-associated ratio comprises performing a spectrophotometric detection of one or more metabolites.

[00204] In some embodiments, methods of the present disclosure employ a chromatography technique.

[00205] In some embodiments, a chromatography technique is employed to determine, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[00206] In some embodiments, the chromatography technique is liquid chromatography-mass spectrometry (LC-MS). In other embodiments, the chromatography technique is gas chromatography-mass spectrometry (GC-MS). In other embodiments, the chromatography technique is high-performance liquid chromatography (HPLC).

[00207] In some embodiments, determining a presence, absence, amount, or alteration of a FXS-associated metabolite, or an alteration of a FXS-associated ratio comprises performing liquid chromatography-mass spectrometry (LC-MS).

[00208] In some embodiments, determining a presence, absence, amount, or alteration of a FXS-associated metabolite, or an alteration of a FXS-associated ratio comprises performing gas chromatography-mass spectrometry (GC-MS).

[00209] In some embodiments, a method comprises determining a level, amount, and/or concentration of a metabolite in a sample (*e.g.*, tissue, bodily fluid, cell, cellular compartment, or organelle).

Treatments

[00210] In some embodiments, a method of treating fragile X syndrome (FXS) in a subject is disclosed herein, the method comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing; and

administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product.

[00211] In some embodiments, a method disclosed herein further comprises treating FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, or the alteration of the FXS-associated ratio, in a sample.

[00212] In some embodiments, treating FXS in a subject in need thereof comprises administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product, restoring metabolite homeostasis, or both.

Agents

[00213] In some embodiments, administering an agent to a subject:

- a) decreases splicing of an aberrant *FMR1* transcript (*e.g.*, between Exons 1 and 2 of *FMR1*-217),

- b) decreases level of an aberrant *FMRI* gene product (*e.g.*, an aberrant *FMRI* transcript such as *FMRI*-217, and/or an aberrant *FMRI*-encoded protein),
 - c) increases splicing of a normal *FMRI* transcript (*e.g.*, between Exons 1 and 2 of *FMRI*-205),
 - d) increases level of a normal *FMRI* gene product (*e.g.*, a normal *FMRI* transcript such as *FMRI*-205 and/or a normal *FMRI*-encoded protein), or
 - e) increases level of FMRP,
- or any combination thereof, in the subject.

[00214] In some embodiments, contacting an agent to a cell:

- a) decreases splicing of an aberrant *FMRI* transcript (*e.g.*, between Exons 1 and 2 of *FMRI*-217),
 - b) decreases level of an aberrant *FMRI* gene product (*e.g.*, an aberrant *FMRI* transcript such as *FMRI*-217, and/or an aberrant *FMRI*-encoded protein),
 - c) increases splicing of a normal *FMRI* transcript (*e.g.*, between Exons 1 and 2 of *FMRI*-205),
 - d) increases level of a normal *FMRI* gene product (*e.g.*, a normal *FMRI* transcript such as *FMRI*-205 and/or a normal *FMRI*-encoded protein), or
 - e) increases level of FMRP,
- or any combination thereof, in the cell.

Polynucleotide Agents

[00215] In some embodiments, an agent comprises a polynucleotide or an analog thereof. In some embodiments, an agent comprises at least two (*e.g.*, three, four, or more) polynucleotides.

[00216] In some embodiments, a polynucleotide is an antisense oligonucleotide (ASO), a small interfering RNA (siRNA), a short hairpin RNA (shRNA), an antisense DNA, an antisense RNA, a microRNA (miRNA), an antagomir, or a guide RNA (gRNA). In some embodiments, a polynucleotide is an ASO.

[00217] In some embodiments, a polynucleotide comprises a sequence that is (fully or partially) complementary to a target sequence (such that a polynucleotide is capable of hybridizing or annealing to target sequence). In some embodiments, a polynucleotide comprises a sequence that is (fully or partially) complementary to a target sequence under physiological

conditions, for example, with a melting temperature (T_m) of at least 45°C, *e.g.*, at least: 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C. T_m is the temperature at which 50% of a target sequence hybridizes to a complementary polynucleotide at a given ionic strength and pH.

[00218] In some embodiments, specific hybridization corresponds to stringent hybridization conditions. In some embodiments, specific hybridization occurs with near complementary of a polynucleotide (*e.g.*, ASO) to a target sequence. In some embodiments, specific hybridization occurs with near or substantial complementary of a polynucleotide to a target sequence. In some embodiments, specific hybridization occurs with exact complementary of a polynucleotide to a target sequence.

[00219] In some embodiments, a polynucleotide is formed of contiguous complementary sequences (to a target sequence). In some embodiments, the polynucleotide sequence is formed of non-contiguous complementary sequences (to a target sequence), for example, when placed together, constitute sequence that spans the target sequence.

[00220] In some embodiments, a polynucleotide has a length of at least about 8 nucleotides, *e.g.*, at least about: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 nucleotides, or about: 10-60, 10-40, 12-80, 12-60, 12-40, 12-38, 12-30, 13-38, 13-36, 14-36, 14-34, 15-80, 15-60, 15-40, 15-34, 15-32, 16-32, 16-30, 17-30, 17-28, 18-28, 18-26, 19-26, 19-24, 20-80, 20-60, 20-40, 20-30, 20-24, or 20-22 nucleotides. In some embodiments, a polynucleotide has a length of about 10-30 or 12-30 nucleotides.

[00221] In some embodiments, a polynucleotide has a length of at least about 12 nucleotides, *e.g.*, at least about: 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides, or about: 12-40, 12-35, 12-30, 12-25, 13-40, 13-35, 13-30, 13-25, 14-40, 14-35, 14-30, 14-25, 15-40, 15-35, 15-30, or 15-25 nucleotides. In some embodiments, a polynucleotide has a length of about 15-25 nucleotides. In some embodiments, a polynucleotide has a length of about 18-22 nucleotides.

[00222] In some embodiments, a polynucleotide is an isolated polynucleotide. An “isolated polynucleotide” refers to a polynucleotide that has been separated from other cellular components normally associated with native nucleotide polymers, including proteins and other

nucleotide sequences. In some embodiments, a polynucleotide is an isolated DNA polynucleotide. In some embodiments, a polynucleotide is an isolated RNA polynucleotide.

[00223] Polynucleotides described herein can be produced recombinantly or synthetically, using methods, techniques, and reagents that are well known in the art, such as routine and well-known molecular cloning techniques and solid-phase synthesis techniques. In some embodiments, a polynucleotide is a recombinant polynucleotide.

[00224] In some embodiments, a polynucleotide comprises a sequence that is complementary to at least 8 contiguous nucleotides (*e.g.*, about 10-30 nucleotides) of a sequence set forth in any one of SEQ ID NOs:6-9, SEQ ID NOs:10-24, or a combination thereof.

[00225] In some embodiments, a polynucleotide comprises a sequence that specifically hybridizes to (*e.g.*, having near, substantial, or exact reverse complementarity to) a portion of a sense strand on X chromosome between base pairs 147,911,919 and 147,921,985, *e.g.*, between 147,911,919 and 147,921,933, between 147,911,919 and 147,912,230, between 147,911,919 and 147,912,123, between 147,911,919 and 147,914,451, between 147,911,919 and 147,912,728, between 147,912,231 and 147,921,932, between 147,912,231 and 147,914,451, between 147,912,231 and 147,912,727, between 147,912,728 and 147,914,451, between 147,912,694 and 147,912,727, between 147,912,710 and 147,912,745, between 147,912,731 and 147,912,766, or between 147,912,694 and 147,912,766. In some embodiments, a polynucleotide has exact reverse complementarity to a portion of a sense strand on X chromosome between base pairs 147,911,919 and 147,921,985.

[00226] In some embodiments, a polynucleotide has at least about: 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to the reverse and complementary sequence of a portion of a sense strand on X chromosome between base pairs 147,912,694 and 147,912,766 (*e.g.*, between base pairs 147,912,731 and 147,912,766). In some embodiments, a polynucleotide has 100% sequence identity to the reverse and complementary sequence of a portion of a sense strand on X chromosome between base pairs 147,912,694 and 147,912,766 (*e.g.*, between base pairs 147,912,731 and 147,912,766).

[00227] In some embodiments, a polynucleotide (*e.g.*, ASO) has at least about 70% sequence identity to the reverse and complementary sequence of a portion of X chromosome between base pairs 147,912,230 and 147,914,451 (*e.g.*, between 147,912,230 and 147,912,728 or between

147,912,728 and 147,914,451), for example, having at least about: 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, having about: 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, or having about: 75-99%, 75-98%, 80-98%, 80-97%, 85-97%, 85-96%, 90-96%, or 90-95% sequence identity to the reverse and complementary sequence of a portion of X chromosome between base pairs 147,912,230 and 147,912,728. In some embodiments, a polynucleotide comprises a sequence having 100% sequence identity to the reverse and complementary sequence of a portion of X chromosome between base pairs 147,912,230 and 147,914,451.

[00228] In some embodiments, a polynucleotide comprises a sequence that is reverse complementary to a contiguous nucleotide sequence (*e.g.*, at least 8 nucleotides, for example, about 10-30 nucleotides) of pre-mRNA of an aberrant *FMRI* transcript (*e.g.*, pre-mRNA of *FMRI*-217).

[00229] In some embodiments, a polynucleotide comprises a sequence that is reverse complementary to at least 8 contiguous nucleotides of a sequence set forth in SEQ ID NOs:6-24.

[00230] UCAGGUCUCCUUUGGCUUCUCUUUCCGGUCUAGCAUUGGGACUUCGG AGAGCUCCACUGUUCUGGGCGAGGGCUGUGAAGAAAGA (SEQ ID NO:6).

[00231] UCAGGUCUCCUUUGGCUUCUCUUUCCGGUCUAGCAUUGGGACUUCGG AGA (SEQ ID NO:7)

[00232] CAUUGGGACUUCGGAGAGCUCCACUGUUCUGGGCGAGGGCUGUGAAGA AAGA (SEQ ID NO:8)

[00233] UGGGACUUCGGAGAGCUCCACUGUUCUGGGCGAGGGCUGUGAAGAA (SEQ ID NO:9)

[00234] UCAGGUCUCCUUUGGCUUCU (SEQ ID NO:10)

[00235] GUCUCCUUUGGCUUCUCUUU (SEQ ID NO:11)

[00236] UGGCUUCUCUUUCCGGUCUAG (SEQ ID NO:12)

[00237] UUCUCUUUCCGGUCUAGCAU (SEQ ID NO:13)

[00238] UCUUUCCGGUCUAGCAUUG (SEQ ID NO:14)

[00239] UCCGGUCUAGCAUUGGGACUU (SEQ ID NO:15)

[00240] UAGCAUUGGGACUUCGGAGA (SEQ ID NO:16)

[00241] UGGGACUUCGGAGAGCUC (SEQ ID NO:17)

- [00242] UCGGAGAGCUCCACUGUUCU (SEQ ID NO:18)
- [00243] GAGCUCCACUGUUCUGGGCG (SEQ ID NO:19)
- [00244] CUCCACUGUUCUGGGCGAGG (SEQ ID NO:20)
- [00245] GGACUUCGGAGAGCUCCACUG (SEQ ID NO:21)
- [00246] GGAGAGCUCCACUGUUCUGGG (SEQ ID NO:22)
- [00247] UGUUCUGGGCGAGGGCUGUG (SEQ ID NO:23)
- [00248] UGGGCGAGGGCUGUGAAGAA (SEQ ID NO:24)
- [00249] In some embodiments, a polynucleotide (*e.g.*, ASO) comprises a sequence having at least about 70% sequence identity to a sequence set forth in any one of SEQ ID NOs:25-43, for example, having at least about: 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, having about: 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, or having about: 75-99%, 75-98%, 80-98%, 80-97%, 85-97%, 85-96%, 90-96%, or 90-95% sequence identity to a sequence set forth in any one of SEQ ID NOs:25-43. In some embodiments, a polynucleotide comprises a sequence having at least about 85% sequence identity to a sequence set forth in any one of SEQ ID NOs:25-43. In some embodiments, a polynucleotide comprises a sequence having 100% sequence identity to a sequence set forth in any one of SEQ ID NOs:25-43.
- [00250] AGAAGCCAAAGGAGACCTGA (SEQ ID NO:25) (W-704).
- [00251] AAAGAGAAGCCAAAGGAGAC (SEQ ID NO:26) (W-705).
- [00252] CTAGACCGGAAAAGAGAAGCCA (SEQ ID NO:27) (W-706).
- [00253] ATGCTAGACCGGAAAAGAGAA (SEQ ID NO:28) (W-707).
- [00254] CAATGCTAGACCGGAAAAGA (SEQ ID NO:29) (W-708).
- [00255] AAGTCCCAATGCTAGACCGGA (SEQ ID NO:30) (W-709).
- [00256] TCTCCGAAGTCCCAATGCTA (SEQ ID NO:31) (W-710).
- [00257] GAGCTCTCCGAAGTCCCA (SEQ ID NO:32) (W-711).
- [00258] AGAACAGTGGAGCTCTCCGA (SEQ ID NO:33) (W-712).
- [00259] CGCCCAGAACAGTGGAGCTC (SEQ ID NO:34) (W-713).
- [00260] CCTCGCCCAGAACAGTGGAG (SEQ ID NO:35) (W-714).
- [00261] CAGTGGAGCTCTCCGAAGTCC (SEQ ID NO:36) (2831).
- [00262] CCCAGAACAGTGGAGCTCTCC (SEQ ID NO:37) (2832).

[00263] CACAGCCCTCGCCCAGAAC (SEQ ID NO:38) (2833).

[00264] TTCTTCACAGCCCTCGCCCA (SEQ ID NO:39) (2834).

[00265] TCTTTCTTCACAGCCCTCGCCCAGAACAGTGGAGCTCTCCGAAGTCCCAAT
GCTAGACCGGAAAAGAGAAGCCAAAGGAGACCTGA (SEQ ID NO:40).

[00266] TCTCCGAAGTCCCAATGCTAGACCGGAAAAGAGAAGCCAAAGGAGACCT
GA (SEQ ID NO:41).

[00267] TCTTTCTTCACAGCCCTCGCCCAGAACAGTGGAGCTCTCCGAAGTCCCAAT
G (SEQ ID NO:42).

[00268] TTCTTCACAGCCCTCGCCCAGAACAGTGGAGCTCTCCGAAGTCCCA (SEQ
ID NO:43).

[00269] In some embodiments, a polynucleotide (*e.g.*, ASO) comprises a sequence having at least about 70% sequence identity to a sequence set forth in any one of SEQ ID NOs:34-39, for example, having at least about: 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, having about: 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity, or having about: 75-99%, 75-98%, 80-98%, 80-97%, 85-97%, 85-96%, 90-96%, or 90-95% sequence identity to a sequence set forth in any one of SEQ ID NOs:34-39. In some embodiments, a polynucleotide comprises a sequence having at least about 85% sequence identity to a sequence set forth in any one of SEQ ID NOs:34-39. In some embodiments, a polynucleotide comprises a sequence having 100% sequence identity to a sequence set forth in any one of SEQ ID NOs:34-39.

[00270] In the sequences (*e.g.*, any one of SEQ ID NOs:25-43), each nucleobase shown as T may independently be T or U. Similarly, each C nucleotide may independently be C or a C analogue such as 5-methyl C, or other substituted C analogue. Other modified nucleobases with equivalent Watson-Crick base pairing properties will be known to one of skill in the art and would also be appropriate for use in the polynucleotides of the instant invention.

[00271] In some embodiments, an agent comprises a first polynucleotide (*e.g.*, ASO) comprising a sequence having at least 70% sequence identity to SEQ ID NO:34, and a second polynucleotide (*e.g.*, ASO) comprising a sequence having at least 70% sequence identity to SEQ ID NO:35.

Modification of Polynucleotides

[00272] Polynucleotides described herein may be modified, including with one or more locked nucleic acid (LNA) nucleotides, one or more 2'-modified ribonucleotides, one or more morpholino nucleotides, or a combination thereof.

[00273] Chemical modifications can be chosen to, *e.g.*, increase nuclease resistance of a polynucleotide (*e.g.*, ASO), to prevent RNase H cleavage of a polynucleotide (*e.g.*, a complementary RNA strand), or to increase cellular uptake of a polynucleotide. For each of these goals, a variety of compatible chemical modifications are available and will be familiar to those skilled in the art.

[00274] In some embodiments, a polynucleotide comprises one or more modified nucleotides. In some embodiments, one or more modified nucleotides each independently comprises a modification of a ribose group, a phosphate group, a nucleobase, or a combination thereof. In some embodiments, a polynucleotide (*e.g.*, ASO) is chemically modified such that a sugar-phosphate backbone is replaced with a phosphorodiamidate morpholino (PMO), a peptide nucleic acid or another pseudopeptide backbone, or a combination thereof.

[00275] In some embodiments, a polynucleotide (*e.g.*, ASO) is chemically modified to comprise:

- a) a locked nucleic acid (LNA), an ethyl-constrained nucleotide, a 2'-(S)-constrained ethyl (S-cEt) nucleotide, a constrained MOE, a 2'-O,4'-C-aminomethylene bridged nucleic acid (2',4'-BNA(NC)), an alpha-L-locked nucleic acid, a tricyclo-DNA, or a combination thereof,
- b) a ribose group comprising 2'-O-methyl, 2'-fluoro, 2'-deoxy, 2'-O-(2-methoxyethyl) (MOE), 2'-O-alkyl, 2'-O-alkoxy, 2'-O-alkylamino, 2'-NH₂, a constrained nucleotide, a tricyclo-DNA modification, or a combination thereof,
- c) a phosphate group comprising a phosphorothioate, a phosphoramidate, a phosphorodiamidate, a phosphorodithioate, a phosphonoacetate (PACE), a thiophosphonoacetate (thioPACE), an amide, a triazole, a phosphonate, a phosphotriester, or a combination thereof,
- d) a nucleobase comprising 2-thiouridine, 4-thiouridine, N⁶-methyladenosine, pseudouridine, 2,6-diaminopurine, inosine, thymidine, 5-methylcytosine, 5-

substituted pyrimidine, isoguanine, isocytosine, halogenated aromatic groups, or a combination thereof,

- e) a sugar-phosphate backbone is replaced with a phosphorodiamidate mopholino (PMO), a peptide nucleic acid or another pseudopeptide backbone, or a combination thereof.

[00276] In some embodiments, modification (*e.g.*, each modification) of a ribose group comprises 2'-*O*-methyl, 2'-fluoro, 2'-deoxy, 2'-*O*-(2-methoxyethyl) (MOE), 2'-*O*-alkyl, 2'-*O*-alkoxy, 2'-*O*-alkylamino, 2'-NH₂, or a constrained nucleotide, or a combination thereof.

[00277] In some embodiments, a constrained nucleotide comprises a locked nucleic acid (LNA), an ethyl-constrained nucleotide, a 2'-(*S*)-constrained ethyl (S-cEt) nucleotide, a constrained MOE, a 2'-*O*,4'-C-aminomethylene bridged nucleic acid (2',4'-BNANC), an alpha-L-locked nucleic acid, and a tricyclo-DNA, or a combination thereof.

[00278] In some embodiments, modification of a ribose group comprises a 2'-*O*-(2-methoxyethyl) (MOE) modification. In some embodiments, every nucleotide of a polynucleotide (*e.g.*, ASO) comprises a 2'-*O*-(2-methoxyethyl) (MOE) modification.

[00279] In some embodiments, modification of a ribose group comprises a tricyclo-DNA modification. In some embodiments, every nucleotide of a polynucleotide (*e.g.*, ASO) comprises a tricyclo-DNA modification.

[00280] In some embodiments, modification of a ribose group comprises a 2'-deoxy modification. In some embodiments, a polynucleotide comprises four or fewer consecutive 2'-deoxy modified nucleotides.

[00281] In some embodiments, each modification of a phosphate group comprises a phosphorothioate, a phosphoramidate, a phosphorodiamidate, a phosphorodithioate, a phosphonoacetate (PACE), a thiophosphonoacetate (thioPACE), an amide, a triazole, a phosphonate, a phosphotriester, or a combination thereof. In some embodiments, each modification of a phosphate group comprises a phosphoramidate.

[00282] In some embodiments, modification of a phosphate group comprises a phosphorothioate modification. In some embodiments, every nucleotide of a polynucleotide (*e.g.*, oligonucleotide) comprises a phosphorothioate modification. In some embodiments, a polynucleotide (*e.g.*, ASO) is a phosphorothioate-modified polynucleotide.

[00283] In some embodiments, a phosphate group of a nucleotide is modified, *e.g.*, by substituting one or more of the oxygens of the phosphate group with sulfur (*e.g.*, phosphorothioates). In some embodiments, a polynucleotide (*e.g.*, ASO) is a phosphorothioate-modified polynucleotide, such as a polynucleotide where each internucleotide linkage is a phosphorothioate, or where at least half of the internucleotide linkages are phosphorothioate. In some embodiments, at least half of internucleotide linkages of a polynucleotide (*e.g.*, ASO) are phosphorothioate. In some embodiments, each internucleotide linkage of a polynucleotide (*e.g.*, ASO) is a phosphorothioate.

[00284] In some embodiments, each modification of a nucleobase comprises 2-thiouridine, 4-thiouridine, N⁶-methyladenosine, pseudouridine, 2,6-diaminopurine, inosine, thymidine, 5-methylcytosine, 5-substituted pyrimidine, isoguanine, isocytosine, halogenated aromatic groups, or a combination thereof. In some embodiments, modification of a nucleobase group comprises a 5-methylcytosine modification.

[00285] In some embodiments, a sugar-phosphate backbone is replaced with a phosphorodiamidate morpholino (PMO) backbone. In other embodiments, a sugar-phosphate backbone is replaced with a peptide nucleic acid or other pseudopeptide backbone.

[00286] In some embodiments, a polynucleotide comprises a mixture of modified nucleotides.

[00287] In some embodiments, a mixture of modified nucleotides comprise two or more modifications selected from the group consisting of: 2'-*O*-methyl, 2'-deoxy, 2'-*O*-(2-methoxyethyl) (MOE), LNA, and tricyclo-DNA.

[00288] In some embodiments, a mixture of modified nucleotides comprise one or more 2'-*O*-methyl modified nucleotides and one or more LNA modified nucleotides.

[00289] In some embodiments, a mixture of modified nucleotides comprises one or more 2'-*O*-(2-methoxyethyl) (MOE) modified nucleotides and one or more LNA modified nucleotides.

[00290] In some embodiments, each ribose group of a polynucleotide (*e.g.*, ASO) comprises 2'-*O*-(2-methoxyethyl) (MOE) and/or each phosphate group of the polynucleotide comprises a phosphorothioate. In some embodiments, each ribose group of a polynucleotide comprises 2'-*O*-(2-methoxyethyl) (MOE). In some embodiments, each phosphate group of a polynucleotide comprises a phosphorothioate. In some embodiments, each ribose group of a polynucleotide comprises 2'-*O*-(2-methoxyethyl) (MOE), and each phosphate group of the polynucleotide

comprises a phosphorothioate.

[00291] In some embodiments, a nucleotide analog comprises a modification to the sugar portion of the nucleotide. For example, the 2' OH— group may be replaced by a group selected from H, OR, R, F, Cl, Br, I, SH, SR, NH₂, NHR, NR₂, COOR, or OR, wherein R is substituted or unsubstituted C₁-C₆ alkyl, alkenyl, alkynyl or aryl.

[00292] In some embodiments, a substituted RNA analogue disclosed herein comprises a methoxyethyl group on the 2'OH.

Polypeptide Agents

[00293] In some embodiments, an agent comprises a polypeptide. In some embodiments, a polypeptide is an isolated polypeptide. In some embodiments, a polypeptide is a recombinant polypeptide.

[00294] In some embodiments, a polypeptide inhibits (directly or indirectly) expression of an aberrant *FMRI* gene product (*e.g.*, *FMRI*-217, and/or its protein product). In some embodiments, a polypeptide activates (directly or indirectly) expression of a normal *FMRI* gene product (*e.g.*, *FMRI*-205, and/or its protein product). In some embodiments, a polypeptide reduces level of an aberrant *FMRI* gene product and increases level of a normal *FMRI* gene product.

[00295] In some embodiments, a polypeptide is an immunoglobulin molecule. In some embodiments, an immunoglobulin molecule an antibody. In some embodiments, an antibody is an antagonist antibody that binds an *FMRI* transcript or protein product associated with FXS. An antibody can be of any species, such as a rodent (*e.g.*, murine, rat, guinea pig) antibody, a primate (*e.g.*, human) antibody, or a chimeric antibody. In some embodiments, an antibody is primatized (*e.g.*, humanized). In some embodiments, an antibody is a polyclonal antibody. In some embodiments, an antibody is a monoclonal antibody. In some embodiments, an antibody (*e.g.*, monoclonal antibody) is multispecific, *e.g.*, bi-, tri-, or quad-specific.

[00296] In some embodiments, a polypeptide is an antigen-binding fragment of an immunoglobulin molecule (*e.g.*, an antibody), that retains the antigen binding properties of the parental full-length immunoglobulin molecule. In some embodiments, an antigen-binding fragment is a Fab, Fab', F(ab')₂, Fd, Fv, disulfide-linked Fvs (sdFv, *e.g.*, diabody, triabody or tetrabody), scFv, SMIP or rlgG.

[00297] In some embodiments, a polypeptide is an antibody mimetic.

[00298] Techniques, assays, and reagents for making and using therapeutic antibodies, or antigen-binding fragments thereof, against a target antigen (*e.g.*, an *FMRI* transcript or protein product associated with a fragile X-associated disorder, such as FXS) are known in the art. See, *e.g.*, *Therapeutic Monoclonal Antibodies: From Bench to Clinic* (Zhiqiang An eds., 1st ed. 2009); *Antibodies: A Laboratory Manual* (Edward A. Greenfield eds., 2d ed. 2013); Ferrara *et al.*, *Using Phage and Yeast Display to Select Hundreds of Monoclonal Antibodies: Application to Antigen 85, a Tuberculosis Biomarker*, PLoS ONE 7(11): e49535 (2012), for techniques and methods of screening, making, purifying, storing, labeling, and characterizing antibodies.

Small Molecule Agents

[00299] In some embodiments, an agent comprises a small molecule. In some embodiments, a small molecule binds to a protein capable of modulating the splicing and/or expression of *FMRI* or a fragment thereof. In some embodiments, a small molecule is an inhibitor of the target protein (*e.g.*, a direct inhibitor, an indirect inhibitor). In some embodiments, a small molecule is an activator of the target protein (*e.g.*, a direct activator, and indirect activator). Non-limiting examples of small molecules include organic compounds, organometallic compounds, inorganic compounds, and salts of organic, organometallic or inorganic compounds.

[00300] In some embodiments, an agent comprises a phosphodiesterase-4D (PDE4D) allosteric inhibitor. In some embodiments, a PDE4D allosteric inhibitor is BPN14770. For additional information on BPN14770, *see, e.g.*, Berry-Kravis *et al.*, *Inhibition of phosphodiesterase-4D in adults with fragile X syndrome: a randomized, placebo-controlled, phase 2 clinical trial*, Nat Med. 27(5):862-70 (2021), the entire teachings of which are incorporated herein by reference.

Gene Editing Systems

[00301] In some embodiments, an agent comprises a gene editing system. In some embodiments, a gene editing system produces deletion, substitution, and/or addition of one or more nucleotides in the *FMRI* gene. In some embodiments, a gene editing system produces a partial or complete deletion in Exon 2 of *FMRI*-217 (*e.g.*, pseudo-exon between base pairs 147,911,919 and 147,914,451 in the human *FMRI* gene).

[00302] In some embodiments, a gene editing system (*e.g.*, a CRISPR/Cas9 gene editing system) is used to decrease *FMRI* CGG expansion (*e.g.*, shortening CGG repeats) in a subject, prior to, during, or after, administering a polynucleotide (*e.g.*, ASO) described herein. In some embodiments, a gene editing system is used to decrease *FMRI* CGG expansion in a subject prior to administering a polynucleotide.

[00303] In some embodiments, a gene editing system is a CRISPR/Cas system, a transposon-based gene editing system, or a transcription activator-like effector nuclease (TALEN) system. In some embodiments, a gene editing system is a CRISPR/Cas system. In some embodiments, the gene editing system is a class II CRISPR/Cas system.

[00304] In some embodiments, a gene editing system comprises a Cas endonuclease or a polynucleotide encoding a Cas endonuclease. In some embodiments, a Cas endonuclease is Cas9, Cpf1, C2C1, or C2C3. In some embodiments, a Cas endonuclease is Cas9 (*e.g.*, of *Streptococcus Pyogenes*). In some embodiments, a Cas endonuclease is Cpf1. In some embodiments, Cpf1 is AsCpf1 (from *Acidaminococcus sp.*) or LbCpf1 (from *Lachnospiraceae sp.*). The choice of nuclease and gRNA(s) will typically be determined according to whether a deletion, a substitution, or an addition of nucleotide(s) to a targeted sequence is desired.

[00305] In some embodiments, a type II Cas endonuclease is Cas9 (*e.g.*, of *Streptococcus pyogenes*). In some embodiments, a modified Cas 9 is nickase Cas9, dead Cas9 (dCas9) or eSpCas9. In some embodiments, a nickase Cas9 is Cas9 D10A. In some embodiments, a dCas9 is D10A or H840A.

[00306] In some embodiments, a gene editing system comprises a double nickase Cas9 (*e.g.*, to achieve more accurate genome editing; *see, e.g.*, Ran *et al.*, Cell 154: 1380-89 (2013)). Wild-type Cas9 generates double-strand breaks (DSBs) at specific DNA sequences targeted by a gRNA. Nickase Cas9 generates only a single-strand break. dCas9 is catalytically inactive. In some embodiments, dCas9 is fused to a nuclease (*e.g.*, a FokI to generate DSBs at target sequences homologous to two gRNAs). Various CRISPR/Cas9 plasmids are publicly available from the Addgene repository (Addgene, Cambridge, MA: addgene.org/crispr/).

