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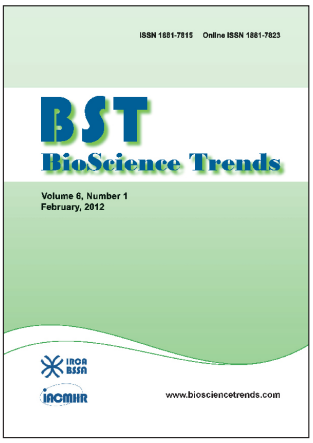

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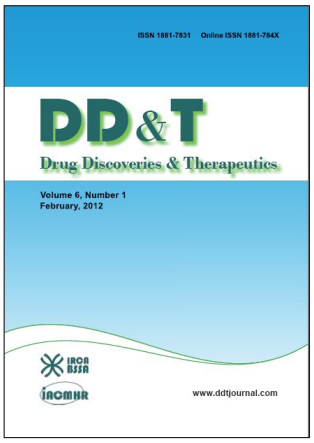

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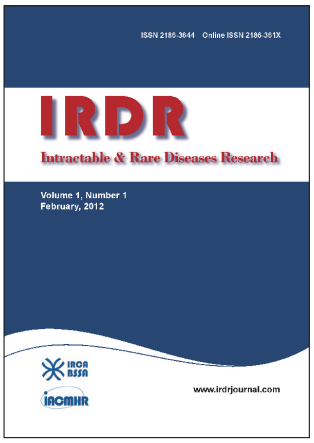

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Editorial Board

CONTENTS

Volume 3, Number 4, 2014

Editorial

Reviews

Original Articles

Case Reports

CONTENTS (*Continued*)

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Editorial

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Translational research guided by animal studies in Fragile X Disorders

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This special issue on fragile X-associated disorders will open your eyes to the broad spectrum of clinical involvement that occurs with mutations in the *FMR1* gene. This gene creates a protein, FMRP, which is a key protein for regulating the translation of hundreds of mRNAs, particularly those involved in synapse formation and plasticity. Fragile X syndrome (FXS) results from the loss or deficiency of FMRP and it is the most common cause of inherited intellectual disability and autism or autism spectrum disorder (ASD). The review of animal models for FXS by Kazdoba-Leach *et al.* (2014) in this issue, demonstrates how these models have led the way to targeted treatments for FXS and for ASD. One of the more promising new treatments for FXS is the use of low dose sertraline in young children 2 years and older with FXS. The paper by Hansen and Hagerman (in this edition) outlines the benefits of sertraline including the enhancement of serotonin neurotransmission, neurogenesis, and BDNF levels that has the potential to improve language and development for these children. GABA agonists and mGluR5 antagonists have also been studied in FXS but the mouse model is easily rescued with many different targeted treatments, whereas the patients with FXS have only responded well to a few new treatments. There is a great need to improve the participation of minorities in the new clinical trials of targeted treatments for FXS and this is reviewed in detail by Chechi *et al.* (2014) in this issue.

Fragile X-associated disorders include both FXS and premutation disorders also. The field of premutation involvement (55 to 200 CGG repeats in the 5'end of *FMR1*) is growing rapidly as reviewed by Lozano *et al.* (2014) in this issue. RNA toxicity from elevated levels of *FMR1* mRNA leads to molecular consequences that affect neurological, endocrine, psychiatric and rheumatological health throughout the lifespan. Problems may begin in childhood, such as anxiety, ADHD and social deficits, with additional issues that complicate adult life including early ovarian insufficiency, hypothyroidism, fibromyalgia, migraines, hypertension, sleep apnea, restless legs syndrome, neuropathy and eventually for some, the fragile X-associated tremor ataxia syndrome (FXTAS). The premutation is common in the general population, approximately 1 in 130-250 women and 1 in 250 to 450 males as reviewed by Muzar *et al.* (2014) in this issue. Both FXTAS and other premutation disorders are under-diagnosed currently and it behooves physicians and other health care providers to read the enclosed papers carefully so that premutation disorders are considered in the differential diagnosis of these common medical problems. Often the diagnosis is considered when the family history includes someone with autism or intellectual disability of unknown etiology or an older relative with a Parkinsonian symptom complex or even dementia. It is easy to order a fragile X DNA test if either a premutation or a full mutation disorder is suspected. Once a diagnosis is made then a treatment plan can be made. Life style changes are important in the treatment of premutation carriers since substance abuse can exacerbate FXTAS as demonstrated in the cases of Muzar *et al.* (2014) in this issue. Other treatment options are discussed in the FXTAS review in this volume.

Once a diagnosis of a fragile X condition is made then genetic counseling is recommended and all family members who are at risk for a premutation or a full mutation should be tested. The risk for a women with the premutation to pass on a full mutation to her offspring is significantly impacted by the number of AGG anchors she has within her CGG repeats. An AGG anchor occurs approximately every 9 to 10 CGG repeats and the more anchors one has the lower the risk for expansion to a full mutation in the next generation. Yrigollin *et al.* (2014) in this issue clarifies this risk in a broad array of international populations. This work guides genetic counselors in their risk assessment for families.

This volume contains a rich array of papers that traverses molecular to animal to human studies to give a full picture of the progress in the fragile X field. Clinicians and bench scientists will all benefit from the research presented in this volume.

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Review

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Current research, diagnosis, and treatment of fragile X-associated tremor/ataxia syndrome

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Summary **Fragile X-associated tremor/ataxia syndrome (FXTAS) is caused by a premutation CGG-repeat expansion in the 5'UTR of the fragile X mental retardation 1 (*FMR1*) gene. The classical clinical manifestations include tremor, cerebellar ataxia, cognitive decline and psychiatric disorders. Other less frequent features are peripheral neuropathy and autonomic dysfunction. Cognitive decline, a form of frontal subcortical dementia, memory loss and executive function deficits are also characteristics of this disorder. In this review, we present an expansion of recommendations for genetic testing for adults with suspected premutation disorders and provide an update of the clinical, radiological and molecular research of FXTAS, as well as the current research in the treatment for this intractable complex neurodegenerative genetic disorder.**

Keywords: FXTAS, tremor/ataxia, premutation carrier, *FMR1*, *FMR1* mRNA, FMPR, late-onset neurological disorder and neurodegenerative disorder

1. Introduction

The fragile X mental retardation 1 gene (*FMR1*) which causes fragile X syndrome if fully mutated (more than 200 CGG-repeats in the polymorphic region- 5'UTR), was discovered in 1991. This discovery led to the description of premutation carriers (individuals with smaller alleles, 55-200 CGG repeats) and was followed by a better understanding of the transition propensity, the expansion of the unstable allele of women with the premutation, and a better genetic counseling for risk of offspring with fragile X syndrome (the most common monogenetic form of autism and intellectual disability). Later on an intermediate allele was described (45-54 repeats) which has a variable risk for disorders that are associated with the premutation. Although before the discovery of the *FMR1* gene, Cronister and colleagues (*1*) had reported a much higher incidence of early ovarian failure (before the age of 40 years) in females premutation carriers (PMC), PMC were generally seen as clinically unaffected (*2-4*). After the description of fragile X-associated primary ovarian insufficiency (FXPOI) in 1991 and FXTAS in 2001, there has been a general recognition that premutation alleles are associated with a wide range of clinical involvement. It is now widely recognized that PMC are at risk to develop a range of mild cognitive and behaviors problems during childhood and neurological, psychiatric and other immune-mediated disorders during adulthood (*5*). The prevalence of the *FMR1* premutation has been described to be 1 in 113 to 259 females and 1 in 260 to 813 males in the general population (*6-11*). This suggests that about 1 in 3,000 men and about 1 in 6,000 women in the general population have fragile X-associated tremor/ataxia syndrome (FXTAS), which could be a common neurodegenerative disorder among the general population; however, more studies are necessary to define the incidence and prevalence of FXTAS. The clinical recommendations for testing of *FMR1* mutation have been expanded after the description of premutation disorders and in this review we provide recommendations of offering testing for adults and will discuss the recent clinical, radiological, molecular and treatment research in FXTAS.

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2. Clinical indications for FXS genetic testing in adults

The family history is crucial to determine whether

there is an X-linked inheritance pattern of intellectual disability (ID) which would be typical for fragile X syndrome. However clinical suspicion of a premutation disorder should also be a consideration for *FMR1* DNA testing. The American Academy of Pediatrics and the American College of Medical Genetics currently recommends *FMR1* DNA testing for all children and adults with undiagnosed developmental delay/ID (*12*) and/or autism (ASD) (*13*). The American College of Obstetricians and Gynecologist (ACOG) also recommends testing in women with a family history of fragile X-related disorders, such as, unexplained ID/developmental delay, ASD or primary ovarian insufficiency (POI). In order to expand the screening criteria and to capture more premutation carriers the ACOG also recommends offering testing to all women who request fragile X carrier screening regardless of their personal and family history and also recommends to offer prenatal testing by amniocentesis or CVS to a known pregnant PMC (*14*). We also recommend considering genetic testing when there is personal medical history of unexplained late onset dementia or parkinsonism with any other associated premutation disorder and to consider testing in individuals with family history of a member with unexplained POI and mood/anxiety disorder, fibromyalgia and mood/anxiety disorder, and undiagnosed dementia or parkinsonism and anxiety/mood disorder (Table 1).

3. FXTAS

Although the prevalence of FXTAS in the general population is uncertain, FXTAS occurs in approximately 40-45% of male PMC and 8-16% of female PMC over the age of 50 (*15-18*). The common features of FXTAS are cognitive decline, autonomic dysfunction, neuropathy, and psychiatric features such as anxiety, depression, and apathy (*16,19-21*). Impairments in executive function abilities including working memory, inhibitory control and visuospatial processing begin as early as middle adulthood, and progressively worsen with increasing age *(22-26*). Subsequently dementia develops in approximately 50% of male PMC and autonomic dysfunction which is thought to be a consequence of involvement of the peripheral nervous system in common (*27,28*). Premutation-associated psychiatric problems are common in adulthood but these problems can worsen before the appearance of tremor and ataxia (*20,29-31*). The increased lifetime prevalence of mood disorders (65%) and of anxiety disorders (52%) in individuals with FXTAS is greater than in those PMC without the FXTAS (*20,31*). The age of onset of FXTAS is typically between the ages of 60 and 65 years; the mean age of onset is 62 years (*32,33*). However, the chance of developing core symptoms of FXTAS (tremor and ataxia) increases with age. From age 50-59 the prevalence of FXTAS in males is 17 percent, from age 60-69 about 38 percent, from age 70-79 about 47 percent, and in males over 80 years old, about 75 percent (*32*).

Men are more frequently diagnosed with a definite diagnosis of FXTAS compared to women (*34*). A previous longitudinal study of progression of tremor and ataxia in 55 male PMCs showed that tremor usually occurs first, with median onset of ~ 60 years of age (*35*). After the tremor onset, the median onset of ataxia was 2 years later; onset of falls was 6 years later; dependence on a walking aid was 15 years later; and death was 21 years later (*35*). The rate of progression of FXTAS varies and life expectancy is between 5 to 25 years after the onset of the symptoms (*36*).

FXTAS in females was initially reported in 2004 (*37*). FXTAS is less common and shows a milder presentation in females because they have a normal X chromosome in addition to the *FMR1*-premutated X-chromosome (*34*). The proportion of normal *FMR1* alleles on the active X chromosome (activation ratio) is thought to modulate the phenotypic severity in females (*38*); however, double heterozygous female have been reported and they seem to have a similar clinical presentation to heterozygous females (*39,40*). Dementia was found in 21-50% males with FXTAS (*32,36*). In females with FXTAS, however, dementia is far less common (*36,37*) and it has been reported in only a few cases (*41-45*). Females with FXTAS may also exhibit parkinsonism, although at a lower rate than in males with FXTAS (*34*). There are associated symptoms in females with FXTAS that usually do not occur in males, including thyroid disorders, fibromyalgia and chronic muscle pain (*46,47*). Conversion disorder has also been reported in a PMC female (*48*). Migraine headache were reported in a higher rate in females (54.2%) when

Table 1. Guidelines to recommend and offer FXS genetic testing

	Recommend Genetic Testing	Offer Genetics Testing
Women	• Premature ovarian insufficiency • Women who request fragile X prenatal carrier screening	• Family history of POI and mood/anxiety disorder • Family history fibromyalgia and mood/anxiety disorder • Prenatal testing by amniocentesis or CVS for known PMC
All Adults	• Intellectual Disability (ID) • Autism Spectrum Disorder (ASD) • Family history of ID or ASD • Family history of FXS • Unexplained late onset tremor and ataxia	• Late onset dementia associated other premutation disorders • Family history of undiagnosed dementia or parkinsonism associated other premutation disorders •"MCP sign" or white matter lesions in the cerebral white matter on MRI

compared with males (26.8%) with the permutation (*49*). Immune mediated disorders have been described at a higher rate in females with FXTAS 72.73% compared with 46.54% female carriers and both rates are higher than published controls (*47*). Females with FXTAS also have a lower frequency of tremor compared to males with FXTAS (*34*).

