A Double-Blind, Placebo-Controlled Trial of Metformin in Individuals with Fragile X Syndrome

1) **Protocol Title:** A Double-Blind, Placebo-Controlled Trial of Metformin in Individuals with Fragile X Syndrome (FXS)

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**Canadian Co-Principal Investigator (Co-PI):** Dr. Sebastien Jacquemont, MD

**Funding Sponsor:** 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 6-25 years), with FXS

**Study duration:** estimated duration of 3 years, starting in Fall of 2018

**Participant duration:** 4 months

**Protocol Version Date:** 19-October-2018

**Protocol Number:** FXSMET-2018 Version 5

2) **Objectives**

1. Assess safety and tolerability of metformin in individuals with FXS ages 6 to 25 who are treated over a 4-month period.

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.

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), behavioral and quality of life measures [Aberrant Behavioral Checklist–Community (ABC-C) fragile X version, Hyperphagia Questionnaire, Child Sleep Habits Questionnaire (CSHQ), Swanson, Nolan and Pelham questionnaire (SNAP-IV), and Pediatric Quality of Life Questionnaire (PedsQL Parent Proxy)], in addition to standard measures of outcome [Clinical Global Impression Scale-Improvement (CGI-I), Visual Analog Scales (VAS), and Vineland Adaptive Behavior Scales–Third Edition].

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, and CYP450 allelic variants, will be studied.

3) **Background**

FXS is the leading monogenic cause of intellectual disability and autism spectrum disorder. Treatment in FXS is currently to 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 Nature Medicine). 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, in press) 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.
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(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 in addition to 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; 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, 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). So we will also assess polymorphism in that gene.

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).
2. Subject is a male or non-pregnant, non-lactating female age 6 to 25 years, inclusive.
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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 parent or caretaker 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 parent/legal authorized representative 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 parent/legal authorized representative 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 IQ, as assessed at Screening on the Leiter-III, is not higher than 79, 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 25 years.
4. History of intolerable adverse events with metformin.
5. Current or recent metformin treatment (within the past year).
6. BMI less than 2 standard deviations 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.
10. Pregnancy at screening or unwillingness to use acceptable method of birth control, if applicable.
11. IQ higher than 79 on the Leiter-III at Screening.

Consenting Process:

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Subjects will be recruited and consented at each site. The research coordinator will speak with both the subject and at least one caregiver (parent or guardian, or other legally authorized representative (LAR)) 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 or legally authorized representative (LAR) (consent labeled subject with parent consenting). For all subjects 7-25, 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 and signed by the study doctor before any other study procedures are done. 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 parents/LARs 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 (labeled adult subject consenting). 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 parent/LAR will need to sign the consent form used for subject with parents consenting. All potential subjects will attend their screening visit with either a parent or LAR able to give surrogate consent.

A separate consent form or separate signatures (at CHU Sainte-Justine) for either subject consenting or LAR consenting will be filled or obtained for the optional biobanking of blood and urine as well as optional skin biopsy and the study data.

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, and once at Week 12/Month 3.
6) Study Endpoints

The primary outcome measure will be the Expressive Language Sampling (ELS) measure at baseline and at the end of treatment, as described in detail in Section 7. All other measures will be secondary outcome measures and are also described in detail in Section 7. Safety endpoints will include safety blood draws and physical exams at each study visits.

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). Blood will also be drawn for safety laboratory tests, including a hemoglobin A1c (HgbA1c), fasting blood glucose, complete blood count (CBC), vitamin B12 level, complete metabolic panel (CMP), cholesterol, LDL, triglyceride, and renal clearance function, blood and urine pregnancy test for female participants in childbearing age, along with biomarker studies described below. We will require 8 hours of fasting prior to the blood draw. Participants will only be allowed water during the fasting period. 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, 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.

Patients will be randomized to either metformin or placebo by the Drug Development and Innovation Center (University of Alberta) 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. In the first 4 weeks, each patient will receive a weekly call to evaluate tolerability of the medication, any adverse events (AEs), and gradual titration of the metformin. 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 to 500 mg daily for 1 week and then to 1000 mg daily after two weeks. As such, the maximum dose of metformin in patients under 50 kg will be 1000 mg daily. We will ensure during the first visit that all patients are taking a multiple vitamin that has B12 to avoid potential anemia. If potentially related AEs such as diarrhea are experienced, the dose can be lowered if necessary to what is tolerated without AEs within the first 4 weeks. Then at 2 months (Week 8-scheduled visit 2), when at their maximum tolerated dose, they will be seen in clinic for an examination, safety labs, biomarkers, assessment of AEs, and limited studies, including a CGI-I, VAS, NIH Toolbox cognitive battery measures, and behavioral and quality of life questionnaires (ABC-C, Hyperphagia Questionnaire, CSHQ,
SNAP-IV, and PedsQL). Subjects will remain at the designated dose through Visits 3 (at Week 16/end of treatment) and will be contacted at Weeks 1, 2, 3, 4, and 12 by study staff to assess for possible adverse events 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, documentation of AEs, and outcome measures will be repeated at the second and final follow-up visit at 4 months/end of treatment. We will also record any seizure activity using a seizure log where the date and time, seizure type, duration and use of abortive medication will be recorded. We will also suggest to families to attempt to video record the episodes when possible. We will record also any change to the seizure medication by the managing physician of the participant.