[00307] CRISPR technology for editing the genes of eukaryotes is disclosed in US Patent Application Publications 2016/0138008A1 and US2015/0344912A1, and in US Patents 8,697,359, 8,771,945, 8,945,839, 8,999,641, 8,993,233, 8,895,308, 8,865,406, 8,889,418,

8,871,445, 8,889,356, 8,932,814, 8,795,965, and 8,906,616. Cpf1 endonuclease and corresponding guide RNAs and PAM sites are disclosed in US Patent Application Publication 2016/0208243 A1. CRISPR technology for generating mtDNA dysfunction in the mitochondrial genome is disclosed in Jo *et al.*, BioMed Res. Int. 2015: 305716 (2015). Co-delivery of Cas9 and sgRNA with nanoparticles is disclosed in Mout *et al.*, ACS Nano 11(3): 2452-58 (2017).

Formulation and Administration

[00308] In some embodiments, an agent (*e.g.*, ASO) is in a form of a pharmaceutical composition, or a pharmaceutically acceptable salt thereof.

[00309] In some embodiments disclosed herein are pharmaceutical compositions comprising ASOs of the present disclosure, and a pharmaceutically acceptable carrier. In some embodiments, pharmaceutical compositions of the present disclosure further comprise one or more additional therapeutic agents. Non-limiting examples of additional therapeutic agents include a modulator of DNA methylation (*e.g.*, by inhibiting DNA methylation and/or promoting DNA demethylation), a metabotropic glutamate receptor 5 (mGluR5) modulator (*e.g.*, Basimglurant or Mavoglurant), a GABAB receptor activator (*e.g.*, arbaclofen), a GABAA or GABAB receptor activator (*e.g.*, acamprostate), an AMPA kinase (*e.g.*, AX516), a CB1 inhibitor (*e.g.*, rimonabant), a RAS signaling inhibitor (*e.g.*, lovastatin), a STEP inhibitor, an S6K inhibitor, a PAK inhibitor (*e.g.*, FRAX486), an MMP9 inhibitor (*e.g.*, minocycline), a GSK3 β inhibitor (*e.g.*, lithium), and combinations thereof.

[00310] Pharmaceutical compositions of the present disclosure may be administered (*e.g.*, to a subject in need thereof) in a number of ways (*e.g.*, depending upon whether local or systemic treatment is desired and upon the area to be treated). Administration may be topical (including but not limited to ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary (*e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes but are not limited to intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.*, intrathecal or intraventricular, administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays,

liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be desirable.

[00311] For oral administration, an agent (e.g., a polynucleotide such as ASO) or a composition may be in the form of, for example, a tablet, capsule, suspension, or liquid. A polynucleotide is preferably made in the form of a dosage unit containing a therapeutically effective amount of an active ingredient. Examples of such dosage units are tablets and capsules. For therapeutic purposes, tablets and capsules can contain, in addition to an active ingredient, conventional carriers such as binding agents, for example, acacia gum, gelatin, polyvinylpyrrolidone, sorbitol, or tragacanth; fillers, for example, calcium phosphate, glycine, lactose, maize-starch, sorbitol, or sucrose; lubricants, for example, magnesium stearate, polyethylene glycol, silica, or talc; disintegrants, for example potato starch; flavoring or coloring agents, or acceptable wetting agents. Oral liquid preparations generally in the form of aqueous or oily solutions, suspensions, emulsions, syrups, or elixirs may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous agents, preservatives, coloring agents, and flavoring agents. Examples of additives for liquid preparations include acacia, almond oil, ethyl alcohol, fractionated coconut oil, gelatin, glucose syrup, glycerin, hydrogenated edible fats, lecithin, methyl cellulose, methyl or propyl para-hydroxybenzoate, propylene glycol, sorbitol, or sorbic acid.

[00312] Pharmaceutical compositions of the present disclosure may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. Compositions of the present disclosure may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for non-limiting example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers. In some embodiments, a pharmaceutical composition described herein is formulated as a solution.

[00313] Pharmaceutical compositions of the present disclosure include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. Pharmaceutical compositions and formulations of the present disclosure may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

[00314] Emulsions include but are not limited to heterogenous systems of one liquid dispersed in another in the form of droplets (*e.g.*, droplets exceeding 0.1 μm in diameter). Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as embodiments of the present disclosure. Emulsions and their uses are well known in the art and are further described in U.S. Pat. No. 6,287,860, which is incorporated herein in its entirety.

[00315] Formulations of the present disclosure include but are not limited to liposomal formulations. As used in the present disclosure, the term “liposome” means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

[00316] Pharmaceutical formulations and compositions of the present disclosure may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Pat. No. 6,287,860, which is incorporated herein in its entirety.

[00317] In some embodiments, various penetration enhancers are employed to effect the efficient delivery of an agent (*e.g.*, ASO) or a composition of the present disclosure. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Pat. No. 6,287,860, which is incorporated herein in its entirety.

[00318] In some embodiments, an agent (*e.g.*, ASO) is administered at a dosage of about 4-20 mg per administration, for example, about: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 mg per administration. In some embodiments, an agent is administered at a dosage of about

12 mg per administration. In some embodiments, an agent is administered at a dosage of about, *e.g.*, up to 50 or 100 mg per injection.

[00319] Administration of an agent or a composition of the present disclosure can be once in a day or more than once in a day (*e.g.*, twice a day or more). Administration of the agent or a composition can be repeated after one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, two months, three months, four months, five months, six months, or longer. Repeated courses of treatment are also possible, as is chronic administration. The repeated administration may be at the same dose or at a different dose.

[00320] In some embodiments, a method disclosed herein comprises administering to a subject two or more polynucleotides (*e.g.*, ASOs), for example, 2, 3, 4, or 5 polynucleotides. In some embodiments, two or more polynucleotides are administered together. In other embodiments, two or more polynucleotides are administered separately.

[00321] Administration of an agent or a composition of the present disclosure to a subject can be by parenteral or non-parenteral means. In some embodiments, an agent (*e.g.*, ASO) is administered intravenously, intra-arterially, intrathecally, intraventricularly, intramuscularly, intradermally, subcutaneously, intracranially, or spinally.

[00322] In some embodiments, an agent (*e.g.*, ASO) or a composition of the present disclosure is delivered locally to the central nervous system. This can include intrathecal or intraventricular injections, including the use of a catheter or Ommaya reservoir. Other methods of delivering agents (*e.g.*, drugs) directly to the cerebrospinal fluid or central nervous system will be known to one skilled in the art.

[00323] In some embodiments, an agent or a composition of the present disclosure is delivered using an approach that enhances bioavailability in the central nervous system after systemic administration. These approaches can include modification of the sugars or phosphate linkages, delivering as a duplex with a ligand-conjugated RNA molecule, formulation into an artificial exosome, liposome, polymer nanoparticle or lipid nanoparticle, or conjugation to lipids, antibodies, peptides, sugars, neuroactive molecules, or other moieties that enhance delivery to the central nervous system. In some embodiments, the agent is delivered after transiently

disrupting the blood-brain barrier. Other methods of enhancing bioavailability in the central nervous system after systemic administration will be known to one skilled in the art.

[00324] In some embodiments, an agent (*e.g.*, ASO) or a composition of the present disclosure is administered as intrathecal bolus injection. In some embodiments, an agent or a composition is delivered systemically, such as via intravenous or subcutaneous injection.

[00325] In some embodiments, an agent (*e.g.*, ASO) or a composition of the present disclosure is delivered using one or more controlled or sustained-release delivery systems (*e.g.*, capsules, biodegradable matrices). Non-limiting example delayed-release delivery systems for drug delivery that would be suitable for administration of a composition described herein are described in U.S. Patent Nos. US 5,990,092 (issued to Walsh); 5,039,660 (issued to Leonard); 4,452,775 (issued to Kent); and 3,854,480 (issued to Zaffaroni), the entire teachings of which are incorporated herein by reference.

Therapeutically Effective Amounts

[00326] In some embodiments, a therapeutically effective amount of an agent is sufficient to:

- a) decrease splicing of an aberrant *FMRI* transcript (*e.g.*, between Exons 1 and 2 of *FMRI*-217),
 - b) decrease level of an aberrant *FMRI* gene product (*e.g.*, an aberrant *FMRI* transcript such as *FMRI*-217, and/or an aberrant *FMRI*-encoded protein),
 - c) increase splicing of a normal *FMRI* transcript (*e.g.*, between Exons 1 and 2 of *FMRI*-205),
 - d) increase level of a normal *FMRI* gene product (*e.g.*, a normal *FMRI* transcript such as *FMRI*-205 and/or a normal *FMRI*-encoded protein), or
 - e) increase level of FMRP,
- or any combination thereof, relative to a reference.

[00327] In some embodiments, a therapeutically effective amount of an agent is sufficient to decrease splicing of an aberrant *FMRI* transcript, relative to a reference, for example, by at least about 5% (*e.g.*, by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%).

[00328] In some embodiments, a therapeutically effective amount of an agent is sufficient to decrease level of an aberrant *FMRI* gene product, relative to a reference, for example, by at least

about 5% (*e.g.*, by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%). In some embodiments, a therapeutically effective amount of an agent is sufficient to decrease level of an aberrant *FMRI* gene product, relative to a reference, by at least about 25%.

[00329] In some embodiments, a therapeutically effective amount of an agent is sufficient to increase splicing of a normal *FMRI* transcript, relative to a reference, for example, by at least about 5% (*e.g.*, by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 100%, 105%, 110%, 120%, or 125%).

[00330] In some embodiments, a therapeutically effective amount of an agent is sufficient to increase level of a normal *FMRI* gene product, relative to a reference, for example, by at least about 5% (*e.g.*, by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 100%, 105%, 110%, 120%, or 125%).

[00331] In some embodiments, a therapeutically effective amount of an agent is sufficient to increase level of FMRP, relative to a reference, for example, by at least about 5% (*e.g.*, by at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 100%, 105%, 110%, 120%, or 125%). In some embodiments, a therapeutically effective amount of an agent is sufficient to increase level of FMRP, relative to a reference, by at least about 25%.

[00332] In some embodiments, a therapeutically effective amount of an agent is sufficient to increase a level of FMRP in a FXS subject to at least about 5% of a level observed in typically developing individuals, *e.g.*, to at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100%, of a level observed in typically developing individuals. In some embodiments, a therapeutically effective amount of an agent is sufficient to increase level of FMRP in a FXS subject to at least about 30% of a level observed in typically developing individuals.

[00333] In some embodiments, splicing of an aberrant *FMRI* transcript, level of an aberrant *FMRI* gene product, splicing of a normal *FMRI* transcript, level of a normal *FMRI* gene product, level of FMRP, or any combination thereof, is measured at least about 1 day after an agent is administered to a subject, *e.g.*, for at least: about: 2 days, 3 days, 4 days, 5 days, 6 days, 8 days, 9 days, 10 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5

months, or 6 months, after administration.

[00334] In some embodiments, splicing of an aberrant *FMRI* transcript, level of an aberrant *FMRI* gene product, splicing of a normal *FMRI* transcript, level of a normal *FMRI* gene product, level of FMRP, or any combination thereof, is measured a tissue or a cell, for example, a white blood cell, a leukocyte, a fibroblast cell (*e.g.*, a dermal derived fibroblast cell or a lung-derived fibroblast cell), a cortex tissue (*e.g.*, a brain biopsy of superficial cortex), or a combination thereof.

[00335] In some embodiments, a therapeutically effective amount of an agent is sufficient to slow progression of FXS, and/or alleviate (*e.g.*, prevent) one or more signs or symptoms of FXS.

[00336] In some embodiments, a therapeutically effective amount of an agent is sufficient to:

- a) improve speech and motor development,
- b) reduce (*e.g.*, prevent) one or more cognitive disabilities (*e.g.*, learning and/or intellectual disability),
- c) alleviate (*e.g.*, prevent) one or more physical and medical features (*e.g.*, a long face, prominent ears and chin, arched palate, large testicles at puberty, low muscle tone, flat feet, hyperextensible joints, sleep problems, seizures, recurrent ear infections, and/or mitral valve prolapse), or
- d) reduce (*e.g.*, prevent) one or more FXS-associated behaviors (*e.g.*, hyperactivity, short attention span, hand biting, hand flapping, poor eye contact, poor social skills, shyness, anxiety, delayed speech, delayed motor development, repetitive speech, sensitivity to sensory stimulation (*e.g.*, touch)),

or a combination thereof.

[00337] In some embodiments, a therapeutically effective amount of an agent is sufficient to raise an intelligence quotient (IQ) score, for example, to at least about 40, *e.g.*, to at least about: 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, or 130, to between about: 40-110, 40-100, 50-105, 60-80, 65-90, 70-80, 75-95, or 70-100, or to about: 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, or 130.

[00338] In some embodiments, a therapeutically effective amount of an agent is sufficient to raise an IQ score by at least about: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or

20 points, by 1-10, 1-15, 2-20, 2-15, 2-10, 5-15, 5-10, 10-20, or 15-20 points, or by about: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 points.

[00339] In some embodiments, a therapeutically effective amount of an agent is sufficient to reduce (*e.g.*, prevent) absent or irregular menses, fertility problems, elevated FSH (follicle-stimulating hormone) levels, premature ovarian failure, primary ovarian insufficiency, and/or hot flashes. In some embodiments, a therapeutically effective amount of an agent is sufficient to reduce (*e.g.*, prevent) intention tremors, parkinsonism, ataxia, memory loss, white matter lesions involving middle cerebellar peduncles, and/or cognitive decline. In some embodiments, a therapeutically effective amount of an agent is sufficient to reduce (*e.g.*, prevent) neuropathy of extremities, mood instability, irritability, explosive outbursts, personality changes, and/or autonomic function problems such as impotence or loss of bladder or bowel functions. In some embodiments, a therapeutically effective amount of an agent is sufficient to reduce (*e.g.*, prevent) high blood pressure, thyroid disorders, and/or fibromyalgia.

Additional Therapeutic Agents

[00340] In some embodiments, one or more additional therapeutic agents are administered to a subject. Non-limiting examples of additional therapeutic agents include a modulator of DNA methylation (*e.g.*, by inhibiting DNA methylation and/or promoting DNA demethylation), a metabotropic glutamate receptor 5 (mGluR5) modulator (*e.g.*, Basimglurant or Mavoglurant), a GABAB receptor activator (*e.g.*, arbaclofen), a GABAA or GABAB receptor activator (*e.g.*, acamprosate), an AMPA/kine (*e.g.*, AX516), a CB1 inhibitor (*e.g.*, rimonabant), a RAS signaling inhibitor (*e.g.*, lovastatin), a STEP inhibitor, an S6K inhibitor, a PAK inhibitor (*e.g.*, FRAX486), an MMP9 inhibitor (*e.g.*, minocycline), a GSK3 β inhibitor (*e.g.*, lithium), and combinations thereof.

[00341] In some embodiments, treating a subject comprises providing the subject with a ketogenic (“keto”) diet.

[00342] In some embodiments, a therapeutically effective amount of a DNA-demethylating compound or DNA demethylase is administered to a subject, prior to, during, or after, administering an agent (*e.g.*, ASO) or a composition described herein to the subject. In some embodiments, a therapeutically effective amount of a DNA-demethylating compound or DNA

demethylase is administered to a subject after administering an agent or a composition disclosed herein to the subject.

[00343] Non-limiting examples of DNA-demethylating compounds include 5-Azacytidine (5-Aza-CR) and 5-aza-2'-deoxycytidine (5-Aza-CdR), dihydro-5-azacytidine (DHAC), zebularine, 5-fluoro-2'-deoxycytidine, Hydralazine, RG108, procainamide, and SGI-1027. In some embodiments, a DNA-demethylating compound is a nucleoside analogue. In some embodiments, a DNA-demethylating compound is a non-nucleoside analogue.

[00344] In some embodiments, a DNA demethylase (*e.g.*, DNA methylation modification enzymes Dnmt or Tet) is fused to a catalytically inactivate Cas9 (dCas9-Dnmt/Tet). Under the guidance of a single guide RNA (sgRNA), a dCas9-Tet1 demethylates the *FMRI* locus and promoter region when *FMRI* has an expanded CGG repeat of 200 or more.

[00345] In some embodiments, a therapeutically effective amount of a DNA-demethylating compound or DNA demethylase is sufficient to demethylate at least about 5% of an *FMRI* gene, for example, at least about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%, about: 10-100%, 10-90%, 15-90%, 15-80%, 15-75%, 20-75%, 20-70%, 25-60%, 25-55%, 25-50%, 30-40%, or 30-35%, or about: 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% of an *FMRI* gene. In some embodiments, a therapeutically effective amount of a DNA-demethylating compound or DNA demethylase is sufficient to demethylate about 25-50% of an *FMRI* gene.

[00346] The term “combination therapy” refers to the administration of two or more therapeutic agents (*e.g.*, one or more agents and additional therapeutic agents described herein) to treat a disease, disorder, or condition described herein. Such administration encompasses co-administration of two or more therapeutic agents in a substantially simultaneous manner, such as in a single capsule having a fixed ratio of active ingredients. Alternatively, such administration encompasses co-administration in multiple, or in separate containers (*e.g.*, capsules, powders, and liquids) for each active ingredient. Such administration also encompasses use of each type of therapeutic agent in a sequential manner, either at approximately the same time or at different times. Therapeutic agents in a combination therapy can be administered via the same administration route or via different administration routes. Powders and/or liquids may be

reconstituted or diluted to a desired concentration (*e.g.*, appropriate for dosage) prior to administration. Typically, a treatment regimen will provide beneficial effects of a drug combination in treating diseases, conditions, or disorders described herein.

[00347] In some embodiments, a method is used for monitoring (*e.g.*, in a clinical trial and/or in a drug screen) effectiveness of an agent. In some embodiments, a method comprises obtaining level of a metabolite from a subject before administering an agent, and monitoring changes in the level as an indication of the effectiveness of the agent in the subject. In some embodiments, a level of a metabolite of a subject is compared to a predetermined reference (*e.g.*, standard).

[00348] In some embodiments, a method comprises altering (*e.g.*, increasing or decreasing) the administration of an agent to a subject. In some embodiments, increased administration of an agent is desirable to increase effectiveness of an agent. In some embodiments, decreased administration of an agent is desirable to decrease effect(s) of an agent.

Methods of Diagnosing, Subclassifying or Predicting a Treatment Outcome of Fragile X-Associated Disorders

[00349] The disclosure provides, among other things, methods of predicting a treatment outcome of, diagnosing, prognosing, or subclassifying fragile X syndrome (FXS) in a subject, the methods comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[00350] The disclosure also provides, among other things, methods of diagnosing FXS in a subject, the methods comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,

- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[00351] The disclosure also provides, among other things, methods of prognosing FXS in a subject, the methods comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[00352] The disclosure also provides, among other things, methods of subclassifying FXS in a subject, the methods comprising determining, in a sample from the subject:

- a) a presence, an absence, or a level of an FXS-associated metabolite,
- b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
- c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,

or a combination of the foregoing.

[00353] The disclosure also provides, among other things, methods of diagnosing, subclassifying or predicting a treatment outcome of FXS in a subject, the methods comprising:

- a) determining a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
- b) determining an alteration of an FXS-associated ratio of two metabolites,

or both a) and b), in a sample from a subject, wherein the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample is indicative of the presence, risk, progression, severity, or treatment outcome of FXS.

[00354] The disclosure also provides, among other things, methods of predicting a treatment outcome of FXS in a subject, the methods comprising:

- a) determining:
 - i. a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii. an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and
- b) predicting the treatment outcome of FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.

[00355] The disclosure also provides, among other things, methods of diagnosing FXS in a subject, the method comprising:

- a) determining:
 - i. a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii. an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and
- b) diagnosing the subject as having or having a propensity to develop FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.

[00356] The disclosure also provides, among other things, methods of prognosing FXS in a subject, the methods comprising:

- a) determining:
 - i. a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii. an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and
- b) prognosing the subject as having a propensity to have a poorer prognosis of FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, or the alteration of the FXS-associated ratio, in the sample.

[00357] For example, if the ratio between levels of two FXS-associated metabolites in a sample from the subject is determined to be 1:1 and the reference ratio of the two FXS-

associated metabolites is 1:5, the subject may be prognosed as having a propensity to have a poorer prognosis of FXS, compared to another subject with a ratio of 1:4.

[00358] A subject is any one or more subjects described herein.

[00359] At least one FXS-associated metabolite is any one or more FXS-associated metabolites disclosed herein.

[00360] In some embodiments, a sample is any one or more samples described herein.

[00361] In some embodiments, an FXS-associated ratio is any one or more FXS-associated ratios disclosed herein.

[00362] In some embodiments, a treatment outcome is any one or more treatment outcomes described herein.

[00363] In some embodiments, an aberrant metabolite level (*e.g.*, an increased level or a reduced level of a metabolite, a presence of a metabolite not usually present, or an absence of a metabolite usually present) indicates a presence of a disease state.

[00364] In some embodiments, a method disclosed herein is used to identify a subject having or at risk for developing FXS.

[00365] In some embodiments, a method is used to determine whether to administer an agent to a subject.

[00366] In some embodiments, the subject is treated when the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the subject's sample, is determined or detected. In some embodiments, the subject's treatment is altered when the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the subject's sample, is determined or detected.

[00367] In some embodiments, a method is used to determine whether a subject is effectively treated with an agent. In some embodiments, a method provides information useful in prognostication, staging and/or management of FXS. In some embodiments, a method provides information useful for designing a treatment regimen.

[00368] In some embodiments, a method is used for monitoring (*e.g.*, in a clinical trial and/or in a drug screen) effectiveness of an agent. In some embodiments, a method comprises obtaining level of a metabolite from a subject before administering an agent, and monitoring changes in the

level as an indication of the effectiveness of the agent in the subject. In some embodiments, a level of a metabolite of a subject is compared to a predetermined reference (*e.g.*, standard).

[00369] In some embodiments, the subject is treated when the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the subject's sample, is determined or detected, for example, in a method disclosed herein. In some embodiments, the subject's treatment is altered when the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the subject's sample, is determined or detected for example, in a method disclosed herein.

[00370] While having described the invention in general terms, the embodiments of the invention will be further disclosed in the following example that should not be construed as limiting the scope of the claims.

Exemplification

Example 1. Metabolite Levels Associated with Fragile X Syndrome (FXS)

[00371] FXS is the most frequently inherited form of intellectual impairment and most common single-gene cause of autism. There are about 83,000 FXS cases in the United States; 1 in approximately 4,000 boys and 1 in approximately 7,000 girls are afflicted. FXS children present a range of symptoms beyond intellectual impairment, including speech and developmental delays, perseveration, hyperactivity, aggression, and epilepsy, among other maladies. FXS is caused by a CGG triplet repeat expansion in a single gene, *fragile X messenger ribonucleoprotein 1 (FMR1)*, which resides on the X chromosome. When the CGG triplet repeat expands to 200 or more, the *FMR1* gene is methylated and thereby transcriptionally inactivated. The loss of the *FMR1* gene product, the protein fragile X messenger ribonucleoprotein protein (FMRP), is the cause of the disorder.

[00372] Treatments for FXS or indeed most other autism spectrum disorders, which are primarily based on animal models, have met with very limited success in human clinical trials (Hagerman *et al.*, Nature Rev Disease Primers 3:17065 (2017); Berry-Kravis *et al.*, Nature Rev Drug Disc. 17:280-299 (2018)).

[00373] There is no widely available therapy that shows even modest efficacy for FXS. Moreover, there are no established molecular readouts with statistical rigor of any FXS

treatment. Because any FXS treatment would be targeted, directly or indirectly, to the brain, a therapeutic biomarker may entail measurement of brain tissue, or a brain-derived fluid – particularly cerebral spinal fluid (CSF), which is derived from the brain ventricles.

[00374] In all FXS model systems so far examined in the field (*Drosophila*, mouse, iPSC-derived neurons), a common theme is dysregulated mitochondrial function. These observations suggest that mitochondrial or indeed other metabolites, molecules weighing <1000 Daltons, might have altered levels in FXS brain/tissue/CSF/cells compared to control (i.e., non-FXS). Metabolites may be determined with extreme accuracy and with strong statistical rigor using liquid chromatography-mass spectrometry (LC-MS).

[00375] Human post-mortem brain cortex samples (University of California at Davis Brain Repository), six FXS and six age-matched typically-developing (TD) controls, were frozen at -80°C and powdered with a mortar and pestle. The metabolites were extracted from ~10 mg of powder material. The samples were vortexed in 800 µL 60% LC-MS-grade methanol in LC-MS-grade water for 10 minutes at 4°C. Five hundred µL of LC-MS-grade chloroform was added to each sample tube and vortexed for 10 minutes at 4°C. The samples were centrifuged at 22,000 x g for 10 minutes at 4°C, creating three layers. The top layer contained polar metabolites, the middle layer was the protein interface, and the bottom layer contained nonpolar metabolites. The polar metabolites were transferred into a new tube and the fluid evaporated in a refrigerated CENTRIVAP® attached to a -105°C cold trap (Labconco Corporation, Kansas City, MO). The bottom layer was discarded, and the protein interface was used to quantify protein. Briefly, 1 mL of RIPA buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150mM NaCl) was added to the tube containing the protein interface and samples were vortexed 10 minutes at 4°C. The samples were centrifuged at 22,000 x g for 10 minutes at 4°C and protein was quantified with a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). Dried down polar metabolite pellets were resuspended in LC-MS-grade water to a concentration of 0.5 µg/µL and the material was vortexed for 10 minutes at 4°C. The samples were centrifuged at 22,000 x g for 10 minutes at 4°C and 20 µL was moved into LC-MS vials.

[00376] Ten mL fresh blood was collected from participants to isolate peripheral blood mononuclear cells (PBMCs). All participants were Caucasian males with a *FMRI* full mutation (CGG repeats >200) or typically-developing (TD) individuals (CGG repeats < 55) as confirmed by DNA analysis. All participants or their legal guardians, as appropriate, signed informed consent to the study. The project was approved by the Rush University Medical Center Institutional Review Board. FXS patients were aged 16–38 years. Age-matched TD individuals for the study were aged 22-29 years. PBMCs were isolated from whole blood using a Cell Preparation (BD VACUTAINER® CPT™) blood tube (BD Biosciences, Franklin Lakes, NJ). PBMCs were pelleted and washed twice with 1X PBS. The PBMCs were cultured in RPMI-1640 medium (Sigma-Aldrich, Burlington, MA), supplemented with 15% fetal bovine serum (FBS) and 2.5% L-glutamine, at 37°C with 5% CO₂ in T25 flasks for 4 days. PBMCs were collected by centrifugation and pellets were frozen at -80°C. For LC-MS, the cells were pelleted and washed twice with 1X PBS. Samples were resuspended in 800 µL 80% LC-MS-grade methanol in LC-MS-grade water and vortexed for 10 minutes at 4°C. Samples were centrifuged at 22,000 x g for 10 minutes at 4°C and supernatants were dried down in a refrigerated CENTRIVAP® attached to a -105°C cold trap (Labconco Corporation). Dried polar metabolite pellets were resuspended in 100 µL of LC-MS-grade water and vortexed for 10 minutes at 4°C. Samples were centrifuged at 22,000 x g for 10 minutes at 4°C and 20 µL was moved into LC-MS vials.

[00377] Lymphoblast cell lines (LCLs) were obtained from Coriell Institute (Camden, NJ) from two FXS individuals (GM07365 and GM06897) and two typically-developing control males (GM07174 and GM06890). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich), supplemented with 15% fetal bovine serum (FBS) and 2.5% L-glutamine, at 37°C with 5% CO₂ in T25 flasks. LCLs were collected by centrifugation and pellets were frozen at -80°C. Samples were resuspended in 800 µL 80% LC-MS-grade methanol in LC-MS-grade water and vortexed for 10 minutes at 4°C. Samples were centrifuged at 22,000 x g for 10 minutes at 4°C and supernatants were dried down in a refrigerated CENTRIVAP® attached to a -105°C cold trap (Labconco Corporation). Dried polar metabolite pellets were resuspended in 100 µL of LC-MS-grade water and vortexed for 10 minutes at 4°C. Samples were centrifuged at 22,000 x g for 10 minutes at 4°C and 20 µL was moved into LC-MS vials.

[00378] Cerebrospinal fluid was collected from non-Fragile X individuals at Rush University Medical Center (Chicago, IL). It was shipped to UMass Chan Medical School and used for LC-MS analysis.

[00379] A Q EXACTIVE® Plus Hybrid Quadrupole-ORBITRAP® mass spectrometer (Thermo Fisher Scientific) equipped with an Ion Max source and a HESI II probe coupled to a VANQUISH® Horizon UHPLC System (Thermo Fisher Scientific) was used to measure polar metabolites on post-mortem brain samples. Prior to operation, the instrument underwent mass calibration for positive and negative ion mode using Calmix (Thermo Fisher Scientific). Two μL of resuspended polar metabolite samples were injected onto a SeQuant ZIC®-pHILIC $5\mu\text{m}$ 150×2.1 mm analytical column equipped with a 2.1×20 mm guard column (MilliporeSigma, Burlington, MA). The column oven was held at 25°C and the autosampler tray was held at 4°C . Buffer A was comprised of 20 mM ammonium carbonate, 0.1% ammonium hydroxide. Buffer B was comprised of 100% acetonitrile. The chromatographic gradient was run at a flow rate of 0.150 mL/min as follows: 0-20 min: linear gradient from 80-20% buffer B; 20-20.5 min: linear gradient from 20-80% buffer B; 20.5-28 min: hold at 80% buffer B. The mass spectrometer was operated in full-scan, polarity switching mode, with the spray voltage set to 4.0 kV and the heated capillary at 350°C . The sheath gas flow was 10 units, the auxiliary gas flow was 1 unit, and the sweep gas flow was 1 unit. Mass spectrometry data was collected in a range of $m/z = 70$ -1000. The resolution was set at 70,000, the automatic gain control (AGC) target at 1×10^6 , and the maximum injection time at 20 msec.

