Protocol Title: A Double-Blind, Placebo-Controlled Trial of Metformin in Individuals with Fragile X Syndrome (FXS)
NCT Number: NCT03862950
Protocol Version Date: 1 November 2022

1) **Protocol Title:** A Double-Blind, Placebo-Controlled Trial of Metformin in Individuals with Fragile X Syndrome (FXS)
Nominated Principal Investigator: Dr. Francois Bolduc, MD PhD FRCPC
Sub Investigators for University of Alberta: Dr. Janette Mailo, MD PhD FRCPC, Dr. Jeffrey Jirsch, MD FRCPC, and Dr. Brenda Clark, MD FRCPC
Canadian Co-Principal Investigators (Co-PIs): Dr. Sebastien Jacquemont, MD, Dr. Sarah Lippé, Ph.D.
Study Sponsor: The Governors of the University of Alberta
Funding Sponsor: Women and Children's Health Research Institute, CHU Sainte-Justine Foundation, and the Azrieli Foundation
Phase: Clinical Trial Phase 2
Sites/Facilities enrolling participants: Stollery Children's Hospital (Edmonton, AB), CHU Sainte-Justine (Montreal, QC)
Study population: 120 children and adults (aged 6-35 years), with FXS and controls
Study duration: estimated duration of 3 years, starting in Spring 2019
Participant duration: 4 months
Protocol Version Date: 1 November 2022
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2) **Objectives**

1. Assess safety and tolerability of metformin in individuals with FXS ages 6 to 35 who are treated over a 4-month period (and an additional 2 years if they participate in the open label extension).
2. Assess the benefit of metformin in the treatment of language deficits, behavior problems, and obesity/excessive appetite in individuals with FXS over a 4-month period (and an additional 2 years if they participate in the open label extension).
3. Assess the utility of innovative outcome measures, including measures of brain processing (event related potential habituation paradigm and social gaze monitoring using the Tobii Eye Tracker), language/cognitive measures (NIH Toolbox cognitive battery measures and expressive language sampling), a measure of memory (The Memory Game), behavioral and quality of life measures [Aberrant Behavioral Checklist–Community (ABC-C) fragile X version, Child Sleep Habits Questionnaire (CSHQ), Anxiety Depression and Mood Scale (ADAMS) Swanson, Nolan and Pelham questionnaire (SNAP-IV), and Pediatric Quality of Life Questionnaire (PedsQL) Parent Proxy], EuroQol-5D (EQ-5D-3L or EQ-5D-Y), Sensory Profile-2 (up to 15 years old) or Sensory Profile Adolescents/Adults (11 years and older), and Care Related Quality of Life (CarerQoL)], a food questionnaire (Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24)), in addition to standard measures of outcome [Clinical Global Impression Scale-Severity (CGI-S), Clinical Global Impression Scale-Improvement (CGI-I), Visual Analog Scales (VAS), and Vineland

Adaptive Behavior Scales–Third Edition (for English speakers) and Second Edition for French speakers)].

4. Assess molecular biomarkers that may predict which patients may be likely to benefit from metformin treatment. A panel of molecular biomarkers, including FMRP level, MMP9, S6 Kinase, EIF4E, *CYFIP1* mRNA, CYP450 allelic variants, and metabolome variants, genomic variants, proteomics and mRNA sequencing will be studied.

3) Background

FXS is the leading monogenic cause of intellectual disability and autism spectrum disorder. Treatment for FXS is currently non-pharmacological or symptomatic interventions. FXS is associated with an increase in body mass index (McLennan et al., 2011), and a subgroup of patients with FXS develops severe hyperphagia, obesity, and hypogonadism or delayed puberty. This presentation has been described as the Prader-Willi phenotype of FXS (PWP) (Nowicki et al., 2007), as it is similar to those with the Prader-Willi syndrome caused by a deletion at 15q11-q13. Unlike Prader-Willi syndrome, the FXS-PWP does not have a deletion of 15q11-q13 or chromosome 15 maternal uniparental disomy (McLennan et al., 2011). However, *FMR1* protein (FMRP) binds to cytoplasmic interacting *FMR1* protein (CYFIP1), a protein that affects synaptic remodeling, including FMRP protein translation (Schenck et al., 2001); CYFIP1 is localized to 15q, the critical region in Prader-Willi-syndrome (Chai et al., 2003). The level of CYFIP1 mRNA expression has been found to be lower in those with the PWP compared to FXS without the PWP (Nowicki et al., 2007), so this appears to be a molecular correlate of the obesity in the PWP. One of the biomarkers to be evaluated in this proposal will be CYFIP1 mRNA expression levels that may change with metformin treatment in those with or without the PWP of FXS.

Metformin is a type 2 diabetes medication that can also improve obesity and excessive appetite. The Food and Drug Administration approved metformin for its effects in lowering blood glucose levels in patients with noninsulin-dependent, type 2 diabetes. Some studies report minimal to moderate decrease in weight in groups of patients with insulin resistance (Klein et al., 2006). Metformin has been shown to be effective and safe in decreasing weight gain associated with atypical antipsychotic use and is well tolerated by children and adolescents with ASD aged 6-17 years old, with up to 500mg twice daily for children aged 6-9 years and 850 mg twice daily for those 10-17 years old (Anagnostou et al., 2016). It has also been utilized for the treatment of obese children and adults who do not have type 2 diabetes with a short-term reduction in BMI (Park et al., 2009; Klein et al., 2006). Metformin is also effective in glycemic control in patients who are not obese (Ong et al., 2006). There is little to no risk of hypoglycemic episodes in the use of metformin because its mechanism of action does not directly stimulate insulin (Bolen et al., 2007; Bodmer et al., 2008).

Metformin has emerged as a candidate drug for the targeted treatment of FXS based on animal studies showing rescue in the FXS model (Monyak et al., 2016; Weisz et al., 2015; Gantois et al., 2017). The fly, Drosophila melanogaster, has a homologue of *FMR1* called dfmr1. The Drosophila FXS model has shown that drosophila insulin-like peptide 2 (Dilp2) in insulin-producing cells results in elevated insulin signaling via the PI3K/Akt/mTOR pathway. The dysregulated insulin signaling in this fly model of FXS leads to defects in circadian rhythm and short- and long-term memory deficits. Use of metformin in this study had been able to rescue and restore memory deficits (Monyak et al., 2016). Further studies performed in the Sonenberg lab (Gantois et al., 2017) showed that adult treatment in the FXS knock-out mouse rescued multiple phenotypes, including social novelty, grooming, dendritic spine morphology, and electrophysiology in eCA1 of the hippocampus. Metformin may contribute to normalizing signaling pathways in FXS in the central nervous system, which may include activities of ERK, mTOR and PI3K, which have shown to be pathogenically overactive in FXS.

In addition, metformin inhibits phosphodiesterase, which would lead to correction of cAMP levels. Moreover, metformin inhibits MMP9 production, which is also elevated in FXS (Hoeffer et al., 2012; Muzar et al., 2016; Dziembowska et al., 2013), and MMP9 as a biomarker will be assessed in this proposal. Looking at the potential signaling pathways, metformin appears to be a good candidate for targeting several of the intracellular functions in neurons disrupted in FXS and therefore possibly rescuing several types of symptoms in individuals with FXS.

Dr. Hagerman's team (MIND Institute) has utilized metformin in the clinical treatment of over 20 individuals with FXS between the ages of 4 and 58 years and have found benefits not only in lowering weight gain and normalizing appetite, but also in language and behavior with doses up to 1000 mg twice a day (Dy et al., 2017). Dr. Hagerman's team has recently reported in Dy et al. (2017) the details of the first 7 patients treated clinically for whom pre and post Aberrant Behavior Checklist results were obtained and documented improvement in weight, language, and behavior. This medication has been well tolerated, even in the 4-year-old patient with FXS, and it is time now to carry out a controlled trial.

Considering these promising preliminary results and the established safety of metformin in children, we propose to carry out a randomized, placebo-controlled trial of metformin to further assess safety and benefits in the areas of language/cognition, eating, and overall behavior, in addition to weight loss. The trial will include a US site at the MIND Institute and two Canadian centers (University of Alberta and CHU Sainte-Justine).

This study also provides an opportunity to carry out novel outcome measures that are capable of documenting improvements in the processing of information in the CNS (event related potentials (ERPs) and Tobii eye tracking), cognitive abilities (NIH Toolbox cognitive battery measures and expressive language sampling), critical biomarkers of involvement by the *FMR1*

mutation (CGG repeats, degree of methylation, and FMRP expression levels), and neurochemical changes in the CNS that can also be measured in blood and fibroblasts (MMP9, S6K, EiF4E, and CYFIP1). Finally, the cytochrome P450 (CYP) isozymes, highly polymorphic drug-metabolizing enzymes, have been demonstrated to be responsible for the metabolism of metformin in humans, mainly via CYP2C11, 2D1, and 3A1/2 (Choi and Lee, 2006). Thus, in this study we will assess if the CYP450 allelic variant genotypes involved in the metformin pharmacokinetics determine the efficiency of response to metformin. Since metformin absorption is done by active transport via the organic cation transport (OCT), we will also assess polymorphism in that gene.

Leveraging the current RCT program and adding an open label extension. New information pointing toward the importance of considering a metformin open-label extension to our current randomized control trial of metformin in individuals with Fragile X syndrome (FXS) has emerged for several reasons. We are therefore adding a 2 year (24 months) optional extension of open-label metformin trial to all participants of our approved RCT of metformin in FXS.

Rationale: **1)** Based on reports from open-label trial papers from Dr. Hagerman's group suggesting potential improvements in development, testicular size, and IQ in 11 patients with FXS, there are important long term tolerance and effects that need to be documented at the end of our controlled trial should the patients continue on an open-label treatment of metformin. **2)** We know that metformin is well tolerated during short term treatments in a majority of individuals with FXS (from our RCT participants in Edmonton and at UC Davis). This supports the initial results observed in individuals with ASD (Dr. Anagnostou trial). In addition, a recent open label trial in Sherbrooke (QC) by Dr. Caku identified no adverse event and pointed toward the need for longer treatment (personal communication, paper in preparation). **3)** There is a strong demand for an extension from families involved so far in our trial at all sites. Previous RCTs in fragile X were also followed by open labels in part because there was a strong demand from families who knew that they had a 50% chance of not receiving the active molecule (e.g. the NIH supported the Neuro NExt trial testing the effect of AFQ056 in a new combined paradigm (PI: Dr. Berry-Kravis) which also includes an open-label extension). **4)** Importantly, adding the open-label extension to the trial allows significant cost-saving compared to a stand-alone project. Indeed, database, server, biobank, and pharmacy cost are not duplicated in this design.

Scientific Merit: In addition to allowing all participants to have a chance to assess the effect of metformin, the extension would allow us to: **1)** Evaluate potential variation in the long-term effect of metformin, particularly on cognition, which is the most important issue for the families. For instance from visit 1 to visit 3, we will measure potential change on the impact of metformin long-term treatment on our primary outcome measure (expressive language sampling), but also activity of daily living abilities (as measured by Vineland and IQ). **2)** Leverage the baseline measures of psychological and biological biomarkers for all participants. This means that every participant will have the opportunity to be on metformin at some point in the trial within a

monitored framework and all patients will have baseline measures (from visit 1 of the RCT) which can be used as internal control. This is very important as most families want to test the effect on their children and will do so outside of the investigational framework if it is not made available to them. Some families will get it prescribed by their general practitioner for instance. This will lead to significant loss of data. **3)** Provide additional longitudinal data on cognitive development, functional performance, the health economics and quality of life for individuals and families with fragile X syndrome with metformin. 4**)** Provide additional longitudinal biological samples (including brain activity) for each given individual with FXS at 1 and 2 years, allowing for a better understanding of potential changes in biomarkers over time. This would make the dataset unique and very useful in integrating the developmental origin of disease concept to the study of FXS.