**Metformin Tritation Schedule ( < 50 kg)**

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<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 4-16</th>
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<tbody>
<tr>
<td>Total daily dose</td>
<td>250 mg</td>
<td>500 mg</td>
<td>1,000 mg</td>
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**Metformin Tritation Schedule ( > 50 kg)**

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<tbody>
<tr>
<td>Total daily dose</td>
<td>500 mg</td>
<td>1,000 mg</td>
<td>1,500 mg</td>
<td>2,000 mg</td>
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The primary outcome measure will be the Expressive Language Sampling (ELS) measure, as described below. 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. Attempts will be made to administer all secondary measures for each participant, to the extent his or her cognitive age and ability allows.

**Treatment Measures:**

**Autism Diagnostic Observation Scale (ADOS-2)** – The ADOS-2 (Lord et al., 2000) is a semi-structured standardized assessment administered directly for the purposes of diagnosing ASD, 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 Leiter-III – 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.

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).

The Anxiety Depression and Mood Scale (ADAMS) - ADAMS is a 28-item questionnaire used to screen for psychiatric disorders in persons with ID. Behaviors are rated on a 4-point Likert scale ranging from 0 (“not a problem”) to 3 (“severe problem”). 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 is completed by the caregiver and takes about 10 minutes.

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). A composite measure is created from computerized analyses of multiple dimensions of the talk produced by the participant (e.g., vocabulary diversity, syntactic complexity). These procedures produce a measure with strong psychometric properties for clinical trials involving individuals with FXS (Berry-Kravis et al., 2013).
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.

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). 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.

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

The ERP will be based on EEG data is acquired using a 72-channel (or 128-channel 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 were 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
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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 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, under review).

NIH Toolbox Measures, Cognitive Battery – The following set of measures will be administered at the first visit, visit 2 (week 8), 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.

**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.

**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 an 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).

**Hyperphagia Questionnaire** – This measure consists of a series of questions relating to the subject’s food intake and overeating habits usually part of routine history. It will be completed by the caregiver at baseline, week 8, and end of treatment/week 16.

**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.

**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.

**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.

**The Memory Game** - This is an online test developed in the Bolduc Laboratory done on a tablet at home. 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 an online consent form followed by a short demonstration video. The participants are
then shown pairs of pictures they must remember. Testing is performed right after the presentation and again 24 hours later. For the 24 hours testing, the participants will receive an email reminder and will be invited to login to the Memory Game site to perform the association test this time without being shown the demonstration video. The testing takes about 20 minutes the first day and 7 minutes the second day.

**Laboratory Evaluations:**

**Safety Lab Tests** – At the first visit (baseline) and at the week 8 (second visit) and end of treatment/week 16 (third visit) follow-up visits, HgbA1c, fasting glucose, CBC (complete blood count), lactate level, vitamin B12 level, CMP (comprehensive metabolic panel), and cholesterol, LDL and triglyceride will be drawn. For those female participants who are both capable of becoming pregnant and sexually active in a way that could lead to pregnancy, pregnancy testing will be performed via blood and urine testing at baseline and repeated in urine at each subsequent visit. Urinalysis will be performed on all participants able to provide a sample.

<table>
<thead>
<tr>
<th>Safety Lab Tests</th>
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<tbody>
<tr>
<td><strong>Chemistry</strong></td>
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<tr>
<td>Complete Metabolic Panel: Light green top (3mL)</td>
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<tr>
<td>Sodium</td>
</tr>
<tr>
<td>Potassium</td>
</tr>
<tr>
<td>Chloride</td>
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<tr>
<td>Carbon Dioxide</td>
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<tr>
<td>BUN</td>
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<tr>
<td>Creatinine</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Calcium</td>
</tr>
<tr>
<td>Total Protein</td>
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<tr>
<td>Albumin</td>
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<tr>
<td>ALP</td>
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<td>AST</td>
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<td>ALT</td>
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<tr>
<td>Total Bilirubin</td>
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<tr>
<td>Cholesterol</td>
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<tr>
<td>LDL cholesterol</td>
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<tr>
<td>Triglyceride</td>
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<tr>
<td>Fasting glucose</td>
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</table>
Molecular Biomarkers – In addition to the blood for safety labs, we will collect approximately 18.5 mL of blood at each visit for biomarker analysis at the protein, RNA and genomic level.