[00380] Changes in polar metabolite levels between post-mortem cortex tissue samples from six FXS males and six age-matched typically-developing (TD) males were detected by liquid chromatography-mass spectrometry (LC-MS) (see FIGs. 1A-1B and Table 1). A significant increase in levels of 4 metabolites in the FXS samples compared to TD samples was detected. The ratio of glutathione (GSH) to glutathione disulfide (GSSG) levels compared between the FXS and TD samples used in FIG. 1A reflected increased oxidative stress in the FXS samples.

Table 1. Changes in Polar Metabolite Levels in Post-Mortem FXS Cortex Tissue

Polar metabolite	$\log_2(\text{fold change})$	$-\log_{10}(\text{p-value})$
NADP	0.027762307	0.009746689
FAD	0.007663653	0.010243425

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
D-ALANINE	0.003405714	0.024242254
GLUTAMATE	-0.018830685	0.033646684
CYTIDINE DIPHOSPHATE	0.09420116	0.035918929
CYTIDINE MONOPHOSPHATE	-0.053958897	0.039364517
PHOSPHOCREATINE	0.240139565	0.048091999
GLUTAMINE	0.030438734	0.053396056
ADENOSINE 5'-DIPHOSPHATE	0.061854864	0.064596258
URIDINE MONOPHOSPHATE	-0.146517939	0.068024466
GLUTATHIONE REDUCED	0.096099818	0.071417279
ADENOSINE-MONOPHOSPHATE	0.127970048	0.076562149
CITRATE	0.092926913	0.079392358
BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE	-0.227771948	0.08659122
ISOCITRATE	0.104482346	0.091287928
HOMOCYSTEINE	-0.315236022	0.170075588
ANSERINE	0.224833961	0.209777683
CREATINE	0.039497664	0.275246878
CYTOSINE	0.590208771	0.324267336
GLYCEROL 3-PHOSPHATE	-0.718041156	0.332025667
S-ADENOSYLHOMOCYSTEINE	0.259585771	0.340027231
URACIL	-0.328214605	0.355147331
CYSTATHIONINE	0.369189958	0.363150303
BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE REDUCED	0.280197869	0.363229253
CYTIDINE	0.627478721	0.373117453
MANNITOL	-0.289653228	0.38884601
KYNURENINE	-0.560015282	0.42128982
CARNOSINE	-0.660353768	0.445115856
HYPOXANTHINE	-0.19536852	0.445686433
MALATE	-0.290011377	0.453315419
FUMARATE	-0.290520811	0.455803575
XYLOSE	0.714800092	0.472595255
ALPHA-D-GLUCOSE	-0.437517751	0.483537482
CYTIDINE TRIPHOSPHATE	0.943972985	0.485118971
CHOLINE	-0.381873415	0.493621709
D-ORNITHINE	-0.79306671	0.537031167
XANTHOSINE	-0.74480199	0.538296415
URIDINE 5'-DIPHOSPHATE	0.604282079	0.562371165
NICOTINAMIDE	0.211018433	0.568390987

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
S-ADENOSYLMETHIONINE	0.600873559	0.574377604
NADPH	-0.764273359	0.603251364
GLUCOSE 6-PHOSPHATE	-0.669871619	0.618856472
GLYCEROL	-0.361114486	0.622568906
METHYLMALONATE	-0.349551379	0.639145303
GALACTOSE	-0.39942912	0.666213769
URIDINE TRIPHOSPHATE	0.961991179	0.667445687
SUCCINATE	-0.355414342	0.669366458
2-PHOSPHOGLYCERATE	0.527871739	0.727000834
3-PHOSPHOGLYCERATE	0.527871739	0.727000834
PYRUVATE	0.600990307	0.777581354
INOSINE	0.622980209	0.794195963
LACTATE	-0.315080509	0.801597826
URIDINE	-0.470094959	0.827582986
CYCLIC AMP	-0.522157888	0.876979136
TAURINE	-0.467446827	0.884739749
HYPOTAURINE	-1.371340985	0.930369799
N-ACETYLASPARTATE	0.263045267	0.996263783
ADENOSINE TRIPHOSPHATE	0.658514616	1.137290377
ITACONATE	-0.374775331	1.23168147
ADENOSINE	1.776931648	1.247202642
GLUCONATE	-1.509849061	1.260792864
ASPARAGINE	-0.917586786	1.293421177
URATE	-0.953872536	1.331473526
GUANOSINE	1.085383722	1.462645335
CYSTINE	-0.955504771	1.472133874
OROTATE	0.726013622	1.51350696
ARGININE	-0.62453692	1.515502208
GSSG	1.420585662	1.548376563
L-CARNITINE	-0.922677805	1.54870619
TYROSINE	-0.698867674	1.611513284
TRYPTOPHAN	-0.984856748	1.763824317
GUANINE	-0.968950345	1.78733277
METHIONINE	-0.958975825	1.806316515
D-SEDHEPTULOSE	-1.014529414	1.83799508
HOMOSERINE	-0.949687637	1.924936416
THREONINE	-0.944338688	1.926829168

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
PHOSPHOENOLPYRUVATE	0.769126219	1.99061885
ISOLEUCINE	-1.147870063	2.052331181
PHENYLALANINE	-1.071997617	2.116278569
SERINE	-0.812598599	2.248303446
LEUCINE	-1.188639819	2.35145698
GLYCINE	-0.658799712	2.420948291
PROLINE	-0.894042223	2.480562141
LYSINE	-1.03798406	2.686367995

[00381] Metabolic changes were observed in FXS patient-derived peripheral blood mononuclear cell (PBMC) samples. Changes in polar metabolite levels between PBMC samples from six FXS males and five age-matched TD males were captured using LC-MS. A significant decrease in levels of 24 metabolites in the FXS samples compared to TD samples was detected (see FIGs. 2A-2B and Table 2). The ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) levels compared between the FXS and TD samples reflected altered mitochondrial metabolism in the FXS samples.

Table 2. Metabolic Changes in FXS Patient-Derived PBMC Samples

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
N-ACETYLMETHIONINE	0.01529662	0.0130453
GLYCOLATE	-0.0662205	0.01942117
METHYL VANILLATE	0.04308646	0.03087431
DEOXYGUANOSINE-MONOPHOSPHATE	0.09523579	0.03922005
ADENOSINE-MONOPHOSPHATE	0.14058924	0.06011119
CYTIDINE MONOPHOSPHATE	-0.27322	0.07775412
LIPOAMIDE	0.07049488	0.0870003
4-HYDROXY-3-METHOXYPHENYLGLYCOL	0.11579325	0.08971228
GUANINE	0.097001	0.09105498
GUANOSINE MONOPHOSPHATE	-0.317401	0.09906664
GSSG	-0.4706428	0.10681032
PIPECOLATE	-0.156792	0.10740771
PYROGLUTAMATE	-0.1588728	0.11017865
OXOPROLINE	-0.1593798	0.11023909
ADENOSINE DIPHOSPHATE RIBOSE	0.228944	0.11614986

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
3-METHYLGLUTACONATE	-0.2408179	0.12009894
METHIONINE	-0.2248315	0.17959854
O-PHOSPHOETHANOLAMINE	-0.4480126	0.19184611
GLYCOCHENODEOXYCHOLATE	-0.136333	0.19962241
AICAR	-0.1844605	0.20343503
BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE REDUCED	1.43191803	0.22251601
VALINE	-0.2391053	0.23289428
ISOLEUCINE	-0.2874232	0.23350985
GLUCOSE 1-PHOSPHATE	-0.4004739	0.25942119
NORVALINE	-0.2633877	0.26086896
SERINE	-0.1473106	0.27323575
ETHYLMALONATE	-0.6168777	0.27628184
1-AMINOCYCLOPROPANECARBOXYLATE	-0.3396032	0.29029844
CDP-ETHANOLAMINE	-0.6743419	0.29619367
URIDINE 5'-DIPHOSPHATE	-0.7651508	0.3055019
PSICOSE	-0.202111	0.30990203
MALTOSE	0.56365778	0.31281396
CYTIDINE DIPHOSPHATE	-1.0614412	0.32047937
INOSINE-MONOPHOSPHATE	-0.9944783	0.3219318
GUANOSINE DIPHOSPHATE	-0.9005488	0.32349363
ADENOSINE 5'-DIPHOSPHATE	-0.6095672	0.324028
DIHYDROBIOPTERIN	-0.4026207	0.3248711
GALACTOSE 1-PHOSPHATE	-0.6799107	0.33309453
3-HYDROXYANTHRANILATE	-0.1879215	0.33328798
BIOTIN	-0.2891638	0.33661189
N-ACETYL SERINE	-0.5063248	0.33785688
GLUTARATE	-0.2963926	0.36164856
DEOXYCARNITINE	-0.3392517	0.36655688
TRYPTOPHAN	-0.4961597	0.40091945
CYSTEINE	-0.367872	0.40685817
URIDINE DIPHOSPHATE GLUCOSE	-1.0183484	0.41067786
URIDINE DIPHOSPHATE GALACTOSE	-1.0183484	0.41067786
SUCROSE	0.51005379	0.41301226
SEROTONIN	-0.3796864	0.41930156
GUANOSINE DIPHOSPHATE MANNOSE	-1.3524779	0.44459475
CELLOBIOSE	0.80425721	0.45219727
4-GUANIDINO BUTANOATE	-0.3991725	0.45240083

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
TAURINE	-0.7380303	0.45335216
BUTANOATE	-0.9042827	0.45632841
QUINATE	0.38990837	0.46057217
D-RIBOSE 5-PHOSPHATE	-0.9926739	0.4607548
GLUTATHIONE	-1.3827259	0.48927467
FOLATE	-0.5331365	0.49163749
ASPARTATE	-0.3669423	0.49760659
CYTIDINE TRIPHOSPHATE	-1.7390948	0.50344571
O-PHOSPHOSERINE	0.37591699	0.51295304
GLYCINE	0.3786294	0.5238252
ALLANTOIN	-0.5473457	0.52497718
URIDINE DIPHOSPHATE-N-ACETYLGLUCOSAMINE	-1.2170889	0.54417263
GLUCONATE	-0.6883157	0.54989155
UROCANATE	-0.9837425	0.55312206
PHOSPHORYLCHOLINE	-1.2669876	0.55954401
METHYLMALONATE	-0.440813	0.56080909
SUCCINATE	-0.440813	0.56080909
ALPHA-D-GLUCOSE	-0.3182155	0.56274191
BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE	-1.6669004	0.56312815
GLUCOSAMINE 6-PHOSPHATE	-0.6545182	0.57100923
D-ORNITHINE	-0.4015304	0.58290279
FRUCTOSE 6-PHOSPHATE	-0.8113538	0.58445288
BETA-GLYCEROPHOSPHATE	-0.7821103	0.58926799
CITRAMALATE	-0.5068396	0.58977179
N-ACETYLASPARTATE	-0.7227142	0.59293777
THEOBROMINE	-0.5114221	0.59458613
THIOUREA	-0.4504796	0.59795653
GLUTAMINE	-0.327084	0.60317619
I-METHYLADENOSINE	-1.8819181	0.60994947
SORBITOL	-0.368355	0.61304836
TYROSINE	-0.4648309	0.62223798
GLUCOSE 6-PHOSPHATE	-0.9121809	0.63103469
GALACTITOL	-0.3760049	0.63129974
MANNITOL	-0.3760049	0.63129974
CYSTINE	-0.613572	0.63732467
GUANOSINE TRIPHOSPHATE	-2.2235531	0.6392944
L-CARNITINE	-0.5425263	0.66478758

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
URIDINE MONOPHOSPHATE	-0.5254017	0.67543034
KYNURENINE	-0.5029857	0.68560259
6-PHOSPHOGLUCONATE	-1.8873197	0.69543279
URIDINE DIPHOSPHATE-N-ACETYLGALACTOSAMINE	-2.4360179	0.69910838
MANNOSE 6-PHOSPHATE	-0.9804876	0.70794511
N-ACETYLNEURAMINATE	-0.8257705	0.71257016
ORNITHINE	-0.3775541	0.71976672
ADENOSINE TRIPHOSPHATE	-1.4615123	0.72212246
HYPOXANTHINE	-0.4521294	0.72901522
ISOCITRATE	-0.5365446	0.76707731
GALACTOSE	-0.384126	0.76874096
TAGATOSE	-0.328693	0.77573622
GLUCOSAMINE	-0.4355869	0.77616945
GALACTOSAMINE	-0.4424054	0.79141783
URIDINE TRIPHOSPHATE	-2.1873007	0.79265892
D-MANNOSAMINE	-0.4502	0.80491143
XYLOSE	-0.6749235	0.80540858
PROLINE	-0.5616502	0.80654671
MELIBIOSE	0.94504295	0.80711648
N,N,N-TRIMETHYLLYSINE	-0.6240628	0.80767067
PYRUVATE	-1.202011	0.80843415
STACHYOSE	-0.8140592	0.82976699
PYRIDOXINE	-0.5948268	0.83650847
L-METHYL-L-HISTIDINE	-0.3988687	0.84192605
SORBOSE	-0.3151285	0.85050709
DIACETYL	-0.4077237	0.85556517
URATE	-0.505949	0.86202213
CHOLINE	-0.7572552	0.86489842
L-ORNITHINE	-0.6114009	0.86792534
MANNOSE	-0.6277571	0.87249345
ARABITOL	-0.5699605	0.8774233
N-ACETYLGLUTAMATE	0.71260686	0.87747495
PALATINOSE	0.38047066	0.88315388
MYOINOSITOL	-0.3370728	0.88711496
TRIGONELLINE	-0.5639564	0.88910617
4-HYDROXYBENZALDEHYDE	-0.6218562	0.89438224
N,N-DIMETHYLARGININE	-0.2982343	0.91931618

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
ITACONATE	-0.7745421	0.92155176
CITRATE	-0.5524125	0.92262822
HIPPURATE	-1.1000264	0.93181455
CREATININE	-0.5916012	0.93322683
HOMOSERINE	-0.6853919	0.93395205
GAMMA-AMINOBUTYRATE	-0.7243194	0.94459303
3-(2-HYDROXYPHENYL)PROPANOATE	-0.5287467	0.94623811
4-PYRIDOXATE	-0.7690999	0.95879418
GULOSE	-0.6663356	0.96173571
THEOPHYLLINE	-1.0395111	0.9738911
GALACTURONATE	-1.3322923	0.97728794
ALLOTHREONINE	-0.7311604	0.97802467
THREONINE	-0.7311604	0.97802467
NICOTINATE	-0.647431	0.99183865
GLUTAMATE	-0.7509187	0.99287631
ALLOSE	-0.666596	0.99715085
NICOTINAMIDE	-0.6435523	1.00581609
CITRULLINE	-0.7885114	1.02567837
LACTATE	-0.7942332	1.03918646
PARAXANTHINE	-0.8736375	1.07549068
PANTOTHENATE	-0.6911859	1.07588012
LACTOSE	0.983016	1.09432157
O-ACETYL SERINE	-0.7592457	1.09535525
SALICYLAMIDE	1.22326481	1.12524399
PHENYLALANINE	-0.6528653	1.13517793
10-HYDROXYDECANOATE	-0.9099607	1.16917356
D-ALANINE	-0.584245	1.17169232
L-ALANINE	-0.6098933	1.23991365
CIS-4-HYDROXY-D-PROLINE	-0.753504	1.24182503
TRANS-4-HYDROXY-L-PROLINE	-0.753504	1.24182503
CREATINE	-0.7542497	1.25178584
HOMOVANILLATE	-0.7394393	1.28281979
HYDROXYPHENYLLACTATE	-0.7394393	1.28281979
GLYCERATE	-0.5875002	1.28550703
AZELATE	-1.3979008	1.30353025
ARGININE	-0.4469878	1.30504096
N-FORMYL-L-METHIONINE	-0.8879052	1.30544519

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
GLUCURONATE	-1.9551136	1.31391138
CAFFEINE	-1.117232	1.31538205
3-METHYL-L-HISTIDINE	-0.7708232	1.32147019
KYNURENATE	-1.1853457	1.32903146
LEUCINE	-0.7597409	1.36144003
D-SEDOHEPTULOSE	0.54455638	1.37292813
URACIL	-0.7948758	1.37616375
METHYL ACETOACETATE	-0.9289906	1.38038814
LYSINE	-0.6901333	1.40806515
MALATE	-0.8069718	1.40962002
MEVALONATE	-0.7907926	1.43835864
ASPARAGINE	-0.8075784	1.44116295
FUMARATE	-1.400301	1.60960015
CYTOSINE	-1.8954697	1.69086108
CARNOSINE	-1.0636184	1.72505067
GLYCEROL	-1.2184031	1.73442135
INDOLEACETALDEHYDE	-0.367473	1.89381767
TYRAMINE	-0.7530834	1.92818951
TREHALOSE	0.94821901	2.02731786
2-HYDROXYBUTYRATE	-0.7745936	2.06434579
XANTHINE	-0.7532739	2.08609044
ARGININOSUCCINATE	-1.1054713	2.21129432

[00382] Significant changes observed in FXS patient-derived PBMC and post-mortem cortex (brain) samples affected some of the same metabolites. Four polar metabolites exhibited significantly changed levels in both PBMC and cortex samples from FXS males compared to TD males (see FIGs. 3A-3C).

[00383] Metabolic changes were observed in FXS patient-derived lymphoblastoid cell lines (LCLs). Changes in polar metabolite levels in two human male FXS LCLs (GM05131 and GM07072) compared to two TD LCLs (GM07174 and GM06890) were captured using LC-MS. A significant decrease in levels of 21 metabolites in the FXS samples compared to TD samples was detected (see FIG. 4 and Table 3).

[00384] Finally, metabolites in cerebrospinal fluid (CSF) detected by LC/MS from individuals who have no known brain or CSF disorder are listed in Table 4.

Table 3. Metabolic Changes in FXS Patient-Derived Lymphoblastoid Cell Lines (LCLs)

Polar metabolite	log₂(fold change)	-log₁₀(p-value)
1-AMINOCYCLOPROPANECARBOXYLATE	-0.5605937	0.32782845
1-METHYL-L-HISTIDINE	-1.1175579	0.45950621
1-METHYLADENOSINE	-0.8197678	0.97259635
10-HYDROXYDECANOATE	-1.120687	0.93979502
2-HYDROXYBUTYRATE	-0.9147371	1.26113404
3-(2-HYDROXYPHENYL)PROPANOATE	-0.0461182	0.0266112
3-HYDROXYANTHRANILATE	-0.4964421	0.55283828
3-METHYL-L-HISTIDINE	-1.0780857	1.2362054
3-METHYLGLUTACONATE	-2.2228819	1.11685569
4-GUANIDINOBUTANOATE	-0.0440077	0.03328756
4-HYDROXY-3-METHOXYPHENYLGLYCOL	-0.7248867	0.22590437
4-HYDROXYBENZALDEHYDE	-1.2127349	0.40708304
4-PYRIDOXATE	-1.5745249	1.0818153
ADENOSINE 5'-DIPHOSPHATE	5.53141347	0.36632947
ADENOSINE DIPHOSPHATE RIBOSE	0.46710356	0.07391796
ADENOSINE TRIPHOSPHATE	3.88946994	0.35625544
ADENOSINE-MONOPHOSPHATE	3.46948557	0.34125739
AICAR	-1.4952196	0.54072024
ALLANTOIN	-2.305798	0.91536637
ALLOSE	-1.1333199	0.78356486
ALLOTHREONINE	-1.0071558	1.47560556
ALPHA-D-GLUCOSE	-0.8142864	1.49407643
ARABITOL	-1.3030392	1.17538038
ARGININE	-0.8126746	1.06746033
ARGININOSUCCINATE	-0.1159048	0.06032677
ASPARAGINE	-0.894519	1.34447737
ASPARTATE	-0.1265315	0.05395481
AZELATE	-1.1396619	1.66607191
BETA-GLYCEROPHOSPHATE	0.7878078	0.12455189
BIOTIN	-0.4599826	0.60448573
BUTANOATE	-0.4452835	0.07039827
CAFFEINE	-0.1416655	0.08425294
CARNOSINE	-1.2364541	1.61476647
CDP-ETHANOLAMINE	3.21372007	0.32917183
CELLOBIOSE	-2.7613739	0.31175904
CHOLINE	-0.7318262	0.95999757

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
CIS-4-HYDROXY-D-PROLINE	-0.9104972	1.68225823
CITRAMALATE	-1.2087148	0.72657526
CITRATE	-0.3864909	0.23580228
CITRULLINE	-1.1305496	1.37347654
CREATINE	-0.920774	1.7391662
CREATININE	-0.7703376	0.95771554
CYSTEINE	-1.4969673	0.95202179
CYSTINE	-1.3643178	0.90043448
CYTIDINE	-1.3564533	0.57374857
CYTIDINE DIPHOSPHATE	6.68327921	0.37015076
CYTIDINE MONOPHOSPHATE	2.15678113	0.27728097
CYTOSINE	0.15874353	0.0454029
D-ALANINE	-0.5629177	1.92472624
D-MANNOSAMINE	-0.8264863	0.94183941
D-ORNITHINE	-0.932118	0.94408828
D-RIBOSE 5-PHOSPHATE	2.40906536	0.29353445
D-SEDOHEPTULOSE	-1.7082446	0.57177921
DEOXYCARNITINE	0.03722687	0.04667008
DEOXYGUANOSINE-MONOPHOSPHATE	3.51051694	0.34189616
DIACETYL	-1.693448	0.75883598
DIHYDROBIOPTERIN	-0.686638	0.6009633
FOLATE	-1.6379475	0.71199232
FRUCTOSE 6-PHOSPHATE	2.07916687	0.27165858
FUMARATE	1.20086425	0.35778688
GALACTITOL	-1.1196375	0.89528124
GALACTOSAMINE	-0.8282764	0.92113571
GALACTOSE	-0.6535339	0.41946116
GALACTOSE 1-PHOSPHATE	2.22268949	0.28181677
GAMMA-AMINOBTYRATE	-1.0261744	0.25097913
GLUCONATE	-0.7360201	0.43092704
GLUCOSAMINE	-0.8172014	0.93213165
GLUCOSAMINE 6-PHOSPHATE	-0.8266942	0.2261935
GLUCOSE 1-PHOSPHATE	0.75955024	0.12022177
GLUCOSE 6-PHOSPHATE	2.16534554	0.27788255
GLUTAMATE	-1.1434519	0.28277795
GLUTAMINE	-0.3393942	0.3328764
GLUTARATE	-1.0755047	0.66764123

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
GLUTATHIONE	7.84116294	0.37547061
GLYCERATE	-1.2146539	0.43878347
GLYCEROL	-0.8836653	1.41192773
GLYCINE	-0.0509777	0.09079733
GLYCOCHENODEOXYCHOLATE	1.50660018	1.13046257
GSSG	2.12815308	0.27524319
GUANINE	-0.5840986	0.69541254
GUANOSINE MONOPHOSPHATE	1.74397475	0.24844305
GULOSE	-1.0732385	0.83711226
HIPPURATE	2.00661335	0.57773708
HOMOSERINE	-0.9875481	1.47531774
HOMOVANILLATE	-0.702034	1.24443339
HYDROXYPHENYLLACTATE	-0.702034	1.24443339
HYPOXANTHINE	-1.4929802	1.41360575
INDOLEACETALDEHYDE	-0.8203768	0.75912441
INOSINE-MONOPHOSPHATE	2.70698818	0.30924514
ISOCITRATE	-0.5808791	0.39699837
ISOLEUCINE	-0.9809216	1.13107545
ITACONATE	-0.8315185	0.22356585
KYNURENATE	-1.8134938	0.88807497
KYNURENINE	-1.1092332	0.4325244
L-ALANINE	-0.5860932	1.32710099
L-CARNITINE	-0.3882935	0.46776321
L-ORNITHINE	-0.7342818	0.64497965
LACTATE	-0.5549742	1.02243507
LACTOSE	-3.0499356	0.585477
LEUCINE	-0.9889538	1.15723771
LIPOAMIDE	-1.6463065	0.74272753
LYSINE	-1.2171899	1.06778931
MALATE	0.39016128	0.105901
MANNITOL	-1.1196375	0.89528124
MANNOSE	-1.1333199	0.78356486
MANNOSE 6-PHOSPHATE	2.1878566	0.27944629
MELIBIOSE	-3.0403401	0.58250599
METHIONINE	-1.0700885	1.16586962
METHYL ACETOACETATE	0.18276496	0.10496223
METHYL VANILLATE	0.29818892	0.23232073

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
METHYLMALONATE	-1.0445177	1.54990777
MEVALONATE	-0.8457716	0.83914444
MYOINOSITOL	-0.9664975	1.20911089
N-ACETYLASPARTATE	-1.1650245	0.81007852
N-ACETYLGLUTAMATE	2.67423671	0.42956854
N-ACETYLMETHIONINE	2.70404658	0.33560134
N-ACETYLNEURAMINATE	-1.6517088	1.34649848
N-ACETYLSERINE	0.88942341	0.22290029
N-FORMYL-L-METHIONINE	-1.0486742	1.18452571
N,N-DIMETHYLARGININE	-0.4231544	1.0094656
N,N,N-TRIMETHYLLYSINE	-1.223234	1.1851627
NICOTINAMIDE	-0.7768741	1.97493655
NICOTINATE	-0.658278	0.78359347
NORVALINE	-0.80354	2.09766958
O-ACETYLSERINE	1.02969462	0.24700943
O-PHOSPHOETHANOLAMINE	2.44729073	0.34232217
O-PHOSPHOSERINE	-0.8262704	0.73706803
ORNITHINE	0.14612288	0.07160501
OXOPROLINE	-0.8915084	1.04256719
PALATINOSE	-3.1367467	0.64879482
PANTOTHENATE	-0.1695989	0.15063705
PARAXANTHINE	0.31292418	0.064371
PHENYLALANINE	-1.0530026	0.97555646
PHOSPHORYLCHOLINE	2.53622001	0.30922125
PIPECOLATE	-0.8784048	1.08807887
PROLINE	-0.4845169	1.17105858
PSICOSE	-1.688711	0.88675158
PYRIDOXINE	-1.1161873	0.99290961
PYROGLUTAMATE	-0.8969546	1.03921459
PYRUVATE	1.19049001	0.24888993
QUINATE	-1.0776104	0.36235444
SALICYLAMIDE	1.69627156	0.6806827
SERINE	-0.4580587	0.24800519
SEROTONIN	-0.739287	0.62907542
SORBITOL	-1.1231322	0.89743366
SORBOSE	-1.1587956	0.74898984
STACHYOSE	-1.115586	0.33724761

Polar metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
SUCCINATE	-1.0445177	1.54990777
SUCROSE	-2.9847648	0.64367996
TAGATOSE	-1.1700265	0.71565563
TAURINE	1.18183522	0.2403167
THEOBROMINE	-0.3499865	0.0649178
THEOPHYLLINE	-0.3499865	0.0649178
THIOUREA	-1.7707056	0.85893558
THREONINE	-1.0071558	1.47560556
TRANS-4-HYDROXY-L-PROLINE	-0.9104972	1.68225823
TREHALOSE	-3.4122117	0.97874739
TRIGONELLINE	-1.1273379	1.27330434
TRYPTOPHAN	-1.1947561	1.22201756
TYRAMINE	-0.7570941	0.66577111
TYROSINE	-1.0829136	1.17725031
URACIL	-1.0585556	0.66899492
URATE	-1.1186772	0.92452065
URIDINE 5'-DIPHOSPHATE	9.11690516	0.37411645
URIDINE DIPHOSPHATE GLUCOSE	3.55468211	0.33893976
URIDINE DIPHOSPHATE GLUCURONIC ACID	4.16624022	0.35136021
URIDINE DIPHOSPHATE-N-ACETYLGALACTOSAMINE	4.28347502	0.35317144
URIDINE DIPHOSPHATE-N-ACETYLGALACTOSAMINE	3.84464077	0.34697371
URIDINE DIPHOSPHATE GALACTOSE	3.55289873	0.34106119
URIDINE MONOPHOSPHATE	1.86337107	0.28811202
UROCANATE	-0.0708077	0.07421462
VALINE	-0.8215089	2.11242945
XANTHINE	-1.1494679	0.74041191
XYLOSE	-0.7189049	0.64828483

Table 4. CSF Metabolites

	CSF (Relative concentration)
1-METHYLADENOSINE	356362.7965
10-HYDROXYDECANOATE	168013.4373
2-DEOXY-D-GLUCOSE	4214264.028
2-HYDROXYBUTYRATE	1664798.003
2-HYDROXYPHENYLACETATE	136351.9078
3-(2-HYDROXYPHENYL)PROPANOATE	329238.8302

	CSF (Relative concentration)
3-METHYL-L-HISTIDINE	4625664.062
4-HYDROXY-3-METHOXYPHENYLGLYCOL	1317372.613
ALLOSE	316172.7725
ALLOTHREONINE	3244311.885
ALPHA-D-GLUCOSE	33263.05266
ARABITOL	907040.7732
ARGININE	24365966.53
ASPARAGINE	6234191.362
AZELATE	164768.4163
CAFFEINE	3862092.019
CELLOBIOSE	69409.44462
CIS-4-HYDROXY-D-PROLINE	133564430.4
CITRAMALATE	218022.4958
CITRATE	4758089.605
CITRULLINE	1450120.415
CREATINE	134986222
CREATININE	259262818
D-ALANINE	3483511.916
D-MANNOSAMINE	3090961.513
DIHYDROBIOPTERIN	224663.2119
ETHYLMALONATE	408009.4504
GALACTITOL	1592387.367
GALACTOSAMINE	3081992.713
GALACTOSE	1937755.694
GLUCONATE	199019.846
GLUCOSAMINE	3081992.713
GLUTAMINE	171356594.2
GLUTARATE	174133.3683
GLYCERATE	274015.7896
GLYCEROL	348288.3221
GLYCOCHENODEOXYCHOLATE	431827.2921
GULOSE	312056.4863
HOMOSERINE	3825098.683
HOMOVANILLATE	111859.3577
HYDROXYPHENYLLACTATE	111859.3577
INDOLEACETALDEHYDE	259191.8373
ISOCITRATE	6548441.782

	CSF (Relative concentration)
L-ALANINE	3557331.371
L-CARNITINE	15065856.78
LACTATE	24397318.89
LACTOSE	48696.61888
LEUCINE	7434165.061
LIPOAMIDE	2511407.324
MALTOSE	41436.44212
MANNITOL	1592387.367
MANNOSE	316172.7725
MELIBIOSE	49532.21725
METHIONINE	2519883.879
METHYL ACETOACETATE	225220.3517
METHYL VANILLATE	0
MYOINOSITOL	34852.93974
N-ACETYLGLUTAMATE	45432.22459
N-ACETYLMETHIONINE	10784.43502
N-ACETYLNEURAMINATE	906299.704
N-ACETYLSERINE	720080.118
N,N-DIMETHYLARGININE	22870190.93
N,N,N-TRIMETHYLLYSINE	1837652.221
NICOTINAMIDE	2595837.7
NORVALINE	33040827.12
O-ACETYLSERINE	706816.9559
O-PHOSPHOSERINE	80919.98936
ORNITHINE	327150.4245
OXOPROLINE	1607361.505
PALATINOSE	53302.7473
PARAXANTHINE	5852153.837
PHENYLALANINE	7313821.864
PHOSPHORYLCHOLINE	1068850.721
PIPECOLATE	1487775.844
PROLINE	0
PSICOSE	402411.6702
PYROGLUTAMATE	1311360.69
PYRUVATE	668445.9069
QUINATE	180393.7698
SALICYLAMIDE	32913.94326

	CSF (Relative concentration)
SORBITOL	1566487.649
SORBOSE	340029.5083
SUCROSE	52589.42633
TAGATOSE	373957.0942
TAURINE	486612.8192
THEOBROMINE	5888517.848
THEOPHYLLINE	5888517.848
THIOUREA	0
THREONINE	3244311.885
TRANS-4-HYDROXY-L-PROLINE	133564430.4
TREHALOSE	80216.79446
TRIGONELLINE	1135756.126
TRYPTOPHAN	547125.5692
TYROSINE	2565267.812
URACIL	605160.7328
URATE	4418459.097
UROCANATE	89799.54758
VALINE	30982386.42
XANTHINE	267408.1883
XYLOSE	180484.3494

[00385] Example 2

[00386] Fragile X Syndrome (FXS) is a neurodevelopmental disorder characterized by a triplet repeat expansion mutation (>200 CGG repeats) in the *FMR1* gene, leading to the loss of FMRP protein expression. This absence results in dysregulated protein synthesis, mitochondrial deficits, gene expression abnormalities, and altered neuronal function, culminating in symptoms like anxiety, seizures, and intellectual disability. (J. D. Richter, X. Zhao, The molecular biology of FMRP: new insights into fragile X syndrome Nat. Rev. Neurosci. (2021), doi:10.1038/s41583-021-00432-0). Most individuals with FXS also exhibit features consistent with autism spectrum disorder (ASD), highlighting shared molecular deficits between the two conditions. (S. Shah, J. D. Richter, Do Fragile X Syndrome and Other Intellectual Disorders Converge at Aberrant Pre-mRNA Splicing? Front. Psychiatry 12, 715346 (2021)).