4) Inclusion and Exclusion Criteria

Inclusion criteria: A subject will be eligible for study participation if subject meets all following criteria:

1. Subject has Fragile X syndrome with a molecular genetic confirmation of the full FMR1 mutation (>200 CGG repeats) or the other loss of function mutations of the FMR1 gene (SNVs and deletions of the gene).
2. Subject is a male or non-pregnant, non-lactating female age 6 through 35 years, inclusive.
3. Subjects who are capable of becoming pregnant must use an acceptable method of birth control for the duration of the study. Acceptable forms of birth control include abstinence (only for subjects who are not sexually active), intrauterine devices in place for at least 3 months, oral contraceptives, surgical sterilization, or adequate barrier methods.
4. Subject must have a caregiver (parent, guardian, or other legally authorized representative) who is willing to participate in the whole study.
5. Subject and caregiver are able to attend the clinic regularly and reliably.
6. Subject and/or subject's caregiver is able to understand, read, write and speak English or French fluently to complete study-related materials.
7. For subjects who are not their own legal guardian, subject's caregiver is able to understand and sign an informed consent to participate in the study.
8. The use of concomitant medication must be stable, in terms of dose and dosing regimen, for at least 4 weeks prior to Screening and must remain stable during the period between first visit (Screening) and the commencement of the study; every effort should be made to maintain stable regimens of allowed concomitant medications from the time of commencement of double-blind study medication until the last study assessment.
9. Behavioral/educational treatments must be stable for 4 weeks prior to first visit (Screening) and must remain stable during the period between Screening and the commencement of randomized double-blind study medication.

10. Overall age equivalent is not higher than 13 and IQ is not higher than 85, as assessed at Screening on the Leiter-III, and subject must speak at least occasional 3-word phrases.

Exclusion criteria:

1. Families that are not cooperative and will not follow through with the demands of this study.
2. Subject has a life-threatening medical problem or other major systemic illness that compromises health or safety and/or would interfere with this study.
3. Age younger than 6 or older than 35 years.
4. History of intolerable adverse events with metformin.
5. Current or recent metformin treatment (within the past 4-months).
6. BMI inferior to 2 standard deviations below the mean for age using the World Health Organization scale.
7. Serum creatinine > 1.4 mg/dl (female) or > 1.5 mg/dl (male).
8. History of metabolic acidosis or a condition with lactic acidosis.
9. Severe Vitamin B12 deficiency.
10. Pregnancy at screening or unwillingness to use acceptable method of birth control, if applicable.
11. Age equivalent higher than 13 or IQ higher than 85 on the Leiter-III at Screening.

No additional inclusion criteria will be assessed for the extension phase. Participants can choose to enter the extension phase after successfully completing the main study (randomized controlled trial - RCT). A washout between the RCT and extension phase will not be required.

Inclusion criteria for the controls providing a stool sample:

1. Able to provide a stool sample
2. Able to read and write in English in order to complete a questionnaire (or have a parent/caregiver able to do this)
3. Age 6-35 years old

Exclusion criteria for the controls providing a stool sample:

1. Diagnosis of Fragile X Syndrome
2. Unable to provide a stool sample
3. Younger than 6 or older than 35 years old
4. Metformin Treatment within the past 4 months

Consenting Process:

Subjects will be recruited and consented at each site. The research coordinator will speak with both the subject and at least one caregiver to explain the research and associated study procedures and assess the subject's understanding of the protocol. Subjects aged 6 will not provide

assent; consent will be obtained from the caregiver (Caregiver (Parent/ Legal Guardian) Consenting for Participants Information & Consent Form). For all subjects 7-35, cognitive ability will be assessed utilizing the Capacity Assessment Checklist for Informed Consent with Cognitively Impaired Subjects, also used at the MIND. Study coordinators will perform the initial capacity assessment, which will be reviewed with the study doctor before any other study procedures are done and signed by the study doctor. If appropriate based on cognitive age and ability, assent will be obtained from subjects aged 7-17; if the assent form is not appropriate for the subject's cognitive age and ability, study staff will instead provide a letter of information that will be read to and reviewed with the subject. In the event of changes to the protocol that could possibly affect subjects' safety or decision to participate in the study, current subjects and/or their caregiver will be informed of the changes and re-consented for continuing participation in the study.

In addition to the assenting process described above, all subjects under the age of 18 will need to have their caregiver sign the consent form. Subjects 18 and older who are cognitively capable of providing consent (based on the Capacity Assessment Checklist for Informed Consent with Cognitively Impaired Subjects mentioned previously) will sign an informed consent form (Adult Participants and Caregiver Information and Consent). For subjects 18 or older at screening/baseline, or for those who turn 18 during the course of study participation, who are not cognitively capable of providing consent, a caregiver will need to sign the consent form used for subject with parents consenting (Caregiver (Parent/ Legal Guardian) Consenting for Participants Information & Consent Form). All potential subjects will attend their screening visit with a caregiver able to give surrogate consent.

Separate consent forms (at University of Alberta) or separate signatures (at CHU Sainte-Justine) for either subject or caregiverwill be filled or obtained for the optional biobanking of blood,urine (cell culture), and stool as well as for the optional skin biopsy.

Extension Consent: The consent for the extension will be shown to participants/caregivers at the beginning of the trial and again at the end of the RCT component. A separate informed consent form and signature page for the extension will be signed for participation in the extension phase of the trial.

Consent for controls providing a stool sample: There will be a separate consent form and signature page for this portion of the study. Adult participants will sign on their own behalf and parents/caregivers will sign on behalf of children. If appropriate based on cognitive age and ability, assent will be obtained from subjects aged 7-17. We will be asking family members of particpants in the trial to complete this portion of the study and we will also recruit controls through the Healthy Infants and Children's Clinical Research Program (HICCUP).

5) Study Timelines

The study plans to enroll 60 participants over a 3-year period at each site. Approximately 20 patients per year with FXS will be randomized at each site to receive either metformin or placebo for a 4-month period. During the 4-month study period, subjects will attend three visits to the recruiting site: screening/baseline, 2-month, and 4-month visits. In addition, routine phone calls will be made once per week during the first month of the study, with the week 4 phone call being conducted by the study physician, and once at week 12/month 3.

Extension Visit schedule: Informed consent will be obtained either concomitantly to visit 3 of the original schedule of the RCT portion or later if the participant finished the RCT already, but would like to enter the open-label extension. A follow up in-person visit will be done at 1 year of the open-label trial, or before if needed, and another in-person visit will occur at 2 years. Scheduled follow up phone calls will be done weekly for the first month of the extension and then every 4 months. If a participant begins the extension study within 1 year of ending the RCT portion of the study, Visit 3 of the RCT will be considered to be Visit 1 of the extension and the participant would be seen at a 1 year follow up from when they had their Visit 3 of the RCT portion. Those participants previously enrolled in the RCT who have been taking metformin off-label for more than a year can be enrolled in the extension phase. These participants will have a 'first visit' for the extension phase in addition to the previously-completed third visit for the RCT (as that visit occurred more than a year before starting the extension phase). Informed consent will be obtained at this 'first visit'.

6) Study Endpoints

The primary outcome measure will be the Expressive Language Sampling (ELS) measure which is conducted at baseline and at the end of treatment, as described in detail in Section 7. The ADOS-2 and Leiter-III will be administered at baseline only for purposes of study population characterization and will not be repeated as outcome measures. All other measures will be secondary outcome measures and are also described in detail in Section 7. Attempts will be made to administer all secondary measures for each participant, to the extent his or her cognitive age and ability allows. Safety endpoints will include safety blood draws and physical exams at each study visits.

For the extension, the primary outcome measure will remain the Expressive language sampling (ELS) measured at the 1 year and 2 year visits. This will provide a connection with the RCT primary outcome. The extension secondary outcome measures will be measured at the 1 year and 2 year visits and will include: Leiter IQ test, Vineland (version 3 for English and version 2 for French), Memory Game (online), NIH Toolbox, CarerQol-Quality of life questionnaires (online), EQ-5D- Health economics questionnaires (online), Pediatric Quality of Life (PedsQL) Parent

Proxy Questionnaire (online), Aberrant Behavior Checklist–Community (ABC-C) (online), Visual Analogue Scale (VAS) (online), Swanson, Nolan and Pelham Questionnaire (SNAP-IV) (online), Clinical Global Impression Scale–Improvement (CGI-I) (online), Anxiety Depression and Mood Scale (ADAMS) (online), Sensory Profile-2 or Sensory Profile Adolescent/Adult, Child Sleep Habits Questionnaire (CSHQ) (online), Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24), biological biomarkers (blood, stool, and urine), resting EEG and event related potentials (ERPs), and eye tracking.

7) Procedures Involved

At the first visit, baseline testing will include IQ testing with the Leiter-III and assessment for autism with the Autism Diagnostic Observation Scale (ADOS-2). Cognitive ability will be derived from the Leiter-III, only at baseline. We have shown that full mutation males often perform at the floor of standardized intelligence tests, such as the Wechsler Scales of intelligence, which severely restricts test sensitivity and variability, and therefore presents major limitations in analyses examining genotype-phenotype associations. The primary reason that we have chosen the Leiter-III is because it is a non-verbal test, and it can be used even with low-functioning patients who have little or no language. The ADOS-2 (Lord et al., 2000) is a semi-structured standardized assessment administered directly for the purposes of diagnosing ASD, and will be done only at baseline. The ADOS-2 uses developmentally appropriate social and object-based interactions in a 30–45 minute assessment to elicit symptoms of ASD in four areas: social interaction, communication, play, and repetitive, restrictive behaviors. The ADOS-2 consists of different modules, each directed at a particular level of language ability, and is thus appropriate to use across subjects of varying ages and functioning levels.

The CGI-S, VAS, ABC-C, ADAMS, CSHQ, SNAP-IV, PedsQL, EQ-5D, CarerQoL, Sensory Profile-2 or Sensory Profile Adolescents/Adults, ASA24, and the memory game will be completed.

Blood will also be drawn for safety laboratory tests, including a hemoglobin A1c (HgbA1c), blood glucose, complete blood count (CBC), vitamin B12 level, comprehensive metabolic panel (CMP), cholesterol, renal clearance function, blood pregnancy test for female participants in childbearing age, along with biomarker studies described below. A small skin biopsy (optional procedure) will be obtained from the back of the shoulder, utilizing a small punch biopsy after numbing the skin with lidocaine or EMLA cream, so that fibroblasts can be grown on each patient at baseline to determine the level of Fragile X Mental Retardation Protein (FMRP), the presence of tissue mosaicism, and other studies described below. At the first visit, a detailed medical history and physical and neurological examination will be carried out, with all medications and medical problems documented. A urine and stool sample will also be collected.

Patients will be randomized to either metformin or placebo by the Alberta Health Services Pharmacy Research Office and by the Service pharmaceutique de support à la recherche (SPSR) of CHU Sainte-Justine (University of Montreal) for their participants after the baseline studies are carried out, as detailed below. For patients 50 kg and over, the initial dose will be 500 mg once a day at dinner by mouth, and then increasing each week by 500 mg until a maximum dose of 2000 mg daily is reached. For those patients who are less than 50 kg, the initial dose will be 250 mg once a day at dinner by mouth, and if this dose is well tolerated, they will increase each week by 250 mg until a maximum dose of 1000 mg daily is reached. As such, the maximum dose of metformin in patients under 50 kg will be 1000 mg daily. We will recommend all patients to take a multiple vitamin that has B12 to avoid potential anemia. In the first 4 weeks following visit 1, each patient will receive a weekly call to evaluate tolerability of the medication, any adverse events (AEs), changes in concomitant medications, and gradual titration of the metformin. If potentially related minor AEs such as diarrhea or gastrointestinal pain are experienced, the dose can be lowered, or medication held until clinically indicated to resume the trial.