**Protein biomarkers**

**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, urine or skin fibroblast. The optional skin biopsy will be carried out after lidocaine is applied topically so that the procedure is painless and then lidocaine (approximately 2 cc is injected and then a small punch biopsy is done covered with a steristrip and then 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. 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. 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.

**Method:** FMRP measurements will be done in Dr. Paul Hagerman’s laboratory at the MIND Institute. They will use 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 (Eu³⁺-Cryptate) and the acceptor is “d2”. Total protein concentration is determined using the Thermo Fisher MicroBCA Assay (23235). Protein levels will be determined
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 is 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 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 children 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 RNA purified.

**Genomic biomarkers**

For additional molecular studies, including genotyping, gene and protein expression, blood and urine samples will be collected at baseline, at 2 months, 4 months, and at the end of the treatment period. Plasma, DNA, and peripheral blood mononuclear cell (PBMCs) will be used for the proposed experiments. The PI also intends to obtain 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. In the event of 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 deidentified upon transfer.

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 variation 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.

**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 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:
### A Double-Blind, Placebo-Controlled Trial of Metformin in Individuals with Fragile X Syndrome

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Visit 1 Week 1, Day 0</th>
<th>Visit 2 Week 8 +/- 14 days</th>
<th>Visit 3 Week 16 +/- 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Informed Consent</td>
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<tr>
<td>Inclusion/Exclusion Criteria</td>
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<td>Medical History</td>
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<td>Physical and Neurological Exam</td>
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<td>Vital Signs</td>
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<tr>
<td>Adverse Events(^a)</td>
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<tr>
<td>Concomitant Medications(^a)</td>
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<td>Autism Diagnostic Observation Schedule (ADOS-2)</td>
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<tr>
<td>Leiter-III</td>
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<td>Clinical Global Impression Scale – Severity (CGI-S)</td>
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<td>Clinical Global Impression Scale – Improvement (CGI-I)</td>
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<td>Expressive Language Sampling (ELS)</td>
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<tr>
<td>Eye Tracking Measures(^b)</td>
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<td>Event Related Potential (ERP) Measures(^b)</td>
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<td>NIH Toolbox Cognitive Battery Measures(^b)</td>
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<td>Visual Analogue Scale (VAS)</td>
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<td>Aberrant Behavior Checklist–Community (ABC-C)</td>
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<tr>
<td>Hyperphagia Questionnaire</td>
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<tr>
<td>Child Sleep Habits Questionnaire (CSHQ)</td>
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<tr>
<td>Swanson, Nolan and Pelham Questionnaire (SNAP-IV)</td>
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<td>X</td>
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<tr>
<td>Pediatric Quality of Life (PedsQL) Parent Proxy Questionnaire</td>
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<tr>
<td>The Memory Game (online)</td>
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<td>Blood Draw</td>
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<tr>
<td>Urine Collection</td>
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<tr>
<td>Skin Biopsy (optional)</td>
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</table>

\(^a\) Ongoing assessments during the study.

\(^b\) Ongoing assessments of the primary outcome measures.
<table>
<thead>
<tr>
<th>Dispense Study Drug</th>
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<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collect Study Drug</td>
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<td>X</td>
</tr>
</tbody>
</table>

- a: Also assessed at phone calls at Weeks 1, 2, 3, 4, and 12
- b: If subject is capable of completing assessment

### 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 from referral from other centers in Canada. The University of Alberta site will also recruit patients from other centers 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 feed and in clinical spaces.

Educational and scientific conferences by the Co-I to health professionals, education system 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 will be created with videos and testimonial from clinicians, researchers, patients and family members to generate interest and facilitate recruitment.

Potential subjects who are interested in participating in the study will be able to contact the study team member to schedule their visit 1. This will also allow potential subjects to ask study-related questions and discuss the study in depth with the research team. Informed consent will be signed at the first visit.

#### 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 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 clinical trial unit or equivalent space dedicated to clinical care or research which will provide as private a setting as possible.

The study monitor board, other authorized representatives of the study sponsor (Azrieli Foundation, 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 as long a period as dictated by the 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.

Analysis will be based on the intention-to-treat principle. The team at the University of Alberta and MIND Institute will collaborate on data analysis. The data will be merged into one dataset shared and analyzed across the different sites Deidentified data will be shared across sites need to be clearly written in the consent form including the consent UC Davis form.