[00387] In FXS patients, repeat expansion in the *FMRI* locus triggers DNA methylation, leading to gene silencing and a subsequent deficiency in *FMRI* RNA production. However, recent research challenges this conventional view by revealing that approximately 70% of FXS individuals express *FMRI* RNA, albeit in a mis-spliced form (*FMRI-217*), rendering it non-functional (S. Shah *et al.*, Antisense oligonucleotide rescue of CGG expansion–dependent FMR1 mis-splicing in fragile X syndrome restores FMRP. Proc. Natl. Acad. Sci. 120, e2302534120 (2023)). (FIG. 5A). To address *FMRI* splicing mis-regulation, splice-switching antisense oligonucleotides (ASOs) were selected over RNaseH recruiting ASOs, which degrade transcripts. Treatment with ASOs in FXS cells led to decreased *FMRI-217* levels (FIG. 5B), increased full-length *FMRI* RNA (FIG. 5C), and most importantly restored FMRP protein (FIG. 5D). Thus, ASO treatments in FXS cells can be used to restore endogenous FMRP expression.

[00388] Previous studies have investigated mitochondrial dysfunction and energy deficits in FXS (B. Liu *et al.*, Regulatory discrimination of mRNAs by FMRP controls mouse adult neural stem cell differentiation. Proc. Natl. Acad. Sci. 115, 201809588 (2018); P. Licznanski *et al.*, ATP Synthase c-Subunit Leak Causes Aberrant Cellular Metabolism in Fragile X Syndrome. Cell 182, 1170-1185.e9 (2020); J. Geng *et al.*, Dereglulation of ER-mitochondria contact formation and mitochondrial calcium homeostasis mediated by VDAC in fragile X syndrome. Dev. Cell 58, 597-615.e10 (2023); M. Shen *et al.*, Species-specific FMRP regulation of RACK1 is critical for prenatal cortical development. Neuron 111, 3988-4005.e11 (2023); M. Shen *et al.*, Reduced mitochondrial fusion and Huntingtin levels contribute to impaired dendritic maturation and behavioral deficits in Fmr1-mutant mice. Nat. Neurosci. 22, 386–400 (2019)), suggesting their potential impact on brain metabolomics).

[00389] Liquid chromatography/mass spectrometry (LC/MS) for polar metabolite analysis was used in post-mortem brain tissues (cortex and cerebellum) from FXS and TD individuals (6 male samples per genotype, age-matched). Reductions in various metabolites were observed, particularly amino acids and their derivatives as highlighted in FIGs. 6A-D. Specifically, 14 of the 20 free amino acids were reduced in the FXS cerebellum tissues, 13 of 20 in the human cortex samples. These reductions encompassed both essential and non-essential amino acids, with notable examples such as decreases in the branched chain amino acids (BCAA) leucine and lysine. Moreover, by utilizing white blood cell (WBC)-derived cell lines from FXS patients

exhibiting different FMRP expression levels, partial FMRP expression might dampen metabolomic disruptions in FXS cells compared to complete loss of FMRP (FIG. 7).

[00390] This initial evidence implies that restoring FMRP in FXS cells may alleviate metabolomic deficits in FXS. Amino acid homeostasis is tightly regulated and reduction in amino acids levels can have multiple consequences such as compromised protein synthesis, disrupted neurotransmitter balance and alterations of numerous signaling pathways and energy production. Neurological symptoms manifest in numerous hereditary disorders associated with amino acid metabolism, emphasizing the need for maintaining amino acid homeostasis in the brain (P. Licznarski, *et al.*, ATP Synthase c-Subunit Leak Causes Aberrant Cellular Metabolism in Fragile X Syndrome. *Cell* 182, 1170-1185.e9 (2020)). Recent studies revealed that mutations of amino acid transporters result in neuronal death and microencephaly in patients with autism. (D. C. Tărlungeanu *et al.*, Impaired Amino Acid Transport at the Blood Brain Barrier Is a Cause of Autism Spectrum Disorder. *Cell* 167, 1481-1494.e18 (2016); L. S. Knaus *et al.*, Large neutral amino acid levels tune perinatal neuronal excitability and survival. *Cell* 186, 1950-1967.e25 (2023)). In a developing brain, amino acids are the main source of energy for neurons, prior to the switch to glucose in a mature brain and, thus, can underlie neurodevelopmental intellectual disability. Thus, alterations in metabolic profiles, specifically amino acid levels, could be an underlying feature of intellectual disability in several neurodevelopmental, neurodegenerative, and metabolic disorders. Thus, the amino acid imbalance observed in the FXS brain may indeed play a significant role in its pathophysiology. The study of how this imbalance occurs and whether it can be reversed by altering FMRP levels is described herein.

[00391] Reduced levels of free amino acids may result from the loss of FMRP expression in FXS. Factors contributing to amino acid imbalances in FXS cells will be explored. Subsequently, whether the restoration of FMRP expression can alleviate amino acid dysregulation in FXS cells will be assessed. To achieve these objectives, established FXS cell lines and ASO-mediated rescue strategies will be utilized, as well as LC-MS.

[00392] One aspect of this study investigates Fragile X syndrome as a metabolic disorder using an ASO-based FMRP rescue paradigm (S. Shah *et al.*, Antisense oligonucleotide rescue of CGG expansion-dependent FMR1 mis-splicing in fragile X syndrome restores FMRP. *Proc. Natl. Acad. Sci.* 120, e2302534120 (2023)) to restore metabolic imbalances in FXS.

Furthermore, analysis of metabolites from bodily fluids can provide an objective measure of therapeutic efficacy in FXS patients.

[00393] Approach

[00394] Preliminary studies:

[00395] LC-MS (polar analysis, <1000kDa) was conducted on post-mortem human brain tissue (cerebellum and cortex) (N=6 males), comparing FXS to age-matched typically developing (TD) individuals. In FIGs. 6A-B, volcano plots display metabolites that exhibit statistical significance ($-\log(p > 1.3)$) in A. cerebellum tissue and B. cortex. FIG. 6C. depicts a bar graph displaying changes in leucine and lysine. Free amino acids are denoted in green, while their derivatives are highlighted in red, constituting most observed changes. The most significant reductions in brain tissues were observed in free amino acids, with 14 out of 20 reduced in the cerebellum and 13 out of 20 in the cortex. Essential neuroactive amino acids crucial for neurotransmission and neuronal function, including glycine and serine, were reduced in both tissues, while aspartate, taurine, and Gamma-aminobutyric acid (GABA) were depleted specifically in the FXS cerebellum.

[00396] Whether FMRP levels might influence the metabolomic disruptions in FXS was studied. The metabolome in white blood cells (WBC) from FXS patients was analyzed, specifically Lymphoblastoid cell lines (LCLs), and compared to a WBC line from a typically developing (TD) individual. These LCLs were cultured in a complete, nutrient-rich medium and then subjected to LC-MS analysis. Two FXS patient-derived cell lines with varying FMRP expression levels were utilized: the FXS2 line displayed 50% FMRP compared to TD cells, whereas the FXS1 line exhibited undetectable FMRP levels via western blot analysis (FIG. 7A). The PCA plot showed distinct clustering based on genotype, with FXS1 further apart from TD compared to FXS2, suggesting that partial FMRP expression in FXS2 cells influences the metabolite profile towards TD (FIG. 7B). The findings revealed a depletion of the free amino acid pool in both FXS LCL cultures (FIG. 7C, D), mirroring the observations in FXS brain tissues (FIG. 6). Indeed, upon comparing the FXS2 vs. FXS1 metabolite profiles, a few amino acids exhibit higher expression levels, which may correlate with the increased FMRP levels in FXS2 line (FIG. 7E). Examples of amino acids changes are depicted in FIG. 7F. These findings suggest that restoring FMRP levels could potentially ameliorate metabolic deficits in FXS.

[00397] Research Strategy:

[00398] Methods:

[00399] a) Cell culture- LCL lines will be grown in RPMI media with 15% FBS as per instructions from Coriell Institute, NJ for all experiments pertaining to 2.1 and 2.2. To assess the metabolome in FXS neurons and perform the ASO rescue paradigm, iPSC derived forebrain neurons via reprogramming of the LCL lines (FIG. 8) have been generated. The forebrain neurons will be cultured in maturation media (STEMCELL Technologies) for a period of 9 days. ASOs resuspended in water will be applied to cultures at 5uM concentration for a minimum of 7 days before testing.

[00400] b) Drug treatments- FXS and TD LCLs will be treated with inhibitors such as Rapamycin, MG-132 or Bortezomib, 3-methyladenine (3-MA) and dexamipexole (DEX) or vehicle control. LC-MS will be performed on the samples.

[00401] c) Protein assays- Western blots will be performed using antibodies against FMRP, GAPDH, phospho-mTOR, mTOR, anti-LC3-I/II, ubiquitin, Hexokinase I.

[00402] d) qPCR- To measure levels of charged vs uncharged tRNAs, the itrap method will be used. This technique involves demethylases to remove hindering base methylations and a β -elimination reaction targeting the 3' end of adenine residues in uncharged tRNA, followed by TaqMan MGB qRT-PCR to distinguish between charged and uncharged tRNA cDNA.

[00403] e) LC-MS- Metabolites will be extracted using an acetonitrile:methanol:water based solution and subjected to a quadrupole orbitrap mass spectrometer coupled to a Vanquish UHPLC system with electrospray ionization.

[00404] f) Data analysis- The LC-MS data will be analyzed using the MAVEN software and Compound Discoverer software.

[00405] g) Power and statistical analysis- All experiments will be performed using at least three biological replicates. Controls will include cells from typically developing individuals cultured parallel to FXS cell lines and treated with the inhibitors or ASOs as well as their respective vehicle treatments. All samples within an experiment will be run simultaneously for LC-MS. All qPCR and western blot data will be analyzed using one-way ANOVA with Tukey's multiple comparisons test.

[00406] 2.1. Investigation of the pathways contributing to metabolome deficits in FXS

[00407] The amino acid pool is influenced by protein synthesis, degradation, autophagy, mitochondrial function, amino acid uptake, and catabolism. Drug treatments will be used to perturb the proteostasis and mitochondrial function in FXS cells and test their effects on the amino acid pool (FIG. 9).

[00408] 2.1A: Effect of increased protein synthesis on amino acid levels in FXS

[00409] Activation of the mTOR (mammalian target of rapamycin) pathway in FXS enhances translation initiation, increases ribosomal biogenesis and alters expression of synaptic proteins (A. Sharma *et al.*, Dysregulation of mTOR signaling in fragile X syndrome. J. Neurosci. 30, 694–702 (2010)). Thus, this study will block mTOR activation using Rapamycin in FXS cells and studying metabolome changes using LC-MS. TD cells will be treated as controls and western blots for phospho-mTOR and mTOR will be used to test the effect of Rapamycin. Reduced amino acids could also alter charged tRNA: uncharged tRNA ratios and impact protein synthesis. A qPCR-based method, i-trap (individual tRNA acetylation PCR), will be used to measure tRNA charging (Y. Tsukamoto *et al.*, i-tRAP (individual tRNA acylation PCR): a convenient method for selective quantification of tRNA charging. Rna 29, 111–112 (2023)). This method includes a demethylation step which makes uncharged tRNAs' 3'-end CC while charged tRNAs' 3'-end CCA, and using TaqMan-MGB probes designed to recognize tRNAs of interest as well as the CC and CCA polymorphisms the charged tRNA fraction will be quantified.

[00410] 2.1B: Effect of protein degradation deficits on amino acid levels in FXS

[00411] Increased activity of the ubiquitin proteasome system (UPS) in FXS model mice, elevates protein degradation. Normalizing proteasome activity with bortezomib or MG-132 inhibitors has been shown to correct FXS phenotypes such as excessive hippocampal neuronal firing (S. R. Louros *et al.*, Excessive proteostasis contributes to pathology in fragile X syndrome. Neuron 111, 508-525.e7 (2023)). Altered protein degradation regulates recycling of free amino acids and hence, whether inhibitors such as MG-132 or bortezomib can restore changes in free amino acids will be tested by LC-MS. Decreased autophagosis is another feature of FXS which can result in reduced breakdown of proteins (J. Yan *et al.*, Activation of autophagy rescues synaptic and cognitive deficits in fragile X mice. Proc. Natl. Acad. Sci. U. S. A. 115, E9707–E9716 (2018)). Inhibitors such as 3-methyladenine (3-MA) may be used to alter autophagy and its effect tested using LC-MS.

[00412] 2.1C: Study of whether restoring mitochondrial deficits can influence amino acid levels in FXS cells

[00413] Mitochondrial deficits, including inner membrane proton leaks, may exacerbate amino acid imbalances by altering their synthesis via the increased flux in the TCA cycle that can be ameliorated by inhibitors such as dexpramipexole (DEX) (P. Licznarski *et al.*, ATP Synthase c-Subunit Leak Causes Aberrant Cellular Metabolism in Fragile X Syndrome. Cell 182, 1170-1185.e9 (2020)) To assess the activity of DEX, western blot will be used against proteins such as HEXOKINASE I and II to measure TCA activity. FXS and TD cells may be treated with dexpramipexole (DEX) and testing the amino acid levels using LC-MS.

[00414] 2.2: Study of whether restoring FMRP can rescue metabolome deficits in FXS cells

Preliminary data using FXS LCL lines indicates an influence of differential FMRP expression on the free amino acid levels (FIGs. 7A-F). In some embodiments, FMRP may be restored in FXS cells and metabolome changes measured using LC-MS (FIG. 10).

[00415] 2.2A: Study of whether FMRP rescue can restore amino acid levels in FXS LCLs

[00416] In FXS2 cells that express the *FMR1-217* mis-spliced RNA, restoration of FMRP levels using antisense oligonucleotides (ASOs) have been shown (S. Shah, *et al.*, Antisense oligonucleotide rescue of CGG expansion–dependent FMR1 mis-splicing in fragile X syndrome restores FMRP. Proc. Natl. Acad. Sci. 120, e2302534120 (2023)). This paradigm in FXS cells may be used to restore FMRP and in TD cells (as controls) and the samples subjected to LC-MS. FMRP rescue upon ASO treatment will be examined using western blots.

[00417] 2.2B: Study of whether FMRP rescue can restore amino acid levels in FXS patient derived forebrain neurons

[00418] FXS is a neurodevelopmental disorder. Thus, FXS LCLs have been re-programmed (FIGs. 7 and 8) to derive forebrain excitatory mature neurons. LC-MS will be used to determine whether the metabolome changes observed in FXS patient derived brain tissues and LCLs (FIGs. 6 and 7) are mirrored in the iPSC derived neurons in culture. Next, neurons will be treated with ASOs (as in 2.2A) and whether the metabolome deficits are rescued by LC-MS will be determined.

[00419] The compounds and strategies proposed in 2.1 and 2.2 will be optimized in FXS LCL lines. Studies of the FXS human tissue samples have provided a list of metabolites other than the

amino acids which can be studied further. Other than increase in proteostasis and mitochondrial dysfunction, there are additional factors, such as cellular uptake of amino acids, catabolism and synthesis of downstream nitrogen compounds that could influence the free amino acid pool.

[00420] Tables 5-10 show the raw data of all the metabolites in the LCL cell lines (TD, FXS1, FXS2).

Table 5. Metabolic changes comparing FXS1 to TD LCLs (cell pellet)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
HISTIDINOL	-4.4532700	3.5617310
SERINE	-3.5436500	2.8650010
HOMOVANILLATE	-2.2447300	3.4316320
INOSINE-MONOPHOSPHATE	-1.9597200	2.2824140
VALINE	-1.9336500	3.6198280
INOSINE	-1.9161400	1.5828230
NORVALINE	-1.9085000	3.7941240
CARNOSINE	-1.6874300	2.8053530
1-METHYL-L-HISTIDINE	-1.5100200	2.5216580
ARGININE	-1.4223400	1.5143100
PHENYLALANINE	-1.3976300	2.1307720
CDP-ETHANOLAMINE	-1.3487200	2.3323650
GLUTAMATE	-1.3220000	2.3973670
ITACONATE	-1.2998700	1.4972240
2-METHYLGLUTARATE	-1.2062100	2.5206120
GALACTOSE	-1.1962700	1.8577900
LYSINE	-1.1925000	1.8073270
ASPARAGINE	-1.1275200	2.3226500
MONOMETHYLGLUTARATE	-1.1268100	3.0337230
CHOLINE	-1.1099500	1.3439610
ASPARTATE	-1.0942500	1.8510570
METHIONINE	-1.0893400	2.9555340
PYRIDOXINE	-1.0655600	1.6615160
PHOSPHORYLCHOLINE	-1.0243400	2.0308620
LEUCINE	-1.0241900	2.1740070
ADIPATE	-0.9942200	3.3829160

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
TYROSINE	-0.9744300	2.3462150
AICAR	-0.9197400	2.8477040
URIDINE DIPHOSPHATE GLUCOSE	-0.8975600	2.2547210
URIDINE DIPHOSPHATEGALACTOSE	-0.8975600	2.2547210
METHYLGUTARATE	-0.8749700	2.9019110
GLUTAMINE	-0.8463200	2.2768240
THREONINE	-0.8295800	3.0652730
ISOLEUCINE	-0.7931300	1.6302980
HOMOSERINE	-0.7601000	2.4457060
CYTIDINE MONOPHOSPHATE	-0.5414800	1.5555210
LACTATE	1.4578430	1.3202000
GUANOSINE TRIPHOSPHATE	2.4672500	1.3564190

Table 6. Metabolic changes comparing FXS1 to TD LCLs (culture media)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
3-HYDROXYMETHYLGLUTARATE	-1.5436827	2.8181856
3-METHYL-L-HISTIDINE	-0.5303698	2.2047053
3-METHYLADENINE	0.9013389	2.1040559
3-METHYLGLUTACONATE	-1.8298101	3.4612618
4-ACETAMIDOBUTANOATE	-0.1691815	1.3236199
ADENOSINE DIPHOSPHATE RIBOSE		1.3804570
Adenosine Monophosphate	2.3578606	1.5262782
AICAR	0.2974893	2.5277272
ALLANTOIN	-0.1947580	1.7918371
ALLOSE	-2.1544866	3.6377858
ALPHA-D-GLUCOSE	-2.1764548	3.6712387
ARABITOL	-0.2196794	2.2750290
ARGININE	-0.2395133	1.7967124
ASCORBATE	0.7829319	3.3518263
ASPARAGINE	-0.4725156	2.2161923
ASPARTATE	-0.6846204	3.9497761
BIOTIN	-0.1899352	1.6573954
BUTANOATE	-1.6836394	2.9291436

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
CARNOSINE	-0.1463210	1.5141962
CHOLINE	-0.4177921	3.5506730
CIS-4-HYDROXY-D-PROLINE	-0.3080191	2.8074738
CITRULLINE	-0.2346998	1.4756586
CREATINE	-0.3072980	2.7909206
CYSTEINE	-0.5480805	2.4305178
CYTIDINE DIPHOSPHATE	2.4473285	1.5509382
CYTOSINE	1.8270789	4.1570216
D-ORNITHINE	-0.3720916	2.6886046
FOLATE	-0.3267952	2.2178154
GALACTITOL	-0.2163428	1.5683552
GALACTOSAMINE	0.3530962	2.7191368
GALACTOSE	-0.3137524	1.6202340
GLUCONATE	-0.3623150	2.9431171
GLUTAMATE	0.1935275	1.5477162
GLUTAMINE	-0.6616074	3.9762210
GLUTARATE	-0.4906173	2.9647522
GUANIDINOACETATE	1.2937534	2.1569694
GUANINE		2.4547670
GUANOSINE DIPHOSPHATE	2.0817384	1.3642134
HOMOSERINE	-1.6424321	4.1356479
HYPOTAURINE	-0.9301541	2.9834981
HYPOXANTHINE	3.1607281	3.2330372
ISOLEUCINE	-0.4834928	2.3293166
ITACONATE	0.4188329	3.2732453
KYNURENINE	-0.3616081	2.0432225
L-CARNITINE	-0.1863363	1.5006249
L-ORNITHINE	-0.3721337	2.6884462
LACTATE	1.1880693	2.7533143
LEUCINE	-0.9859969	3.8980659
LYSINE	-1.3542454	4.2102240
MANNITOL	-0.2163428	1.5683552
MANNOSE	-2.1764548	3.6712387

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
METHIONINE	-0.9095272	3.4960272
METHYGLUTARATE	-0.8714170	4.2599317
METHYLMALONATE	-0.3875720	3.1238363
N-ACETYLNEURAMINATE	-1.5045201	2.0927643
N-METYL-ALANINE	-0.2002387	1.5207320
NICOTINAMIDE	-0.2248861	2.0511395
NICOTINATE	-0.2153483	1.9054251
OXOGLUTARATE	0.9681144	1.6835675
OXOPROLINE	-0.2348109	1.6440123
PHENYLALANINE	-0.9834999	3.7800365
PHENYLETHANOLAMINE		4.7352752
PHOSPHORYLCHOLINE	1.4270795	2.7047923
PROLINE	0.3948594	2.1599232
PSICOSE	-2.1544866	3.6377858
PYRIDOXAL	1.4576834	2.5865693
PYRIDOXINE	-0.4683378	2.3144200
PYROGLUTAMATE	-0.2348109	1.6440123
PYRUVATE	0.5781288	2.3105763
RIBITOL	-0.2821011	4.3189487
S-ADENOSYLMETHIONINE	2.8044275	1.6912205
SERINE	-2.4808637	3.9427101
SORBITOL	-1.1548225	2.9126683
SUCCINATE	-0.3875720	3.1238363
THREONINE	-1.6981005	4.1077132
THYMINE	-0.4064502	2.0242273
TRIGONELLINE	-4.8256024	4.2979860
TRYPTOPHAN	-1.1054245	3.1993839
TYROSINE	-0.9502213	3.5254669
URATE	-0.2541366	2.1306533
URIDINE DIPHOSPHATE GLUCOSE	1.5930427	1.8767735
URIDINE DIPHOSPHATE-N-ACETYLGLUCOSAMINE	3.8805224	2.0080170
URIDINE DIPHOSPHATEGALACTOSE	1.5930427	1.8767735
XYLITOL	-0.2196794	2.2750290

Table 7. Metabolic changes comparing FXS2 to TD LCLs (cell pellet)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
O-PHOSPHOETHANOLAMINE	-2.6872600	3.3875600
HISTIDINOL	-2.4165700	3.5407010
GSSG	-2.1359600	1.5069490
LYSINE	-1.9937500	1.6082610
INOSINE-MONOPHOSPHATE	-1.7307100	2.2843440
N-ACETYLASPARTATE	-1.4527700	1.5629940
SERINE	-1.1692400	2.3471110
PHOSPHORYLCHOLINE	-1.0697800	1.7936300
BETA-NICOTINAMIDE ADENINE DINUCLEOTIDE	-0.9751200	2.6032690
TAURINE	-0.8818400	1.8499040
MONOMETHYLGLUTARATE	-0.8812700	2.1687170
GLUTATHIONE REDUCED	-0.8802800	1.6572610
CDP-ETHANOLAMINE	-0.8795900	1.6015380
GLUTAMATE	-0.8526800	1.5772810
PHENYLALANINE	-0.8279000	1.3684020
1-METHYL-L-HISTIDINE	-0.8085100	1.4161430
CYS-GLY	-0.7946100	1.3752300
GLUTAMINE	-0.7292900	1.6730570
HYPOTAURINE	-0.7119900	1.4230200
2-METHYLGLUTARATE	-0.6927500	1.9410780
CHOLINE	-0.6745100	1.5330860
ASPARAGINE	-0.6687100	1.8614590
ADIPATE	-0.6278600	1.6464210
GUANIDINOACETATE	-0.6128100	1.6427330
URIDINE DIPHOSPHATE GLUCOSE	-0.6019200	1.4257830
URIDINE DIPHOSPHATE GALACTOSE	-0.6019200	1.4257830
METHIONINE	-0.5213600	1.3286730
TYROSINE	-0.5203900	1.3843550
CYTIDINE MONOPHOSPHATE	-0.3671000	1.3438310
L-CARNITINE	1.0839370	1.5275230

Table 8. Metabolic changes comparing FXS2 to TD LCLs (culture media)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
3-METHYLGLUTACONATE	-1.3559872	3.764998
ALLOSE	-1.4372882	4.3322291
ALPHA-D-GLUCOSE	-1.4482602	4.4328737
CYTOSINE	2.8371294	3.2712526
GUANIDINOACETATE	1.1810920	3.2229419
MANNOSE	-1.4482602	4.4328737
METHYGLUTARATE	-0.9985798	3.2982634
PHENYLETHANOLAMINE		4.7352752
PSICOSE	-1.4372882	4.3322291
SERINE	-1.7043050	3.6763093
THREONINE	-1.3632587	3.0733015
TRIGONELLINE		4.8863057

Table 9. Metabolic changes comparing FXS2 to FXS1 (cell pellet)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
3-METHYL-2-OXOVALERATE	0.6428880	1.4926930
INOSINE	2.7041320	2.7094220
MALATE	0.7831920	1.7142650
HISTIDINOL	2.0367060	2.1393990
TRIGONELLINE	1.4413480	2.3283920
PHENYLETHANOLAMINE	1.4683570	2.3153050
SERINE	2.3744140	2.4405990
HOMOVANILLATE	2.1622560	2.4774590
TAURINE	-0.6866100	1.5010080
2-METHYLGLUTARATE	0.5134620	1.3104990
METHIONINE	0.5679710	1.4024500
NORVALINE	1.5256990	2.2491080
VALINE	1.5037690	2.3436660
BETA-ALANINE	-0.7226700	1.4096910

Table 10. Metabolic changes comparing FXS2 to FXS1 (culture media)

Metabolite	log ₂ (fold change)	-log ₁₀ (p-value)
AICAR	0.0704180	1.3042006
ASCORBATE	0.9802342	1.8671102
CYTOSINE	1.0100505	2.3592504
GALACTOSAMINE	-0.5488815	1.9005105
GLUCONATE	0.2880639	1.3847824
GLUTAMATE	0.3539717	1.3003242
GUANINE		2.4547670
GUANOSINE DIPHOSPHATE	-1.7939540	1.4381014
HOMOVANILLATE	0.6250486	1.7876558
PHOSPHORYLCHOLINE	-0.5959697	1.4405430
PYRUVATE	0.6705328	2.1372435

[00421] Example 3

[00422] Wild-type (WT) and FXS2 cells were incubated with RPMI media (without glutamine) + FBS + 2mM of ¹³C₅, ¹⁵N₂ glutamine isotope for 8hrs. The cells and media were collected post incubation, and LC-MS was performed. Increased glutamine incorporation into citrate in FXS (FIG. 16A) is indicative of increased reverse carboxylation of glutamine in the citric acid cycle. In FXS cells, increased percent of glutamine incorporation into glutamate was observed which accumulated more in the extracellular media than intracellularly (FIGs. 16B-E). Increased ion count of glutamine-derived glutamate was observed in media in FXS cultures (FIGs. 16B and 16D) but a smaller increase was observed intracellularly (FIGs. 16C and 16E).