4. Radiological findings

Cerebral magnetic resonance imaging (MRI) in patients with FXTAS shows global brain atrophy, enlargement of ventricular volume, white matter disease and heightened signal intensity with lesions in the middle cerebellar peduncles (*50-53*) (Figure 1). The middle cerebellar peduncle (MCP) sign presents as white matter hyperintensities in the middle cerebellar peduncles, and it is a cardinal radiological sign for the diagnosis of FXTAS (*16*). The MCP sign includes fronto-cerebellar tracts connecting to orbitofrontal and dorsolateral prefrontal cortices that are critical for cognitive control (*54*). Correspondingly, those with FXTAS and the MCP sign are likely to have more severe cognitive deficits and a longer history of symptoms than those without the MCP sign (*55*). Asymptomatic *FMR1* premutation carriers show white matter alterations (demyelination and axonal damage) of the afferent projections of the MCPs and superior cerebellar peduncles (*53,56,57*), which may be the earliest neuroanatomical marker of the onset of cognitive and motor symptoms associated with FXTAS (*58*).

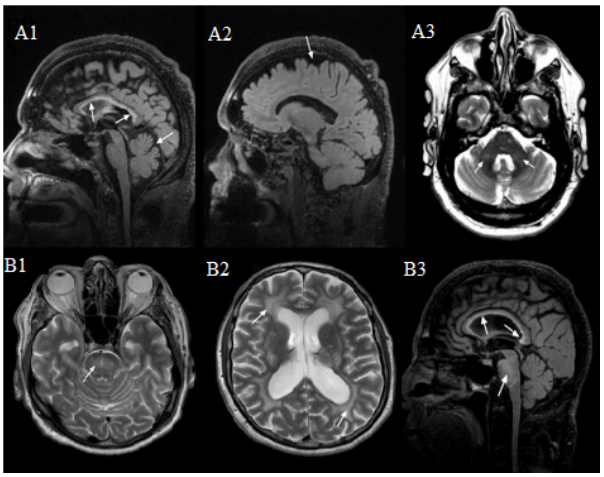

Figure 1. MRI features of FXTAS. **(A1)** Moderately thin truncus of the corpus callosum with severe increased signal intensity in both the truncus and the splenium, and moderate cerebellar and cerebral **(A2)** volume loss. **(A3)** Mild white matter changes in the middle cerebellar peduncles (MCPs). **(B1)** Severe increased T2 signal intensity in the pons (can also be seen in **B3**). **(B2)** Severe diffuse increased T2 signal intensity in the deep white matter of the cerebrum, as well as periventricular. **(B3)** Moderately thin truncus of the corpus callosum with severe increased T2 signal intensity in both the truncus and the splenium.

Other common neuroimaging signs of FXTAS include white matter hyperintensities in the pons, insula, splenium of the corpus callosum, and periventricular region (*59,60*). T2-weighted and FLAIR corpus callosum splenium (CCS) hyperintensity was as frequent (68%) as MCP hyperintensities (64%) and it may be a marker of severe disease progression in FXTAS (*34*). Women with FXTAS have less white matter disease and brain atrophy on MRI, as well as less dementia in late-stages of FXTAS than men with FXTAS (*36,50*). The MCP sign was demonstrated in 13% of females compared with 58% of males with FXTAS (*50*). Corpus Callosum Splenium (CCS) hyperintensities were present in 50% of females versus 72% males (*34*).

5. Diagnosis and clinical severity stage of FXTAS

The FXTAS diagnostic revised criteria are presented in Table 2 (*16,17*). The clinical severity of FXTAS is estimated by using an empirical staging system, which incorporates the motor signs of FXTAS. The system gives an indication of the impact of motor aspects of the disease on activities of daily living as described in Table 3.

6. Molecular mechanisms of FXTAS

PMC were initially described with normal FMRP levels (*61-64*). However new molecular techniques led Tassone and colleagues (2000) (*65*) to the identification of increased *FMR1* mRNA levels; Kenneson and colleagues (2001) (*66*) also demonstrated low FMRP levels in PMC. Current research shows that as the premutation increases from 55 to 200, particularly

Table 2. Current diagnostic criteria of FXTAS

Molecular: *FMR1* gray mutation, premutation or full mutation (Mandatory for all categories).

Diagnostic	Definite	one major clinical + one major radiological or one major clinical + intranuclear inclusions (postmortem)
	Probable	two major clinical or one minor clinical + one major radiological
	Possible	one major clinical + one minor radiological
Clinical	Major	intention tremor; cerebellar ataxia
	Minor	Parkinsonism; moderate to severe short term or executive function deficits; neuropathy
Radiological	Major	MCPs; MRI white matter lesions in splenium of the corpus callosum (or postmortem intranuclear inclusions)
	Minor	MRI lesions in the cerebral white matter; moderate to severe generalized atrophy

MCPs; white matter lesions in middle cerebellar peduncle sign.

Table 3. Clinical staging of FXTAS

Stage	Clinical Description
0	Normal functions
1	Subtle or questionable signs such as subtle tremor or mild balance problems and no interference with ADLs
2	Clear tremor and/or balance problems and minor interference with ADLs
3	Moderate tremor and/or balance problems and occasional falls and significant interference with ADLs
4	Severe tremor and/or balance problems with at least intermittent use of a cane or a walker
5	The use of a wheelchair on a daily basis
6	Bedridden

ADL: activities of daily living.

more than 110 CGG repeats, the level of *FMR1* mRNA increases and the levels of FMRP start to decline (*67,68*). The CGG repeat size also correlates with the age of onset and the age of death from FXTAS (*38,67*). The elevated level of mRNA in PMCs led to the hypothesis of "*FMR1* mRNA toxicity" in FXTAS, however the causative mechanism of increase transcription by the CGG repeat remains unclear as well as the mechanism of neuronal toxicity by the accumulation of the *FMR1* mRNA. There are a few suggested pathological models including; "RNA toxicity"; a sequestration model which suggests that the RNA expanded CGG repeats are pathogenic by toxic sequestration of crucial transcriptional proteins (DROSHA-DGCR8, hnRNP A2/B1, SAM68, Purα, Rm62, and CUGBP1) (*69-72*); a non-canonical translation the CGG repeats which may result in the expression of toxic polyglycine products (*73,74*); and lastly the presence of antisense *FMR1* transcription which may lead to toxicity by antisense transcripts products (*75*).

7. Neuropathology and neurobiology of FXTAS

The neuronal toxicity is thought to be led by the formation of pathognomonic eosinophilic and ubiquitin-positive intranuclear inclusions in neurons and astrocytes throughout the brain, peripheral nervous system and other organs such as the adrenals, thyroid, heart, Leydig cells and pancreas (*28,76-78*). Other findings include mild brain atrophy and involvement of the cerebellum (MPC sign), loss of Purkinje neuronal cells, spongiosis of the deep cerebellar white matter, Bergman gliosis, and swollen axons (*51,77*). Neurons of heterozygous female mice with the premutation showed shorter dendritic lengths and fewer branches between 7 and 21 days compared with wild-type (WT) littermates, display lower viability, and express elevated stress protein levels (*79*). Furthermore altered embryonic neocortical development (*80*) and abnormal spontaneous clustered calcium bursts (*81,82*) with glutamate hyper-responsiveness have been described (*81*); thus suggesting a clear state of neuronal vulnerability.

Mitochondrial abnormalities have also been found in PMC (*83,84*) and recently a decreased immune responses and immune dysregulation in both humans and mice with the premutation were described (*85*). It is unknown how the premutation alters mitochondrial and immunological responses, and if these abnormalities contribute to FXTAS and other associations found in PMC, such as, autoimmune and rheumatologic disorders (*47*).

8. Treatment of FXTAS

There are as yet no effective targeted therapies for the treatment of FXTAS; however there are many medications that have been use to ameliorate some of the symptoms associated to FXTAS. However the use of these medications rely on very few small trials and case studies that showed improvements only in some individuals (*86,87*). The only clinical targeted trial for FXTAS utilized memantine (NMDA receptor antagonist, FDA approved for treatment of moderate to severe Alzheimer's disease since 2003). Memantine was thought to selectively block the excitotoxic effects associated with abnormal transmission of glutamate while allowing for the physiological transmission associated with normal cell functioning. In this randomized, double-blind, placebo-controlled trail, 94 individuals aged 34-80 years with probable or possible FXTAS diagnosis and clinical stages 1-5 were enrolled for one year. Primary outcome measures were the Behavioral Dyscontrol Scale (BDS) score and CATSYS intention tremor severity. Intention-to-treat analysis showed no improvement with respect to intention tremor severity nor BDS scores (*88*). However of those (94 participants) 41 completed longitudinal ERP studies (20 placebo/21 memantine group) and the use of this compound showed improvements on cued-recall memory and N400 repetition effect amplitude; thus suggest that the treatment may have benefits on verbal memory (*88*). More frequent mild adverse events were observed in the placebo group, while more frequent moderate adverse events occurred in the memantine group and these included dizziness, headache and constipation amongst others. As mention before other treatments are directed to symptom reduction. For anxiety and depression selective serotonin and selective norepinephrine reuptake inhibitors are effective (*5,86*) as well as psychotherapy (*31*). Atypical antipsychotics are effective in individuals with psychosis and agitation

(*89*). Propranolol and primidone may improve tremor (*86,87,90*). Deep brain stimulation has shown benefits for tremor and in few cases for ataxia (*91*); however the general outcome for FXTAS patients was poor (*92*). As previously mentioned the premutation causes neuronal susceptibility and therefore other treatments for PMC focus on preventive measures, such as, avoidance of toxins including smoking alcohol and some types of anesthesia, healthy diet and vitamins/antioxidants supplementation, exercise and cognitive training, and stress reduction (*93*).

9. Current research on FXTAS

Phenotype studies aim to determine early signs of disease for early diagnosis and treatment as well as to determine timing and reversibility of the pathological mechanism. A preliminary study shows that oculomotor inhibitory control impairments (measured by eye tracking) might precede FXTAS, and thus indicating elevated risk for motor impairment associated with FXTAS (*94*). Magnetic resonance imaging is useful for non-invasive testing; functional MRI for brain activation during cognitive tasks, and structural MRI for quantification of volume changes, morphometry, and white/gray matter integrity and connectivity. In fact, verbal working memory in male and female premutation carriers (*95*) has been associated with reduced activation in the right inferior frontal cortex and left premotor cortex in both asymptomatic premutation carriers and carriers with FXTAS. Reduced activation was found in right premotor/inferior frontal cortex in individuals with FXTAS. Individuals with FXTAS also showed diffuse gray matter loss most prominent in areas important for working memory, including prefrontal cortex, anterior cingulate cortex, and cerebellum (*96*). Molecular studies aim to determine the early molecular mechanisms that induce neurodegeneration including cellular stress and toxicity. The mechanism for inclusion formation and identification the intranuclear inclusions proteins are also a fertile area of research. Current targeted treatment research focuses on reversing the neurobiological abnormalities in FXTAS with pharmaceutical compounds (*e.g*. allopregnanolone) and other molecular mechanisms of disease modification (oligonucleotide-based therapies to reduce *FMR1* mRNA) as well as designing a mechanism that will allow blood-brain cross-transportation of pharmacological compounds.

Animal models for the fragile X premutation have been developed to understand the molecular mechanism of FXTAS (*97*). Mice models have shown increased *FMR1* mRNA levels, decreased FMRP levels and ubiquitin-positive intranuclear inclusions (*98*). In addition, mice models showed neurocognitive deficits in spatial and temporal memory processes, impaired motor performance, and anxiety traits (*99*). In order to determine timing and reversibility of disease and their associate molecular mechanism, a doxycycline-inducible premutation mouse has been created (R. Hukema, Abstracts of the 1st Premutation Meeting, Perugia, Italy, 2013). Animal models are crucial in the testing of preclinical therapies, for instance the acute administration of the neurosteroid allopregnanolone mitigated cluster burst firing in mouse hippocampal premutation-neurons and identified allopregnanolone as a potential targeted treatment for premutation disorders (*100*).