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 provide the power for the t-test and power for the ANCOVA will be higher depending on this factor. We estimate clinically relevant effects sizes from our preliminary data at follow-up visit for (metformin/placebo) as follows: 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 will be established for the Canadian centers and located at the University of Alberta. The Canadian DSMB will collaborate and exchange information with the DSMB of the US site at the MIND Institute. The 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 event 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 IRB of each site respectively and the FDA 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.

Biological samples (blood, urine and skin) 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 blood draw volume of up to 25 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 the study consent form, participants 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 at the University of California Davis or 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 volume of up to 16 mL and will be processed through the Clinical trial Lab at the University of Alberta. Safety labs from the Universite de Montreal will be assessed locally as well. Banked samples will be stored and could be used for future studies by our study team or other scientists approved by our team.

Urine:

Urine samples for safety and biomarker analysis will be collected at three points throughout the study. Samples will be stored in one of the biobank facilities described above. Banked samples will be stored and could be used for future studies.

Skin:

Skin sample will be collected at the baseline visit only. As described above with regards to blood specimens, participants may indicate permission to allow for their skin sample to be kept and stored indefinitely at each biobank facility for use in future research; otherwise, samples will be destroyed upon completion of the study.
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 preparing 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.

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, hunger, participants will be instructed to proceed to their local emergency room and contact the study doctor.

11) Withdrawal of Subjects

All subjects and their parent/legal authorized guardian 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 withdraw consent to participate. Investigator 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, as well as any blood and stool samples, will be kept for analysis unless the subject’s caregivers specifically ask for this information to be removed from the analysis. Caregivers will be both informed of this as a part of the consent process and also reminded of this in the event of an early termination.

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 will be applied to numb the area.

Metformin is an 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 regular release and extended release formulations of metformin, as well as 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.

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.

In case of serious adverse event, the blind will be removed by contacting the study doctor who will then open the envelop containing the subject identifier treatment status (placebo or metformin). Individualized sealed envelop will be given to the site study doctor from the DDIC (University of Alberta) and SPSR (CHU Sainte-Justine, University of Montreal) who will proceed to the randomization for their respective site. The DDIC and the SPSR will retain a copy of the randomization codes.

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 a potential significant and actionable result is found and that preventive measures or treatments are available for the participants, they will be notified through a the study Doctor. In this situation, the study investigator will recommend to repeat these tests to confirm the research results. A meeting with a genetic counsellor will be organized to discuss the pros and cons of further testing in the clinical set up. In addition, a medical genetic doctor will be consulted. 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 will be informed of clinically significant findings from either the medical exam or CBC as appropriate.
4. will 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 anticipates obtaining 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. In the case of a material transfer agreement, deidentified data as well as deidentified 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. The pharmacy and randomization for this study for the Montreal and Edmonton sites will be coordinated in each respective site.

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 parent/guardian. Participants will not be unblinded at any point during their enrollment. This is to prevent unconscious biases that may present as the trial progresses and to ensure quality data. Subjects will be unblinded once all study procedures and all statistical analyses for all subjects have been completed. Unblinding will be conducted by a study
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physician. If a subject has to be unblinded for emergency purposes, then the subject 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.

16) Drugs or Devices

We will treat children with Fragile X syndrome aged 6 to 25 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 Drug development and Innovation Center (DDIC) at the University of Alberta and SPSR at CHU Sainte-Justine (University of Montreal), and dosing will be determined based on patient weight (see below). This is a controlled, double-blind study of metformin. Metformin pills will be purchased, split in half, and encapsulated by the each Canadian site (Strathcona pharmacy for University of Alberta site and SPSR 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 SPSR at CHU Sainte-Justine for the Universite de Montreal site.

Subjects under 50 kg will start at 250 mg at dinner for 1 week and then increased to 250 mg twice a day with meals for 1 week and then increased to 1000 mg twice a day with meals for the remaining of the study. Subjects 50 kg and over will start at 500 mg at dinner for 1 week and then increased to 500 mg twice a day for 1 week then increased to 500 mg AM and 1000 mg at dinner for 1 week and then increased to 1000 mg twice a day for the remaining of the study. If the dosing is not tolerated due to minor side effects (such as gastrointestinal-GI) the dose will be held for 4 days and then reassessed by the study coordinator or doctor. 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. After the dose titration period, for the remaining 12 weeks of the study, only one dose modification will be allowed by protocol at the discretion of the study doctor; more than one dose modification after the first 4 weeks will be cause for early termination from the study.
The University of Alberta pharmacy will buy, store and dispense the study drugs for the Edmonton site. The SPSR at CHU Sainte-Justine (Université de Montreal) will buy, store and dispense the study drugs for the Montreal site.

17) ClinicalTrials.gov Registration

A description of this clinical trial will be available on Clinicaltrials.gov. This website will not include information that can identify the participants. At most, the website will include a summary of the results of the research.

References Cited:


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