EMBODIMENTS

1. A method of diagnosing, subclassifying, or predicting a treatment outcome of fragile X syndrome (FXS) in a subject, the method comprising:
 - a) determining a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - b) determining an alteration of an FXS-associated ratio of two metabolites,

- or both a) and b), in a sample from the subject, wherein the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample is indicative of the presence, risk, progression, severity, or treatment outcome of FXS.
2. A method of predicting a treatment outcome of fragile X syndrome (FXS) in a subject, comprising:
 - a) determining:
 - i) a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii) an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and
 - b) predicting the treatment outcome of FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.
 3. A method of diagnosing fragile X syndrome (FXS) in a subject, comprising:
 - a) determining:
 - i) a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii) an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and
 - b) diagnosing the subject as having or having a propensity to develop FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.
 4. A method of prognosing fragile X syndrome (FXS) in a subject, the method comprising:
 - a) determining:
 - i) a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or
 - ii) an alteration of an FXS-associated ratio of two metabolites, or both i) and ii), in a sample from the subject, and

- b) prognosing the subject as having a propensity to have a poorer prognosis of FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.
5. A method of treating fragile X syndrome (FXS) in a subject, the method comprising:
- a) determining a presence, absence, amount, or alteration of at least one FXS-associated metabolite, or an alteration of an FXS-associated ratio of two metabolites, in a sample from the subject, and
 - b) treating FXS based on the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, and/or the alteration of the FXS-associated ratio, in the sample.
6. The method of Embodiment 1, 2, or 5, wherein treating FXS comprises administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product.
7. The method of Embodiment 6, wherein the therapeutically effective amount of the agent decreases an aberrant *FMR1* transcript, a protein encoded by the aberrant *FMR1* transcript, or both.
8. The method of Embodiment 6 or 7, wherein the aberrant *FMR1* gene product comprises *FMR1*-217.
9. The method of any one of Embodiments 6-8, wherein the therapeutically effective amount of the agent decreases *FMR1*-217 by at least 25%.
10. The method of any one of Embodiments 6-9, wherein the therapeutically effective amount of the agent increases the expression of fragile X messenger ribonucleoprotein (FMRP) by at least 25%.

11. The method of any one of Embodiments 6-10, wherein the agent targets a contiguous nucleotide sequence in a polynucleotide sequence set forth in any one of SEQ ID NOs:6-24.
12. The method of Embodiment 11, wherein the contiguous nucleotide sequence is at least 12 nucleotides in length.
13. The method of any one of Embodiments 6-12, wherein the agent is an antisense oligonucleotide (ASO) comprising a nucleotide sequence having at least 85% sequence identity to a sequence set forth in any one of SEQ ID NOs:25-43.
14. The method of Embodiment 13, wherein the ASO comprises a nucleotide sequence set forth in any one of SEQ ID NOs:25-43.
15. The method of Embodiment 13, wherein the ASO comprises a nucleotide sequence having at least 85% sequence identity to a sequence set forth in any one of SEQ ID NOs:34-39.
16. The method of Embodiment 15, wherein the ASO comprises a nucleotide sequence set forth in any one of SEQ ID NOs:34-39.
17. The method of any one of Embodiments 13-16, wherein the ASO is about 18-22 nucleotides in length.
18. The method of any one of Embodiments 13-17, wherein the ASO comprises a modification of a ribose group, a modification of a phosphate group, a modification of a nucleobase, or a combination thereof.
19. The method of any one of Embodiments 13-17, wherein the ASO is chemically modified to comprise:
 - a) a locked nucleic acid (LNA), an ethyl-constrained nucleotide, a 2'-(S)-constrained ethyl (S-cEt) nucleotide, a constrained MOE, a 2'-O,4'-C-aminomethylene bridged nucleic acid (2',4'-BNA(NC)), an alpha-L-locked nucleic acid, or a tricyclo-DNA, or a combination thereof,

- b) a ribose group comprising 2'-O-methyl, 2'-fluoro, 2'-deoxy, 2'-O-(2-methoxyethyl) (MOE), 2'-O-alkyl, 2'-O-alkoxy, 2'-O-alkylamino, 2'-NH₂, a constrained nucleotide, or a tricyclo-DNA modification, or a combination thereof,
 - c) a phosphate group comprising a phosphorothioate, a phosphoramidate, a phosphorodiamidate, a phosphorodithioate, a phosphonoacetate (PACE), a thiophosphonoacetate (thioPACE), an amide, a triazole, a phosphonate, or a phosphotriester, or a combination thereof,
 - d) a nucleobase comprising 2-thiouridine, 4-thiouridine, N⁶-methyladenosine, pseudouridine, 2,6-diaminopurine, inosine, thymidine, 5-methylcytosine, 5-substituted pyrimidine, isoguanine, isocytosine, or halogenated aromatic groups, or a combination thereof,
 - e) a phosphorodiamidate morpholino (PMO), a peptide nucleic acid, or another pseudopeptide backbone,
- or a combination of the foregoing.
20. The method of any one of Embodiments 13-19, wherein the ASO is a phosphorothioate-modified polynucleotide.
21. The method of any one of Embodiments 13-20, wherein at least half of the internucleotide linkages of the ASO are phosphorothioate.
22. The method of any one of Embodiments 13-21, wherein each internucleotide linkage of the ASO is a phosphorothioate.
23. The method of any one of Embodiments 1-22, wherein the sample comprises a bodily fluid selected from cerebrospinal fluid, blood, serum, plasma, or urine.
24. The method of any one of Embodiments 1-23, wherein the sample comprises cerebrospinal fluid (CSF).
25. The method of any one of Embodiments 1-24, wherein the sample comprises peripheral blood mononuclear cells (PBMCs).

26. The method of any one of Embodiments 1-25, comprising determining an alteration of at least one FXS-associated metabolite in the sample.
27. The method of any one of Embodiments 1-26, wherein the at least one metabolite has an altered level in a sample from a subject, relative to a control sample and/or reference level.
28. The method of Embodiment 27, wherein the at least one metabolite comprises n-acetylneuraminate, D-alanine, L-alanine arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, L-carnitine, carnosine, citrulline, creatine, cystine, cytosine, fumarate, alpha-D-glucose, glucuronate, oxidized glutathione (GSSG), reduced glutathione (GSH), glycerol, glycine, guanine, 3-methyl-L-histidine, homoserine, hypoxanthine, indoleacetaldehyde, isoleucine, kynurenate, leucine, lysine, malate, methionine, methyl acetoacetate, methylmalonate, mevalonate, n-formyl-L-methionine, nicotinamide, phenylalanine, proline, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, D-sedoheptulose, serine, succinate, threonine, allothreonine, tryptophan, tyramine, tyrosine, uracil, urate, valine, norvaline, or xanthine, or a combination thereof.
29. The method of any one of Embodiments 1-28, wherein the at least one metabolite comprises:
- a) lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or reduced glutathione (GSH), or a combination thereof,
 - b) argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof, or
 - c) valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol,

- citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof,
- or a combination thereof.
30. The method of any one of Embodiments 1-29, wherein the at least one metabolite comprises arginine, leucine, or lysine, or a combination thereof.
31. The method of Embodiment 29 or 30, wherein the at least one metabolite has a reduced level in the sample from the subject, relative to a control sample and/or reference level.
32. The method of Embodiment 31, wherein a level of the at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq(-0.50)$ in the sample, relative to a control sample and/or reference level, optionally, wherein the $\log_2$ fold reduction is $\leq(-0.90)$.
33. The method of Embodiment 31 or 32, wherein the control sample:
- a) is a sample from an age-matched typically-developing individual, or
 - b) is a baseline sample from the subject.
34. The method of Embodiment 29 or 30, wherein the at least one metabolite has an increased level in the sample from the subject, relative to a control sample and/or reference level.
35. The method of Embodiment 34, wherein a level of the at least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.50 in the sample, relative to a control sample and/or reference level, optionally, wherein the $\log_2$ fold increase is ≥ 0.90 .
36. The method of Embodiment 34 or 35, wherein the control sample:
- a) is a sample from an age-matched typically-developing individual, or
 - b) is a baseline sample from the subject.
37. The method of any one of Embodiments 1-28, wherein the at least one metabolite comprises:
- a) phosphoenolpyruvate, oxidized glutathione disulfide (GSSG), orotate, or guanosine, or a combination thereof, or

- b) trehalose, or D-sedoheptulose, or both,
or a combination thereof.
- 38. The method of Embodiment 37, wherein the at least one metabolite has an increased level in the sample from the subject, relative to a control sample and/or reference level.
- 39. The method of Embodiment 38, wherein a level of at least one FXS-associated metabolite has a $\log_2$ fold increase of ≥ 0.50 in the sample, relative to a control sample and/or reference level, optionally, wherein the $\log_2$ fold increase is ≥ 0.90 .
- 40. The method of Embodiment 38 or 39, wherein the control sample:
 - a) is a sample from an age-matched typically-developing individual, or
 - b) is a baseline sample from the subject.
- 41. The method of Embodiment 37, wherein the at least one metabolite has a reduced level in the sample from the subject, relative to a control sample and/or reference level.
- 42. The method of Embodiment 41, wherein a level of the at least one FXS-associated metabolite has a $\log_2$ fold reduction of $\leq (-0.50)$ in the sample, relative to a control sample and/or reference level, optionally, wherein the $\log_2$ fold increase is $\leq (-0.90)$.
- 43. The method of Embodiment 41 or 42, wherein the control sample:
 - a) is a sample from an age-matched typically-developing individual, or
 - b) is a baseline sample from the subject.
- 44. The method of any one of Embodiments 1-43, wherein the FXS-associated ratio is indicative of increased oxidative stress in the sample from the subject.
- 45. The method of Embodiment 44, wherein the FXS-associated ratio is a ratio of reduced glutathione (GSH) to oxidized glutathione disulfide (GSSG).
- 46. The method of any one of Embodiments 1-43, wherein the FXS-associated ratio and/or metabolite level is indicative of suppressed mitochondrial metabolism in the sample from the subject.

47. The method of Embodiment 46, wherein the FXS-associated ratio is a ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP).
48. The method of any one of Embodiments 44-47, wherein the FXS-associated ratio is reduced in the sample from the subject, relative to a control sample and/or reference level.
49. The method of Embodiment 48, wherein the control sample:
 - a) is a sample from an age-matched typically developing individual, or
 - b) is a baseline sample from the subject.
50. The method of any one of Embodiments 44-47, wherein the FXS-associated ratio is increased in the sample from the subject, relative to a control sample and/or reference level.
51. The method of Embodiment 50, wherein the control sample:
 - a) is a sample from an age-matched typically-developing individual, or
 - b) is a baseline sample from the subject.
52. The method of any one of Embodiments 1-51, wherein the subject is a human male.
53. The method of any one of Embodiments 1-51, wherein the subject is a human female.
54. The method of any one of Embodiments 1-53, wherein determining the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, or the alteration of the FXS-associated ratio, comprises performing liquid chromatography-mass spectrometry (LC-MS).
55. The method of any one of Embodiments 1-53, wherein determining the presence, absence, amount, or alteration of the at least one FXS-associated metabolite, or the alteration of the FXS-associated ratio, comprises performing gas chromatography-mass spectrometry (GC-MS).

56. A method of diagnosing, subclassifying, or predicting a treatment outcome of fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
- a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,
- or a combination of the foregoing, wherein a), b), or c), or a combination of the foregoing is indicative of the presence, risk, progression, severity, or treatment outcome of FXS.
57. A method of predicting a treatment outcome of fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
- a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,
- or a combination of the foregoing; and
predicting the treatment outcome of FXS based on a), b), or c), or a combination of the foregoing.
58. A method of diagnosing fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
- a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,
- or a combination of the foregoing; and

diagnosing the subject as having or having a propensity to develop FXS based on a), b), or c), or a combination of the foregoing.

59. A method of prognosing fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
- a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,
- or a combination of the foregoing; and
prognosing the subject as having a propensity to have a poorer prognosis of FXS based on a), b), or c), or a combination of the foregoing.
60. A method of treating fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
- a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,
- or a combination of the foregoing; and
treating FXS based on a), b), or c), or a combination of the foregoing.

REFERENCES

1. J. D. Richter, X. Zhao, The molecular biology of FMRP: new insights into fragile X syndrome, *Nat. Rev. Neurosci.* (2021), doi:10.1038/s41583-021-00432-0.
2. S. Shah, J. D. Richter, Do Fragile X Syndrome and Other Intellectual Disorders Converge at Aberrant Pre-mRNA Splicing? *Front. Psychiatry* **12**, 715346 (2021).
3. S. Shah, K. J. Sharp, S. Raju Ponny, J. Lee, J. K. Watts, E. Berry-Kravis, J. D. Richter, Antisense oligonucleotide rescue of CGG expansion-dependent FMR1 mis-splicing in fragile X syndrome

restores FMRP. *Proc. Natl. Acad. Sci.* **120**, e2302534120 (2023).

4. S. Shah, G. Molinaro, B. Liu, R. Wang, K. M. Huber, J. D. Richter, FMRP Control of Ribosome Translocation Promotes Chromatin Modifications and Alternative Splicing of Neuronal Genes Linked to Autism. *bioRxiv* (2019), doi:10.1101/801076.

5. B. Liu, Y. Li, E. E. Stackpole, A. Novak, Y. Gao, Y. Zhao, X. Zhao, J. D. Richter, Regulatory discrimination of mRNAs by FMRP controls mouse adult neural stem cell differentiation. *Proc. Natl. Acad. Sci.* **115**, 201809588 (2018).

6. P. Licznarski, H. A. Park, H. Rolyan, R. Chen, N. Mnatsakanyan, P. Miranda, M. Graham, J. Wu, N. Cruz-Reyes, N. Mehta, S. Sohail, J. Salcedo, E. Song, C. Effman, S. Effman, L. Brandao, G. N. Xu, A. Braker, V. K. Gribkoff, R. J. Levy, E. A. Jonas, ATP Synthase c-Subunit Leak Causes Aberrant Cellular Metabolism in Fragile X Syndrome. *Cell* **182**, 1170-1185.e9 (2020).

7. J. Geng, T. P. Khaket, J. Pan, W. Li, Y. Zhang, Y. Ping, M. I. Cobos Sillero, B. Lu, Deregulation of ER-mitochondria contact formation and mitochondrial calcium homeostasis mediated by VDAC in fragile X syndrome. *Dev. Cell* **58**, 597-615.e10 (2023).

8. M. Shen, C. L. Sirois, Y. Guo, M. Li, Q. Dong, N. M. Méndez-Albelo, Y. Gao, S. Khullar, L. Kissel, S. O. Sandoval, N. E. Wolkoff, S. X. Huang, Z. Xu, J. E. Bryan, A. M. Contractor, T. Korabelnikov, I. A. Glass, D. Doherty, J. E. Levine, A. M. M. Sousa, Q. Chang, A. Bhattacharyya, D. Wang, D. M. Werling, X. Zhao, Species-specific FMRP regulation of RACK1 is critical for prenatal cortical development. *Neuron* **111**, 3988-4005.e11 (2023).

9. M. Shen, F. Wang, M. Li, N. Sah, M. E. Stockton, J. J. Tidei, Y. Gao, T. Korabelnikov, S. Kannan, J. D. Vevea, E. R. Chapman, A. Bhattacharyya, H. van Praag, X. Zhao, Reduced mitochondrial fusion and Huntingtin levels contribute to impaired dendritic maturation and behavioral deficits in *Fmr1*-mutant mice. *Nat. Neurosci.* **22**, 386-400 (2019).

10. D. C. Tărlungeanu, E. Deliu, C. P. Dotter, M. Kara, P. C. Janiesch, M. Scalise, M. Galluccio, M. Tesulov, E. Morelli, F. M. Sonmez, K. Bilguvar, R. Ohgaki, Y. Kanai, A. Johansen, S. Esharif, T. Ben-Omran, M. Topcu, A. Schlessinger, C. Indiveri, K. E. Duncan, A. O. Caglayan, M. Gunel, J. G. Gleeson, G. Novarino, Impaired Amino Acid Transport at the Blood Brain Barrier Is a Cause of Autism Spectrum Disorder. *Cell* **167**, 1481-1494.e18 (2016).

11. L. S. Knaus, B. Basilico, D. Malzl, M. Gerykova Bujalkova, M. Smogavec, L. A. Schwarz, S. Gorkiewicz, N. Amberg, F. M. Pauler, C. Knittl-Frank, M. Tassinari, N. Maulide, T. Rüllicke, J.

Menche, S. Hippenmeyer, G. Novarino, Large neutral amino acid levels tune perinatal neuronal excitability and survival. *Cell* **186**, 1950-1967.e25 (2023).

12. A. Sharma, C. A. Hoeffler, Y. Takayasu, T. Miyawaki, S. M. McBride, E. Klann, R. Suzanne Zukin, Dysregulation of mTOR signaling in fragile X syndrome. *J. Neurosci.* **30**, 694–702 (2010).

13. Y. Tsukamoto, Y. Nakamura, M. Hirata, R. Sakate, T. Kimura, i-tRAP (individual tRNA acylation PCR): a convenient method for selective quantification of tRNA charging. *Rna* **29**, 111–112 (2023).

14. S. R. Louros, S. S. Seo, B. Maio, C. Martinez-Gonzalez, M. A. Gonzalez-Lozano, M. Muscas, N. C. Verity, J. C. Wills, K. W. Li, M. F. Nolan, E. K. Osterweil, Excessive proteostasis contributes to pathology in fragile X syndrome. *Neuron* **111**, 508-525.e7 (2023).

15. J. Yan, M. W. Porch, B. Court-Vazquez, M. V. L. Bennett, R. S. Zukin, Activation of autophagy rescues synaptic and cognitive deficits in fragile X mice. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E9707–E9716 (2018).

[00423] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[00424] While example embodiments have been particularly shown and described, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the embodiments encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of predicting a treatment outcome of, diagnosing, prognosing, or subclassifying fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
 - a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,or a combination of the foregoing.
2. A method of treating fragile X syndrome (FXS) in a subject, the method comprising determining, in a sample from the subject:
 - a) a presence, an absence, or a level of an FXS-associated metabolite,
 - b) an alteration or a ratio of a level of an FXS-associated metabolite, relative to a reference level of the same metabolite,
 - c) a ratio between levels of two FXS-associated metabolites, or an alteration thereof relative to a reference ratio of the two FXS-associated metabolites,or a combination of the foregoing; and administering to the subject a therapeutically effective amount of an agent that decreases an aberrant *fragile X messenger ribonucleoprotein 1 (FMR1)* gene product.
3. The method of claim 1 or 2, wherein the FXS-associated metabolite has a reduced level in the sample from the subject, relative to the reference level of the same metabolite.
4. The method of any one of claims 1-3, wherein the alteration of the level of the FXS-associated metabolite, relative to the reference level of the same metabolite, is a log₂ fold reduction of from about -10 to about 0 or a log₂ fold increase of from about 0 to about 10.

5. The method of any one of claims 1-4, wherein the alteration of the level of the FXS-associated metabolite, relative to the reference level of the same metabolite, is a $\log_2$ fold reduction of equal to or less than about -0.50 or a $\log_2$ fold increase of equal to or greater than about 0.50.
6. The method of any one of claims 1-5, wherein the alteration of the ratio between levels of two FXS-associated metabolites relative to the reference ratio of the two FXS-associated metabolites is a decrease of at least about 20%.
7. The method of any one of claims 1-6, wherein the ratio between the levels of two FXS-associated metabolites is the ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG) or the ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP).
8. The method of any one of claims 1-7, wherein the reference level is based on:
 - a sample from an age-matched typically-developing individual, or
 - a baseline sample from the subject.
9. The method of any one of claims 1-8, wherein the sample comprises a bodily fluid selected from cerebrospinal fluid (CSF), blood, serum, plasma, or urine.
10. The method of any one of claims 1-9, wherein the sample comprises peripheral blood mononuclear cells (PBMCs).
11. The method of any one of claims 1-10, wherein the FXS-associated metabolite comprises n-acetylneuraminate, D-alanine, L-alanine, arginine, argininosuccinate, asparagine, azelate, 2-hydroxybutyrate, caffeine, L-carnitine, carnosine, citrulline, creatine, cystine, cytosine, fumarate, alpha-D-glucose, glucuronate, oxidized glutathione (GSSG), reduced glutathione (GSH), glycerol, glycine, guanine, 3-methyl-L-histidine, homoserine, hypoxanthine, indoleacetaldehyde, isoleucine, kynurenate, leucine, lysine, malate, methionine, methyl acetoacetate, methylmalonate, mevalonate, n-formyl-L-methionine, nicotinamide, phenylalanine, proline, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, D-sedoheptulose, serine, succinate, threonine, allothreonine, tryptophan,

tyramine, tyrosine, uracil, urate, valine, norvaline, xanthine, phosphoenolpyruvate, orotate, guanosine, trehalose, or a combination thereof.

12. The method of any one of claims 1-11, wherein the FXS-associated metabolite comprises:
- a) lysine, proline, glycine, leucine, serine, phenylalanine, isoleucine, threonine, homoserine, D-sedoheptulose, methionine, guanine, tryptophan, tyrosine, L-carnitine, arginine, cystine, urate, or reduced glutathione (GSH), or a combination thereof,
 - b) argininosuccinate, xanthine, 2-hydroxybutyrate, tyramine, indoleacetaldehyde, glycerol, carnosine, cytosine, fumarate, asparagine, mevalonate, malate, lysine, methyl acetoacetate, uracil, leucine, kynurenate, 3-methyl-L-histidine, caffeine, glucuronate, n-formyl-L-methionine, arginine, or azelate, or a combination thereof,
 - c) valine, norvaline, nicotinamide, D-alanine, creatine, cis-4-hydroxy-D-proline, trans-4-hydroxy-L-proline, azelate, carnosine, methylmalonate, succinate, alpha-D-glucose, allothreonine, threonine, homoserine, hypoxanthine, glycerol, citrulline, n-acetylneuraminate, asparagine, or L-alanine, or a combination thereof,
- or a combination of a) to c).
13. The method of any one of claims 1-12, wherein the FXS-associated metabolite comprises arginine, leucine, or lysine, or a combination thereof.
14. The method of any one of claims 1-11, wherein the FXS-associated metabolite comprises:
- a) phosphoenolpyruvate, oxidized glutathione disulfide (GSSG), orotate, or guanosine, or a combination thereof, or
 - a) trehalose, or D-sedoheptulose, or both,
- or a combination thereof.

15. The method of any one of claims 2-14, wherein the therapeutically effective amount of the agent decreases an aberrant *FMRI* transcript, a protein encoded by the aberrant *FMRI* transcript, or both.
16. The method of claim 15, wherein the aberrant *FMRI* gene product comprises *FMRI*-217.
17. The method of any one of claims 2-16, wherein the agent is an antisense oligonucleotide.
18. The method of any one of claims 1-17, wherein determining b) or c) comprises determining the statistical significance of the alteration based on a significance threshold.
19. The method of claim 18, wherein the significance threshold is a p-value equal to or less than about 0.05.
20. The method of any one of claims 1-19, wherein the method employs a chromatography technique.