10. FXTAS

The identification of the *FMR1* gene has led to characterization of risk alleles and recently there are a variety of disorders associated with the premutation in children and adults. Although FXTAS is described to occur in premutation carriers only, recent reports identified FXTAS in individuals with grey zone/ intermediate alleles (*101,102*), as well as in individuals with unmethylated full-mutation alleles (*103*) and in a few patients with full-mutation/premutation mosaicism (*104*). These findings increase the number of patients that are at risk for FXTAS with elevated *FMR1* mRNA besides only those with the premutation. The description of FXTAS as an intractable disorder, has led to expansion of recommendations for genetic testing in adults which in turn have caused ethical concerns for the identification of individuals at risk of FXTAS. These is a concern especially in males with the suspicion of the premutation because males do not have increased risk of having children with fragile X syndrome, but have about a 40% chances to develop FXTAS, if they are determined to be premutation carriers. However, the documentation of the premutation is helpful for both males and females because these individuals can be treated for many of the childhood and adult problems related to the premutation such as anxiety, depression, ADHD, hypertension, hypothyroidism, fibromyalgia, sleep apnea, and can be counseled to avoid toxicity from the environment that has the potential to bring on FXTAS at an earlier age. The identification of radiological signs of FXTAS is used by clinicians to make a clinical diagnosis of FXTAS; however, the phenotypic variability and progression of FXTAS should be taken in consideration as many adults will not meet all clinical criteria until advanced age, particularly females. There are also radiological and clinical gender variations, while males are more prone to develop dementia, females are more likely to develop other autoimmune-related disorders. The phenotypic variability of the premutation is partially explain by CGG expansion size, *FMR1* mRNA levels, decrease FMRP and mosaicism; however, other mechanisms are now being consider including protein synthesis alterations, non-AUG translation, and antisense transcription, as well as, additional

genomic variants and environmental exposures (*5*). Further genotype to phenotype studies are necessary to determine the relative contribution of these pathological processes in this complex disorder. Many FDA approved medications have shown to improve some of the symptoms of FXTAS; however there are limited clinical trials and none that can prove the efficacy of these treatments. It is crucial to undertake further clinical trials of drugs that anecdotally have shown positive results in individuals with FXTAS. There has been only one targeted clinical trial for FXTAS and there is an urgent need to identify more compounds that target the pathogenesis of FXTAS, which in theory may reverse, treat or prevent the development of FXTAS.

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Review

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Serotonin dysregulation in Fragile X Syndrome: implications for treatment

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Summary **Fragile X Syndrome (FXS) is a trinucleotide repeat disorder that results in the silencing of the Fragile X Mental Retardation 1 gene (*FMR1*), leading to a lack of the FMR1 protein (FMRP). FMRP is an mRNA-binding protein that regulates the translation of hundreds of mRNAs important for synaptic plasticity. Several of these pathways have been identified and have guided the development of targeted treatments for FXS. Here we present evidence that serotonin is dysregulated in FXS and treatment with the selective serotonin reuptake inhibitor (SSRI) sertraline may be beneficial for individuals with FXS, particularly in early childhood.**

Keywords: Fragile X Syndrome, fragile X mental retardation protein, selective serotonin reuptake inhibitors, sertraline

1. Introduction

Fragile X Syndrome (FXS) is the leading inherited cause of intellectual disability and autism. A hallmark feature of FXS is delay in receptive and expressive language development and this is often the presenting sign of FXS in early childhood (*1,2*). Symptoms of anxiety, attention deficit hyperactivity disorder (ADHD), and hyperarousal with sensory stimuli are all typical of children with FXS (*3-8*).

FXS is a monogenic disorder caused by an expanded CGG repeat in the 5' untranslated region of the *FMR1*, located on the long arm of chromosome X (*9*). It is considered normal to have between 5-40 CGG repeats in *FMR1*. The premutation is characterized by 55 to 200 CGG repeats and a full mutation occurs at > 200 CGG repeats (*10*). In the full mutation, *FMR1* becomes methylated, resulting in significantly reduced or absent levels of the FMR1 protein (FMRP). FMRP is a selective, inhibitory, mRNA-binding protein that regulates the translation of mRNAs into their respective proteins (*11*). It is expressed throughout the body, but is especially critical in neuronal soma and dendrites because most of the proteins that are regulated by FMRP are important for synaptic plasticity (*12,13*). Since FMRP expression depends on age, the lack of FMRP in FXS is particularly disruptive in early development, when synapse formation is especially dynamic (*14*).

As a result of the loss of FMRP expression, many neurochemical pathways are disrupted in patients with FXS (*15,16*). For example, there is up-regulation of the metabotropic glutamate receptor 5 (mGluR5) pathway leading to enhanced long term depression (LTD), down-regulation of GABA pathways (*17*), and dysregulation of dopamine and cholinergic pathways (*12,18*). Here we discuss evidence that serotonin (5-hydroxytryptamine, 5-HT) represents another potential target for mechanistic therapy.

2. Serotonin in FXS mouse models

Findings in animal models of FXS provide evidence that serotonin can be specifically helpful in treating the dysregulated pathways in FXS.

One of the pathways known to be dysregulated in FXS is the mGluR-regulated LTD pathway (*11,19,20*). In the mGluR-mediated LTD mechanism, stimulation of postsynaptic group 1 (Gp1) mGluRs at a dendrite rapidly evokes local protein synthesis that results in the

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internalization of AMPA receptors (AMPARs), such as GluA1 (GluR-A) from the synapse. Among the proteins synthesized upon stimulation at the dendrite is FMRP. FMRP is an mRNA translation repressor and serves as the negative feedback to the increased protein synthesis (*20*). Without FMRP, such as in FXS, evoked protein synthesis runs unchecked, leading to excessive AMPAR internalization and, thus, exaggerated LTD in response to a stimulus. Using hippocampal slices from the FXS mouse model, Costa *et al*. (*21*) showed that stimulation of postsynaptic 5-HT_7 serotonin receptors successfully ameliorates the exaggerated mGluR5-mediated synaptic LTD in FXS to wild-type levels.

GluA1-dependent long-term potentiation (LTP) is also disrupted in FXS (*22*), and can be partly corrected by serotonin. Lim *et al*. (*23*) showed this with an experiment measuring synaptic GluA1 delivery in hippocampal slice preparations from *Fmr1* knockout (KO) and wild-type mice. Although GluA1 delivery to the synapse is normally impaired in the FXS model, application of a $5HT_{2B}$-R agonist restored about 20% of GluA1 synaptic delivery.

3. Serotonin in patients with FXS and autism

There is limited research concerning serotonin levels in people with FXS. One study done by Hessl *et al*. (*24*) found that genetic polymorphisms in the gene encoding serotonin reuptake transporter protein correlated with levels of aggression in patients with FXS. Those individuals with polymorphisms conferring higher reuptake (a 44 base pair insertion in the promoter region at 17q11.2 of the 5-HTT receptor) correspond to a more aggressive FXS phenotype.

Although there have been few studies specifically in the FXS population, significant research has been done in children with autism. This research is still highly relevant since there is significant overlap between autism and FXS (*3,25*). An analysis of de novo gene mutations resulting in autism showed that 30-50% of autism genes are regulated by or associated with FMRP (*26*). As previously mentioned, FXS is the leading monogenetic cause of autism. One third of patients with FXS are diagnosed with autism and another third meet criteria for autism spectrum disorder (ASD) (*25,27*). Children with FXS that did not meet ASD criteria still had autistic features such as poor eye contact, hand flapping or hand stereotypies, in addition to shyness or social anxiety (*5,27*).

There is ample evidence that normal serotonin synthesis is disrupted in patients with autism. For example, it has been shown that metabolism of tryptophan, the amino acid precursor to serotonin, is decreased in patients with autism (*28*). Additionally, studies in which adults with autism were deprived of tryptophan found that this diet worsened autistic symptoms (*29*). Tryptophan metabolism occurs in mitochondria and follows one of two pathways, leading to either the creation of serotonin/melatonin or kynurenin-quinolinic acid. Both pathways also lead to nicotinamide adenine dinucleotide (NADH) production. In experiments done by Boccuto *et al*. (*28*), comparisons between lymphoblastoid cells from patients with autism and controls revealed a uniting abnormality in the cells from patients with autism: reduced ability to process tryptophan. The origin of autism in the study patients included both syndromal and non-syndromal cases. Subsequent genetic analysis revealed abnormally low levels of key enzymes involved in mitochondrial tryptophan metabolism (Figure 1). These proteins include SLCA5 and SLC7A8 (enzymes involved in tryptophan transport into mitochondria), WARS2 (tryptophanyl tRNA synthetase), TPH2 (tryptophan hydroxylase 2, rate-limiting enzyme in the serotonin/melatonin pathway inside mitochondria), as well as TDO2 (tryptophan 2,3-dioxygenase) and AADAT (aminoadipate aminotransferase), enzymes involved in the kynurenin-quinoloinic acid pathway. It is interesting to note that children with FXS often have sleeping difficulties (*30*), which may be related to dysfunction of melatonin due to ineffective tryptophan processing. Therefore, is not surprising that patients with FXS usually show improvements in their sleep patterns with melatonin treatment (*31*).

Children with ASD also have a significantly different capacity for serotonin production compared to children without ASD during development. Serotonin levels are normally relatively high in the developing brain compared to adults. This peak appears between ages 2-5 years, when brain serotonin synthesis capacity reaches twice the levels found in the adult brain (*32*). After age 5, synthesis ability declines until age 15, when it reaches adult levels. Children with autism, however, do not reach the same 2-5 year old peak. Instead, their serotonin levels increase slowly, resulting in a relatively low level during the 2-5 year old period and ending up at a higher level in adulthood (*32*). This suggests that therapeutic intervention with an SSRI may be more beneficial during this critical window in early childhood as opposed to later in life for children with autism, including those with FXS.

Children with ASD also display abnormal cortical asymmetry in serotonin synthesis capacity (*33*). Notably, the specific pattern of asymmetry correlates with symptom presentation. For example, children with decreased left-sided serotonin synthesis have a higher rate of language impairment.

4. Serotonin and up-regulation of brain derived neurotropic factor (BDNF)

An intricate relationship seems to exist between serotonin and BDNF. Treatment with an SSRI can up-regulate BDNF levels (*34,35*), and BDNF can also

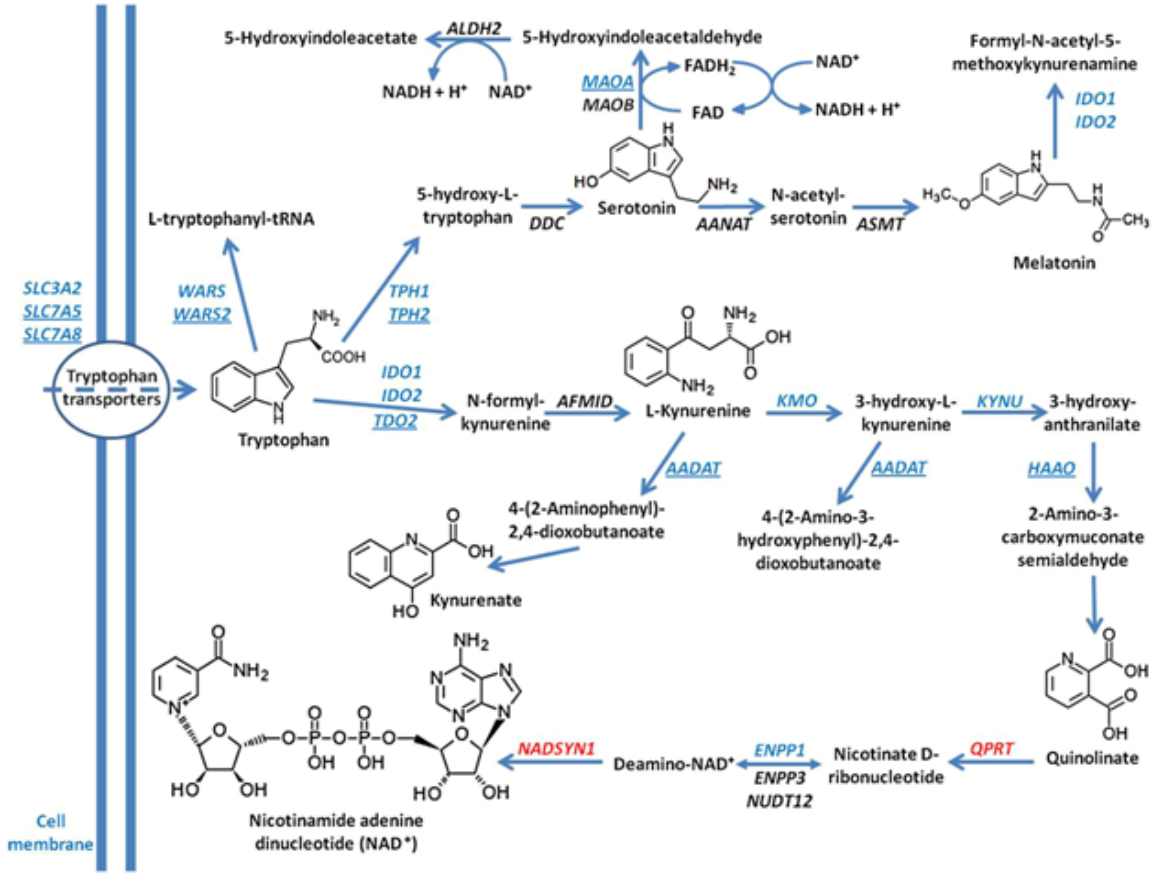

Figure 1. Tryptophan pathways in patients with autism. The figure illustrates the main intracellular pathways involving tryptophan. The microarray dataset of Boccuto *et al*. 2013 (consisting of patients with autism) are in blue, genes with increased expression are in red. Genes with statistically significant reduction of expression in patients with autism are underlined. (*Note*: Figure reprinted and legend adapted from "Decreased tryptophan metabolism in patients with autism spectrum disorders" by Boccuto L, *et al*., 2013, Molecular autism, 4(1), page 7. Copyright 2013 by BioMed Central. Reprinted with permission.)

stimulate serotonin synthesis (*36*).