At 2 months (Week 8 +/- 7 days, scheduled visit 2), when at their maximum tolerated dose, they will be seen in clinic for an examination, fasting safety labs (consisting of the lipid panel which includes cholesterol, LDL, and triglycerides; complete blood count; and comprehensive metabolic panel), urine, biomarkers, assessment of AEs, and limited studies, including a CGI-I, VAS, and behavioral and quality of life questionnaires (ABC-C, ADAMS, CSHQ, SNAP-IV, PedsQL, EQ-5D, Sensory Profile-2 or Sensory Profile Adolescents/Adults, and CarerQoL). We will require 8 hours of fasting prior to the visit 2 blood draw. Participants will only be allowed water during the fasting period.

Subjects will remain at the designated dose through Visit 3 (at Week 16/end of treatment +/-7 days) and will be contacted at week12 by study staff via phone to assess for possible adverse events, changes in concomitant medications, and verification of proper dosing. Study participants will be dispensed study medication at Visits 1 and 2 and will discontinue dosing after completing Visit 3. Any change in medication or new medication will also be documented. The examination, safety labs, biomarkers, urine and stool collection, documentation of AEs, and outcome measures will be repeated at the final follow-up visit at 4 months/end of treatment. We will also record any seizure activity that occurs throughout the study using a seizure diary (which the participant/caregiver will take home) where the date, time, seizure type, duration, whether the participant was awake or asleep during the seizure, use of baseline and abortive medication, as well as any changes to medication. We will also suggest to families to attempt to video record the episodes when possible (on a phone or other camera). They will be asked to show the study doctor or their primary care physician the video as part of standard of care for patients. We will not retain a copy of the video.

Remote visits may be utilized for visit 2 if deemed appropriate by the PI. It will save on travel cost and burden to families. When this is the case, the urine dipstick and research blood work will not be performed. Questionnaires will be sent to the family and the physical exam will be performed virtually via tele/videoconference, for example via Zoom. Study medication will be mailed to participants.

Metformin Titration Schedule (< 50 kg)

	Week 1	**Week 2**	**Week 3**	**Weeks 4-16**
Morning Dose		250mg	250mg	500mg
Evening Dose	250mg	250mg	500mg	500mg
Total daily dose	250mg	500mg	750mg	1,000mg

Metformin Titration Schedule (≥ 50 kg)

	Week 1	**Week 2**	**Week 3**	**Weeks 4-16**
Morning Dose		500mg	500mg	1,000mg
Evening Dose	500mg	500mg	1,000mg	1,000mg
Total daily dose	500mg	1,000mg	1,500mg	2,000mg

Optional open label Metformin extension treatment regimen: We will use the same treatment regimen as for the RCT portion of the trial. The extension component would begin after the end of the 4 month RCT. It will include a progressive ramping up of metformin over 4 weeks based on weight as for the RCT. Treatment will be based on weight (measured at the time of entering the open label phase and can be adjusted during the 2 year period based on change in weight) as shown below:

Metformin Titration Schedule (< 50 kg)				
	Week 1	**Week 2**	**Week 3**	**Weeks 4-104**
Morning Dose		250mg	250mg	500mg
Evening Dose	250mg	250mg	500mg	500mg
Total daily dose	250mg	500mg	750mg	1,000mg

Metformin Titration Schedule (≥ 50 kg)				
	Week 1	**Week 2**	**Week 3**	**Weeks 4-104**
Morning Dose		500mg	500mg	1,000mg
Evening Dose	500mg	500mg	1,000mg	1,000mg
Total daily dose	500mg	1,000mg	1,500mg	2,000mg

To maintain consistency with the trial and our current Health Canada approval, metformin 500mg (Sandoz) will be used for all participants. Medication will be dispensed at visit 1 and then every 3 months either in person or via secure mail service. Participants that have been taking off-label metformin after completing the RCT (but before being given the opportunity to join the extension phase) will not be required to ramp up the dosage of the drug.

For the controls providing a stool sample: Participants will only be asked to provide a stool sample and complete the ASA24 questionnaire as well as provide information about medcations/supplements they are on.

Treatment Measures:

Expressive Language Sampling (ELS) – Expressive language samples will be collected from each participant twice, at study baseline and at the end of treatment/week 16. At each assessment, samples will be collected in two different contexts: conversation and narration. In conversation, the examiner engages the participant in talk on a variety of topics (e.g., school) according to guidelines that specify the order of topics and the ways in which topics are introduced and maintained. In narration, the participant tells the story in a wordless picture book. The examiner prompts and responses are scripted. In both contexts, the examiner follows a script that minimizes his/her participation, maximizes the participant's contribution, and avoids the use of examiner language that would constrain the participant's talk. In both conversation and narration, all talk is digitally recorded and transcribed by highly experienced transcriptionists following procedures that yield high intertranscriber agreement (Abbeduto et al., 1995). Computerized analyses of the transcripts characterize multiple dimensions of the talk produced by the participant (e.g., vocabulary diversity, syntactic complexity). The mean Number of Different Words (NDW) in conversation and in narration will be computed (and statistically adjusted through analysis of covariance for differences in talkativeness as suggested by Conners et al., 2018) and serve as the primary outcome measure. This measure has been shown to have excellent test-retest reliability, display negligible practice effects, be free of floor and ceiling effects, have good construct validity for individuals with FXS, and improve with treatment (Berry-Kravis et al., 2013; Channell et al., 2018; McDuffie et al., 2016). For French speaking participants, we will use a French translation of the ELS.

Clinical Global Impression Scales of Severity (CGI-S) and Improvement (CGI-I) – These scales are standard assessment for medication studies because it allows the clinician to utilize the history from the parent or caretaker and incorporate it into a clinical rating for the clinical follow up of the patient through the treatment trial. In the initial evaluation of the patient, we will use the CGI-S (severity) to judge the severity of the symptoms with a scale of normal, not at all ill; borderline ill; mildly ill; moderately ill; markedly ill; severely ill; or among the most extremely ill. The CGI-I scale will be utilized at the week 8 and end of treatment/week 16 follow-up visits. We

will use with CGI-I to look at improvement or worsening of symptoms with a scale of very much improved; much improved; minimal improvement; no improvement; minimally worse; or very much worse (Guy, 1976; Psychopharmacology, 1985).

Eye Tracking Measures: Social Gaze and Pupillometry – For individuals with FXS, we have demonstrated that the social gaze measure shows decreased visual fixations on the eye region while viewing human faces (with greater fixation to the nose region), and these individuals show abnormal pupillary dilation, an indication of sympathetic nervous system reactivity, compared with controls (Farzin et al., 2009). While these measures do not appear to map directly onto caregiver-reported anxiety of the individuals level of anxiety, the results are consistent with high rates of social anxiety (Cordeiro et al., 2011) and sympathetic reactivity in FXS (Miller et al., 1999). In a subsequent replication study, we showed that the gaze abnormalities (fixation to eye region, pupillary reactivity) are stable in FXS across a 9-10 day period, with intraclass correlations of 0.70–0.91 for pupillary response to faces and 0.94 for proportion of looking time to the eye region. All stimuli are presented on a Tobii T120 binocular eye-tracker monitor (Tobii Technology AB, Sweden). This eye-tracking system consists of a high-resolution camera embedded in a 17-inch TFT monitor (1280 X 1024 pixels resolution, 120 Hz sampling rate, average precision of 0.5 degrees of visual angle). The Tobii system has several benefits that make it conducive to testing individuals with developmental disorders, including approximately 30 cm of tolerance to head-motion in any direction without requiring any head apparatus or restraints. Stimuli consist of sixty colored photographs of adult human faces (equal numbers of males and females, different races and ethnicities) from the NimStim Face Stimulus Set (Tottenham et al., 2009), each showing a calm, happy, or fearful expression, and sixty scrambled versions of the face images. Since it is critical that pupil responses following the onset of the face stimulus be independent of a pupillary light reflex, each face and its scramble are matched on mean luminance confirmed using a photometer (Minolta, LS-100, Osaka, Japan). Face images subtend a 12.12 degree by 17.19 degree region (the size of an actual human face) when viewed from a distance of 60 cm, and are presented on a standard 50% gray background. Percent duration of fixation to the eye, nose, mouth, and other regions are calculated for each participant, as well as mean pupil size calculated for each 250 ms interval.

This assessment will be administered twice, at study baseline and at the end of treatment/week 16.

Event Related Potential (ERP) Measures – The ERP measures described below will be carried out twice, at study baseline and at the end of treatment/week 16. We will use the 3000-0751 this is a 128 channel GES300 system. This is already approved by Health Canada (https://www.egi.com/images/stories/company/documents/Health_Canada_GES_300_issued_2014_03_11.pdf)

In a recently published study (Schneider et al., 2013), researchers were able to obtain EEG recordings and ERP responses during a passive auditory oddball paradigm from 12 patients with FXS enrolled in a controlled trial of minocycline (4 females, 8 males, mean age 10.5 years, SD 3.7) at baseline, 3 months, and after 6 months at the end of the trial. Current source density (CSD) and ERP analysis at baseline showed high amplitude, long latency components in the temporal regions. After 3 months of treatment with minocycline, the temporal N100 and P200 amplitudes were significantly reduced. There was a significant amplitude increase at the Cz electrode position on minocycline treatment. Electrocortical habituation to the auditory stimuli was improved with minocycline treatment (Figure 1). This preliminary study demonstrated the potential feasibility and sensitivity of ERPs as an indicator of cortical processing changes in a targeted treatment trial. It appears to provide a biomarker and measure for the human equivalent of cortical hyperexcitability.

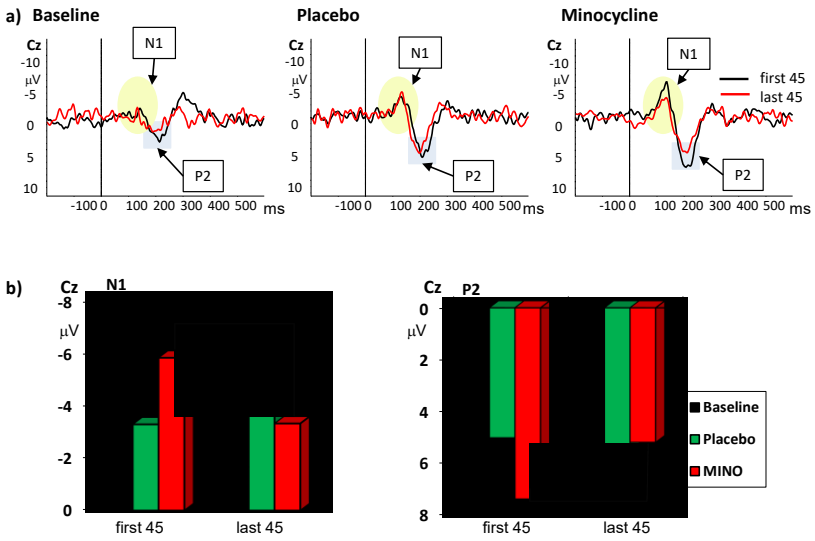

Figure 1. (a) Grand average waveforms at Cz, highlighted in yellow the N1 component, the P2 component in blue. Black line signifies first 45 stimuli, the red line last 45 stimuli potentials. Negative plotted upwards. (b) Significantly improved attenuation of N1 and P2 on minocycline condition, not significant for baseline and placebo.