FIG. 1A

Human Volcano Plot

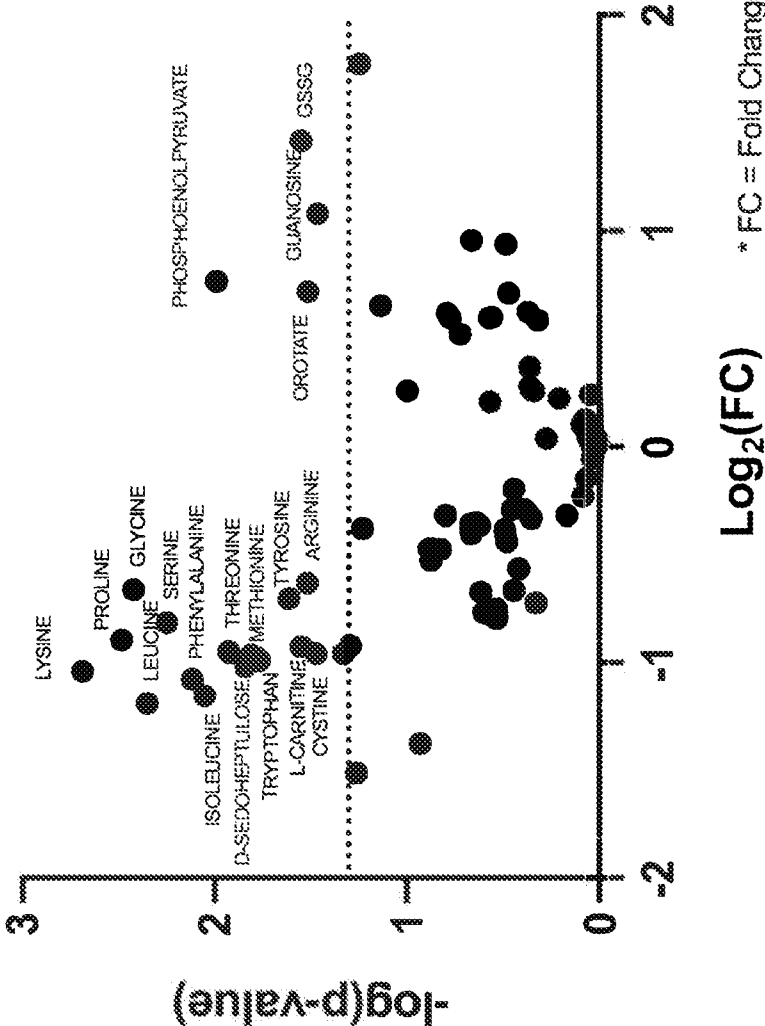


FIG. 1B

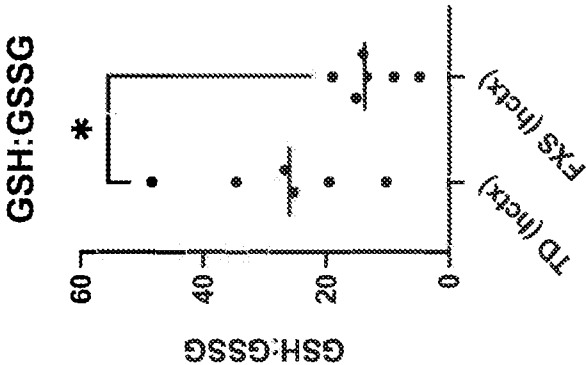


FIG. 2A

PBMC Volcano Plot

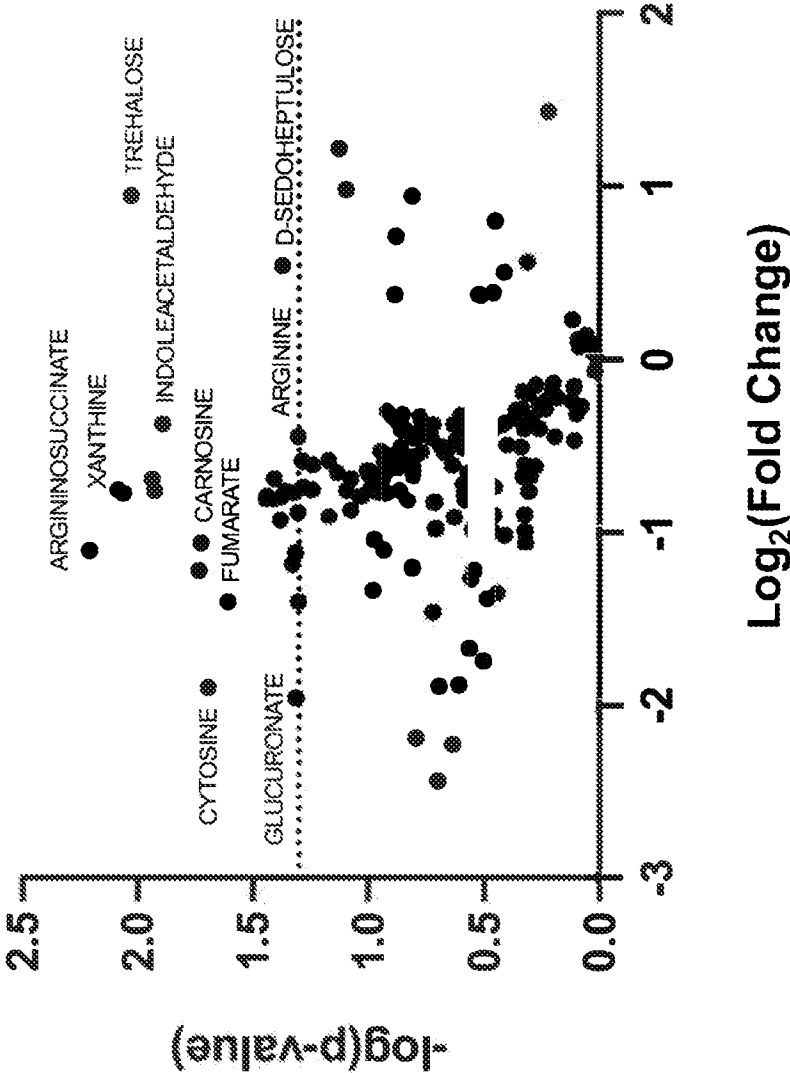


FIG. 2B

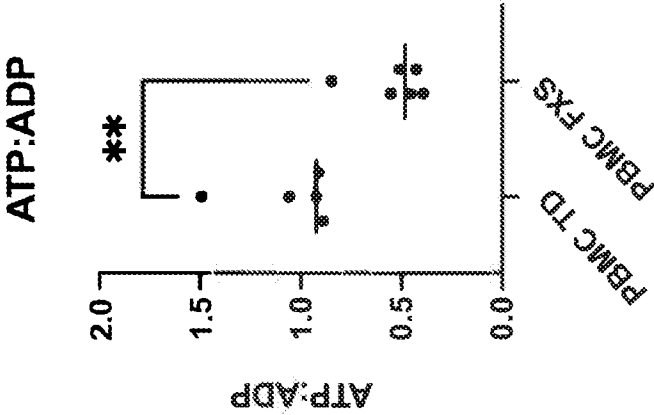


FIG. 3A

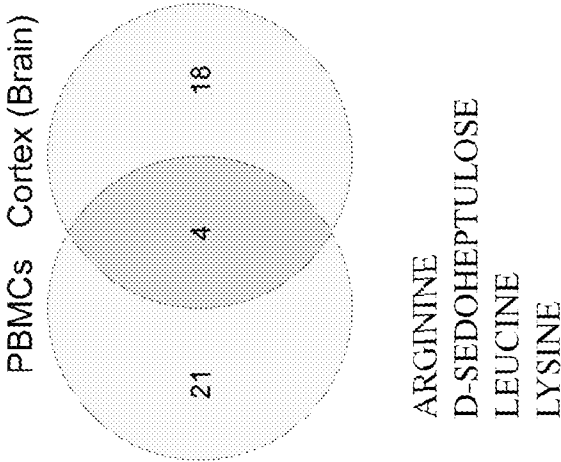


FIG. 3B

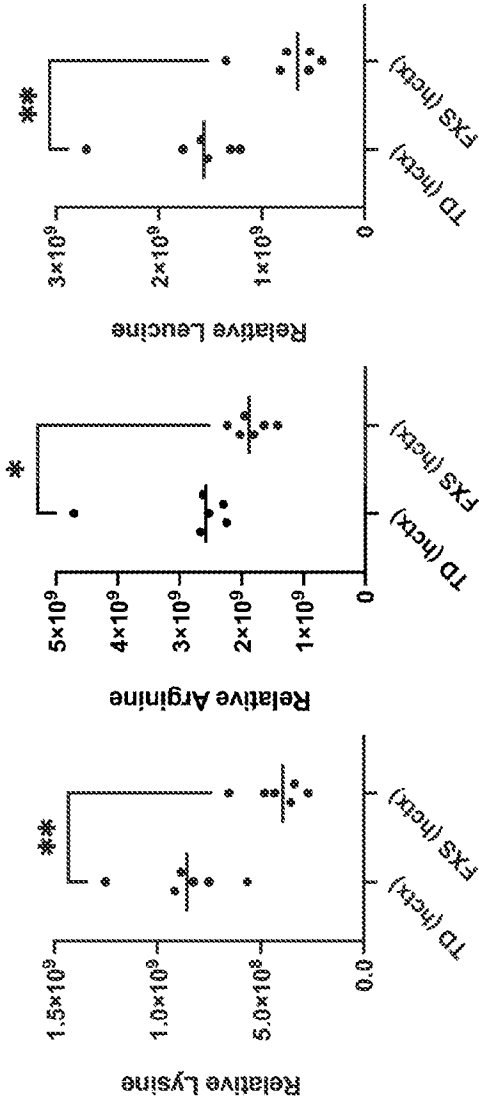


FIG. 3C

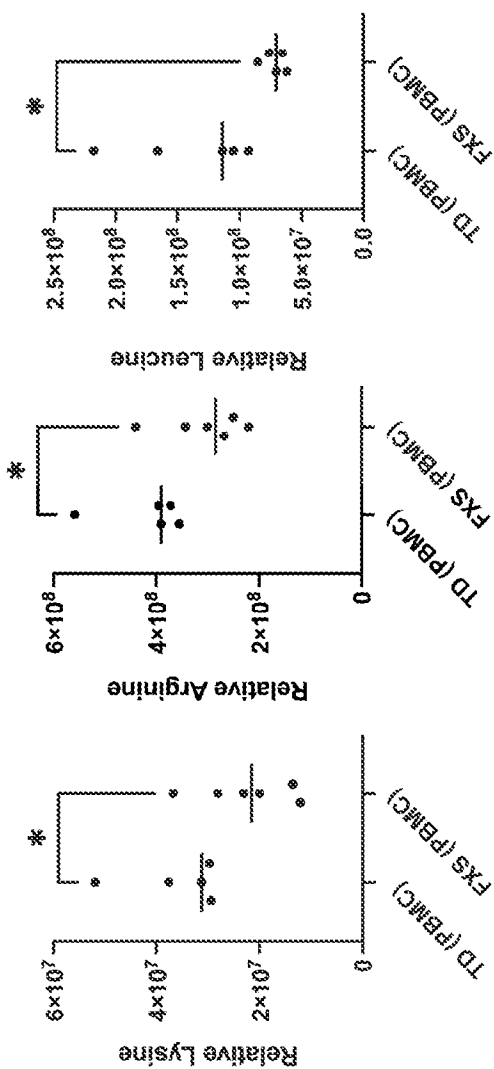


FIG. 4

Lymphoblast Volcano Plot

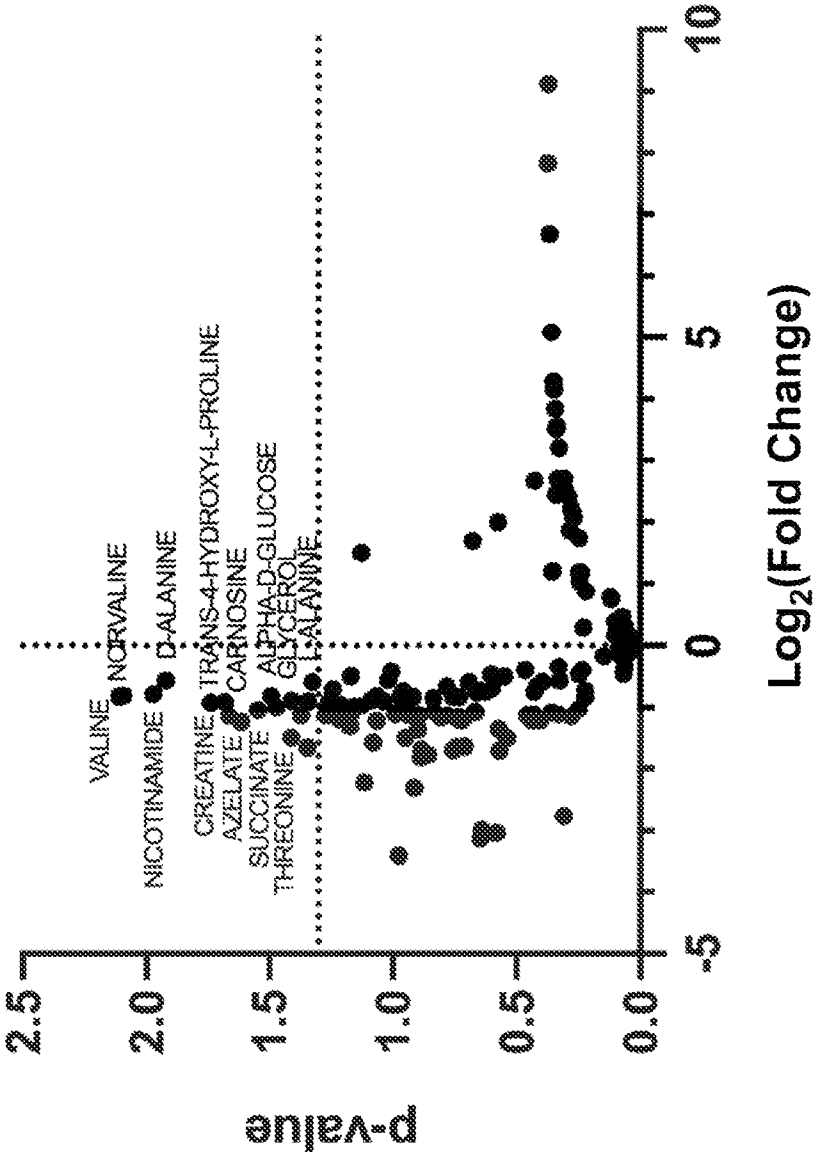


FIG. 5A

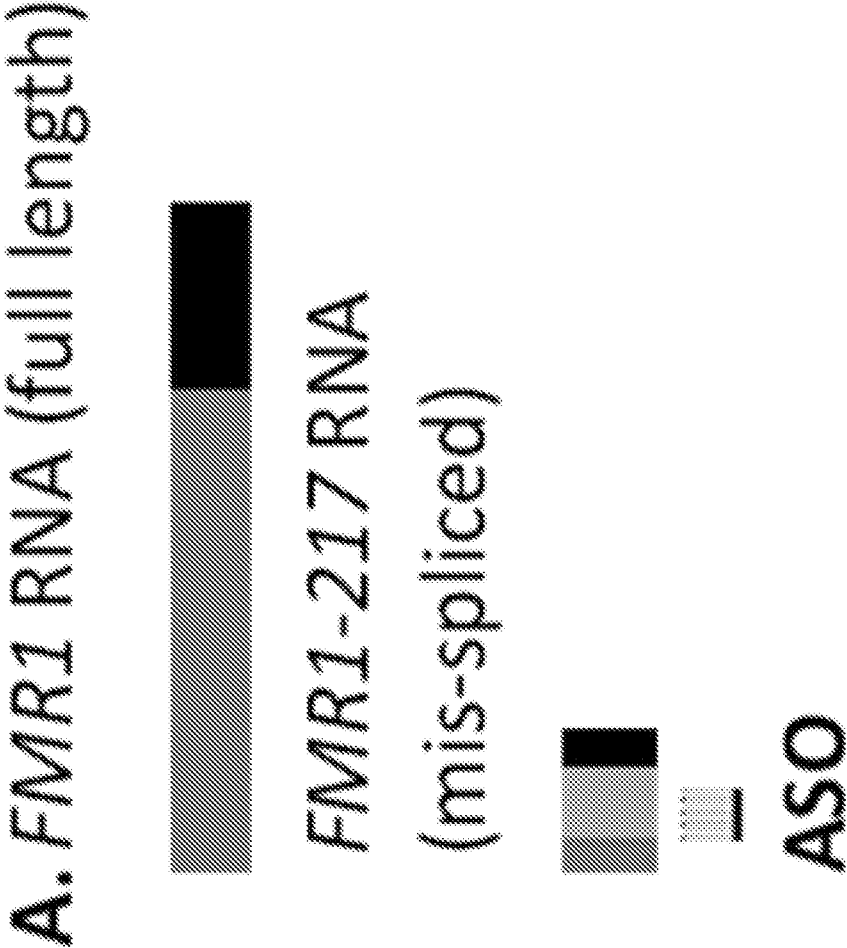


FIG. 5B

B. mis-spliced RNA levels

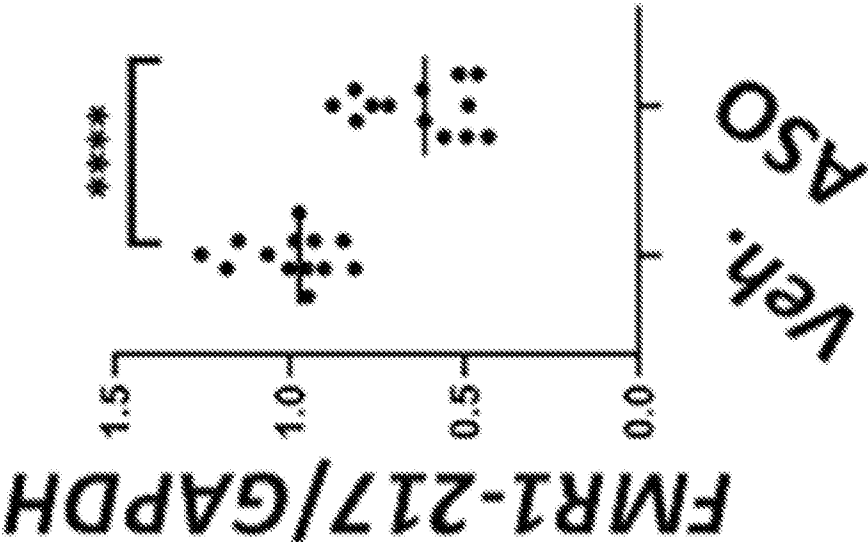


FIG. 5C

C. Full length RNA levels

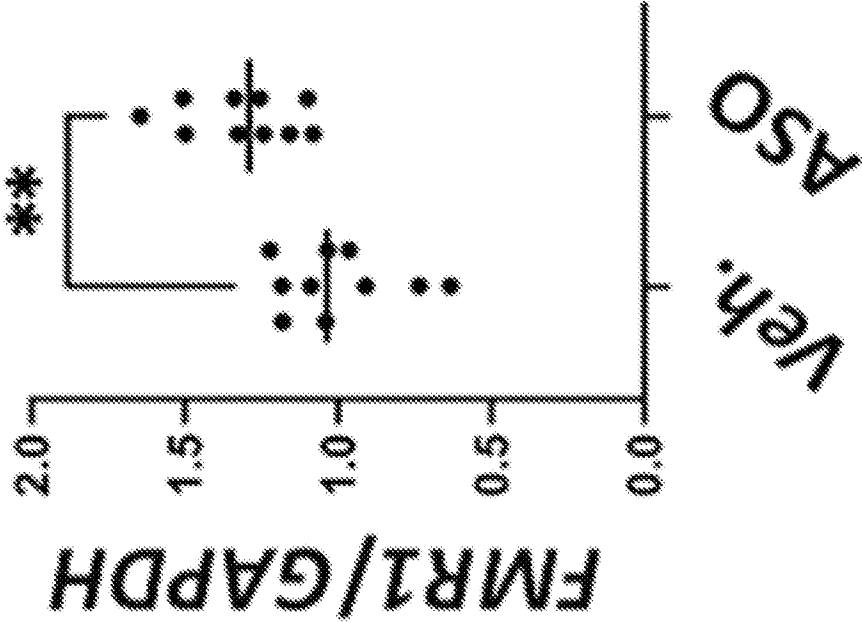


FIG. 5D

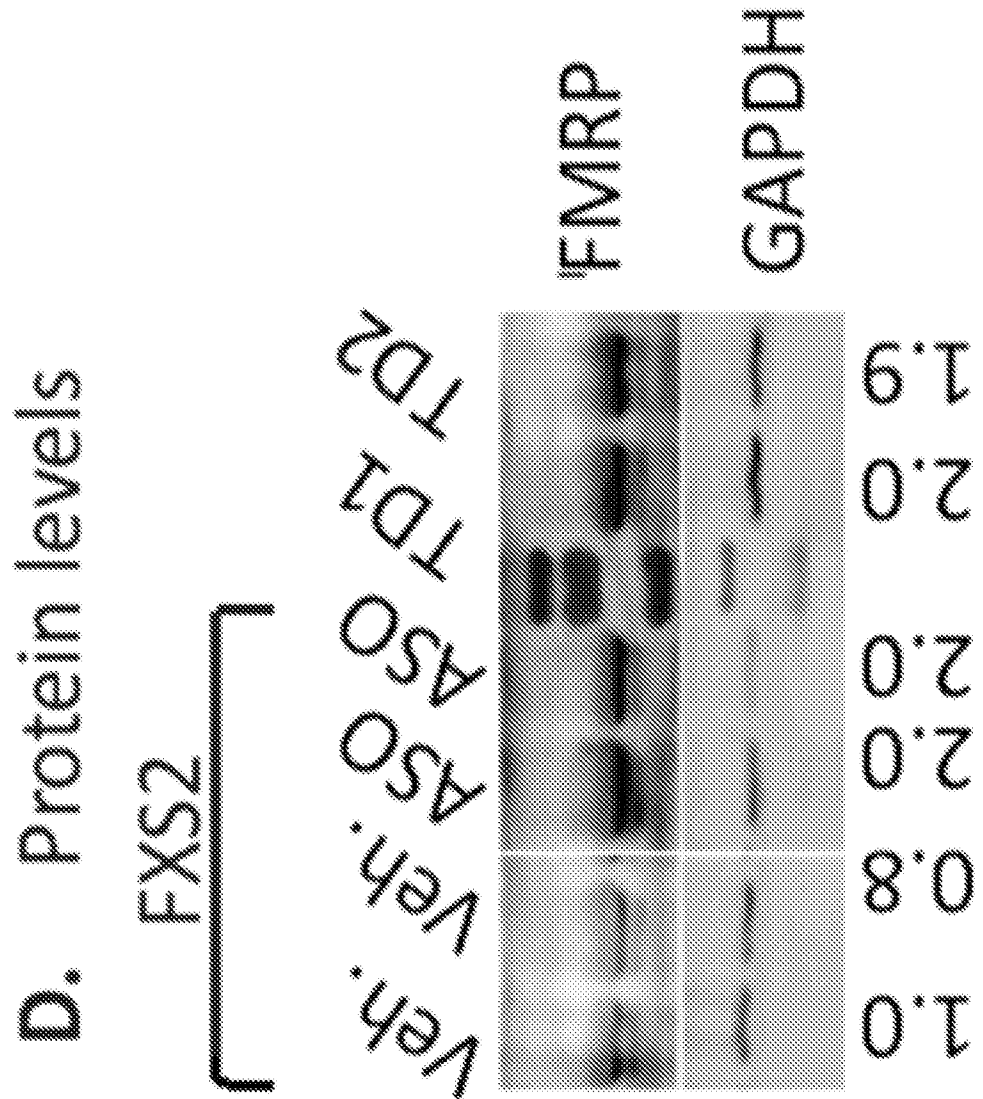


FIG. 6A

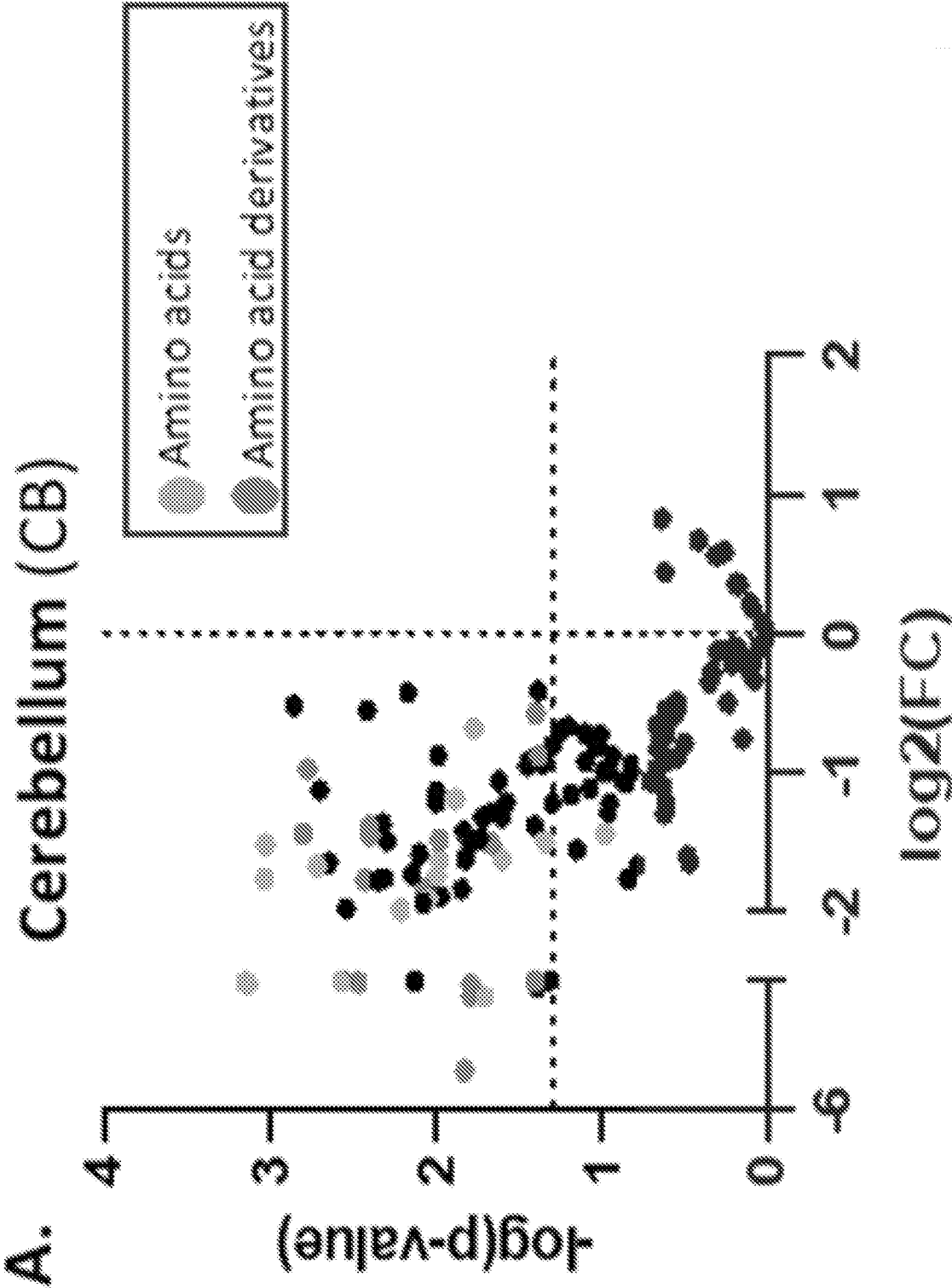


FIG. 6B

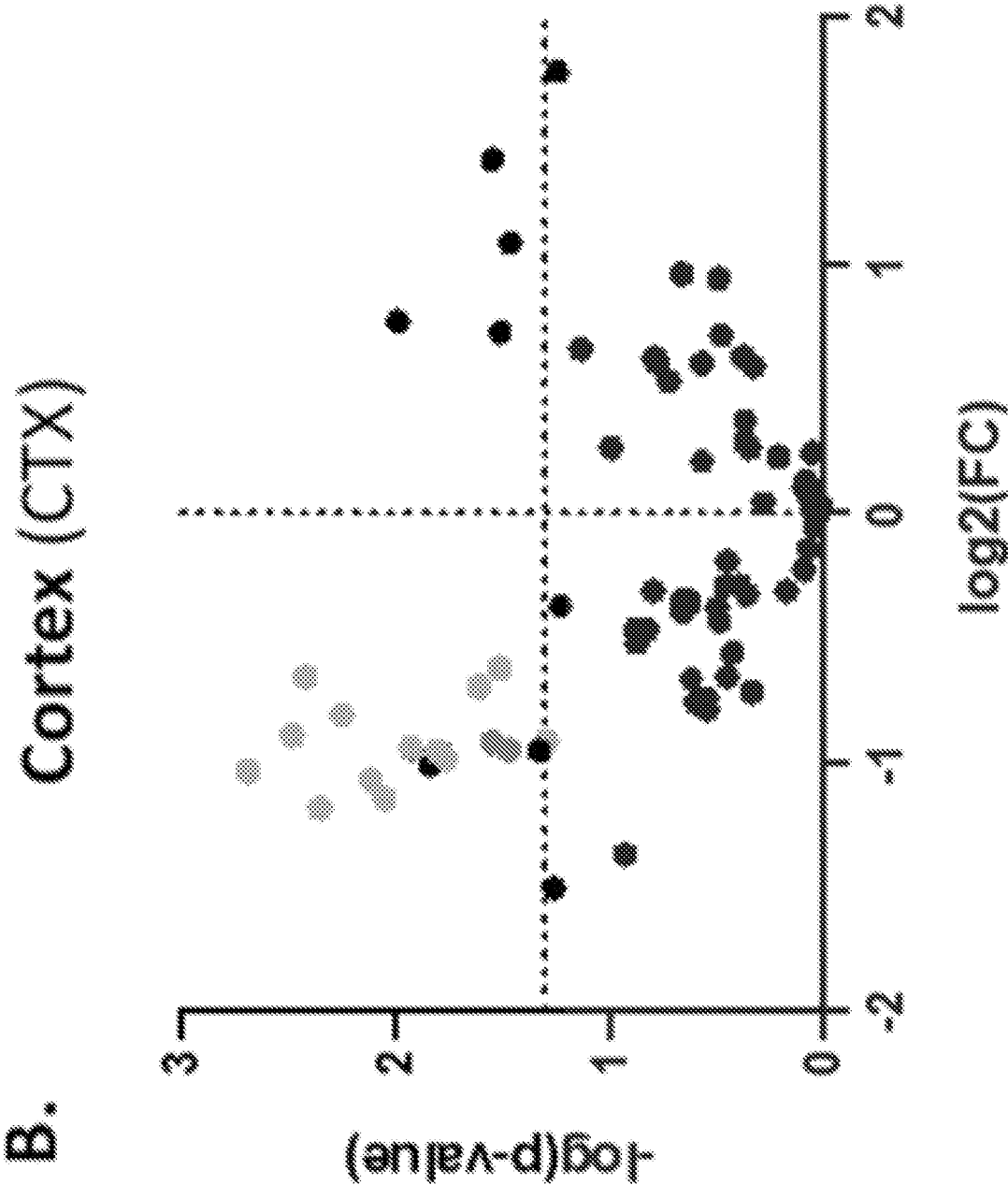
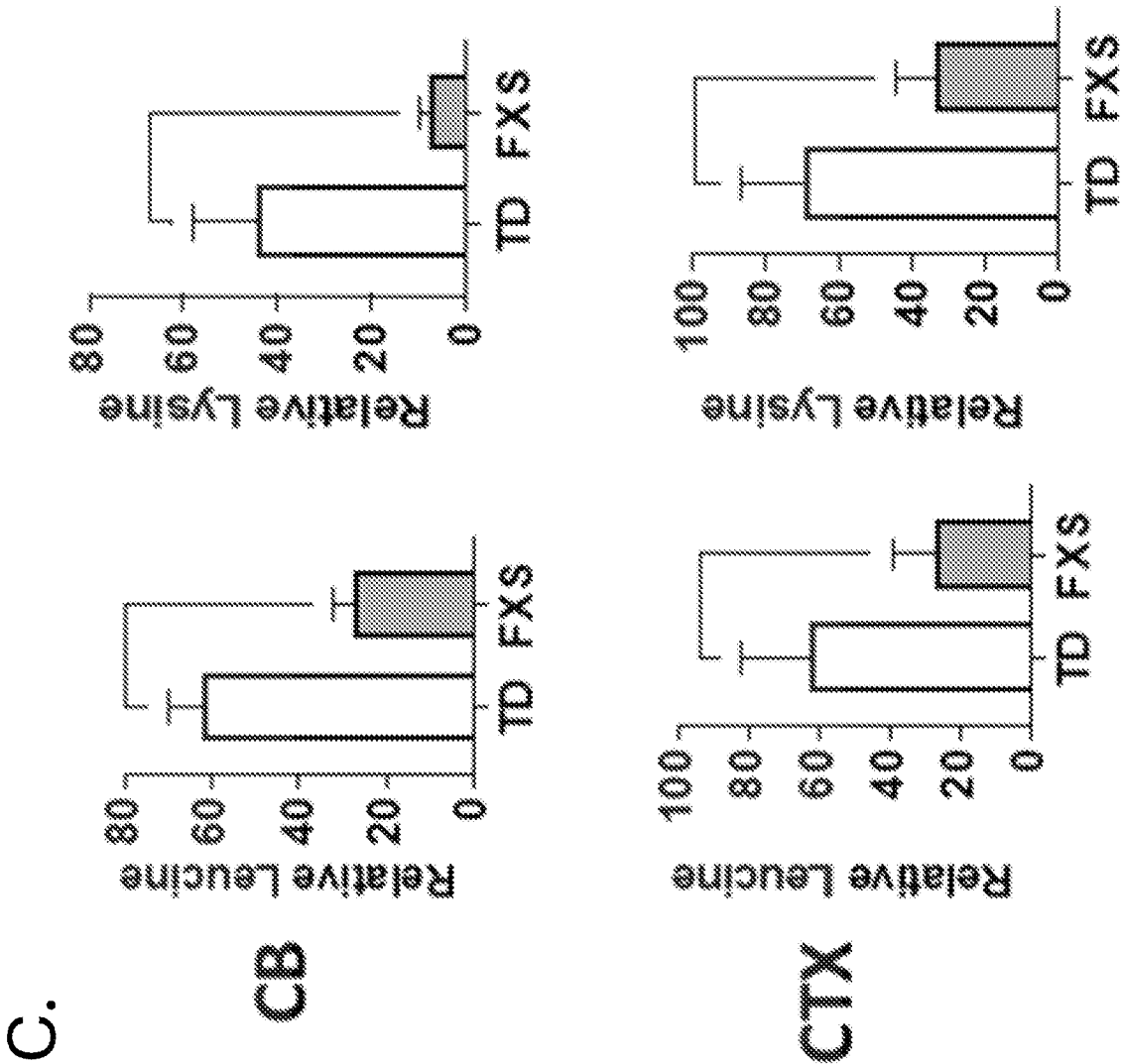