BDNF is a critical component of synaptic maturation, synaptic plasticity, and neurogenesis (*37-40*). FXS can be classified as a disorder of the synapse (*14,41,42*). FMRP is highly expressed in neurons and plays an important role in dendritic plasticity (*41,43-45*). Without FMRP, dendrites do not develop normally. A hallmark morphological finding in patients with FXS is an abundance of immature dendritic spines (*11,41,46-48*). Dendritic-dependent changes involved in long-term depression and potentiation are impaired, contributing to the cognitive deficits seen in these patients. Given the synaptic abnormalities seen in patients with FXS, BDNF has been a focus of many FXS-related studies.

Serum levels of *BDNF* mRNA and BDNF protein are overall lower in patients with autism (*49*) and serum *BDNF* mRNA levels may positively correlate with IQ in patients with ASD (*49*). A crucial experiment by Lauterborn *et al*. (*44*) showed that impaired LTP (long term potentiation) in the *Fmr1*-KO mouse model is rescued when hippocampal slices are bathed in BDNF. This experiment evidenced that BDNF is affected by the absence of FMRP (*47,50*).

Though much attention is given to mature neurons, FMRP regulates proliferation and differentiation of adult neural stem/progenitor cells (*51*) and neurogenesis in early development (*52*). For example, FMRP is thought to play a crucial role in maintenance of radial glial cells (RGCs) in the neocortex during early development (*53*). Without FMRP, the RGC population is significantly reduced due to cell fate change from RGC to intermediate progenitor cell. In newborn neurons derived from neural progenitor cells lacking FMRP, basal levels of *BDNF* mRNA are increased (*52,54*). Levels of catalytic *TrkB* (tropomyosin-related kinase B), a receptor for BDNF, are also higher in the murine *Fmr1* KO neural progenitor cells (*54*). This enhanced BDNF/TrkB signaling in FMRP-deficient progenitor cells likely contributes to the abnormal neural differentiation and migration patterns seen in the *Fmr1* KO (*43*), such as the premature differentiation of neural progenitor cells which gives rise to neurons with small soma and short neurites (*43*). In addition, neural progenitor cells that lack FMRP also give rise to less glia (*47*).

However, the profile of BDNF expression appears

to change significantly with age. In early mouse brain development, hippocampal expression of BDNF in the KO is still increased compared to WT (wild type) (*54,55*). However, by age 3-4 months, BDNF expression in the murine hippocampus is reduced compared to WT (*52,55*). Defects in hippocampal neurogenesis lead to cognitive deficits in the adult *Fmr1* KO (*56*) and correlates with the hippocampal neurogenesis defects observed in individuals with FXS (*57*).

Experiments performed by Uutela *et al*. (*55*) provide mixed evidence as to whether BDNF is beneficial in the FXS mouse model. When *Fmr1* KO mice were crossed with *Bdnf*$^{+/-}$ mice, the double transgenic mice showed roughly half of WT BDNF levels and deficits in water maze learning, contextual fear learning, and hippocampal neurogenesis. However, the double transgenic mice also showed improvements in locomotor activity, sensorimotor learning, and startle response in comparison to *Fmr1* KO mice. Additionally, histological analysis of cultured neural progenitor cells showed that the double transgenic mice did not have the immature and abundant dendrites characteristically found in *Fmr1* KO mice (*55*).

These mixed findings may be partially explained by considering the changing profile of BDNF levels during different stages of development. The double transgenic mice had relatively lower BDNF levels during early development when BDNF may be detrimentally overactive due to absence of normal reciprocal regulation by FMRP. In contrast, BDNF levels in the double transgenics are low in adulthood when its presence could be beneficial, as evidenced by Lauterborn *et al*. (*44*). It is unclear when in childhood BDNF stimulation would be beneficial and whether this is a critical mechanism for improvement with sertraline treatment.

5. SSRI treatment in FXS

Effective targeted treatments for FXS are being researched with a focus on mechanism-based approaches (*58,59*). These include agents targeting mGluR5, GABAA, the endocannabinoid system, and other signaling pathways such as insulin growth factor (IGF), MAPK/Erk, and BDNF (*12,18,19,60-63*). Symptom-based treatments currently include stimulants, antidepressants (*e.g*. selective serotonin reuptake inhibitors; SSRIs), and atypical antipsychotics which are useful in treating symptoms such as hyperactivity, anxiety, and aggression (*59,64*). SSRIs are sometimes prescribed for patients with FXS to relieve symptoms of anxiety (*59*). Anxiety is a classic feature in FXS throughout life and particularly in childhood (*4,5*). Recent evidence shows that SSRI treatment may confer non-classical benefits to patients with FXS as well (*65*). A core symptom in this patient population is difficulty in language acquisition and communication (*1*). Individuals may have abnormal speech rate, stuttering or exaggerated repetition, and a limited vocabulary. Oftentimes, patients fixate on a particular topic, word, or phrase and perseverate on these phrases or topics. Treatment with an SSRI may additionally benefit communication abilities in patients with FXS.

In 2011, Winarni *et al*. (*65*) performed a retrospective chart review of 45 children with FXS, aged 12-50 months. This analysis found that children with FXS who received the SSRI sertraline had significantly improved receptive and expressive language development compared to those not treated with sertraline. A subsequent controlled trial of sertraline in children with FXS ages 24 to 68 months is currently enrolling at the UC Davis MIND Institute (ClinicalTrials.gov identifier: NCT01474746) to assess the effects of sertraline in three general domains: early language/developmental abilities, sensory processing abilities, and symptoms relating to cognition, anxiety, and ASD.

6. Unique aspects of sertraline among the SSRIs

Clinical results and theoretical knowledge support the usefulness of SSRIs in treating patients with FXS (*65*). Sertraline may be relatively more effective than other SSRIs for this patient population. Sertraline has been approved by the Food and Drug Administration (FDA) as a treatment for OCD in children (age 6-17 years old) and main side effects are worsening of mood and/or behavior, irritability, aggression and suicidal thoughts. Other side effects include drowsiness, fatigue, dizziness, and sleep problems.

6.1. *Dopamine reuptake inhibition*

There is evidence that sertraline has unique neurochemical properties when compared to other SSRIs. Along with paroxetine, sertraline is considered one of the most potent inhibitors of serotonin reuptake (*66*). Additionally, sertraline significantly prevents dopamine reuptake (*66*). In a study done by Kitaichi *et al*. (*67*), researchers compared extracellular levels of serotonin, dopamine, and noradrenaline found in the prefrontal cortex, nucleus accumbens, and striatum of rats following administration of therapeutic doses of sertraline, fluvoxamine, or paroxetine. All agents successfully up-regulated serotonin in these areas, but sertraline was the only agent that also up-regulated dopamine, specifically in the nucleus accumbens and striatum.

Dopamine dysregulation is implicated in many neuropsychiatric conditions (*66*). Irregularities in dopamine production and/or dopamine receptors are linked to disorders such as autism, schizophrenia, depression, ADHD, and substance abuse. Abnormally high or low levels of dopamine negatively impact

dendritic morphology (*22,68,69*). In a thorough review of the impact of dopamine on brain disorders and neurodevelopment, Money and Stanwood (*68*) state that examination of all the evidence points to dopamine playing "a crucial role…in formation and stabilization of synaptic connections in the striatum and frontal cortex".

In vitro experiments done by Wang *et al*. (*70*) showed that dopamine receptor-mediated synaptic modulation is impaired in cells lacking FMRP. Normally, D1 stimulation leads to changes in AMPA receptor expression and phosphorylation necessary for LTP, both of which were significantly blunted in prefrontal cortical neurons derived from *Fmr1* KO mice. This deficit was reversed when FMRP expression was induced in the cells *via* transfection. Furthermore, Wang *et al*. (*70*) showed that treating *Fmr1*$^{-/-}$ mice with a dopamine agonist specifically ameliorated the hyperactive behavior normally seen in these mice.

Further evidence of the importance of dopamine in FXS comes from the study discussed earlier by Lim *et al*. (*23*) that showed treatment of FXS hippocampal preparations with serotonin partially ameliorated *in vitro* LTP deficits by 20%. The researchers actually experimented further to discover that a particular low dose combination of a $5HT_{2B}$-R agonist and D_1 receptor agonist restored GluA1-mediated LTP in hippocampal slices to 100% (wild-type levels). This finding was subsequently tested *in vivo*, yielding impressive results. *Fmr1* KO mice were treated with either 5HT, the dopamine agonist, or both. The mice then underwent an associative learning task. Though there was mild improvement in each of the monotherapy groups, only the FXS mice receiving the combined 5HT and D1 cocktail were able to perform at WT levels, far superior to the abilities of their untreated FXS counterparts (*23*).

6.2. *Neuroprotective effects*

Taler *et al*. (*35*) analyzed in vitro cell (SHSY5Y human neuroblastoma cells) survival after 24 hours of antidepressant drug exposure including multiple SSRIs. Results showed that a low dose preparation (1-10 microgram) of sertraline or its derivative desmethylsertraline was the most beneficial SSRI in terms of cell survival. Compared to controls, sertraline improved cell survival rate by 50%. Paroxetine was the second most effective compound for cell survival, increasing viability by 40%. The rest of the drug candidates, which included fluoxetine, citalopram, reboxetine, venlafaxine, clomipramine, and mirtazapine showed no significant effects on cell survival. In a follow-up experiment, Taler *et al.* (*35*) compared the effects of sertraline on neuroblastoma cells exposed to stress (in the form of FCS-deprived media) *vs*. non-stressed conditions. The results showed that sertraline and desmethylsertraline administration during stress conditions increase cell survival, suggesting that sertraline has a neuroprotective effect (Figure 2).

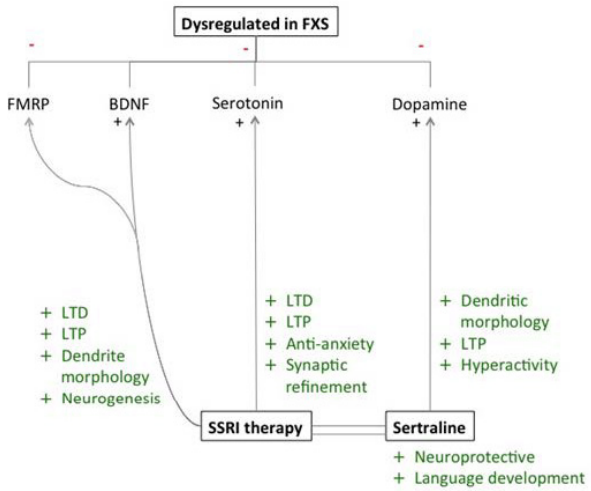

Figure 2. Neurochemical effects of sertraline therapy in FXS. FMRP, BDNF, serotonin, and dopamine are all dysregulated in patients with FXS. Abnormal levels of FMRP and BDNF in FXS cause atypical dendritic morphology, LTD, LTP, and neurogenesis, all of which have been shown to normalize with serotonin application. Serotonin treatment may also directly benefit patients with FXS as an anxiolytic and by ameliorating defects in LTD, LTP and synaptic architecture. Sertraline may be an especially beneficial SSRI agent for FXS treatment because of its neurprotective effects and positive impact on language development. In addition, sertraline prevents reuptake of dopamine, another neurotransmitter thought to be dysregulated in FXS. Increasing dopamine levels in patients with FXS may help to improve hyperactivity and irregularities in LTP and dendritic morphology.

In subsequent *in-vivo* experiments by Taler *et al*. (*35*), four to six week old wild-type mice treated with 1mg/kg daily sertraline for 3 weeks showed improved performance on the Morris Water Maze (MWM) reacquisition phase. Older mice (12-14 months) performance on the reacquisition phase improved most when dosed at 10 mg/kg/day. Interestingly, no differences were observed in treated mice in the acquisition and extinction phases of the MWM. Compared to controls, sertraline-treated mice had increased BDNF expression in the hippocampus when dosed at 5 and 10 mg/kg/day. Additionally, phosphorylated ERK and Bcl-2 expression was up-regulated in young mice receiving 5 mg/kg/day, though not in older mice at any of the measured dosages. It is noteworthy that the more beneficial results occurred in the younger mice, lending more support to the theory that early intervention with sertraline may be more beneficial.

7. Conclusion

Serotonin enhances synaptic modulation and refinement (*71*). There is evidence that during the peak of synaptogenesis in brain development (birth to 5 years of life), there is a reduction of serotonin synthesis (*28,32*). In mice and humans, SSRIs can upregulate

neurogenesis in the hippocampus. Pertinent to FXS, serotonin levels are likely affected by the lack of FMRP (*24,28,33*). Furthermore, other proteins that can be influenced by serotonin deficiency, such as BDNF, may contribute to the neurobiological deficits observed in FXS (*34-36*).

SSRIs are considered a symptomatic treatment for patients with FXS, but they may be working in a targeted manner as well. We have discussed evidence here that increasing serotonergic signaling can potentially rescue the neurobiology that is disrupted in FXS by upregulating levels of BDNF, increasing the number of GluA1 receptors and GlutA1-LTP, increasing levels of serotonin in the synapse, and by enhancing the dopaminergic system. These mechanisms are thought to improve synaptic plasticity and brain development. Other effects may include balancing cortical asymmetry of serotonin and overall neuroprotective effects.