The ERP method to be followed in this study is as follows. Subjects are presented with an auditory oddball paradigm using Presentation software (Neurobehavioral Systems, Albany, CA). The auditory stimuli are sinusoidal tones with frequencies of 1000 Hz (standard tone) and 2000 Hz (target/oddball). The tones have a 10ms rise/fall, 50ms plateau, and a sound pressure intensity of 70 dB. The randomized order of both auditory stimuli consists of first six 1000 Hz standard tones, then one target tone (2000 Hz) either at 7th, 8th, 9th, or 10th position, with standard tones presented in the remaining positions. The tones are presented with a consistent interstimulus interval (ISI) of 1000ms over stereo speakers. Standard stimuli are presented 80% of the time and deviant stimuli are presented 20% of the time. The auditory oddball task includes a total of 240 standard and 60 deviant stimuli, which are presented in three blocks, each containing 80 standards and 20 deviants. After a 3-minute resting phase, the passive auditory oddball paradigm will be administered, and then following the experimental compliance and depending on the functioning level of the individual, the auditory oddball paradigm will be administered as an active paradigm, requiring a button press for the deviant stimuli. This behavioral data can be used for an analysis of

attentional performance (Van der Molen et al., 2012). Following the experimental compliance, auditory steady-state of a 1000Hz tone at 6 and 40 Hz (3 min task) and visual steady state of abstract shapes at 6, 15 Hz (2.5 min tasks) will be administered. Before the experiment, the subjects pick a favorite movie, which is shown during the preparation and the oddball task. The movie is required to provide a comforting environment for the patients and provide a fixation point for their eyes. During the oddball paradigm, the movie is muted. Before the experiment, we collect 2 minutes of resting EEG, and in compliant subjects, we include an Alpha-block paradigm with four 30-second blocks of alternating eyes-open and eyes-closed continuous EEG recording.

In addition to the oddball paradigm, we will administer a recently developed task looking at EEG habituation to pseudowords to assess word learning with EEG (Knoth et al., 2018). Fifty pseudowords will be successively repeated 6 times each with an inter-stimulus interval of 1000ms. Each sequence of pseudowords appears in randomized order.

Throughout the protocol, positive reinforcement through age appropriate gifts (stickers and/or reward sheet) will be provided.

EEG data is acquired using a 72-channel (or 128-chanel modified) Brainproducts Quickamp system with an Acticap 32-channel Ag+/Ag+Cl- active EEG electrode array [International 10-20 system, positions (Fp1, Fp2, F7, F3, Fz, F4, F8, FC5, FC1, FC2, FC6, T7, C3, Cz, C4, T8, TP9, CP5, CP1, CP2, CP6, TP10, P7, P3, Pz, P4, P8, PO9, O1, Oz, O2, PO10] using a common average reference and a ground electrode positioned between Fz and Pz sites. Electrode impedances are maintained below 10 kΩ and electrical activities amplified and recorded with Brain Vision Recorder and Quickamp® amplifier (Brain Products, Germany). During the recording, bandpass filters are set at 0.3–100 Hz, and data are digitized continuously at 250-500 Hz. Raw data is than imported into Brainvision Analyzer software (Version 2.01) for analysis.

The continuous data is segmented according to the event type (standard or target tone with a 1000ms time window, -100ms before the event until 900ms after the event) and filtered (Butterworth Zero Phase Filters with low cutoff 0.5 Hz, time constant 0.3, 12 dB/oct, high cutoff: 80 Hz, 12 dB/oct, a notch filter is not included because of the active shield technology).

For artifact rejection, we define the maximal allowed voltage step in a segment to 50 µV/ms, with a maximal allowed difference of values in intervals of 1000 µV, minimal allowed amplitude -500 µV, maximal allowed amplitude: 500 µV, lowest allowed activity in intervals 0.5 µV. For the detection and correction of blinks we use the electrode sites Fp1 and Fp2 as source for an independent component analysis (ICA) Infomax restricted slope algorithm. The components relevant for vertical activity are selected by computing the global field power. The number of ICA steps and convergence bound will be selected individually according to the quality of the data; in general, the ocular correction ICA converged between 90–120 steps, with the last step's matrix modification usually smaller than 9.575E-08. In general, there was a loss of 6% of all trials in both

control and FXS participants. We exclude participants without a sufficient number of artifact free trials (>30). ERPs are baseline corrected using the 100 ms prestimulus interval and averaged for standards and target tones separately. Peak amplitude and latency of the N100, P200, N200, and P300 components are determined at the Fz, F3, F4, Cz, C3, C4, Pz, P3, and P4 electrode positions by the largest voltage deflection within the 1000ms time window relative to stimulus onset, depending on the specific latency range for each component (N100 = 50-150ms, P200 = 150-250ms, N200 = 150-250ms, P300 = 250-400ms). The peak detection is performed semiautomatically, and a large voltage deflection also determined as a peak if it is outside the predefined latency range.

We also analyze the EEG data at resting state for the oddball paradigm according to frequency distribution and the occurrence of spontaneous oscillations, and pseudoword habituation with ERP (similar to oddball), energy and time-frequency analyses (Rigoulot et al, 2017; Knoth et al., 2018).

NIH Toolbox Measures, Cognitive Battery – The following set of measures will be administered at the first visit and end of treatment/week 16:

Picture Sequence Memory [PSM; Episodic Memory; (Bauer et al., 2013)] – This test involves recalling increasingly lengthy series of illustrated objects and activities around different themes (e.g., "playing at the park," "working on the farm") that are presented in a particular order on the screen. For each trial, pictures appear in the center of the computer screen and then are moved one at a time into a fixed spatial order, as an audio file simultaneously describes the content of each (e.g., "Plant the tomatoes") and until the entire sequence is displayed on the screen. Then the pictures return to the center of the screen in a random display and the participant moves them into the sequence that was shown. The score is derived from the cumulative number of adjacent pairs of pictures remembered correctly over 2-3 learning trials. Level of task difficulty is adjusted for the various age groups. Administration time is about 10 minutes. Encoding, storage, and retrieval of episodic memories depend on a neural network including the temporal lobe (especially the hippocampus), the prefrontal cortex, and limbic/temporal association areas (Eichenbaum, 2001; Zola and Squire, 2000). Patients with FXS perform approximately two standard deviations below normal on PSM, which demonstrates good feasibility, test-retest reliability [ICC= 0.76 in both healthy children and those with ID; (Hessl et al., 2016)] and moderate correlation with FSIQ. Although this is not a major relative weakness, compared to their executive function (EF) deficits, we note that *Fmr1* knock-out mice demonstrate memory deficits and hippocampal dependent long term depression (LTD) that are rescued by metformin in the Sonenberg studies and by the CB1 receptor antagonist/inverse agonist rimonabant. We therefore included PSM in the current battery.

Flanker Inhibitory Control and Attention Test (Zelazo et al., 2013) – Flanker is a measure of inhibition and visual attention. On each trial, a central directional target (fish for mental age younger than 8, arrows for ages 8 and older) is flanked by similar stimuli on the left and right. The participant chooses the direction of the central stimulus. On congruent trials, the flankers face the same direction as the target. On incongruent trials, they face the opposite direction. A scoring algorithm integrates accuracy, a suitable measure in early childhood/low mental ages, and reaction time, a measure more relevant to adult performance on this task, yielding computed scores from 0 to 10. There are 40 trials, and the test duration is about 4 minutes. Patients with FXS demonstrate profound deficits on Flanker, performing about 7 standard deviations below normal, and significantly worse than IQ-matched controls with Down syndrome (Hessl et al., 2016). This test demonstrates excellent test-retest reliability (ICC=0.94) and correlates significantly with FSIQ and dialing functioning in children and adolescents with ID. Like the Dimensional Change Card Sort test describe below, Flanker is an EF task dependent on prefrontal cortex activity. Because EF deficits are consistently observed in patients with FXS, significantly affecting their daily functioning, we predicted that metformin, if it normalizes aspects of brain function in the disorder, would improve EF as demonstrated in the KO mice in the Sonenberg lab.

Dimensional Change Card Sort Test (Zelazo et al., 2013) – Dimensional Change Card Sort Test is a measure of cognitive flexibility. Two target pictures are presented that vary along two dimensions (i.e., shape and color). Participants are asked to match a series of bivalent test pictures (e.g., yellow balls and blue trucks) to the target pictures, first according to one dimension (e.g., color) and then, after a number of trials, according to the other dimension (e.g., shape). "Switch" trials are also employed, in which the participant must change the dimension being matched. For example, after four trials matching on shape, the participant is asked to match on color in the next trial and then switch back to matching by shape. Scoring is based on a combination of accuracy and reaction time (computed score, ranging from 0–10), and the test duration is about 4 minutes. Dimensional Change Card Sort Test demonstrates good test-retest reliability (ICC=0.74) and correlates well with FSIQ (r=0.66) in children and adolescents with ID, including FXS (Hessl et al., 2016). Aside from having general EF deficits, individuals with FXS have notable impairments in cognitive flexibility and show perseveration in response patterns (Abbeduto and Hagerman, 1997; Hooper et al., 2008).

List Sorting Working Memory Test (Zelazo et al., 2013) – This test requires immediate recall and sequencing of different visually and orally presented stimuli. Pictures of different foods and animals are displayed with accompanying audio recording and written text (e.g., "elephant"), and the participant is asked to state the items in size order from smallest to largest, first within a single dimension (either animals or foods, called 1-

List) and then on 2 dimensions (foods, then animals, called 2-List). The raw score is the number of items recalled and sequenced correctly, and the test takes approximately 7 minutes to administer.

Pattern Comparison Processing Speed Test (Zelazo et al., 2013) – This test measures speed of processing by asking participants to discern whether two side-by-side pictures are the same or not the same by touching "yes" or "no" (or a happy or frowning face for lower mental age). Participants' raw score is the number of items correct in a 90-second period. The items are designed to be simple to distinguish. The test takes approximately 3 minutes to administer.

Oral Reading Recognition Test (Zelazo et al., 2013) – The participant is asked to read and pronounce letters and words as accurately as possible. The test administrator scores them as right or wrong. The items are administered by computer adaptive testing (CAT; continuously adapted depending on performance), and participant responses are scored by the examiner. For the youngest children, the initial items require identification of letters (as opposed to symbols) and identification of a specific letter in an array of 4 symbols. The test requires approximately 3 minutes. A theta score is calculated for this test.

Picture Vocabulary Test (Zelazo et al., 2013) – This measure of receptive vocabulary is administered in a CAT format. The respondent is presented with an audio recording of a word and four photographs on the screen and is asked to select the picture that most closely matches the meaning of the word. The test takes approximately 4 minutes to administer. A theta score is calculated for this test.

Speeded Matching Test (introduced in 2018) – This test measures speed of processing by asking participants to discern whether four side-by-side pictures match the target image at the top of the screen. The items are presented one group at a time on the iPad screen, and the participant is given 119.999 seconds of actual presentation time (excluding transitions between items) to respond to as many items as possible (up to a maximum of 130). The items are designed to be simple so as to most purely measure processing speed. Speeded Matching gives a raw score, which is the number of items the participant correctly responds to (up to 130) in 119.999 seconds. Overall, the test takes approximately 3 minutes to administer.

Because the NIH toolbox has not been validated in French, we will use the original English version and only administer the non-verbal tasks : Flanker inhibitory control, Dimensional card change sort task, and Pattern comparison processing speed. We will say the instructions verbally to the participants, in French.

Visual Analogue Scale (VAS) – Parents will mark on a visual line measuring 10 cm with one side marked "worst behavior" and the other side marked "best behavior." They will mark three key behaviors that we are targeting with this study: behavior problems (parents can note which problems are most severe and track on them throughout the study), language abilities, and eating behavior. For each behavior, they will mark their impression of the behavior at baseline and during the follow-up visits at week 8 and end of treatment/week 16. The horizontal marks are measure in centimeter distance where they fall from the worst behavior side so that we can see improvements or worsening of behavior over this time period.