FIG. 6C



D. FIG. 6D

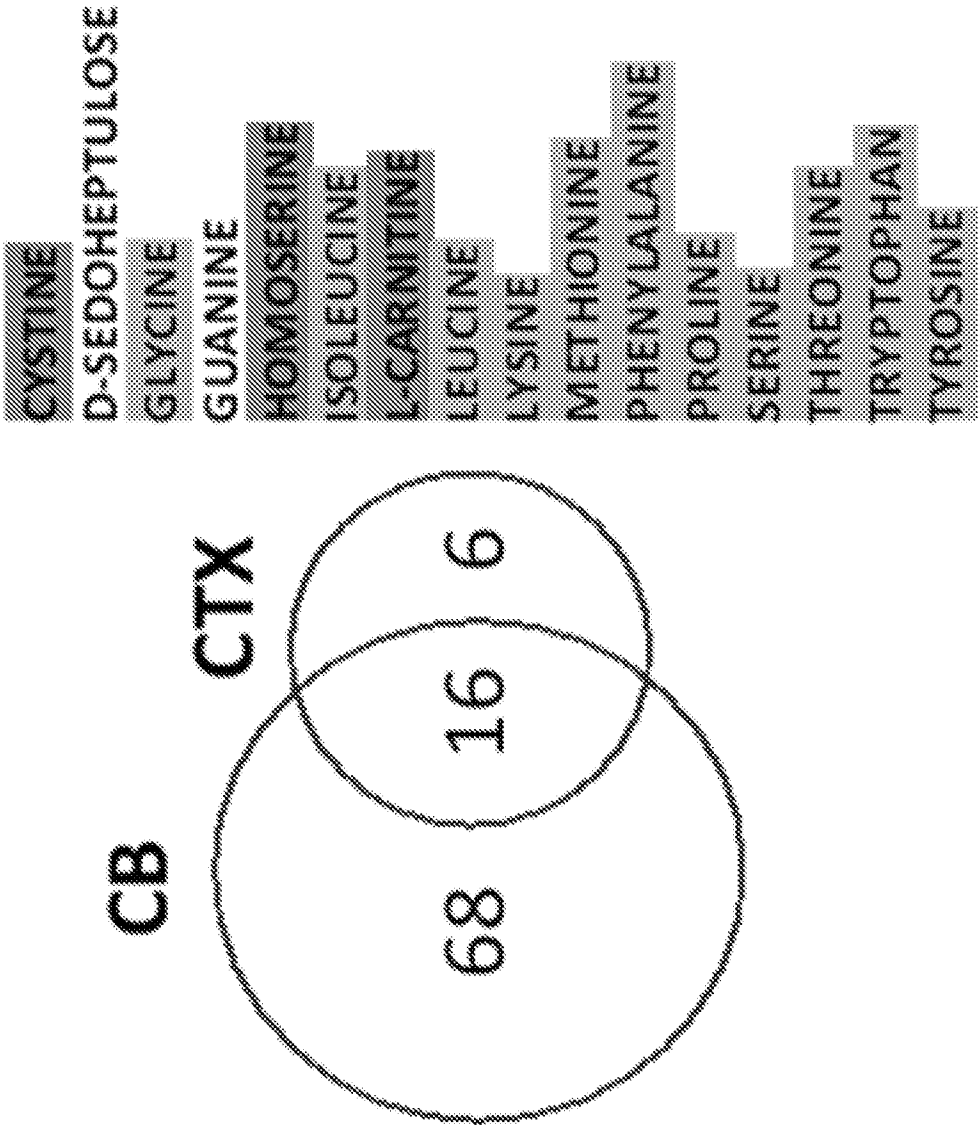


FIG. 7A

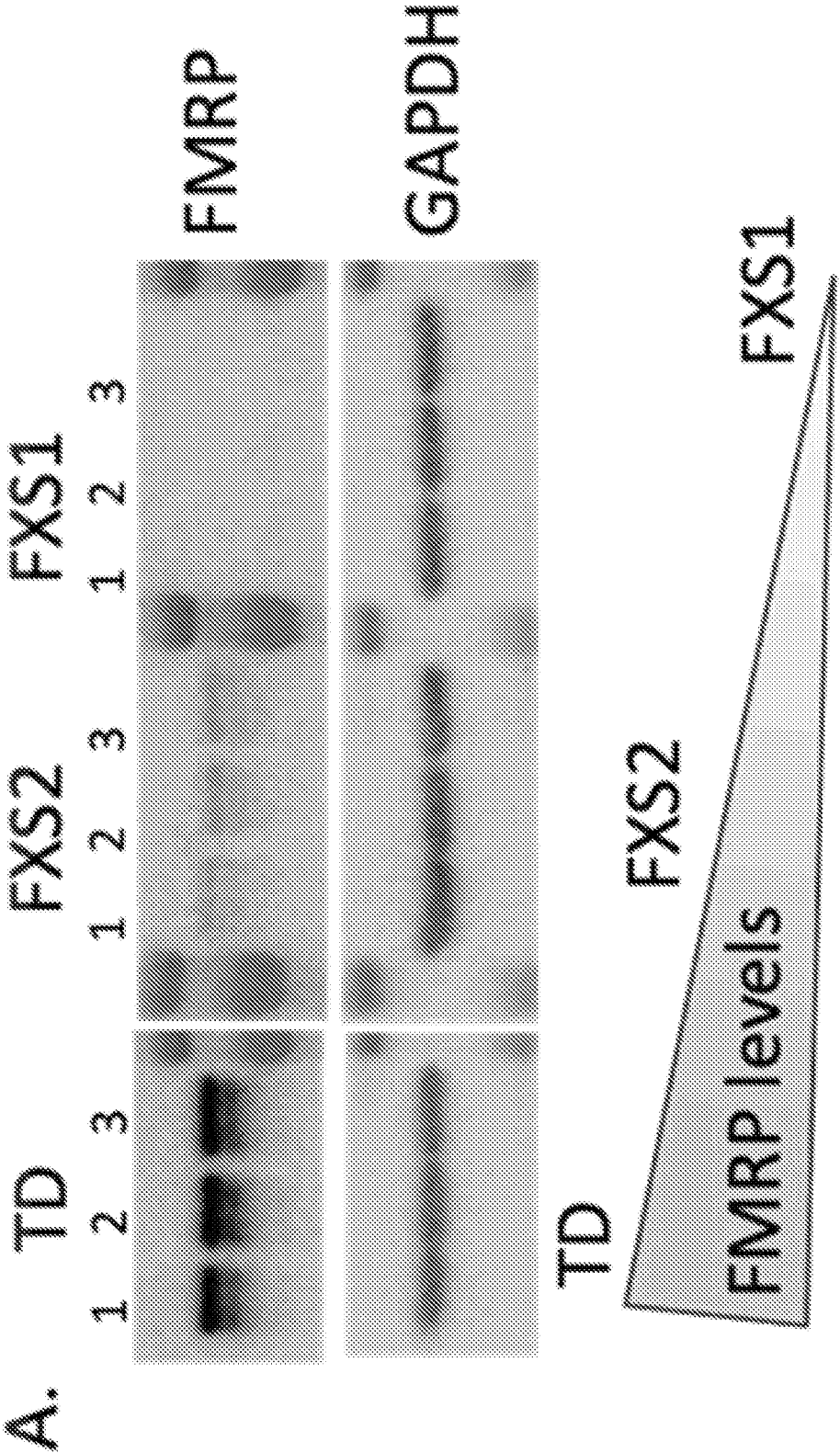


FIG. 7B

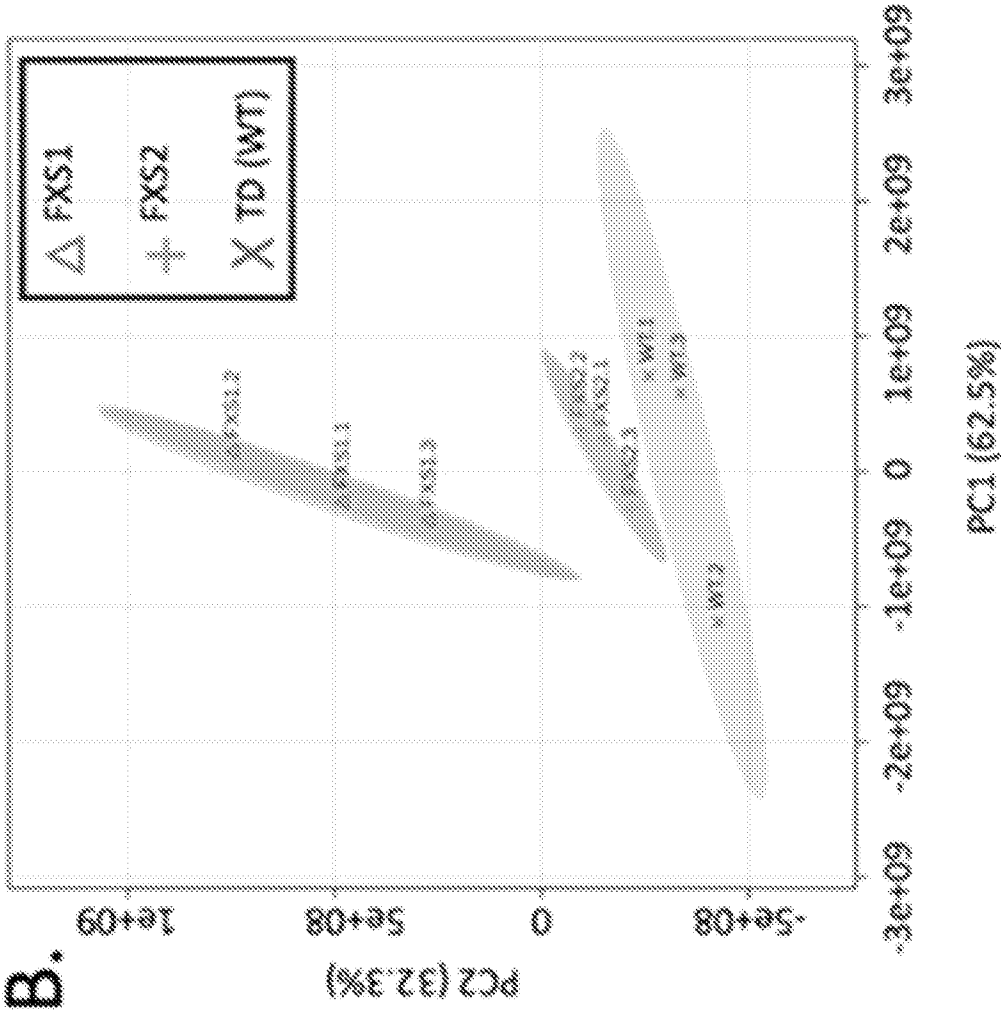


FIG. 7C

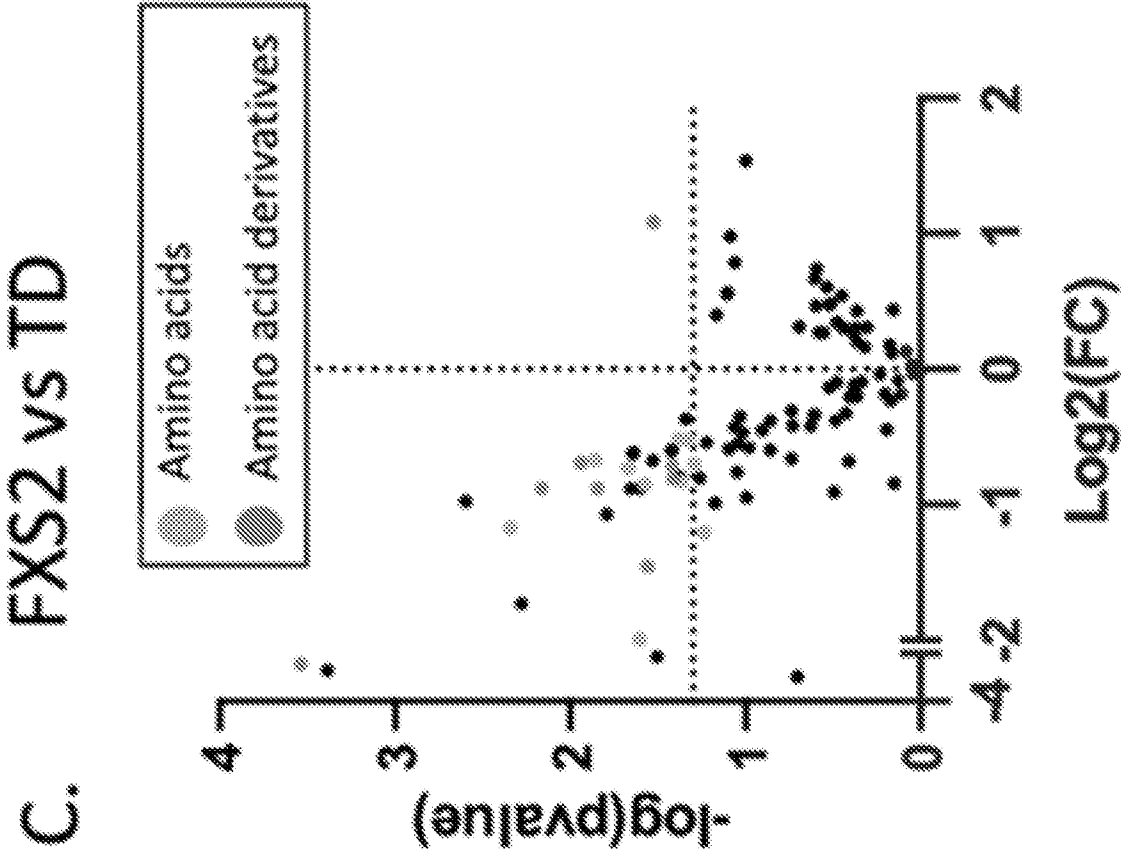


FIG. 7D

D. FXS1 vs TD

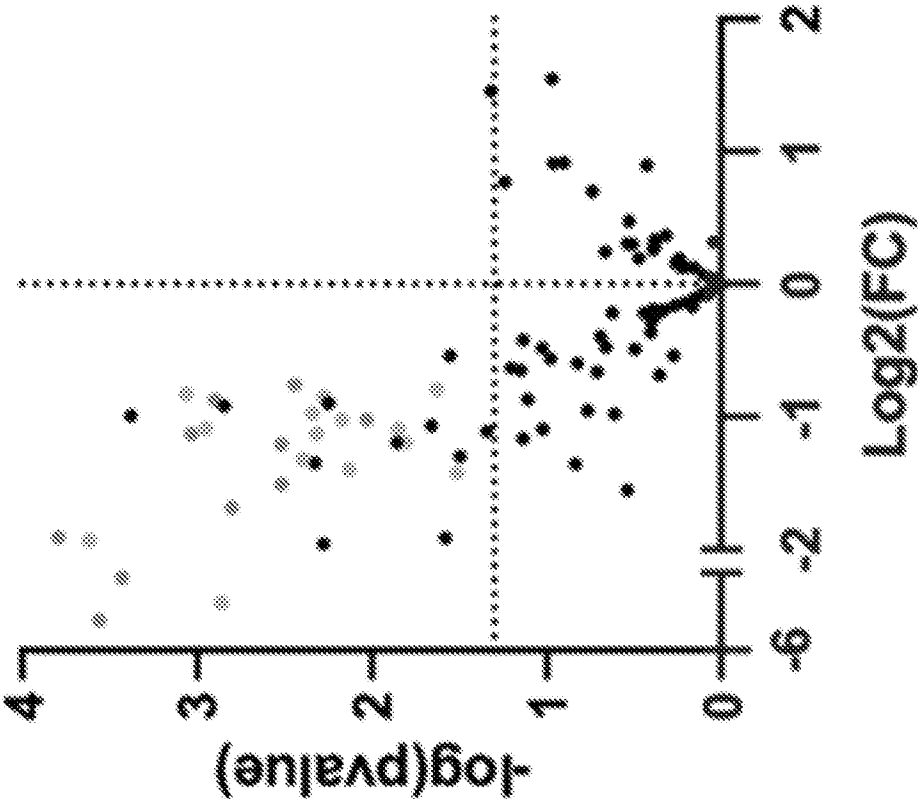


FIG. 7E
E. FXS2 vs FXS1

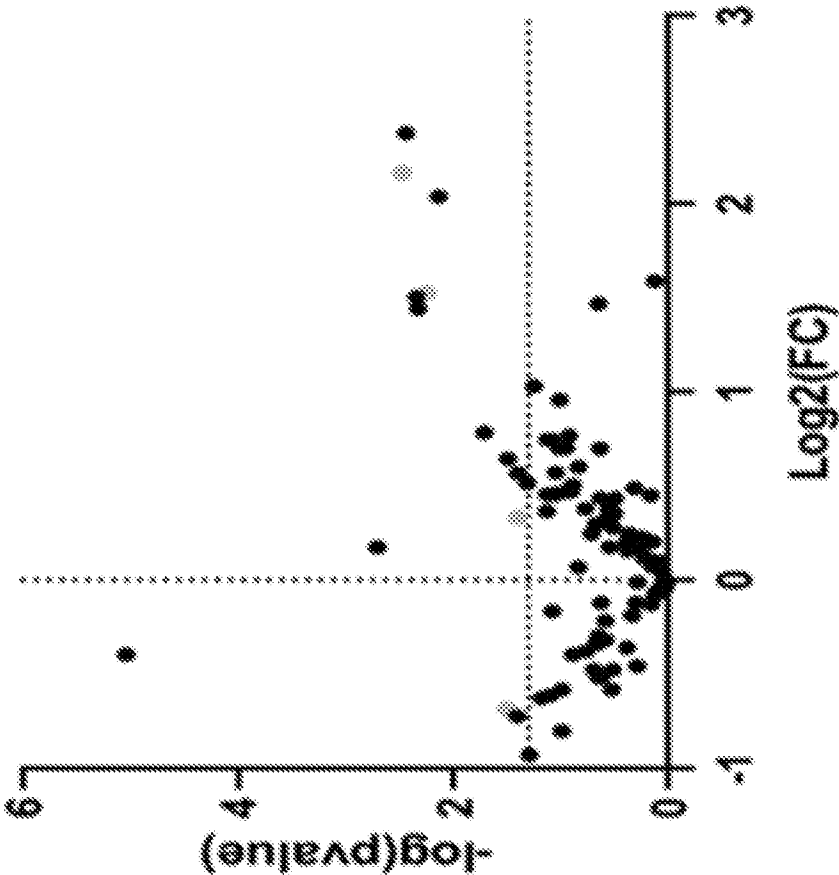
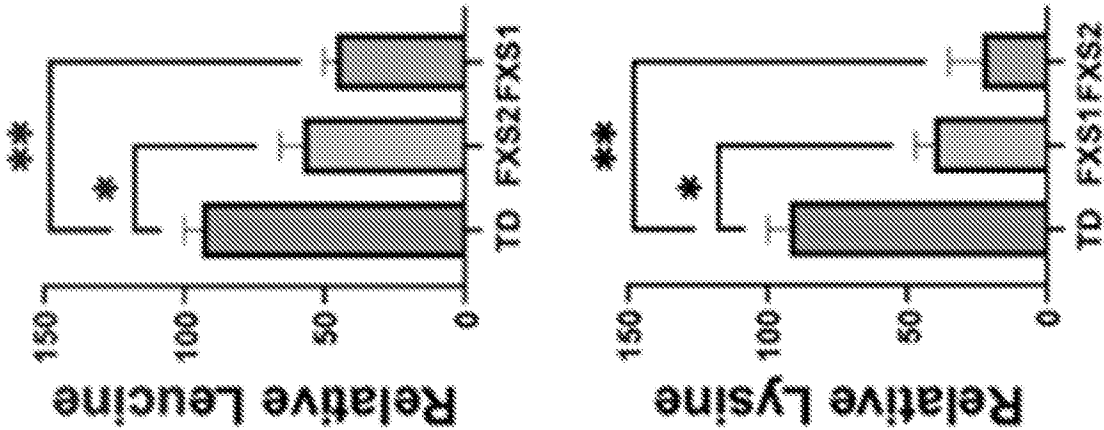
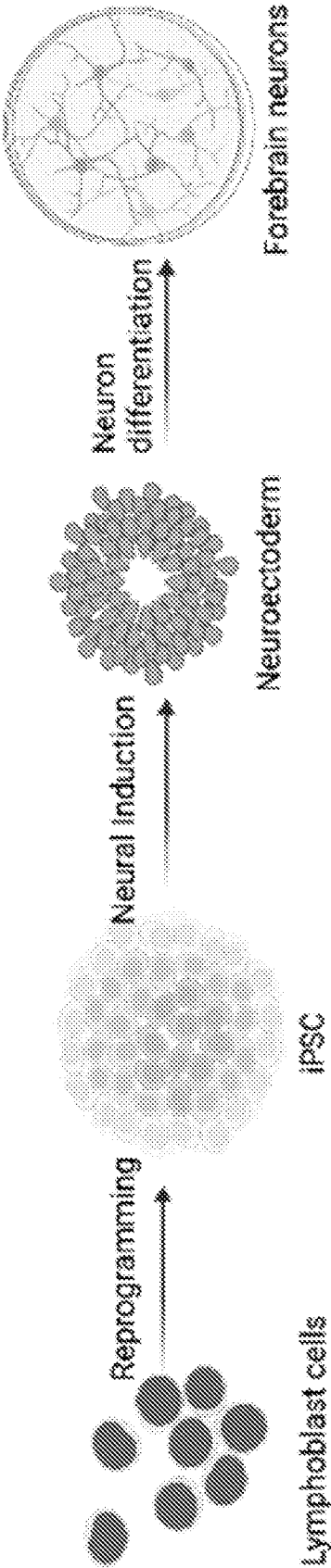


FIG. 7F



F.

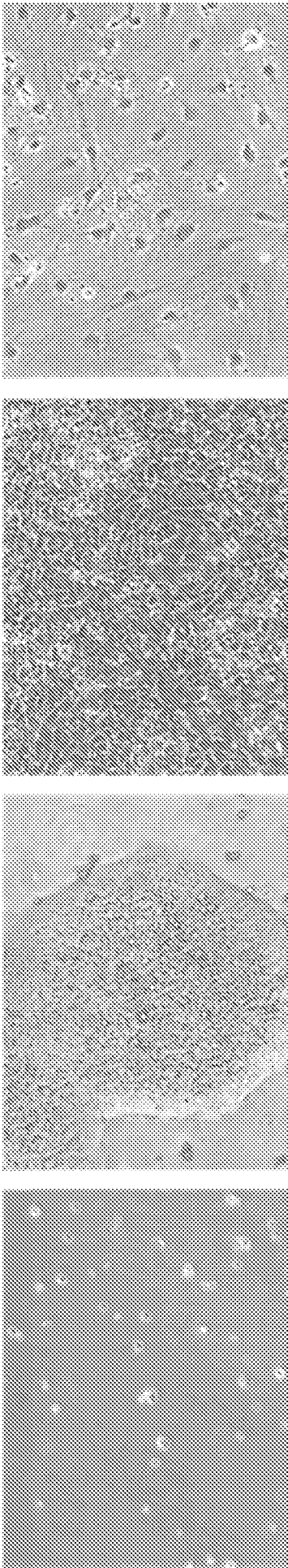
FIG. 8A



A.

FIG. 8B

B.



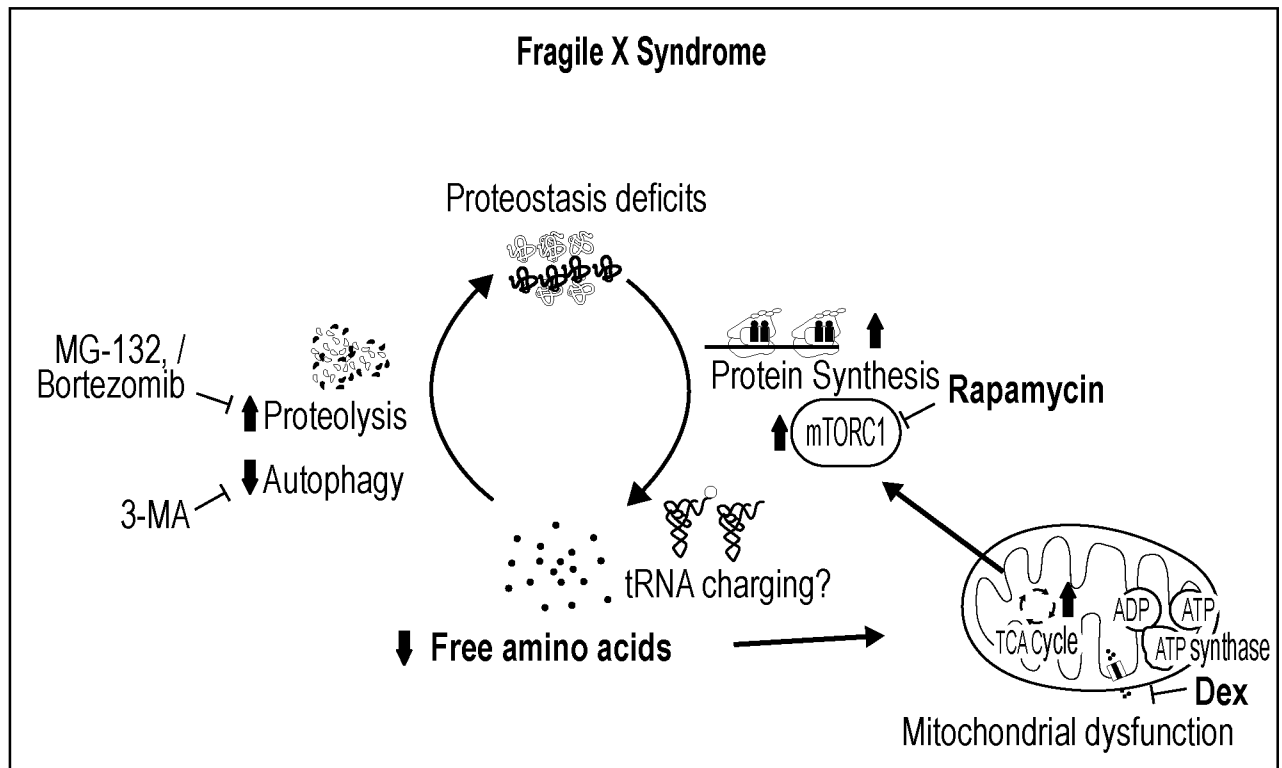


FIG. 9

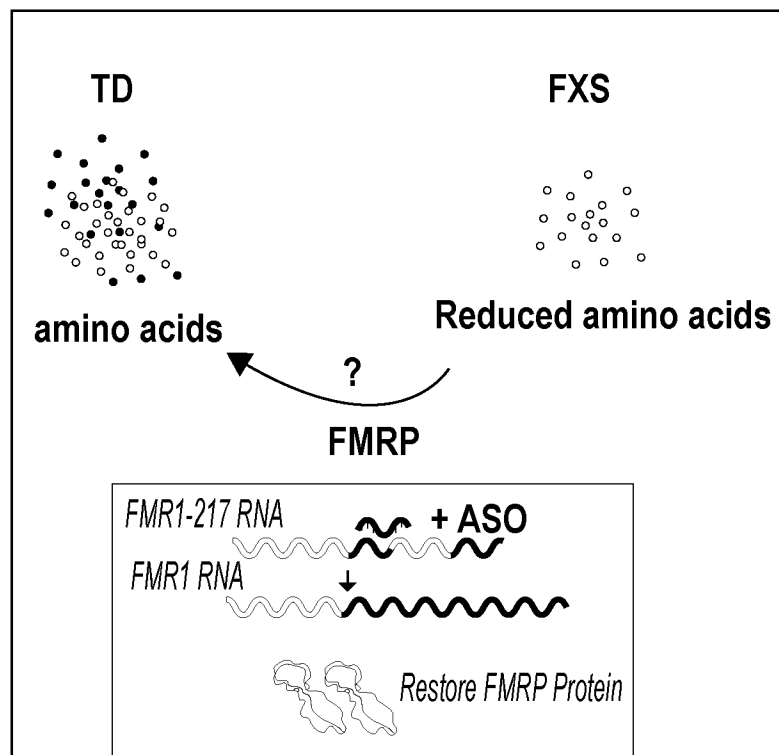
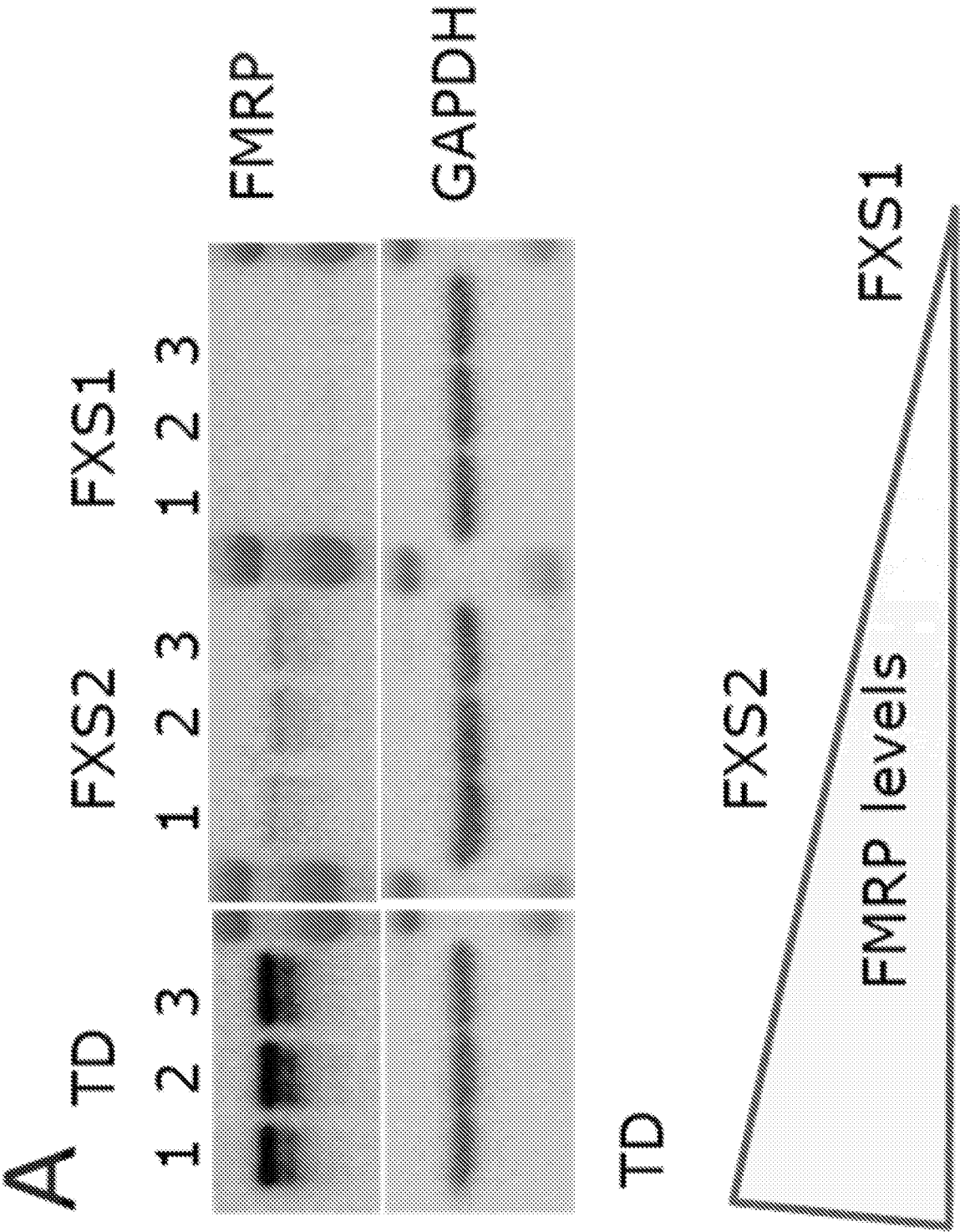