Among the SSRIs, sertraline may be especially beneficial to patients with FXS due to its potency and ability to block the reuptake of dopamine, a neurotransmitter known to be dysregulated in FXS (*71*) and other neuropsychiatric conditions (*68*). Experiments done on murine FXS models show that treatment benefits vary depending on age (*14*). Serotonin and BDNF profiles change over time, and may be pathologically low in early development. Therefore, the timing of therapy with serotonergic agents may be extremely important in patients with FXS. Similarly, the consequences of FMRP expression depend on age (*14*). This is consistent with evidence from a retrospective chart review done by Winarni *et al*. (*65*), which found that one to four year old children with FXS who received sertraline showed improved receptive and expressive language outcomes. It is critical that this therapeutic opportunity is further investigated with controlled trials, as it could lead to significant improvements in symptoms, cognition, and quality of life for patients with FXS.

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Review

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Modeling fragile X syndrome in the *Fmr1* knockout mouse

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Summary **Fragile X Syndrome (FXS) is a commonly inherited form of intellectual disability and one of the leading genetic causes for autism spectrum disorder. Clinical symptoms of FXS can include impaired cognition, anxiety, hyperactivity, social phobia, and repetitive behaviors. FXS is caused by a CGG repeat mutation which expands a region on the X chromosome containing the *FMR1* gene. In FXS, a full mutation (> 200 repeats) leads to hypermethylation of *FMR1*, an epigenetic mechanism that effectively silences *FMR1* gene expression and reduces levels of the *FMR1* gene product, fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that is important for the regulation of protein expression. In an effort to further understand how loss of *FMR1* and FMRP contribute to FXS symptomology, several FXS animal models have been created. The most well characterized rodent model is the *Fmr1* knockout (KO) mouse, which lacks FMRP protein due to a disruption in its *Fmr1* gene. Here, we review the behavioral phenotyping of the *Fmr1* KO mouse to date, and discuss the clinical relevance of this mouse model to the human FXS condition. While much remains to be learned about FXS, the *Fmr1* KO mouse is a valuable tool for understanding the repercussions of functional loss of FMRP and assessing the efficacy of pharmacological compounds in ameliorating the molecular and behavioral phenotypes relevant to FXS.**

Keywords: Fragile X Syndrome, *Fmr1* knockout mouse, behavior, phenotyping, anxiety, social behaviors, cognition, attention

1. Introduction

Fragile X Syndrome (FXS) is one of the most commonly inherited forms of intellectual disability and monogenic causes of autism spectrum disorder (ASD) (*1,2*). Prevalence estimates for FXS are approximately 1:4,000 males (*3,4*) and 1:8,000 females (*5*), although a recent epidemiological meta-analysis reports FXS prevalence to be lower (1:7,143 males and 1:11,111 females) (*6*). This neurodevelopmental disorder is caused by a CGG repeat mutation on chromosome Xq27.3 (*7*), expanding the 5'-non-coding region of the fragile X mental retardation 1 (*FMR1*) gene. The *FMR1* gene encodes the fragile X mental retardation protein (FMRP) which regulates protein expression *via* its interaction with mRNA (*8*), associating with up to 4% of mRNA in the mammalian brain (*9,10*). The full mutation (> 200 CGG repeats) leads to hypermethylation of the *FMR1* promoter, an epigenetic mechanism which transcriptionally silences *FMR1* and reduces FMRP levels (*11*). FMRP is widely expressed throughout the body, but is enriched in neurons and testes (*12-14*). FMRP's binding targets include several synaptic proteins crucial for neurotransmission and structure (*15,16*), including postsynaptic density-95 (PSD-95), AMPA receptor subunits GluR1 and GluR2, and microtubule-associated protein 1b (MAP1b) (*17-22*), and further, binds to its own *Fmr1* mRNA (*23-25*). Through its association with target mRNAs, FMRP is thought to assist in the localization, transport, stabilization and translational regulation of the mRNA for these proteins (*10,16,26-29*). Loss of FMRP is also associated with elevated mTOR signaling (*30*), which is vital to cellular growth, energy metabolism and protein synthesis (*31*).

Due to the X-linked nature of its inheritance, FXS phenotypes are heterogeneous and vary considerably

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between males and females (*32,33*). In general, females typically display milder symptoms than males due to compensation by the second non-affected X chromosome (*34*). Common characteristics of individuals with FXS include intellectual impairment, increased anxiety, hyperarousal to stimuli and unusual physical features (*e.g.*, an elongated face, flat feet and hyperextendable finger joints) (*35*). In individuals carrying the full mutation, the severity of the physical and behavioral phenotypes correlates with lower levels of FMRP (*36*). To be noted, there are limitations in FMRP quantification, as many techniques utilize immunohistochemistry to label peripheral white blood cells (*37,38*) or hair roots (*39,40*) with monoclonal antibodies to indirectly measure FMRP levels. These methods cannot quantify FMRP protein levels, which is essential for understanding how the degree of FMRP loss relates to FXS clinical phenotypes. Development of additional detection methods, such as quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (*41*), time-resolved Förster's resonance energy transfer immunoassay (*42*) and semi-quantitative western blot protein analysis (*43*), has provided additional tools for the detection and quantification of FMRP protein levels, allowing for further investigation of the relationship between FMRP and FXS phenotypes.

Animal models of FXS have been developed in various species, such as the Drosophila fruit fly, zebrafish, mouse, and rat (*44-48*). Much effort has focused on the characterization of mouse models of FXS, in particular the *Fmr1* knockout (KO) mouse. The *Fmr1* KO mouse was created and initially characterized by the Dutch-Belgian Fragile X Consortium (*48*). The first *Fmr1* KO mice were generated using embryonic stem cells and C57BL/6J (B6) wildtype mice, a commonly used inbred mouse strain. A targeting vector containing a disrupted *Fmr1* DNA sequence with an insertion in exon 5 (the knockout allele) was inserted into embryonic stem cells and transferred into pseudo-pregnant female mice. These founder mice yielded offspring that were crossed with B6 mice to generate experimental animals. *Fmr1* KO mice harboring this mutation did not produce FMRP protein, but did possess detectable levels of *Fmr1* mRNA (*49*). Subsequently, these mice were bred into different background strains, such as the FVB inbred mouse strain. Since its initial description in 1994, many labs continue to use *Fmr1* KO mice to further understand the outcomes of functional FMRP loss in mice, and how it relates to FXS clinical symptoms. The goal of this review is to outline the progress to date, and discuss which areas will benefit from future research.

2. The *Fmr1* KO mouse

2.1. *Physiology of the Fmr1 KO Mouse*

Males with FXS tend to possess certain dysmorphic features, such as prominent ears, narrow face, loose joints, smooth skin and macroorchidism (enlarged testes) (*35,50*). The presence of macroorchidism is due to the loss of FMRP, which is highly expressed in the testes (*13*). *Fmr1* KO mice have significantly heavier testes than wildtype controls, but normal structural morphology (*48,51*). This is likely due to an increase in the proliferative activity of Sertoli cells found in the seminiferous tubules, which increases the number of germs cells in the testicles, and therefore, their weight (*51*). Other physical features, such as core temperature and body weight, and neurological reflexes did not differ between genotypes, suggesting otherwise normal gross physical and neural development (*48,52*). The presence of enlarged testes mirrors the macroorchidism found in male individuals with FXS, and therefore lends face validity to the *Fmr1* KO mouse model in this aspect of the clinical disorder.

2.2. *Dendritic spine morphology and neurotransmission*

FMRP is an RNA-binding protein that is enriched in neurons, particularly in the cell body, dendrites and postsynaptic spines (*14,28,53,54*). Dendritic spines, small protrusions along neuronal dendrites, are sites of excitatory synaptic input, which contain receptors and signaling molecules that are essential for synaptic neurotransmission (*55*). Postmortem analysis of human cortical tissue revealed that individuals with FXS have an increased density of dendritic spines relative to controls, with a majority of spines appearing elongated and immature (*56-63*). Directly analogous deficits in spine number and morphology have been found in *Fmr1* KO mice bred onto both B6 and FVB genetic backgrounds (*64-67*), providing additional face validity to the *Fmr1* KO mouse model. Developmental analysis of the barrel cortex of young (1 week old) *Fmr1* KO mice revealed an increase in spine density and length in mutant mice compared to controls, which was not present at 4 weeks of age (*65*). This absence of spine abnormalities at 4 weeks of age was also detected in the developing somatosensory cortex of *Fmr1* KO mice by the Greenough laboratory (*63*). In addition, in the same study, adult *Fmr1* KO mice exhibited increased density of immature, thin spines compared to controls (*63*). Therefore, there may be a period of synaptic development during which dendritic spine morphology briefly normalizes in the absence of FMRP, but is not sustained. In other brain regions, similar structural deficits in dendritic spines were seen at older ages of *Fmr1* KO mice. For example, *Fmr1* KO mice possess greater densities of elongated spines in the visual cortex at 16 weeks of age compared to wildtype controls (*66*). These data suggest that FMRP expression is necessary for the development of normal dendritic spine morphology, and that the loss of FMRP negatively impacts the physical structure of the synapse.

As a negative regulator of mRNA translation, FMRP influences protein synthesis and can therefore affect the synaptic components located in dendritic spines. Long term potentiation (LTP) and depression (LTD) are the long lasting enhancement and reduction, respectively, of signal transduction between two neuronal synapses (*68,69*). These activity-dependent cellular events rely on translational regulation of synaptic proteins in order to rapidly respond to synaptic activity and maintain cognitive function. Analyses of LTP and LTD, which are considered to represent electrophysiological correlates of learning and memory (*69*), have revealed abnormalities in the neurotransmission of mice lacking the *Fmr1* gene. LTD, which is dependent on protein synthesis and metabotropic glutamate receptor (mGluR) activation, was enhanced in *Fmr1* KO hippocampus and hippocampal neuron cultures (*70-72*). LTP, along with decreased AMPA receptor surface expression and selective increases in NMDA receptor subunit protein expression, was impaired in *Fmr1* KO mice (*17,21,71,73,74*), although these findings are inconsistent (*17,21,61,70,74,75*). *Fmr1* KO2 mice, another *Fmr1* null mouse model that lacks both FMRP protein and *Fmr1* RNA due to deletion of the *Fmr1* promoter and first exon (*76*), also displays abnormal synaptic plasticity. In the *Fmr1* KO2 hippocampus, a lower ratio of AMPA to NMDA receptors was detected early in development compared to wildtype controls (*77*). The upregulation of NMDA receptors in the *Fmr1* KO2 hippocampus resulted in increased NMDA receptor-dependent LTP. These data demonstrate that lack of *Fmr1* produces alterations in normal synaptic activity, which likely contributes to the FXS phenotype. Given the importance of FMRP for the regulation of proteins integral to synaptic function, it is unsurprising that loss of FMRP results in abnormalities in the structure and functionality of neuronal synapses.

2.3. *Seizure and stimuli hypersensitivity*

Approximately 10-20% of individuals with FXS with full mutations exhibit childhood seizures (*78-81*). Seizures associated with FXS are infrequent, are often partial, and are typically controlled with medications (*82,83*). *Fmr1* KO mice have not been reported to display spontaneous seizures, but are more susceptible to audiogenic seizures, induced by exposure to a 125 decibel, high-intensity siren (*48,81,84-93*). Audiogenic seizure vulnerability in *Fmr1* KO mice may reflect seizure susceptibly in FXS, although audiogenic seizure severity in *Fmr1* KO mice varied in degree depending on age and background strain (86,91,94,95).

Individuals with FXS report hyperarousal and heightened sensitivity to sensory stimuli (*7*). For example, subjects with FXS had stronger and more frequent responses and reduced habituation to sensory stimulations (*e.g.*, olfactory, auditory, visual, tactile, and vestibular stimuli) as measured by electrodermal responses (*96*). Electrophysiological recordings in the auditory cortex demonstrated enhanced responses to auditory tones in *Fmr1* KO mice, indicating that auditory neurons of *Fmr1* KO mice are hyper-responsive to stimuli (*97*). These data are consistent with the increased responses to pure tones seen in individuals with FXS (*98,99*).

Prepulse inhibition (PPI), a measure of sensorimotor gating, occurs when a weak pre-stimulus attenuates the response to a sudden strong stimulus (pulse) within 100 milliseconds (*100,101*). Deficits in PPI have been noted in FXS, correlating with other clinical FXS features, such as IQ severity and attention (*102-104*). Studies of *Fmr1* KO mice have yielded mixed results. The majority of studies indicate *Fmr1* KO mice exhibit enhanced PPI and reduced startle (*89,90,105-107*); this is a significant effect but in the opposite direction to the results in human FXS. In contrast, others report impaired PPI in *Fmr1* KO mice (*108*), increased startle responses to low intensity auditory stimuli (*109*), or minimal or no PPI differences between genotypes (*49,91,109,110*). As has been previously discussed, *Fmr1* KO behavior phenotypes are influenced by genetic background (*89,107*). Explanations for the divergent findings on PPI in *Fmr1* mice reported by different laboratories include use of different murine genetic backgrounds and differences in testing protocols (*111*). Of greater concern are the contrasting phenotypes between the majority of PPI studies in the *Fmr1* KO mouse and FXS human studies. These data suggest that while certain aspects of FXS are recapitulated in the *Fmr1* KO mouse, other clinical features are not reproduced.