Vineland Adaptive Behavior Scales–Third Edition (VABS-III) – The Vineland, which is a gold standard test for assessing adaptive behavior that is widely used in clinical trials, will be administered to the parent/caregiver at baseline and end of treatment/week 16. Subtests include Communication, Daily Living Skills, Socialization, Motor Skills, and Adaptive Behavior Composite. The Vineland has been normed for individuals with intellectual disability and ASD. The third edition includes updated item content to streamline similar items and reduce redundancy, to reflect changes in daily living (e.g., technology) and in conceptions of developmental disabilities (e.g., ASD), and to allow for potential cultural differences by using more generalized wording of certain items. French participants will perform the Vineland II Échelles de comportement adaptatif as version III is not available in French.

The Aberrant Behavior Checklist – Community Edition (ABC-C), scored using the FXS-specific factoring system [ABC-FX; (Sansone et al., 2012)] – This measure will be completed by the parent/caregiver at the first visit (baseline), week 8, and end of treatment/week 16. This parent/caregiver report measure is the gold standard measure of problem and interfering behaviors in clinical trials in developmental disabilities (Aman et al., 1995; Kerr et al., 2015). The ABC is actively used in over 70 countries and has been translated into over 30 languages, including Spanish and French. The ABC asks responders to rate behaviors from 0 "not a problem at all" to 3 "the problem is severe in degree" across 58 questions. Its use has been validated in a variety of clinical populations, including in ASD and FXS, has been used extensively in clinical trials, and is a Health Canada and FDA-vetted endpoint used in the Health Canada and FDA approvals for use of risperidone and aripiprazole targeting irritability in youth with ASD (Owen et al., 2009). It has been subjected to utility analysis in FXS and linked to caregiver stress in families (Kerr et al., 2015; Bailey et al., 2012). Scores will be analyzed using the FXS-specific factor structure such that 54 of the items resolve into 6 subscales (irritability, lethargy, social avoidance, stereotypic behavior, hyperactivity, and inappropriate speech) (Sansone et al., 2012). For French speaking participants we will use Questionnaire sur les comportements anormaux. Version 28 Jan 1997.

Child Sleep Habits Questionnaire (CSHQ) – This measure consists of a series of questions relating to the sleep habits of children. It will be completed by caregivers of all subjects, regardless

of age, at baseline, week 8, and end of treatment/week 16. For French speaking participants we will use Questionnaire sur les habitudes de sommeil de l'enfant (enfants d'âge préscolaire et scolaire) Non validé.

The Anxiety Depression and Mood Screen (ADAMS) – This measure is an informant report, 28-item questionnaire, that serves as a screening instrument to determine anxiety, mood, and depression among individuals with intellectual disability. The respondent is asked to rate the extent to which their child displays target symptoms (mild/moderate/severe/profound structure). The ADAMS yields 5 subscale scores: General Anxiety, Social Avoidance, Depression, Manic/Hyperactive and Obsessive/Compulsive Behavior. It was psychometrically evaluated and normed using 265 individuals and validated with 129 psychiatric patients with ID (Esbensen et al. 2003). It will be completed at baseline, week 8, and end of treatment/week 16. For French speaking participants we will use Anxiété Dépression et troubles de l'humeur Non validé.

Swanson, Nolan and Pelham Questionnaire (SNAP-IV) – This measure, based on DSM-V criteria for ADHD, is a caregiver-rated questionnaire that effectively identifies those with and without ADHD and accurately predicts presentation specifier (inattention, hyperactivity/ impulsivity, and combined). Its psychometric properties and clinical utility have been demonstrated in multiple studies since its introduction in 2001, and it has been found to be reliable and well validated with normative data from both parents and teachers. It will be completed by caregivers of all subjects at baseline, week 8, and end of treatment/week 16. For French speaking participants we will use SNAP-IV version Fr April 2008.

Pediatric Quality of Life Questionnaire (PedsQL) Parent Proxy – This measure consists of a series of questions relating to a child's quality of life and is administered to the caregiver of the child. The parent proxy module designed for children 8-12 years of age will be administered to the caregivers of all subjects, regardless of age, because the questions therein are most appropriate for the overall study population's cognitive age and ability. It will be completed at baseline, week 8, and end of treatment/week 16. For any subjects not in school, questions pertaining to "school" will be replaced with references to "work" or other activities in their life. For French speaking participants we will use PedsQL : inventaire de la qualité de vie pédiatrique.

EuroQol-5D (EQ-5D) – This is a standardized instrument developed by the EuroQol Group as a measure of health-related quality of life that can be used in a wide range of health conditions and treatments. The EQ-5D consists of a descriptive system and the EQ VAS. We will use the EQ-5D-Y, EQ-5D-Y parent proxy, EQ-5D-3L, and EQ-5D-3L parent proxy depending on the participants age and ability.

The descriptive system comprises five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. The EQ VAS records the patient's self-rated health on a

vertical visual analogue scale. This can be used as a quantitative measure of health outcome that reflects the patient's own judgement. The scores on these five dimensions can be presented as a health profile or can be converted to a single summary index number (utility) reflecting preferability compared to other health profiles.

Care Related Quality of Life (CarerQoL) – The CarerQol was designed to measure and value the impact of providing informal care on carers. It combines a subjective burden measure that provides a comprehensive description of the caregiving situation (CarerQol-7D) with a valuation of informal care in terms of well-being (CarerQol-VAS).

Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24) – This is a free, web-based tool that enables multiple, automatically coded, self-administered 24-hour diet recalls and/or single or multi-day food records, also known as food diaries. The ASA24 system consists of a respondent website used to collect dietary intake data and a researcher website used to manage study logistics and obtain nutrient and food group data files. We will collect information related to food intake for the 24 hours prior to when the participant provides a stool sample.

Sensory Profile-2 or Sensory Profile Adolescents/Adults – The Sensory Profile 2 family of assessments evaluates a child's sensory processing patterns in the context of everyday life. The forms are completed by caregivers, who are in the strongest position to observe the child's response to sensory interactions that occur throughout the day. The Adolescent/Adult Sensory Profile was developed to assess sensory processing abilities in individuals beyond childhood. Applicable in a variety of typical settings, such as schools, clinics, and programs that use client-centered practices, it is designed to promote self-evaluation of behavioural responses to everyday sensory experiences. The profile is most appropriate for individuals 11 to 65+ years of age and is intended as a trait measure of sensory processing. Questions are answered in a way how an individual generally responds to sensations, as opposed to how he or she responds at any given time. This enables the instrument to capture the more stable and enduring sensory processing preferences of an individual. The assessment tool includes 60 items, covering the sensory processing categories of Taste/Smell, Movement, Visual, Touch, Activity Level, and Auditory. The Sensory Profile Adolescents/Adult is a self report, however, if the participant is unable to complete it themself, we will ask their caregiver to complete it and we will make note of who completed it. The French versions are the Profil sensoriel 2 and the Profil sensoriel—adolescent/adulte.

The Memory Game- This is an online test developed in the Bolduc Laboratory done on a tablet. Participants will have access to French and English versions. Participants are asked to remember association between sets of pictures. The test has been done in typical development and developmental delay for participants 4 years and up. The test has a short demonstration video and a practice session. The participants are then shown pairs of pictures they must remember. Testing is performed right after the presentation and again approximately 24 hours later. For the 24 hours

testing, the participant will perform the association test this time without being shown the demonstration video or doing the practice session. The testing takes about 20 minutes the first day and 7 minutes the second day. It will be completed before treatment starts (at visit 1) and after treatment ends (at visit 3).

Questionnaires will be done on a tablet device directly into REDCap when possible. Paper copies will be available when needed.

Laboratory Evaluations:

Safety Lab Tests – At baseline and at the end of treatment/week 16 follow-up visit, comprehensive metabolic panel, glucose, vitamin B12, folate, lactate, pregnancy testing (for those female participants who are both capable of becoming pregnant and sexually active in a way that could lead to pregnancy), complete blood count, HgbA1c, and urinalysis (if able to provide a sample) will be done. A total of 3 tubes (6mL of blood) are drawn for the safety labs at each visit.

At the second visit complete metabolic panel, fasting glucose, folate, lactate, pregnancy testing, complete blood count, lipid panel (including cholesterol, LDL, and triglycerides), and urine analysis will be done.

Extension Safety labs: The same safety labs included in RCT visit 3 will be measured at 1 year and 2 years (after the beginning of the open-label extension phase).

A summary of all tests is provided below.

Safety Lab Tests

Chemistry	**Hematology**	**Urinalysis**
Comprehensive Metabolic Panel: *Light green top (3mL minimum)*	Complete Blood Count: *Purple top (2mL minimum)*	Urine Dipstick Analysis: *Sterile Urine Container*
Sodium	Hemoglobin	pH
Potassium	Hematocrit	Color
Chloride	WBC	Clarity
Carbon Dioxide	RBC	Specific Gravity
BUN	MCV	Occult Blood
Creatinine	MCH	Urobilinogen
Glucose	MCHC	Ketones
Calcium	RDW	Protein
Total Protein	Platelet Count	Glucose
Albumin	Neutrophils	
ALP	Lymphocytes	
AST	Monocytes	
ALT	Eosinophils	
Total Bilirubin	Basophils	
Lipid Panel:		
Cholesterol	HgbA1c	
LDL cholesterol		
Triglyceride		
Fasting glucose		
Vitamin B12		
Folate		
Pregnancy test (bHCG)		
Lactate *Gray top (1mL minimum)*		

Molecular Biomarkers – In addition to the blood for safety labs, we will collect a minimum of 24.5 mL of blood at each visit for biomarker analysis including FMRP level, MMP9, S6 Kinase, EIF4E, *CYFIP1* mRNA, CYP450 allelic variants, proteomics, mRNA sequencing and metabolome variants. The Edmonton site will collect four EDTA tubes (2mL, 2 x 3mL, and 6mL), one PAXgene tube (2.5mL), and one CPT tubes (8mL). Due to differences in laboratory processing protocols, the Montreal site will collect 4 EDTA tubes (2mL, 3mL, and 2 x 5mL) and one PAXgene tube (2.5mL).

Protein biomarkers

Plasma, DNA, and peripheral blood mononuclear cells (PBMCs) will be used for the proposed experiments. We consider testing various target proteins including the following.

FMRP level. The level of FMRP has been correlated to the IQ in individuals with Fragile X. Significant variation in FMRP levels can be observed in FXS individuals based on sex, type of tissue examined, degree of FMR1 methylation and presence of mosaicism. FMRP measurements will be performed on lymphocytes, fibroblasts, and epithelial cells (obtained from urine sample) at baseline.

Tissue type: The cells will be obtained from lymphocytes derived from blood, cells from urine, or skin fibroblast. 1) The optional skin biopsy will be carried out after lidocaine or EMLA cream is applied topically and approximately 2 cc of lidocaine is injected so that the procedure is painless. A small punch biopsy is done and then covered with a steristrip and a 4X4 gauze. This will only be done at baseline. However, the aforementioned blood sample and the urine sample (described below) will be obtained at baseline, week 8, and end of treatment/week 16. 2) The urine sample, a non-invasive method, will provide epithelial cell cultures that are alternatives to fibroblast cultures and can also later be derived in stem cells. Methods involve fresh urine samples (< 5h) being centrifuged and the cell pellets being re-suspended and then plated into 24-well tissue culture plates. Urine derived cells are isolated and characterized as previously described (Zhou, T et al. 2011). Numbers of cell clones are counted from each urine sample after they are cultured for 2 weeks. Cell colonies from fresh urine samples often appeared as a cluster of 5–12 cells within 5–7 days. 3) If done with blood, peripheral blood mononuclear cells (PBMC) are used as described later.