FIG. 10

FIG. 11A



B PCA plot of polar metabolites

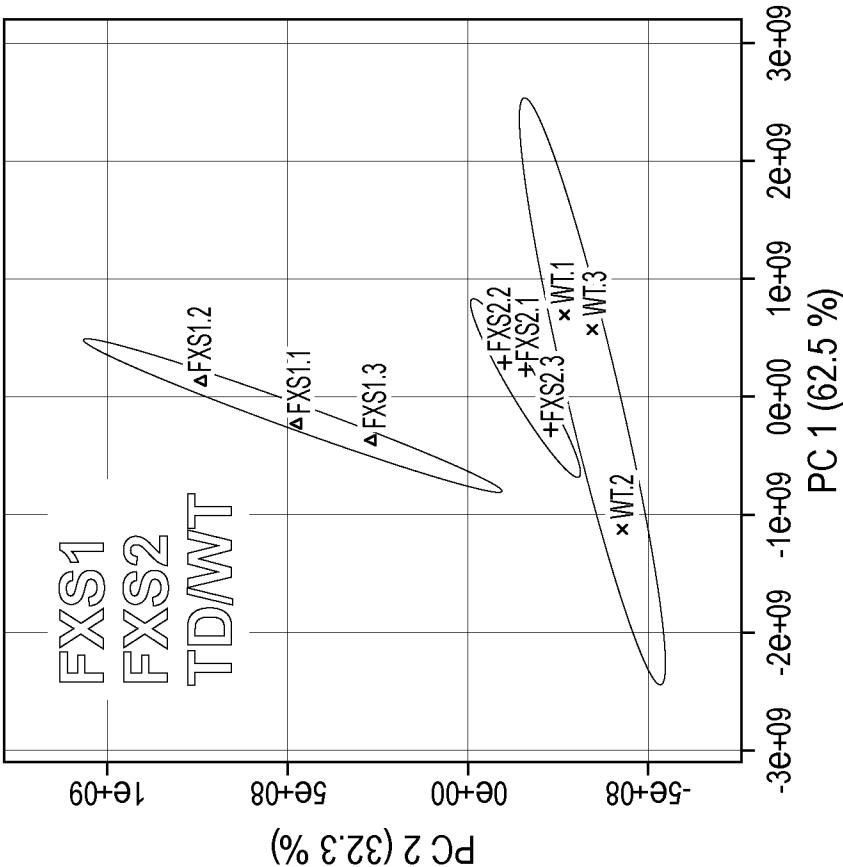


FIG. 11B

FIG. 11C

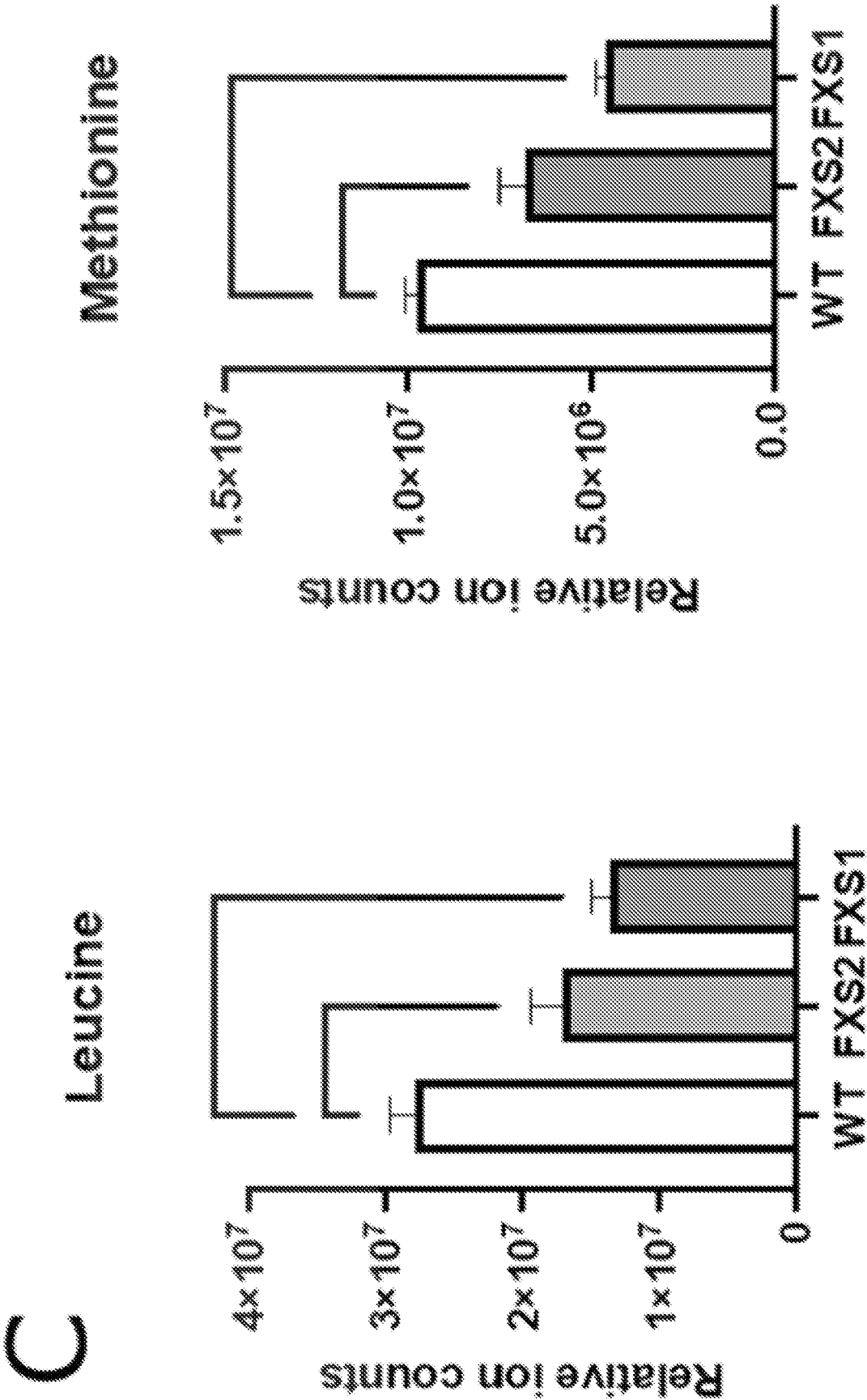
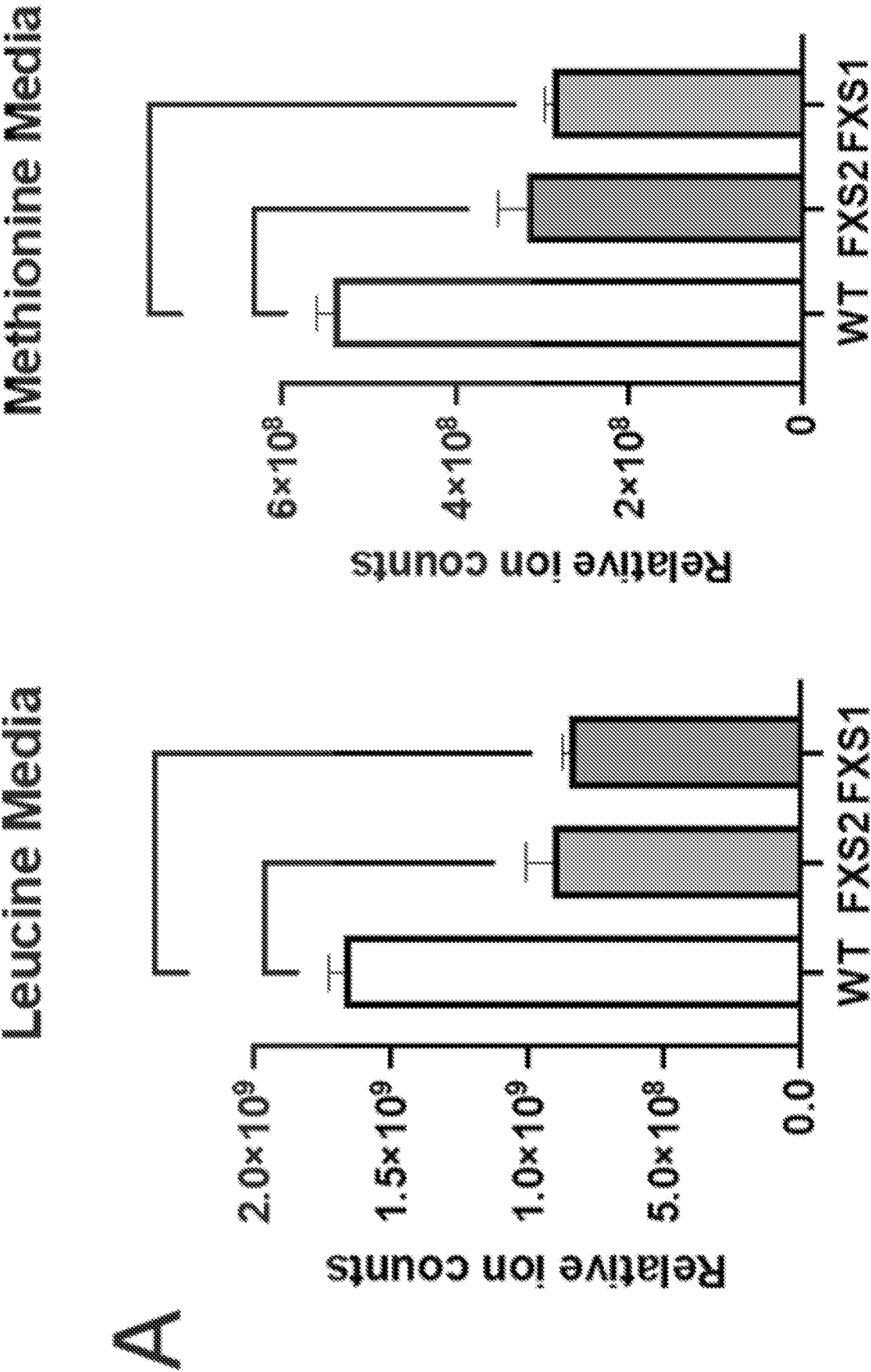


FIG. 12A



B PCA plot: culture media

FXS1
FXS2
TD/WT

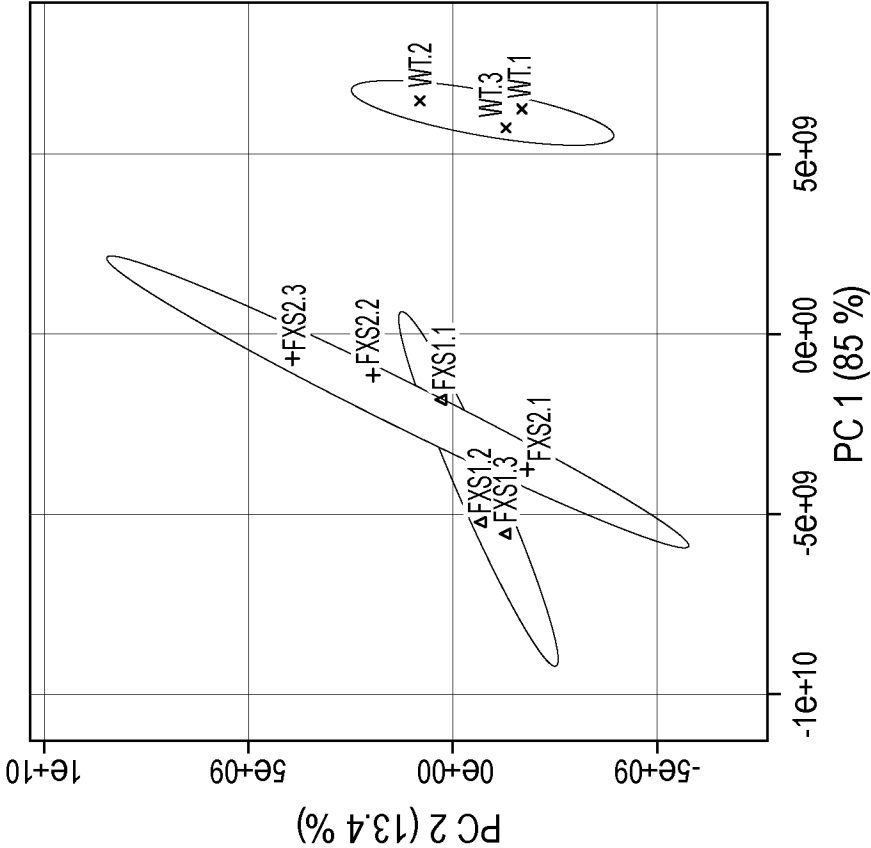


FIG. 12B

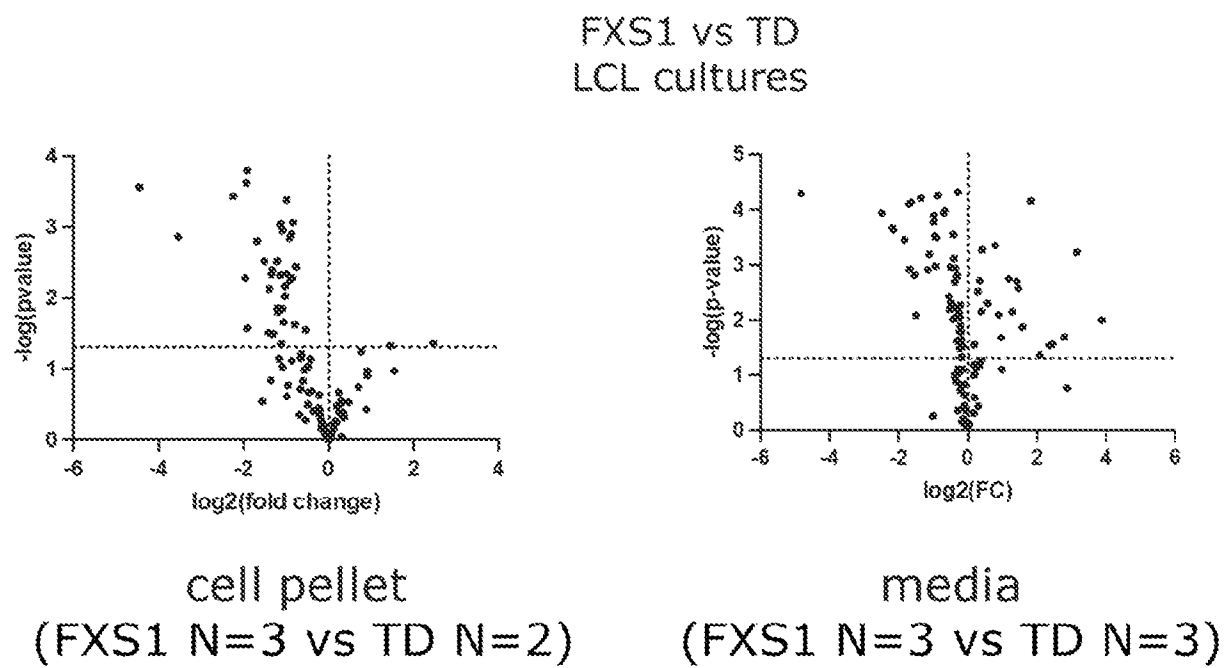


FIG. 13

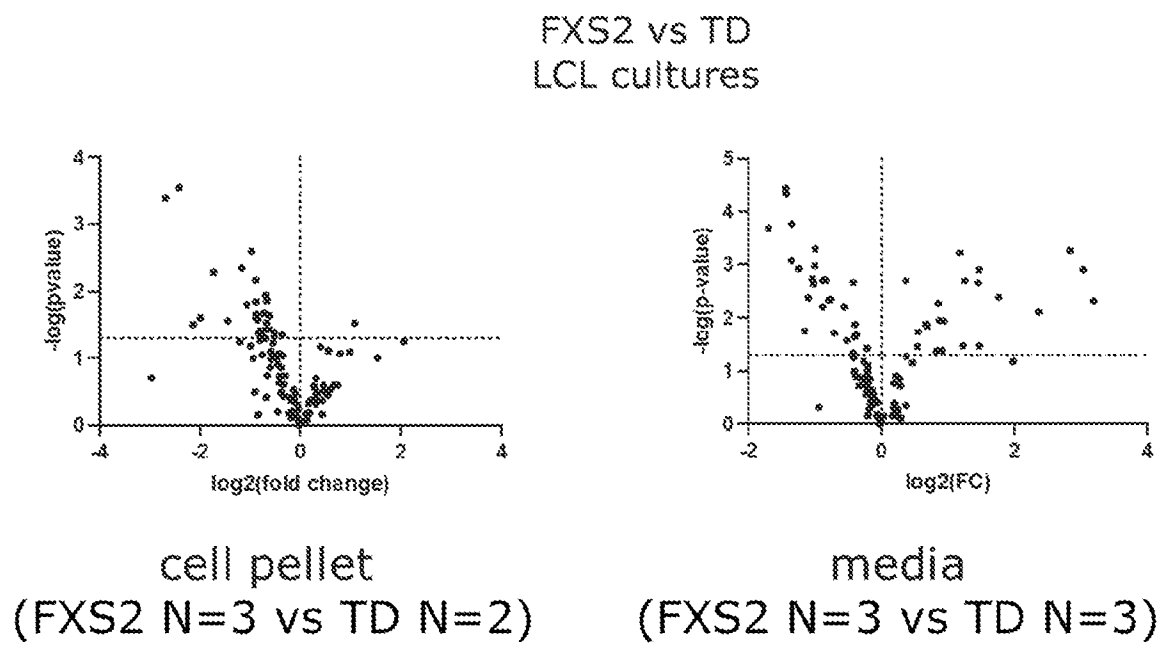


FIG. 14

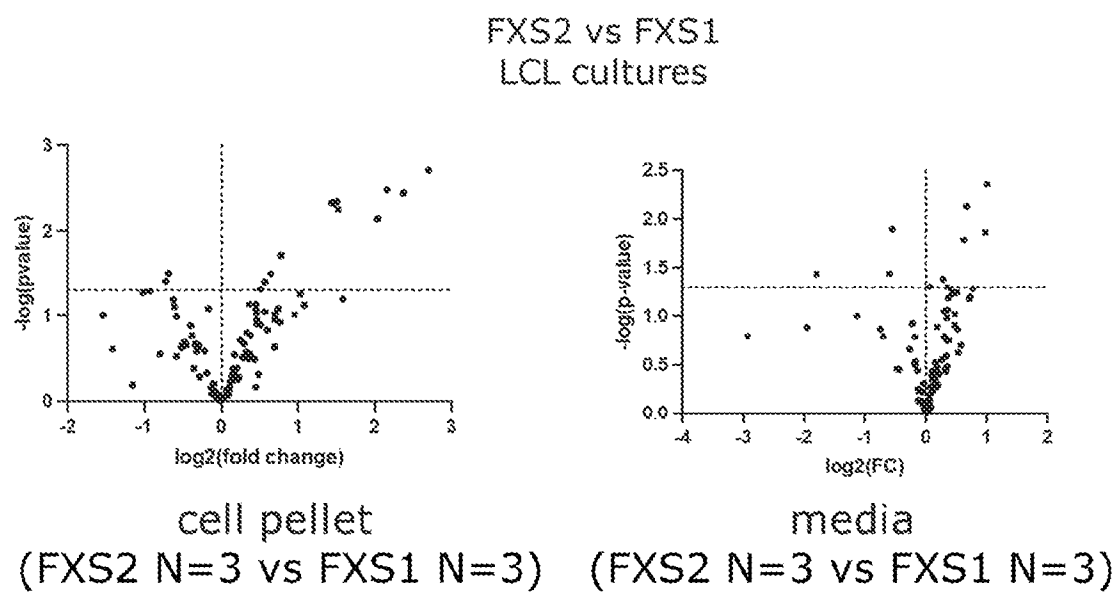


FIG. 15

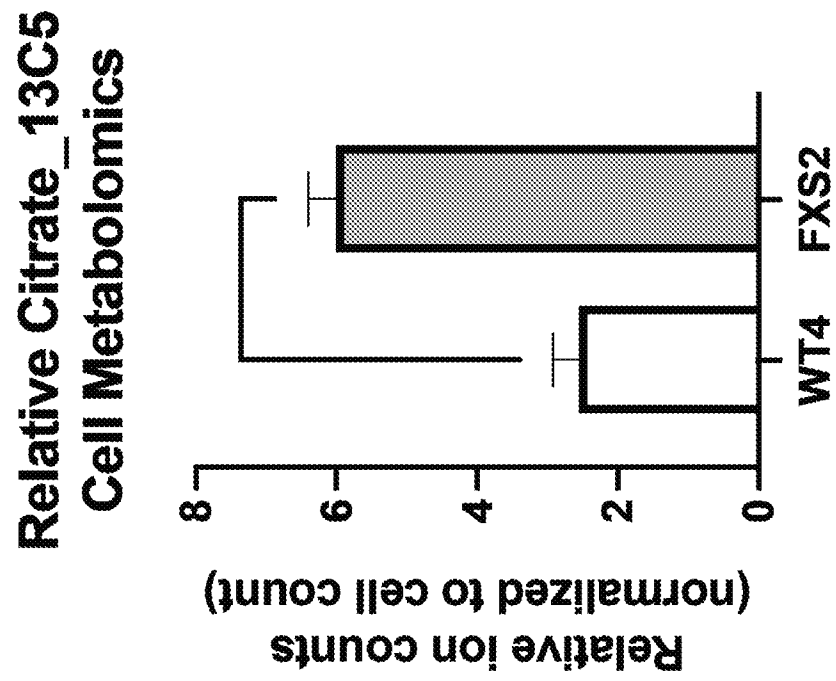


FIG. 16A

%Glutamate_13C5_15N1/%Glutamine_13C5_15N2
Media Metabolomics

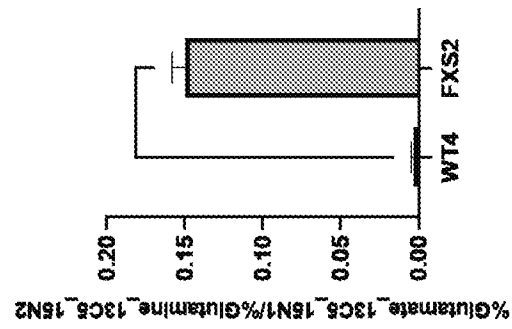


FIG. 16B

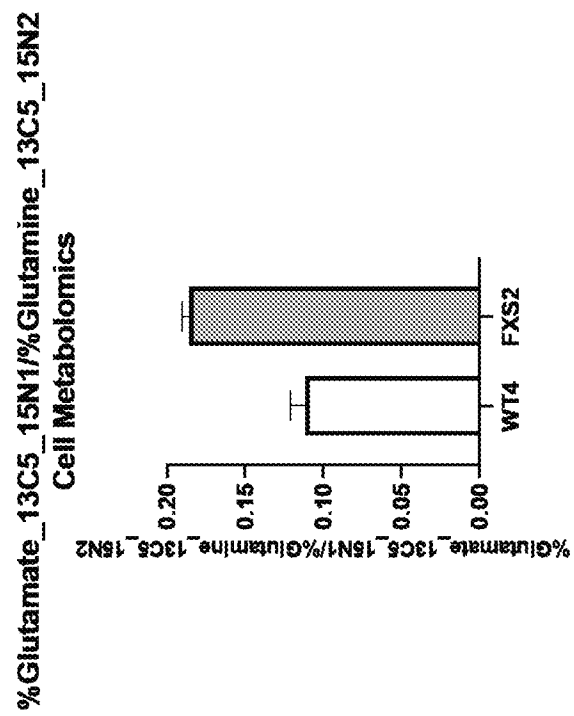


FIG. 16C

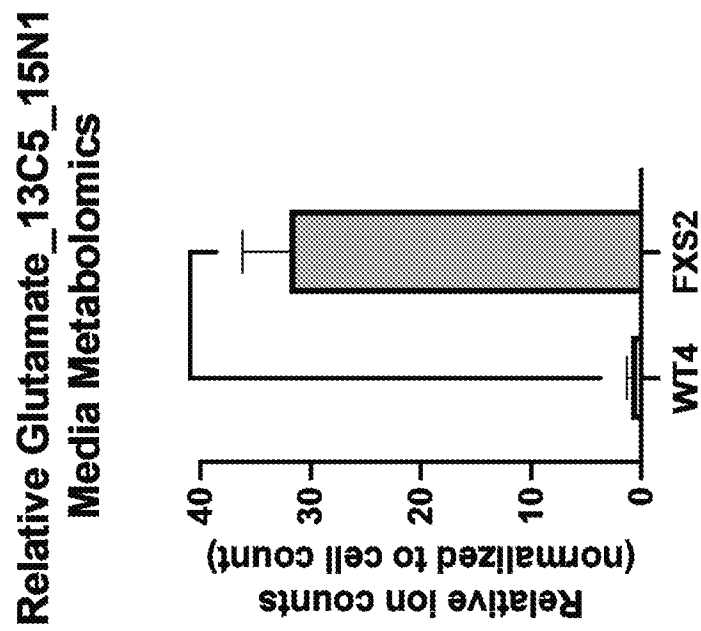


FIG. 16D

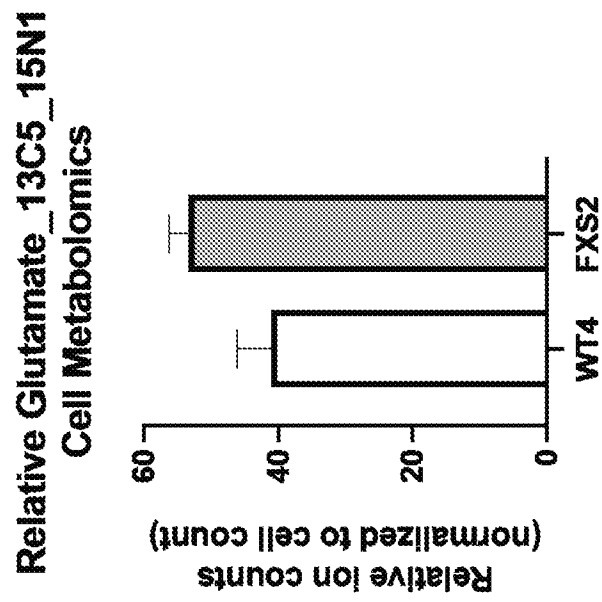


FIG. 16E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 24/36262

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12Q 1/6883 (2024.01)

ADD. A61K 39/395, A61P 25/00, A61P 25/14 (2024.01)

CPC - INV. C12Q 1/6883

ADD. A61K 39/3955, C12Q 2600/156, C12Q 2600/158

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.
Y	WO 2023/122802 A2 (UNIVERSITY OF MASSACHUSETTS et al.) 29 June 2023 (29.06.2023) Abstract; Claim 1; Claim 18; para [00285]	1-3
Y	US 2019/0231871 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 1 August 2019 (01.08.2019) Abstract; Claim 1; para [0041]; 0042]; 0045]	1-3



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

"D" document cited by the applicant in the international application

"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

"I" 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

7 September 2024 (07.09.2024)

Date of mailing of the international search report

SEP 23 2024

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

Kari Rodriguez

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 24/36262

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 24/36262

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.: 4-20
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.