2.4. *Attention and hyperactivity*

Individuals with FXS are hyperactive and have difficulties with attention and impulse control (*35,112-115*). Subjects with FXS performed better than learning disabled controls on selective attention, but the subjects with FXS had deficits similar to the learning disabled controls in sustained attention and working memory (*116*). Further, studies have found that FXS confers more drastic attentional deficits as task difficulty increases, such that individuals with FXS have more difficulty inhibiting/switching responses (*117*). In light of clinical FXS symptomology (*i.e.*, its comorbidity with ADHD), *Fmr1* KO mice were evaluated in the five-choice serial reaction time task, considered the gold standard task for attention and impulsivity in rodents (*118*). Although *Fmr1* KO mice were impaired in select phases of a visual-spatial discrimination task, they did not differ from wildtype controls in the five-choice serial reaction time task (*119,120*). Specifically, Krueger and colleagues found that *Fmr1* KO mice took longer to reach criterion during the

second phase of training (> 50% correct of > 15 trials for 2 consecutive days), when nose-pokes in a signaled nose-poke hole were correct and non-signaled nose-pokes were incorrect, but this effect did not replicate in subsequent studies (*121*). Sidorov and colleagues instead demonstrated augmented extinction of nose-poke responses in *Fmr1* KO mice. In another series of attention tasks, *Fmr1* KO mice had impaired inhibitory control, exhibiting a higher rate of premature responses than wildtype mice (*122*). This was associated with changes in task contingencies, suggesting inhibitory control in *Fmr1* KO mice may be affected by stress or novelty. Additionally, *Fmr1* KO performance was disrupted by olfactory distracters, with mutant mice making more inaccurate responses during distracter presentations (*122*). A consistent behavioral finding in *Fmr1* KO mice is their increased locomotor activity compared to wildtype controls in the open field test (*48,52,89,90,123-130*). It is important to note that the robust hyperactivity phenotype seen in *Fmr1* KO mice could be a confounding factor for the assessment of sustained attention, given that the general activity of mutant mice may interfere with task engagement.

2.5. *Repetitive behaviors*

Perseveration and repetitive behaviors, such as hand flapping, are associated with the full mutation in FXS (*33,35,131,132*). In the five-choice serial reaction time task, *Fmr1* KO mice demonstrated heightened perseveration and responding during novel rule acquisition, which normalized with training (*119*). *Fmr1* KO mice also exhibited higher levels of self-grooming, a repetitive behavior, than wildtype controls (*89,133*). Additionally, *Fmr1* KO mice buried more marbles in the marble burying test (*93,107,124*), a measure of repetitive behavior (*134*). However, marble burying was not significantly different between genotypes in some studies (*91,110,135*). Genotype differences in marble burying in *Fmr1* KO mice appear to be dependent on background strain (*107*). Overall, these data suggest that *Fmr1* KO mice show signs of repetitive behaviors, which parallels FXS clinical features.

2.6. *Anxiety*

Anxiety is one of the core behavioral features of FXS, in both children and adults (*35,132,136*). The evaluation of anxiety-related behaviors in *Fmr1* KO mice has generated inconsistent results, ranging from less anxiety-like scores in *Fmr1* mutant mice to no genotype differences to increased anxiety-like scores on several tasks. The elevated plus-maze is an anxiety-related task that utilizes a mouse's preference for dark spaces by evaluating the amount of time and entries made into dark, enclosed arms as compared to open arm runways (*137,138*). *Fmr1* KO mice spent significantly more time in the open arms and less time in the closed arms, but also traveled more throughout the maze, which may indicate higher general locomotion (*52,84,129,130*). In the zero-maze, *Fmr1* KO mice spent more time in the open quadrants (*130,139*). In the open field, the time or distance spent in the center of the open arena is sometimes considered an indicator for anxiety-related behavior, since wildtype mice prefer to remain in the perimeter when introduced to a novel environment. *Fmr1* KO mice spent a greater portion of their distance traveled in the center area of the open field compared to wildtype control mice (*49,52,123,129*). Together, these publications indicated a profile of lower anxiety-related behaviors in *Fmr1* KO mice, which is contrary to the FXS clinical phenotype. In contrast, others have shown that *Fmr1* KO mice exhibited increased anxiety-like responses in the mirrored chamber task (*123*), avoidance of the center of the open field (*128*) and reduced open arm time in the elevated plus-maze (*140*). In the light↔dark exploration test, an anxiety-related task in which a subject mouse typically spends more time in a dark chamber than a well-lit chamber (*141*), and in which number of transitions between compartments is increased by anxiolytic drug treatments (*142*), *Fmr1* KO mice made more transitions between the chambers (*90,107*), but did not differ from wildtype mice in time spent in the light chamber. In some studies, no genotype differences were detected in *Fmr1* KO mice as compared to wildtype littermates in the elevated plus-maze (*49,109,127*), in light↔dark exploration (*107*), or on center time in the open field (*91,93,135*). These differing results could potentially be explained by differences in testing and housing conditions, genetic background, and age at testing, as these factors can influence performance on conflict tests in mice (*143*). Given the sensitive nature of anxiety-related assays, it is imperative that similar testing protocols are used across labs to determine the robustness of the *Fmr1* KO genotype on anxiety-related phenotypes.

2.7. *Sociability and social communication*

Along with increased anxiety, individuals with FXS are often diagnosed with social phobia and avoidance (*35,132,144,145*). In the three-chambered sociability task, a subject mouse is evaluated for its exploration of a novel social stimulus (*e.g.*, novel mouse) versus a novel object stimulus (*146*). Wildtype mice will preferentially explore a novel mouse when given the choice between a novel mouse and a novel object with no social valence. Results using the three-chambered social approach with *Fmr1* KO mice to evaluate their sociability vary in the literature. For example, several groups report that *Fmr1* KO mice have normal sociability, preferring to explore the novel mouse over the novel object (*89,130,133,139*). Similarly, direct

social interactions with freely moving juvenile mice of the same sex, or in adult male subjects interacting with estrus females, were reported as normal (*89,147*) or even enhanced, as evidence by greater sniffing duration and interaction time of a partner mouse by *Fmr1* KO mice (*123,148*). In contrast, other research suggests that the sociability of *Fmr1* KO mice is abnormal, such that mutants do not exhibit a preference for a novel mouse over an object (*126*) and have reduced sniffing duration of the novel mouse compared to wildtype mice (*133*). Furthermore, additional studies demonstrate *Fmr1* KO mice spent less time engaging in affiliative behaviors, such as nose-to-nose sniffing, nose-to-anogenital sniffing and crawling over or under the partner's body during social interaction with a female mouse (*89*). Social scores appeared to be dependent on the background strain into which the *Fmr1* mutation had been bred (*107,149*). Although individuals with FXS are described as having social interaction deficits and social phobia, it has been suggested that these social deficits are due to hyperarousal and heightened anxiety rather than a lack of social understanding (*i.e.*, the "Fragile X handshake" in which an initial gesture, such as brief eye contact or social remark, is paired with active gaze avoidance (*150,151*)). The rodent models described here may differentially account for these factors.

Children with FXS are delayed in their language development, but this is associated with other cognitive delays (*152-154*). Rodent pup ultrasonic vocalizations are considered to be biologically meaningful (*155,156*), as they are emitted in young pups during stressful situations (*157*) and elicit retrieval behaviors by the parents. Adult male mice and rats emit ultrasonic vocalizations during interaction with females and in response to urine from estrus females (*158*). Studies focusing on ultrasonic vocalizations of *Fmr1* KO mice have been inconsistent in their findings. While there are reports of increases (*107*) or no differences in the number of calls of *Fmr1* mutant and wildtype mice (*89*), other labs observe a significant reduction in vocalizations in *Fmr1* KO mice (*124,147*), including call-type specific deficits (*159*). Together, data suggest that while *Fmr1* KO mice exhibit some aspects of normal sociability, they exhibit some abnormalities in social behavior and communication.

2.8. *Cognitive deficits*

A majority of individuals with FXS exhibit intellectual impairment, which can range from mild to severe. IQ scores decrease over time, which is likely a result of delayed development in individuals with FXS (*160,161*). Novel approaches to intelligence testing have found that traditional IQ tests can be modified to reveal subtle differences within this select population (*162*). Starting with the Dutch-Belgium Fragile X Consortium, many researchers have conducted thorough characterizations of *Fmr1* KO mice to compare their phenotypes to the intellectual disabilities displayed by individuals with FXS. One cognitive test conducted very early on in the development of the *Fmr1* KO mouse model was passive avoidance, a task that utilizes association of a footshock with a dark chamber to assess memory for the aversive event. Passive avoidance learning relies on the dorsal hippocampus (*163*) but also requires the amygdala (*164*). Dependence of passive avoidance performance on the dorsal hippocampus and amygdala would predict that animals deficient in the function of either or both of these brain regions would be impaired in this task, but the data are mixed. While amygdala volumes are not generally affected in subjects with FXS, affected individuals with FXS have difficulty with emotion regulation. A recent study revealed that individuals with FXS demonstrated less activation of the amygdala while viewing fearful faces than neurotypical subjects (*165*). Passive avoidance learning was not altered in *Fmr1* KO mice in some studies (*48,93,135,166*) but was disrupted in others (*90-92,129,167,168*). Interestingly, passive avoidance extinction may occur more rapidly in *Fmr1* KO mice (*92,166*), which is consistent with augmented extinction in *Fmr1* KO mice in other assays (*121*). It may be that cognitive deficits combined with augmented fear responses are working in opposition, explaining some of the disparate results in fear-associated tasks such as passive avoidance.

Fear conditioning studies were used to further elucidate whether other specific cognitive domains are disrupted in *Fmr1* KO mice. Fear conditioning can be parsed out into several distinct subtypes that rely on the amygdala, hippocampus, and prefrontal cortex to different extents. Contextual fear conditioning requires both the amygdala and hippocampus, while delay-cued fear conditioning requires the amygdala but not the hippocampus (*169-172*). Contextual and delay-cued fear conditioning can be acquired during the same training session and assessed in independent settings to reveal hippocampus-dependent and hippocampus-independent memory effects, respectively. In amydgala-dependent delay-cued fear conditioning, a deficit was reported in *Fmr1* KO mice (*75,90*), but other studies did not observe this effect (*173-175*). In hippocampus-dependent contextual fear conditioning, one report indicated a deficit (*75*) and another identified a context-discrimination deficit (*176*); other studies did not detect genotype differences in contextual fear conditioning in *Fmr1* KO mice (*52,173,175*). Trace-cued fear conditioning requires hippocampus and prefrontal cortex (*177,178*) and may or may not be independent of the amygdala (*179,180*). Trace fear conditioning, a more difficult task in which the tone and shock are not simultaneous during training, indicated that *Fmr1* KO mice may have deficits (*74*) but others showed that

Fmr1 KO mice appeared equal or superior to wildtype mice in the acquisition of trace fear conditioning (*106*).

The hippocampus is larger in individuals with FXS (*181,182*) and functional deficits in the hippocampal domain in subjects with FXS (*183,184*) would suggest that any fear task requiring the hippocampus would show a deficit. The FXS association with larger hippocampal volumes (*182*) and/or subjectively assessed hippocampal morphology differences in affected individuals (*185*) may or may not relate to deficits in hippocampal-dependent memory. Further, while individuals with FXS have normal amygdala and prefrontal cortex volumes, they have altered behavioral responses to tasks requiring the amygdala (*165*), frontal lobe (*186*) and prefrontal cortex (*187*). This may represent another instance in which behavioral tasks that require functional circuits (*i.e.*, the limbic system) may lead to variable results when multiple neural substrates within that system are affected (*i.e.*, prefrontal cortex, amygdala, and hippocampus).

Decades of research characterizing the cognitive abilities of individuals with FXS predict that deficits in a FXS mouse model should occur in short-term (visual) memory, visual-spatial abilities, sequential information processing, executive function and attention (*188-191*). The Morris water maze, a hippocampus-mediated task, was used to evaluate *Fmr1* KO visual-spatial abilities to determine whether subject mice could locate a submerged platform using spatial cues (*48*). The study did reveal subtle genotype differences, such that *Fmr1* KO performance was significantly worse in reversal (*i.e.*, a change in platform location) than wildtype littermates, specifically during the first trials after location-switching. This may indicate difficulty in changing reinforcement contingencies. Interestingly, however, there were no performance differences in the probe trial when the platform was removed, suggesting no impairment in visual-spatial memory. Kooy and colleagues (*192*) added additional animals (22 KO and 17 wildtype mice) to the original Consortium study (14 KO and 11 wildtype mice) and pooled these results. The larger sample sizes revealed similar results on Morris water maze reversal, with the additional finding of a genotype effect during the initial spatial memory acquisition. However, no significant probe trial differences were observed, indicating that while there are some differences in Morris water maze performance, they may not be functionally relevant to the FXS condition. Despite the *Fmr1* KO deficit occurring in reversal trials, a similar reversal learning task conducted in an E-shaped maze revealed no such genotype difference. However, while *Fmr1* KO mice did not show a persistent perseveration phenotype across cognitive modalities (*i.e.*, impaired reversal in Morris water maze, but not E-shaped maze (*192*)), a cross-shaped maze replicated the Morris water maze acquisition deficit (*173,175*). These acquisition deficits have been replicated (*106*), but not consistently (*75,174*). Similarly, deficits in reversal learning in *Fmr1* KO mice were replicated in some studies (*106,193*), but not all (*75*). Based on the variable results across laboratories, the spatial learning deficits identified in earlier studies may require very specific conditions in order to reproduce these results. In the majority of published studies, however, probe trial analyses revealed no differences between *Fmr1* KO and wildtype mice, indicating limited and selective deficits in spatial learning and memory (*48,75,174,192,193*). However, some probe trial differences have been observed in *Fmr1* KO mice (*106*). Some researchers have observed task-specific impairments in spatial cognition rather than global impairments (*183,184*), although global cognitive impairments in individuals with FXS have also been reported (*160-162*). The mild deficits in spatial learning and memory observed in *Fmr1* KO mice may support the idea of task-specific cognitive deficits and not global dysfunction.