Method: FMRP measurements will be done using the Cisbio Human FMRP assay (63ADK038PEC0). The assay uses HTRF (homogeneous time-resolved fluorescence) technology in which fluorescence resonance energy transfer (FRET) occurs between antibody-bound donor and acceptor groups located on the same FMRP molecule via the two fluorophores-conjugated antibodies (Degorce et al., 2009). Operation in a time-resolved fashion eliminates any background fluorescence. The donor is labeled with europium cryptate (Eu^{3+}-Cryptate) and the acceptor is "d2". Total protein concentration is determined using the Thermo Fisher MicroBCA Assay (23235). Protein levels will be determined in triplicate in a 384 well format following the instructions on the assay protocol. Samples are read on the PerkinElmer VictorX5.

Global protein synthesis rate. *Fmr1* KO mouse model of FXS show that protein synthesis rates measured *in vivo* are increased in many regions of the brain (Qin et al., 2005). Both genetic manipulation and pharmacological treatment of *Fmr1* KO mice with drugs that normalize rates of protein synthesis have been shown to correct some molecular and behavioral phenotypes (Bhattacharya and Klann, 2012; Henderson et al., 2012; Liu et al., 2012; Michalon et al., 2012;

Osterweil et al., 2013). Preliminary data shows that altered protein synthesis can be reliably measured in human fibroblast cultures of patients with Fragile X syndrome and data suggest that it may be correlated to clinical outcomes. Protein synthesis rates will be measured using cell culture and SuNSET assay. During this trial, we aim to measure the critical "molecular phenotype" to understand how it may contribute or co-vary with clinical manifestations and respond to treatment.

We propose to perform Data-independent acquisition mass spectrometry (DIA-MS) analysis on a subset of lymphocytes and fibroblasts to determine the influence of metformin treatment on the cell proteome. DIA-MS is a recently developed MS approach in which all ions within a selected *m/z* range are fragmented and analyzed in a second stage of tandem mass spectrometry (Doerr et al., 2015). Mass spectra are acquired either by fragmenting all ions that enter the mass spectrometer at a given time (called broadband DIA) or by sequentially isolating and fragmenting ranges of *m/z*. Metformin is known to alter the translation of numerous proteins, and therefore that part of its molecular response should be gauged by the nature and extent of the proteomic response. The analysis requires approximately 20-40 ug of total protein, which would be obtainable from approximately one-third of blood from a CPT tube. For lymphocytes, the DIA-MS analysis would be performed both before (baseline) and immediately following metformin treatment. For fibroblasts, cells would be subjected to DIA-MS prior to, and following addition of metformin. In this instance, we would be looking for differences in the basic proteomic response to metformin, and whether the nature of this response would correlate with the magnitude of the clinical response.

In addition, we will use mass spectroscopy to study specific metabolites. Indeed, we will establish metabolome variants integrating data from three different platforms into a coherent view on regulation of metabolites in participants. In all three methods ((1) Complex lipids by CSH-QTOF accurate mass / high resolution mass spectrometry, (2) Primary metabolites by the cold injection GC-TOF mass spectrometry, and (3) Exogenous metabolites and biogenic amines by HILIC-QTOF mass spectrometry) we will use 'metabolite targets' for arrays of identified metabolites, with known quantification ions and retention times and specialized internal standards, in addition to detecting 'novel metabolite signals' which may be important to innovate biomarkers and mechanistic understanding of diseases.

Gene expression biomarkers (RNA). Loss of FMRP has been shown to affect gene expression in FMR1KO mice and human. We will therefore obtain RNA from participants at each visit to assess levels of candidate targets.

The loss of FMRP is believed to cause dysregulated translation of its target mRNAs (including MMP9), many of which are critical for synaptic plasticity, maintaining neuronal function, and regulatory control of protein synthesis (Darnell and Klann, 2013). We will perform RNA sequencing on RNA isolated from leukocytes isolated in the blood of participants on either

placebo or metformin at each visit. mRNA sequencing (RNAseq) – As with DIA-MS, RNAseq would be performed before/after treatment of subjects with metformin, and pre- and post-metformin treatment from a subset of participants. mRNA would be purified in the participating laboratories and submitted for RNAseq analysis sequencing/expression core. As with DIA-MS, the mRNA would be isolated from one aliquot of pelleted lymphocytes, either the same pellet used for the DIA-MS experiment or from a portion of the purified RNA.

Genomic biomarkers

Molecular measures at the FMR1 locus will include CGG sizing (using a combination of PCR and Southern blot analysis), methylation status, and FMR1 and CYFIP1 mRNA expression using procedures outlined and detailed in our previous studies (Tassone et al., 2000; Tassone et al., 2008; Filipovic-Sadic et al., 2010).

To determine whether the efficiency of response to metformin is dependent on the type of CYP450 allelic variants, we will determine the genotype in DNA samples obtained from lymphocytes of participants and correlate them to the clinical response measures outlined above. Analysis of Cytochrome P450s polymorphisms will be obtained by the xTAGv3 Kits (Luminex Corporation, Austin, TX) on a Luminex 100/200 instrument. Expression levels of targets in the mTOR/p70S6 kinase signaling pathways (Renard et al., 2016) (total and phosphorylated forms) will be measured by western blot analysis, as described in Hoeffer et al. (2012). MMP9 activity will be measured using a Milliplex assay (EMD-Millipore, Billerica, MA) in plasma samples isolated from whole blood collected in EDTA-containing tubes, followed by centrifugation for 10 minutes at 1000 x g within 30 minutes of blood collection. Samples will be diluted 100-fold with assay buffer. Overnight incubation will be carried out for 17 hours at 4°C with shaking. Samples will be measured within one hour of finishing protocol using Luminex bead reader. MMP9 activity will be correlated to clinical improvement measures, as described above.

In addition, several variations in genes involved directly with metformin pharmacokinetics have been identified and will be assessed using next-generation sequencing and Sanger sequencing confirmation. Indeed, transport of metformin into the body is determined by several transporters. We will analyze the sequence for the transporter OCT1 and OCT2 (coded by the genes SLC22A1 and SLC22A2) and Multi-drug and toxic extrusion 1 (MATE1). A recent genomic analysis of response to metformin also identified other candidates including Ataxia telangiectasia mutated (ATM) and other candidate genes who did not reach statistical significance in the study (Zhou, K et al, 2011).

Furthermore, other genes involved in pathways related to FXS, metformin, metabolism or other genes not directly related could also be associated with variation in the response to metformin will be assessed using unbiased exome or genome next-generation sequencing.

Gut Microbiome

Stool collected from participants will be stored in the biobank and used by researchers in the future to, for example, look at the differences in the gut microbiome before and after treatment. The OMNIgene GUT OMR-200 stool collection kit will be utilized by the participant (with assistance from the caregiver if needed) to collect a sample at visits 1 and 3 (and the extensions visits after 1 and 2 years) and once from controls. The kit allows for transport and storage at room temperature for 60 days.

Other, Non-Outcome Measure Procedures:

Demographics – Basic demographic information will be collected on all study subjects at the screening/baseline visit. This information includes, but is not limited to, date of birth, race, ethnicity, socioeconomic status, education of parents, parental occupation, and parental salary range.

Medical Examination – This will be carried out by the Co-PI and the nursing team at each site and may include growth percentiles, vitals, a detailed medical history, general physical and neurological examination, and review of medical records (paper, electronic), laboratory testing, and educational record, as applicable. In addition, a detailed family history may be obtained if medically necessary. A medical history and exam will be conducted at the screening/baseline visit. A medical exam and a review of recent history will take place at each subsequent visit.

The complete schedule of all study procedures is also listed in a table format below:

	Main Study			Open-label Extension		
Assessment	**Visit 1 Week 1, Day 0**	**Visit 2 Week 8 ±7 days**	**Visit 3 Week 16 ± 7 days**	**Visit 1* Week 1**	**Visit 2 Week 52 ±7 days**	**Visit 3 Week 104 ±7 days**
Informed Consent	X			X**		
Inclusion/Exclusion Criteria***	X					
Medical History	X				X	X
Physical and Neurological Exam	X	X	X	X	X	X
Vital Signs	X	X	X	X	X	X
Adverse Events[a]	X	X	X	X	X	X
Concomitant Medications[a]	X	X	X	X	X	X
Autism Diagnostic Observation Schedule (ADOS-2)	X					
Leiter-III	X				X	X
Clinical Global Impression Scale – Severity (CGI-S)	X					
Clinical Global Impression Scale – Improvement (CGI-I)		X	X	X	X	X
Anxiety Depression and Mood Scale (ADAMS)	X	X	X	X	X	X
Expressive Language Sampling (ELS)	X		X	X	X	X
Eye Tracking Measures[b]	X		X	X	X	X
Event Related Potential (ERP) Measures[b]	X		X	X	X	X
NIH Toolbox Cognitive Battery Measures[b]	X		X	X	X	X
Visual Analogue Scale (VAS)	X	X	X	X	X	X
Vineland Adaptive Behavior Scales–Third Edition (VABS-III)	X		X	X	X	X
Aberrant Behavior Checklist–Community (ABC-C)	X	X	X	X	X	X

Child Sleep Habits Questionnaire (CSHQ)	X	X	X	X	X	X
Swanson, Nolan and Pelham Questionnaire (SNAP-IV)	X	X	X	X	X	X
Pediatric Quality of Life (PedsQL) Parent Proxy Questionnaire	X	X	X	X	X	X
EuroQol-5D (EQ-5D)	X	X	X	X	X	X
Care Related Quality of Life (CarerQoL)	X	X	X	X	X	X
Sensory Profile-2/Sensory Profile Adolescents/Adults	X	X	X	X	X	X
Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24)	X		X	X	X	X
The Memory Game (online)[b]	X		X	X	X	X
Blood Draw	X	X	X	X	X	X
Urine Collection	X	X	X	X	X	X
Stool Collection	X		X	X	X	X
Skin Biopsy (optional)	X					
Dispense Study Drug	X	X			X	
Collect Study Drug		X	X		X	X

a : Also assessed at phone calls at Weeks 1, 2, 3, 4, and 12 and during the optional open label extension at weeks 1, 2, 3, 4, 20, 36, 52, 68, 84, 100

b : If subject is capable of completing assessment

*** If a participant begins the extension study within 1 year of ending the RCT portion of the study, Visit 3 of the RCT will be considered to be Visit 1 of the extension and the participant would be seen at a 1 year follow up from when they had their Visit 3 of the RCT portion. So assessments, safety laboratory and biological sample collections (blood, urine, stool) will not be duplicated if no change in medical condition has occured.**

**** Informed consent for the open label extension will be done at Visit 3 of the RCT portion if possible, otherwise if a participant in the RCT portion decides to participate in the open label extension later, consent will be done then.**

***** Inclusion criteria, which are met at the beginning of the RCT, will be used to participate in the open label extension as well. If a participant is over 35 by the start of the extension or any time during the extension they will still be included.**

8) Data and Specimen Management and Confidentiality

Recruitment:

Subjects will be recruited through the FXS clinics at the University of Alberta and CHU Sainte-Justine as well as by referral from other centers in Canada. The University of Alberta site will also recruit patients from other centers across Canada (including in Alberta, Manitoba, Saskatchewan, and British Columbia) referred to the study. The Universite de Montreal site will also recruit patients from the Maritimes, Quebec, and Ontario referred to their center. This way, any FXS patient from Canada will be able to participate in the study if they want. Approved study information material will be posted on intellectual disability, autism, and FXS support group websites and social media feeds, and in clinical spaces.

Educational and scientific conferences by the Co-I to health professionals, education systems, and other individuals interacting with FXS patients and families will also be used to disseminate knowledge about the study and recruitment. A website for the study has been created with videos and testimonial from clinicians, researchers, patients, and family members to generate interest and facilitate recruitment.