The mixed results in cognitive assays to date has initiated a debate as to whether the *Fmr1* KO mouse is a sufficient model of FXS in humans, since the primary symptom of intellectual impairment is not prominent in the mutant mouse model. In an effort to find cognitive tasks with more ethological relevance, recent studies have included novel object recognition as well as spatial and temporal order object recognition tasks. Novel object recognition, which is typically conducted as a short-term memory task, relies on rodents' natural tendency to investigate novelty. A mouse is placed into an arena with two identical copies of an object, where their species-typical response is to explore and investigate the objects. After a certain interval, subject mice are returned to the arena with one familiar object and a novel object. If the mouse recognizes the previously seen object, it preferentially investigates the novel object. *Fmr1* KO mice have a deficit in this task (*194,195*), but as with the previously discussed cognitive domains, this impairment has not always been replicated (*49*). A recent study identified hippocampus-dependent spatial object recognition deficits in *Fmr1* KO mice (*195*), such that *Fmr1* mutant mice did not preferentially explore an object when it was moved to a new location.

Working memory deficits have been suggested as being a core feature of FXS (*196*). In several human clinical studies, individuals with FXS had low performance on specific working memory tasks under low-control conditions (*i.e.*, verbal and visual-spatial (*116,185,197,198*), or visual-spatial alone (*199*)). A recent study identified working memory deficits under high-control conditions (*i.e.*, a dual task request; for example, selective word recall only when a stimulus with particular properties was presented) in individuals with FXS that were specific to another component of working memory, central executive functioning

(*200*). Further, while central executive processing was impaired in individuals with FXS, both verbal and visual-spatial working memory modalities were intact. While these studies and others (*183,184*) suggest that human cognition deficits in FXS are task-specific and not global in nature, additional research has revealed impairments in all components of working memory in FXS (*i.e.*, visual-spatial sketchpad, central executive, and phonological loop) (*198*). Similarly, a study in young boys with FXS revealed working memory deficits regardless of task complexity and modality (*196*). The differing results on specific versus general working memory deficits in FXS may be due to task-specific differences (*e.g.*, the type of stimuli used), as individuals with FXS have more accurate recall with familiar stimuli rather than abstract material (*189*). In rodents, working memory tasks, such as olfactory working memory and radial arm maze, can rely heavily on other brain regions (*i.e.*, olfactory bulb or hippocampus, respectively). In several tasks, including the radial arm maze, *Fmr1* KO mice did not show robust working memory deficits (*49*), although others have identified a working memory impairment in *Fmr1* KO mice in a serial reversal version of the Morris water maze (*106*). It is possible that the olfactory bulb and hippocampus in *Fmr1* KO mice are compensating for deficiencies in working memory in some of these tasks. Therefore, identification of a behavioral task that is less reliant on other brain regions is necessary to determine if *Fmr1* KO mice exhibit a reliable working memory impairment, as this would add further face validity to the model.

3. Conclusions

The development of FXS animal models has furthered our understanding of several molecular and synaptic deficits underlying FXS, including abnormal dendritic spine morphology, protein dysregulation and neurotransmission. In addition, animal models provide an opportunity to evaluate novel drug targets to ameliorate FXS symptoms. Indeed, gene therapy (*124*) and pharmacological compounds such as minocycline (*147,201*), mGluR5 antagonists (*202*), arbaclofen (*203*), ganaxolone (*84*), lovastatin (*204*) and lithium (*195,205*) have shown efficacy in ameliorating some of the phenotypes detected in *Fmr1* KO mice. Thorough evaluation of the *Fmr1* KO mouse on numerous genetic backgrounds across a multitude of labs indicates that several phenotypes, such as neuronal morphology and hyperactivity, are robust and consistent across studies. In contrast, several aspects of cognition, anxiety and social phenotypes of *Fmr1* KO mice are highly variable across published reports (Table 1). Additionally, many reported *Fmr1* KO phenotypes are in direct opposition to the clinical FXS phenotype, such as a lack of robust cognitive impairments, enhanced prepulse inhibition and reduced anxiety in the mouse model. The *Fmr1* KO mouse was generated by genetically modifying the *Fmr1* DNA sequence to reduce FMRP protein levels. This is contrast to the human FXS condition, which is generally caused by expansion of the *FMR1* gene region and subsequent promoter hypermethylation, although there are rare instances of FXS being due to point mutations and partial or complete deletion of the *FMR1* gene (*206-208*). Given that FXS clinical symptomology is associated with lower levels of FMRP, one would expect that complete disruption of *Fmr1* and resulting loss of FMRP would recapitulate the most severe clinical phenotypes of FXS. However, this is not the case for the *Fmr1* KO mouse model, which may limit its utility. The mechanistic differences between the mouse model and the human genotype underlying loss of FMRP, *i.e.* deletion and expansion, respectively, could be a contributing factor to the phenotypic differences seen between *Fmr1* KO mice and individuals with FXS. Therefore, in order to more fully recapitulate the clinical features of FXS, such as severe intellectual disability and social anxiety, it will be important to explore other mechanisms associated with FXS in combination, such as CGG expansion and hypermethylation of the *Fmr1* gene, as well as loss of FMRP protein.

It is possible that the variance seen in the *Fmr1* KO phenotype reflects the range of FXS clinical symptoms, rather than being due to subtle differences in methodology or genetic background influence alone. The variability in the strength and direction of phenotypic differences observed in the *Fmr1* KO mouse may at first seem unsettling and worthy of discarding the model altogether. However, the heterogeneity of FXS is such that affected individuals exhibit a range of cognitive impairments, with affected males presenting with mild to severe cognitive symptoms (*162,209*). This poses a challenge for FXS animal models, but it also might be considered a strength. If the *Fmr1* KO model is expected to primarily encompass only the most severe symptoms of FXS, then more is expected of the model than exists in the human syndrome. Instead, if the model is looked at through a clinician's lens, one would expect a heterogeneous population with a portion of the animals showing severe impairments with others displaying mild to moderate effects or none at all. Indeed, it is a challenge to think of how variable FXS symptomology in both the human syndrome and the animal model can be leveraged toward the identification of successful treatments for individuals with FXS. Despite these challenges, pharmacological interventions using the *Fmr1* KO mouse have demonstrated predictive validity for this model, as results from several drug studies in *Fmr1* KO mice parallel findings from human FXS open-label treatment trials (*e.g.* minocycline (*210*) and lithium (*211*)). As research of the molecular and behavioral dysfunction in

Table 1. Summary of behavioral and cognitive phenotypes of *Fmr1* knockout mice (↓ = decrease; ↑ = increase; ↔ = no change)

Domain	Fragile X Syndrome Clinical Phenotype	Rodent Assay	*Fmr1* Knockout Mouse		References
			Direction	Phenotype	
Cognition	Intellectual disability; working memory deficits	Passive avoidance	↓	Impaired performance; augmented extinction	*90-92,129,166-168*
			↔	No genotype differences	*48,93,135,166*
		Fear conditioning	↓	Deficits in delay-cued and contextua fear conditioning; deficits in trace fear conditioning	*74, 75, 90, 176*
			↔	No genotype differences	*52,106,173-175*
		Morris water maze	↓	Impaired performance during acquisition and/or reversal	*48,106,192,193*
			↔	No genotype differences	*49,75,174*
		Maze learning	↓	Impaired acquisition of a cross-shaped maze	*173,175*
			↔	No genotype differences in radial arm maze	*49*
		Reversal task	↔	No genotype differences in E-shaped maze	*192*
		Novel object recognition	↓	No preference for novel object	*194,195*
			↔	No genotype differences	*49*
Anxiety	Increased anxiety	Elevated plus-maze and zero-maze	↑	Reduced open arm time	*140*
			↓	Increased open arm and open quadrant time	*52,84,129,130,139*
			↔	No genotype differences	*49,109,127*
		Light↔dark exploration test	↓	Increased transitions	*90,107*
			↔	No genotype differences	*107*
		Center area of open field	↑	Avoidance of center area	*128*
			↓	More distance traveled in the center area	*49,52,123,129*
			↔	No genotype differences	*91,93,135*
		Mirrored chamber task	↑	Increased anxiety responses	*123*
Communication	Delayed language development	Ultrasonic vocalizations	↓	Reduction in vocalizations	*124,147,159*
			↑	Increased vocalizations	*107*
			↔	No genotype differences	*89*
Social	Social phobia and avoidance	Three-chambered sociability task	↓	No preference for novel mouse; social preference with reduced sniffing of novel mouse compared to wildtype mice	*126,133*
			↔	No genotype differences	*89,130,133,139*
		Direct social interactions with juvenile or with estrus female mice	↓	Reduction in affiliative behaviors	*89*
			↑	Greater sniffing duration and interaction time with partner mouse	*123,148*
			↔	No genotype differences	*89,147*
General Activity	Hyperactivity	Open field	↑	Increased locomotor activity	*48,52,89,90,123-130*

(To continue)

Table 1. Summary of behavioral and cognitive phenotypes of *Fmr1* knockout mice (↓ = decrease; ↑ = increase; ↔ = no change) (continued)

Domain	Fragile X Syndrome Clinical Phenotype	Rodent Assay	*Fmr1* Knockout Mouse		References
			Direction	Phenotype	
Attention and Impulse Control	Deficits in attention, particularly as difficulty increases; difficulty in response inhibition and rule switching	Visual-spatial discrimination task	↓	Longer to reach criterion; augmented extinction	*120, 121*
		Attentional task with odor distractors	↓	Impaired inhibitory control, with a higher rate of immature responses associated with rule changes	*122*
		Five-choice serial reaction time task	↔	No differences	*119, 120*
Repetitive Behaviors	Perseveration and repetitive behaviors	Five-choice serial reaction time task	↑	Increased perseveration and responding during novel rule acquisition	*119*
		Cage observations	↑	Higher levels of self-grooming	*89, 133*
		Marble burying	↑	Higher number of buried marbles	*93, 107, 124*
Stimuli Sensitivity	Hyperarousal and heightened sensitivity to sensory stimuli	*In vivo* single unit extracellular electrophysiology	↑	Enhanced responses to auditory tone	*97*
		Auditory startle response	↑	Increased startle response to low intensity auditory stimuli	*109*
Sensorimotor Gating	Reduced prepulse inhibition	Prepulse inhibition	↓	Impaired prepulse inhibition	*108*
			↑	Enhanced prepulse inhibition; reduced startle response	*89, 90, 93, 105-107*
			↔	Minimal or no differences in prepulse inhibition	*49, 91, 109, 110*

the *Fmr1* KO model accumulates, our understanding of how these molecular differences translate into observed behavioral dysfunction will continue to increase, providing a platform for the future identification of targeted FXS treatments.

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Review

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Fragile X spectrum disorders

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Summary **The fragile X mental retardation 1 gene (*FMR1*), which codes for the fragile X mental retardation 1 protein (FMRP), is located at Xp27.3. The normal allele of the *FMR1* gene typically has 5 to 40 CGG repeats in the 5' untranslated region; abnormal alleles of dynamic mutations include the full mutation (> 200 CGG repeats), premutation (55-200 CGG repeats) and the gray zone mutation (45-54 CGG repeats). Premutation carriers are common in the general population with approximately 1 in 130-250 females and 1 in 250-810 males, whereas the full mutation and Fragile X syndrome (FXS) occur in approximately 1 in 4000 to 1 in 7000. *FMR1* mutations account for a variety of phenotypes including the most common monogenetic cause of inherited intellectual disability (ID) and autism (FXS), the most common genetic form of ovarian failure, the fragile X-associated primary ovarian insufficiency (FXPOI, premutation); and fragile X-associated tremor/ataxia syndrome (FXTAS, premutation). The premutation can also cause developmental problems including ASD and ADHD especially in boys and psychopathology including anxiety and depression in children and adults. Some premutation carriers can have a deficit of FMRP and some unmethylated full mutation individuals can have elevated *FMR1* mRNA that is considered a premutation problem. Therefore the term "Fragile X Spectrum Disorder" (FXSD) should be used to include the wide range of overlapping phenotypes observed in affected individuals with *FMR1* mutations. In this review we focus on the phenotypes and genotypes of children with FXSD.**

Keywords: Fragile X syndrome, autism spectrum disorder, intellectual disability, developmental delay, premutation

1. Introduction

A variety of disorders are associated with mutations in the fragile X mental retardation 1 (*FMR1*) gene including fragile X syndrome (FXS) caused by a full mutation (> 200 CGG repeats in the 5' untranslated region of *FMR1* gene) leading to absence or deficiency of the FMR1 protein (FMRP) and premutation (55 to 200 CGG repeats) disorders characterized by elevation of *FMR1* mRNA 2 to 8 times normal. Although these 2 types of disorders are distinct in their phenotypes and molecular pathology, recent studies have demonstrated significant overlap that has been fertile areas for research. The term fragile X spectrum disorder (FXSD) has been developed to emphasize the continuity of clinical involvement from the gray zone (45 to 54 repeats) throughout the premutation and into the full mutation range. *FMR1* mutations are dynamic in that they usually expand between generations particularly when passed on by a female to her children when it can expand from a premutation to a full mutation (*1*) .