Potential subjects/caregivers who are interested in participating in the study will be able to contact a study team member to schedule their visit 1. This will also allow potential subjects/caregivers to ask study-related questions and discuss the study in depth with the research team. The research team will use a pre-screen form where questions will be asked to the participant/caregiver over the phone to determine if the participant will be eligible to continue to visit 1. Informed consent will be signed at the first visit. Informed consent includes authorization to access and use their health information for the research study. For participants followed at CHU Ste-Justine, the study team is authorized to access medical records of CHU Ste-Justine patients to verify eligibility prior to getting their consent for the trial.

Confidentiality:

Participant confidentiality and privacy are strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. This confidentiality is extended to cover to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without the prior written approval of the sponsor. Study files will be kept in a locked cabinet in a locked room with access restricted to research staff.

All research activities will be conducted in the Stollery Children's Hospital and the Biological Sciences building at the University of Alberta in as private a setting as possible.

The study monitor board, other authorized representatives of the study sponsor (Women and Children's Health Research Institute, Fondation CHU Ste-Justine), representatives of the

Research Ethics Board (REB) or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for the period dictated by Health Canada regulation (25 years).

De-identified study participant research data, which is for purposes of statistical analysis and scientific reporting, will be stored in the REDCap electronic data capture system at the Women and Children's Health Research Institute (WCHRI) Data Coordinating Centre (DCC) at the University of Alberta. This data will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. Permission to store data at the WCHRI DCC will be included in the informed consent.

WCHRI's REDCap installation is housed in a secure data centre at the University of Alberta Hospital and is behind the Faculty of Medicine & Dentistry's firewall. Data is entered through a web based interface using 128 bit SSL encryption. Login is via a username/password pair with additional two factor authentication (2FA). Additional information is available in WCHRI's privacy document (*https://redcap.ualberta.ca/privacy.pdf*).

Coordinators and investigators at each study site will only have access to data relating to their own study participants. Study management staff (PI and research coordinator) at the lead site will have access to data for all study participants. Staff at the WCHRI DCC will have access to data for all study participants. This access is required to perform system management functions, data cleaning, and analysis. Once the study database is "locked" and data has been extracted for analysis, read only access to the study database will be granted to the PI and/or their designate if requested.

We will have a material transfer agreement between the Canadian sites and a research institute in the United States (UC Davis MIND Institute) that plans to collect similar data, with the hopes that it may contribute to the research endpoints. This collaboration may include additional biomarker studies. With a material transfer agreement, each site may have access to blood epithelial cells cultured from urine and fibroblasts of patients at other sites for future research, if the patient consents to this use, in which case all such specimens would be de-identified upon transfer.

Analysis will be based on the intention-to-treat principle. The team at the University of Alberta, Ste-Justine, and MIND Institute will collaborate on data analysis. The data will be merged into one dataset shared and analyzed across the different sites. De-identified data will be shared across sites as stated in the consent form including the UC Davis and Ste-Justine forms. Data will

also be analyzed in the lab of Dr. Arnaud Droit using machine learnig approaches and advanced statistical analysis, as well as proteomics on urine samples.

The analysis of treatment efficacy will be based on the analysis of covariance (ANCOVA) with outcomes (receptive and expression language at 4-month follow-up with corresponding baseline measure as a covariate). The chosen endpoint at 4-month, based on preliminary data, provides a reasonable treatment time period to assess changes in the response. The proposed method adjusts for potential baseline differences between treatment arms despite randomization, if any. The sample size/power for the ANCOVA is based on sample size needed for a t-test by a variance inflation factor 1-2 where there is the correlation between baseline and follow-up measures. From our preliminary data, the receptive and expressive scores have ranges from approximately 0.2 to 0.5. Thus, we predictthe power for the t-test and power for the ANCOVA will be higher depending on this factor. We estimate clinically relevant effects sizes, based on our preliminary data, at follow-up visit as follows (metformin/placebo): receptive language score mean 18.1/23.8, SD=7.0; expressive language score mean 13.6/20.7, SD=8.4. For these effect sizes of the 2 primary measures, the ANCOVA will have power of at least 80% (or have between 0.2-0.5) at level alpha=0.016 with the proposed total sample size n = 60 for each group (placebo and treatment).

Data Safety and Monitoring Board (DSMB):

A DSMB for the Canadian centers located at the University of Alberta will collaborate and exchange information with the DSMB of the US site at the MIND Institute. The Canadian DSMB will include a minimum of two physicians who are independent of this study but who have significant expertise in carrying out psychopharmacological interventions in individuals with FXS. In addition, an independent statistician not involved with the study will be a member of each DSMB. The DSMBs will examine unblinded adverse events and clinical data on a semi-annual basis to review study progress and to advise whether the trial can be safely continued. The investigators and study staff will remain blinded throughout the trial. Any serious adverse events (SAEs) will be reported to the REB of each site and Health Canada in accordance with their respective reporting guidelines.

9) Data and/or Specimen Banking

Retention of Records:

Data will remain in REDCap system at the WCHRI DCC until all data management and statistical analysis activity has been completed. Following study completion and publication, the data will be deleted from the REDCap system.

The WCHRI DCC will facilitate the deposit of de-identified data in a publicly accessible, secure and curated repository for discovery and reuse by others in accordance with the Tri Agency Statement of Principles on Digital Data Management (2017). This will include creating a public

metadata in an open source system, such as Dataverse or Dryad. This could then be used by other investigators as a starting point to contact you if they want to collaborate and/or access de-identified data sets.

The PI will be responsible for storing copies of the data and other study materials in a secure archival facility in compliance with Health Canada and local institutional research data retention policy. At the end of this retention period these materials will be destroyed.

Specimen Banking:

All collected samples are de-identified and will only contain the subject's study ID number and date/time of collection. Samples will be used by our team and could be shared with other investigators with appropriate ethics approval for investigation.

Biological samples (blood, urine, skin, and stool) will be processed, stored, and destroyed in accordance with protocols in place for biological samples. Biological samples for participants enrolled through the University of Alberta will be stored in the Canadian Biosample Repository at the University of Alberta; biological samples for participants enrolled in Quebec will be stored at the CHU Sainte Justine in the Institutional biobank under the name "Biobanque sur les troubles neuro-développementaux et les conditions associées".

Blood:

Blood will be drawn at three points throughout the study with a total minimum blood draw volume of 30.5 mL at each draw. Serum samples will be frozen immediately and stored at the respective biobanks.

Samples may be used in the future for purposes related to this research. On a separate consent form, the participant/caregiver may indicate permission to allow for samples to be kept and stored indefinitely after the end of the study for use in future research. Alternatively, samples may be destroyed upon completion of the study.

The samples are stored in the respective biobank facilities described above. A part of the sample will be used for analysis locally or shipped for analysis to other scientists approved by the study investigators. Samples collected for safety analyses will not be used for any other purpose. Safety labs drawn at each visit will have a minimum volume of 6mL and will be processed through the Alberta Health Services Clinical Trial Lab. Safety labs from the Universite de Montreal will be assessed locally as well. Banked samples will be stored and will be used in biomarker studies.

Urine:

Urine samples for safety labs, biomarker analysis, and cell culture will be collected at three points throughout the main study (and at extension visits). Urine derived cell lines will be obtained locally at Ste-Justine and/or at the Universite Laval. We will only collect urine for cell culture/biomarker studies until we have a viable sample and then urine will only be collected for

safety labs. Samples will be stored in one of the biobank facilities described above. Banked samples will be stored and used in biomarker studies.

Skin:

Skin sample will be collected at the baseline visit only if the participant/caregiver chooses to participate in this optional part of the study (separate consent form). As described above with regards to blood specimens, the participant/caregiver may indicate permission to allow for their skin sample to be kept and stored indefinitely at the biobank facility for use in future research; otherwise, samples will be destroyed upon completion of the study.

Stool:

Stool samples will be collected at visits 1 and 3 of the main study (and at extension visits) and once from control participants at any point to be stored in the biobank facility for use in biomarker/other gut microbiome research if there is consent from the participant/caregiver to do so.

10) Provisions to Monitor the Data to Ensure the Safety of Subjects

It is the responsibility of the investigators to oversee the safety of the study for subjects seen at each site. This safety monitoring will include careful assessment and appropriate reporting of physical exam and lab abnormalities, adverse events, and SAEs as noted above. Adverse events, SAEs, and laboratory results will be reviewed by the DSMB after each SAE. The WCHRI DCC can do the ongoing data monitoring for both sites and prepare reports for the DSMB. Unanticipated problems posing risks to subjects or others and serious, unexpected adverse events associated with the research will be brought to the attention of the DSMB as soon as possible, accompanied by the evaluation of the event conducted by the site investigator.

The role of the DSMB will be to evaluate the participant risk versus benefit, consider factors external to the study when relevant information becomes available, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the trial, and make recommendations concerning continuation, termination, or other modifications of the trial based on the observed beneficial or adverse effects of the treatment under study, particularly in regards to a maximum tolerated dose in this study population. The PI will abide by the recommendation of the DSMB regarding stopping the study.

11) Withdrawal of Subjects

All subjects and their caregiver will be advised that they are free to withdraw from participation in this study at any time, for any reason, and without prejudice. Every reasonable attempt should be made by the investigator to keep subjects in the study; however, subjects must be withdrawn from the study if they/their caregivers withdraw consent to participate. The

investigator and/or the Research Coordinator must attempt to contact subjects who fail to attend scheduled visits by telephone or other means to exclude the possibility of an AE being the cause of withdrawal. Should this be the cause, the AE must be documented, reported, and followed as described in Section 10.

The site investigator also has the right to withdraw subjects from the study at any time for lack of therapeutic effect that is intolerable or otherwise unacceptable to the subject, for intolerable or unacceptable AEs, inter-current illness, noncompliance with study procedures, administrative reasons, or in the investigator's opinion, to protect the subject's best interest. If a subject is withdrawn before completing the study, the reason for withdrawal and the date of discontinuation will be recorded on the appropriate case report form (CRF). Whenever possible and reasonable, the evaluations that were to be conducted at the completion of the study should be performed at the time of premature discontinuation. Information gathered about a subject who has terminated the study early will be kept for analysis unless the subject/subject's caregivers specifically ask for this information to be removed from the analysis. Any unused biological samples will be destroyed.

Participants/caregivers will be informed of this as a part of the consent process and also reminded of this in the event of an early termination.

In addition, the study staff will explain to the participant/caregiver that, although it happens rarely, the sponsor may stop the trial early. This could be due to a number of reasons and would involve discontinuation of the participant's and caregiver's involvement in the study.

12) Risks to Subjects

The most common anticipated risks due to participation in the study include anxiety, frustration, fatigue, or embarrassment during the answering of questionnaires, study assessments and testing, as well as during the medical history and exam. Breaks will be offered to subjects as needed.

Risks associated with blood draws include bruising, soreness, and slight risk of infection at the needle entry site for the blood draw. This site will be carefully cleaned prior to the draw and an appropriate dressing will be applied to the area. Risks associated with skin biopsies include bleeding, bruising, scarring, and slight risk of infection at the biopsy site. This site will be carefully cleaned prior to the biopsy and lidocaine or EMLA cream will be applied to numb the area.

Metformin is a Health Canada and FDA-approved medication with an established adverse events profile explained in the package insert. The clinical data suggest that metformin is safe and well-tolerated in diverse populations. The more common observed side effects during treatment with metformin are: diarrhea, nausea/vomiting, flatulence, asthenia, and abdominal or stomach

discomfort. The less common observed side effects during treatment with metformin are: headache, blurred vision, chest discomfort, cold sweats, coma, confusion, difficult or labored breathing, dizziness, feeling of warmth, or a heartbeat or pulse that is fast, irregular, pounding, or racing. Adverse events and toleration are similar between the liquid and tablet formulations of metformin. Safety labs will help rule out candidate at risk of serious side effects such as lactic acidosis and anemia. Patients and parents will be instructed about the symptoms of hypoglycemia and advised to consult urgently in case of such symptoms.

Adverse events

Serious Adverse Reactions associated with metformin include:
Lactic acidosis (rare in absence of renal insufficiency)
Anemia, megaloblastic
Hepatotoxicity
Hypoglycemia (rare in absence of concomitant use of sulfonylurea)

Common Reactions include:
Diarrhea
Nausea/vomiting
Flatulence
Asthenia (fatigue)
Indigestion
Abdominal discomfort
Anorexia
Headache
Metallic taste
Rash
Ovulation induction

Seeing symptoms of lactic acidosis (eg, weakness, fatigue, drowsiness, unusual muscle soreness, difficulty breathing, stomach pain with nausea, vomiting or diarrhea, feeling cold, dizziness, lightheadedness, slow or irregular heartbeat), requires discontinuation of metformin and immediate medical care. In case of suspicion of lactic acidosis, participants will be instructed to proceed to their local emergency room and contact the study doctor. Similarly, for signs of hypoglycemia such as shakiness, dizziness, sweating, and hunger, participants will be instructed to proceed to their local emergency room and contact the study doctor.

To monitor for any adverse events between visits, weekly phone calls during the first month will be made by study personnel to evaluate the presence of side effects. One additional phone call

will be made at Week 12 (Month 3), midway between visits 2 and 3. Phone calls will be made during the optional extension at weeks 1, 2, 3, 4, 20, 36, 52, 68, 84, and 100.

In case of serious adverse event, the blind will be removed by contacting the study doctor who will then open the envelope containing the subject identifier treatment status (placebo or metformin). Individualized sealed envelope will be given to the site study doctor from the Alberta Health Services Pharmacy Research Office (University of Alberta) and SPSR (CHU Sainte-Justine, University of Montreal). The Pharmacy Research Office and the SPSR will retain a copy of the randomization codes. The randomization of participants will be done directly in the REDCap shared database of the trial.

During the course of a clinical trial, Health Canada and the REB shall be informed of any serious unexpected adverse drug reaction:

a. if it is neither fatal nor life threatening, within 15 days after becoming aware of the information; and
b. if it is fatal or life threatening, within seven days after becoming aware of the information.

Other unanticipated risks:

Drug-related risk: Prediction of drug effects or side-effects in any individual cannot be done with certainty, and unexpected potentially harmful effects could possibly occur. The close clinical and laboratory monitoring of subjects is intended to detect any such unanticipated side-effects so that appropriate corrective measures can be implemented in a timely manner.

Genetic testing risk: Since genetic analysis will be performed to identify the basis of clinical variation and differential response to metformin in both a targeted and unbiased manner, it may be possible to discover some "incidental findings" i.e. information that we were not looking for. This information related to the participant's health could have a direct impact on their clinical care in some cases. We will follow the guidelines from the American College of Medical Genetics reported previously and widely adopted including in Canada (Genet Med. 2013 Jul; 15(7): 565–574).

In the event that a potential significant and actionable result is found and that preventive measures or treatments are available for the participants, they will be notified through the study doctor. In this situation, the study doctor will recommend to repeat these tests to confirm the research results. A meeting with a geneticist and/or a genetic counsellor will be organized to discuss the pros and cons of further testing in the clinical set up If the clinical testing confirms the research results, the medical geneticist will proceed as he/she would do normally in the clinical setting and the health information will be recorded in the participant's medical chart.

Genetic test results for children will be communicated to the family if there is actionable

conduct. As suggested by the American College of Medical Genetics, results for which an adult onset or for which there is no interventions will not be disclosed as this is a research study. In addition, results related to non-paternity will not be disclosed in the context of this research testing for genes affecting the clinical presentation and drug response.

Possible ineffectiveness of treatment: Some patients may not benefit from the treatment provided. If their condition deteriorates to the point that requires immediate effective treatment, they will be discontinued from the study and referred for such treatment.

Duty to report:

If the investigators or study staff learn that a patient is in immediate danger to themselves or others as a result of a mental disorder or for any other reason, the investigator is obligated to contact the appropriate facility (i.e. mental health facility) for immediate referral, which may include involuntary hospitalization.

13) Potential Benefits to Subjects

The potential benefits of study participation are that subjects with FXS:

1. may experience an improvement in physical health, behavioral symptoms, and/or cognitive abilities as a result of treatment with metformin.
2. will undergo neuropsychological assessments, the results of which may be made available to the family of participants on request.
3. will receive medical exams offered through the study. Additionally, a complete blood count will be conducted as a part of this study. Participants/caregivers will be informed of clinically significant findings from either the medical exam or CBC as appropriate.
4. may understand that they are contributing to the scientific knowledge that may lead to expansion of the targeted treatment options for subjects with FXS.
5. will have a direct access to health professionals and will be followed very closely during the study.

No other benefits of participation are anticipated.

14) Multi-Site Research

This trial has a multi-site design. The study sponsor/PI has obtained a material transfer agreement with an additional international research institution (UC Davis MIND Institute) that will collect similar data to contribute to the research endpoints. With the material transfer agreement, de-identified data as well as de-identified biological samples including blood, urine endothelial cells, and fibroblast lines would be shared to compare biomarkers that are outlined in this study (see Section 7). All results and data will be de-identified to protect the confidentiality of

the research subjects. Allocation tables generated by the Data Coordinating Centre statistician have been uploaded to REDCap and sent to the Alberta Health Services Pharmacy Research Office (University of Alberta) and SPSR (CHU Sainte-Justine, University of Montreal). Once the study coordinator has randomized a participant in REDCap (done separately for each site), the randomization number that is generated will be sent to the respective pharmacy, which will then prepare the drug or placebo based on the number they are given and the table that has been provided to them by the DCC.

15) Sharing of Results with Subjects

At the conclusion of the study, the possible medical benefit from the study will be reviewed with the subject and his or her caregiver. Participants/caregivers will not be unblinded at any point during enrollment. This is to prevent unconscious biases that may present as the trial progresses and to ensure quality data. Participants/caregivers will be unblinded once all study procedures and all statistical analyses for all subjects have been completed. Unblinding will be conducted by a study physician. If a subject/caregiver has to be unblinded for emergency purposes, then the subject/caregiver will be notified by the PI or site investigator.

The results of the study will also be published in a peer reviewed scientific journal that will be made publicly available through PubMed Central. In addition, after publication the results will be posted on the MIND Institute website under clinical trials.

Preliminary data may be presented annually at scientific meetings. Preliminary data may also be shared with the network of 22 USA National Fragile X Foundation (NFXF), Fragile X Clinical and Research Consortium clinics, and with the 20 countries involved with the NFXF International Consortium. Permission from Drs. Bolduc and Jacquemont will be required prior to the presentation of Canadian derived data.

16) Prior Approvals

Initial submission was approved by Health Canada on 24-OCT-2018 and the REB on 24-DEC-2018. Amendments were approved by Health Canada on 6-MAY-2019, 15-JAN-2021, and 6-JUN-2021 and the REB on 7-MAY-2019, 13-JUN-2019, 26-JUN-2019, 25-JUL-2019, 20-FEB-2020, 7-MAY-2020, 6-OCT-2020, 19-NOV-2020, 24-DEC-2020, 11-FEB-2021, 15-MAR-2021,

12-APR-2021, 26-APR-2021, 8-JUN-2021, 17-JUN-2021, 16JUL-2021, 27-OCT-2021, 11-JAN-2022, 22-MAR-2022, 6-APR-2022, and 5-JUL-2022.

17) Economic Burden to Subjects

There is no charge for the subject to participate in this study. Neither the subject nor his or her caregiver will be charged for taking part in the research. All costs associated with the study will be paid by the sponsor/department.

Possible expenses to the subject's family include time spent at the clinic as well as expenses for travelling. Subjects will not be paid just for taking part in this study (i.e., compensated). However, all eligible research subjects, who would not otherwise be able to participate due to the cost of travelling to the University of Alberta (e.g., flights, gas, lodging), will be offered reimbursement per visit for actual costs incurred.

18) Drugs or Devices

We will treat children with Fragile X syndrome aged 6 to 35 years old in a randomized, double-blind, placebo-controlled trial of metformin for 4 months. Randomization to metformin or placebo will be carried out by the Alberta Health Services Pharmacy Research Office (University of Alberta) and SPSR at CHU Sainte-Justine (University of Montreal) after they are given the randomization number that is generated in REDCap by their respective site, and dosing will be determined based on patient weight (see below). Metformin is available in a 500 mg tablet, which Strathcona Pharmacy staff will split into 250 mg halves and over-encapsulate to create both 250 mg and 500 mg capsules. Capusules will be white for a dose of 250mg and pink for a dose of 500mg. Strathcona Pharmacy staff will also develop identical placebo capsules. Metformin pills will be purchased, split in half, and encapsulated by each Canadian site (Strathcona pharmacy for University of Alberta site and the central pharmacy at CHU Sainte-Justine for Universite de Montreal site). Matching placebo capsules will be manufactured by the Strathcona Pharmacy for the University of Alberta site and the central pharmacy at CHU Sainte-Justine for the Universite de Montreal site.

Subjects under 50 kg will start at an initial dose of 250 mg once a day at dinner by mouth and increase to 250 mg twice a day after the first week. If well tolerated, after the second week the dose will be increased to 250 mg at breakfast and 500 mg at dinner, and then to 500 mg twice a day after the third week for a maximum dose of 1000 mg per day.

Subjects at and over 50 kg will start at an initial dose of 500 mg once a day at dinner by mouth and increase to 500 mg twice a day after the first week. If well tolerated, after the second

week the dose will be increased to 500 mg at breakfast and 1000 mg at dinner, and then to 1000 mg twice a day after the third week for a maximum dose of 2000 mg per day.

Since the maximum tolerated dose may vary between individuals, dose modifications may be made at the discretion of the study doctor as well during the treatment dose titration period for the first 4 weeks of the study; for instance, if a subject experienced adverse events after increasing from 500 mg to 1000 mg, decreasing the dose back to 500 mg would be allowed per protocol. The dose may then increase again at the discretion of the study doctor. After the dose titration period, for the remaining 12 weeks of the study, dose adjustments may be performed considering intercurrent illnesses associated with gastrointestinal symptoms.

The Strathcona pharmacy will buy, store, and dispense the study drugs for the Edmonton site. Drug will be dispensed to the Alberta Health Services Research Pharmacy, where the study staff can pick it up to dispense to each participant. The SPSR at CHU Sainte-Justine (Universite de Montreal) will buy, store, and dispense the study drugs for the Montreal site.

Randomization:

Allocation tables generated by the Data Coordinating Centre statistician have been uploaded to REDCap and sent to the Alberta Health Services Pharmacy Research Office (University of Alberta) and SPSR (CHU Sainte-Justine, University of Montreal). Once the study coordinator has randomized a participant in REDCap (done separately for each site), the randomization number that is generated will be sent to the respective pharmacy, which will then prepare the drug or placebo based on the number they are given and the table that has been provided to them by the DCC.

Participants will be randomized to treatment or control arm with a 1:1 allocation ratio, stratified by sex and using permuted blocks of randomly varied sizes 2 and 4. Participant randomization will be performed in REDCap, based on allocation tables that have been generated and uploaded by the Data Coordinating Centre statistician. REDCap maintains an automated audit trail which includes the assigned study identification number, treatment allocation, and date and time of the transaction.

The randomization process will be performed the same way at both sites, i.e. randomization to treatment or control arm with a 1:1 allocation ratio stratified by sex and using permuted blocks of randomly varied sizes 2 and 4.

19) ClinicalTrials.gov Registration

A description of this clinical trial is available on Clinicaltrials.gov (NCT03862950). This website does not include information that can identify the participants/caregivers. At most, the website will include a summary of the results of the research.

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