FXS was the first identified disorder in this spectrum and it was discovered in association with the fragile site of the X chromosome in two brothers in 1969 by Lubs and colleagues (*2*). In retrospect the first X- linked pedigree of intellectual disability (XLID) reported by Martin and Bell in 1949 turned out to be a fragile X pedigree when tested by the *FMR1* DNA test that was developed after the discovery of *FMR1* in 1991 (*3,4*). The fragile site was characterized by not only the CGG expansion to > 200 repeats, but also methylation of the cytosine bases leading to silencing of translation and little or no production of *FMR1* mRNA and FMRP. Since FMRP is a critical protein for regulation of translation

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for hundreds of mRNAs into their respective proteins, most of them involved with synaptic plasticity (*5*), the lack or severe deficiency of FMRP almost always leads to intellectual deficits as seen in males with FXS. In females with FXS the normal X produces FMRP so only 25% will have an IQ below 70 and an additional 50% will have an IQ in the borderline range (*6*).

Premutation disorders were first identified with the discovery of an increased incidence of early menopause (prior to the age of 40) in female carriers in 1991 (*7*). This has been confirmed by multiple investigators and has now been named fragile X-associated primary ovarian insufficiency (FXPOI) (*8*). Approximately 20% of female carriers have FXPOI, although the rate varies in a curvilinear fashion with CGG repeat number; the greatest prevalence of FXPOI is between 70 to 100 CGG repeats (*9*).

The next premutation disorder identified was the fragile X-associated tremor ataxia syndrome (*10,11*) seen initially in older male carriers (> 50 years) involving an intention tremor and cerebellar gait ataxia in addition to autonomic dysfunction, Parkinsonism, neuropathy, memory and executive function deficits followed by cognitive decline. This is a neurodegenerative disorder that occurs in approximately 40% of men and 16% of women with the premutation (*12,13*). FXTAS is hypothesized to be caused by mRNA toxicity from the elevated *FMR1* mRNA levels (*14*) leading to the production of pathognomonic inclusion formation in neurons and astrocytes throughout the CNS, peripheral nervous system and even in some organs such as the adrenals, heart and pancreas (*15*).

Currently there are numerous additional medical, neurological and psychiatric problems associated with the premutation both with and without FXTAS including depression (*16*), anxiety (*17,18*) , migraines (*19*) hypertension (*20*), immune mediated disorders including fibromyalgia and hypothyroidism (*21,22*), sleep apnea (*23*), restless legs syndrome (RLS) (*24*), and neuropathy (*25,26*) often associated with chronic pain symptoms. Since the prevalence of the premutation is much higher (1 in 130-250 females and 1 in 250-810 males) (*27*) than those with the full mutation (1 in 4,000-7,000) the impact of multiple medical and neurological problems in premutation carriers is far more significant in the population than the full mutation (*28,29*). The association of other disorders in adults with the premutation led to multiple studies in children and here we present a review of the manifestations in children with FXSD.

2. Full mutation - Fragile X syndrome

The *FMR1* gene, which codes for the fragile X mental retardation protein (FMRP, a major negative translation regulator), is located at Xp27.3 from base pair 146,993,469 to base pair 147,032,647 (GRCh37/hg19). The *FMR1* gene is highly expressed in the brain and testis (*30*). FXS is associated with a variety of neurological, cognitive and behavioral deficits, and less frequent dysmorphic features. Males with the full mutation and full methylation have little to no *FMR1* mRNA and little to no FMRP contributing to the clinical phenotype of FXS. The range of involvement in females is determined by the X-chromosome activation/inactivation ratio (the percentage of cells with active normal X chromosome) because this will determine how much FMRP is produced by the normal X chromosome depending on whether it is active or not.

2.1. *Physical findings*

The physical phenotype and dysmorphology of FXS include signs of a connective tissue disorder such as a long and narrow face, large and prominent ears, a high arched palate, hyperextensible finger joints, pectus excavatum, flat feet, soft skin and mitral valve prolapse. Other features include low muscle tone, and pubertal macroorchidism (*31,32*). Noteworthy approximately 30% of young children with FXS will not have obvious dysmorphic features; the physical features are associated with the FMRP deficits. The most evident effects of lower levels of FMRP in both males and females are prominent ears and hypermobility of the metacarpal-phalangeal (MP) joints (*33,34*). In males FMRP deficits are associated with a narrow face and large ears, while in females the FMRP deficits are associated with increased ear prominence and jaw length (*35*). In about 5-10% of children with FXS a Prader-Willi phenotype is observed including severe obesity, hyperphagia, hypogonadism and in some cases delayed puberty (*36,37*) (Figure 1). The reduced expression of the cytoplasmic interacting FMR1 protein

Figure 1. A female adolescent with FXS Prader-Willi-like phenotype.

gene (*CYFIP*, located at 15q11-13) is believed to be the cause of this phenotype (*37*).

2.2. *Neurological disorders*

In a national survey of caregivers of individuals with FXS (1,394 individuals), 14% of males and 6% of females were reported to have seizures (*38*). The seizures were easily treated, often partial and infrequent; however they were associated with more severe developmental and behavioral problems (*38*). Remarkably those with seizures are more likely to have ASD. The seizures may add to the severity of the phenotype because animal studies of early life seizures have shown that the FMRP leaves the dendrites and migrates to the perinuclear area during seizures, thereby depleting the dendrites of the regulatory effects of FMRP (*39*). Hypersensitivity to audiogenic stimuli and hyperarousal are also characteristics of children with FXS. These children have enhanced amplitude to sensory stimuli measured by electrodermal studies and a lack of habituation to repetitive stimuli (*35*). In addition, MEG studies also demonstrate an enhanced electromagnetic response to stimuli (*36*).

2.3. *Cognition deficits*

Male and female individuals with FXS present a wide range of learning disabilities in a context of normal, borderline IQ or mild to severe ID. The average IQ of males with the full mutation is 40 (*40*). Intellectual and developmental disability occurs in 85% of males and 25% of females. The level of FMRP correlates directly with IQ (*41*); males with the full mutation with unmethylated or only partially methylated alleles produce more FMRP than those with fully methylated alleles (*35*). The higher levels of FMRP explain the typically higher IQ (above 70) in high-functioning individuals with FXS. Similarly those individuals with "size-mosaicism" (full mutation plus premutation, gray zone or normal alleles) have a higher IQ than those without mosaicism. Therefore full mutation cells have a deficit of FMRP and the premutation cells produce an excess of *FMR1* mRNA, leading to mRNA toxicity but relatively normal levels of FMRP ("dual mutation effects", pathological involvement from two different mechanisms). Higher rates of psychotic thinking have been observed in individuals with this type of mosaicism leading to dual mutation effects (*42*). In females with FXS the normal X typically produces 25% to 50% of the normal FMRP level and these females have IQ scores that range from normal to moderate intellectual disability (*6*). Working and short-term memory (*43*), executive function (*44*), visual memory, visual-spatial processing (*45*) and verbal deficits are common in FXS (verbal comprehension and vocabulary) (*46*). Almost all males and approximately 30% of females with FXS have impaired speech (*47*).

In general, overall IQ declines with age in those with FXS because of the deficits in abstract reasoning which cannot keep up with the intellectual growth seen in typical children and adolescents (*48*). The adaptive skills also decline in FXS from adolescence into adulthood (*49*). This emphasizes the importance of early intervention with intensive behavioral/cognitive programs and targeted treatments early in life to improve or prevent cognitive decline.

2.4. *Behavioral phenotype*

FXS accounts for approximately 2-5% of all individuals diagnosed with FXS accounts for approximately 2-5% of all individuals diagnosed with ASD (*50*) . In FXS about 60% of males have an ASD (*51,52*). About 80% of males and 30% of females with FXS have symptoms of attention deficit hyperactivity disorder (ADHD) (*53*). Sleep disturbances, such as difficulty falling asleep and/or interrupted sleep are also characteristic of individuals with FXS (*54*). Altered sleep patterns and dysregulated melatonin profiles were found in 13 boys with fragile X when compare with age-matched normal controls (*55*). Results showed greater variability in total sleep time, difficulty in sleep maintenance, and significantly greater nocturnal melatonin production in the boys with FXS.

A hallmark feature of FXS that can also occur in some premutation carriers is social anxiety. This behavior leads to the characteristic "Fragile X handshake"; where the individuals may shake the interviewer's hand or acknowledge his/her presence but will avoid eye contact until the interviewer looks away (*56*). Additional behavioral features include stereotypies such as hand-flapping and hand-biting, shyness, perseveration, mood instability, aggression and impaired speech (*52*). Cross-sectional analyses suggest that dimensions of problem behavior, anxiety, and hyperactivity are age-related; thus, age should serve as an important control variable in behavioral studies in FXS. Measures of anxiety, attention, and hyperactivity are highly associated with other behavior problems (*29*). There is evidence that autism scores decreased with time, particularly in communication and social aspects of adaptive behavior (*57*). However, emotional symptoms, behavioral difficulties, problems with peers and social behaviors may remain relatively stable over time (*58*). These trajectories may be associated with variations of FMRP, which in turn can be related to epigenetic changes, but there have been no large longitudinal studies that assess the molecular variations and behavior/cognitive correlations. Further longitudinal studies are necessary to assess the developmental trajectories of FXS across the lifetime and relate the outcomes to molecular and environmental factors.

2.5. *Genotypes*

The unstable dynamic *FMR1* mutation can result in "size-mosaicism", but cells of individuals who have only one size allele may also show different patterns of methylation (none, partial, and full methylation) referred as "methylation mosaicism". Some individuals may have the presence of three or more populations of cells with different size-alleles and methylation-patterns. Therefore, the complex molecular mechanism and multiple possibilities of genotypes results in the wide variety of clinical characteristics of individuals with FXS and may also relate to different responses to standard and targeted treatments but this has not been well studied (*59*).

2.6. *Neurobiology*

At the cellular level, FXS is associated with immature dendritic spine morphology (*60,61*). FMRP is an essential protein for synaptic development and plasticity because it is a key negative regulator mRNA translation and subsequent protein synthesis that can down-regulate and/or up-regulate their targets at the synapse (*62*). FMRP inhibits protein synthesis that is needed for internalizing the AMPA receptors leading to long term depression (LTD); thus without FMRP there is enhanced LTD in the hippocampus (*63*). The *Fmr1*-KO mouse shows enhanced protein translation and protein synthesis in the hippocampus (*64*), LTD is significantly increased and this leads to deficits in synaptic plasticity and weakening of synaptic connections (*65*). Protein synthesis promotes synaptic plasticity activation, which is thought to be mainly coordinated by the action of metabotropic glutamate receptors (mGluRs) (*66*). This is the basis of the "mGluR theory of fragile X syndrome" (*63*). The neurobiology and several symptoms of FXS were rescued when the mGluR heterozygous mouse was crossed with the *Fmr1*-KO mouse (*63,67*).

Currently there are many other pathophysiological mechanisms described that are thought to be the result of absence or low FMRP. The lack of FMRP can also up-regulate PI3K, an important signaling molecule downstream of the activation of mGluR (*31*). Recently Matic *et al*. (2014), showed a global down-regulation of the MAPK/ERK pathway and decrease in phosphorylation level of ERK1/2 in the murine *Fmr1* KO. However, others show an increase in this system in patient fibroblasts (*68*). A differential expression of many proteins involved in the p53 pathway, Wnt and calcium signaling was also found and led to postulate that calcium imbalance is part of pathophysiology of FXS (*69*). Although FMRP is mainly a negative regulator, there is evidence that it can up-regulate the translation of some mRNAs, such as those encoding GABAA receptor subunits (α1, α3, α4, β1, β2, ɤ1, ɤ2, and δ), which were significantly reduced in neocortex and cerebellum of the *Fmr1*-KO mice (*70*). Other proteins required for GABA synthesis (Glutamate decarboxylase, GAD), transport (GABA transporter, GAT) and catabolism (GABA transaminase, GABA succinic semialdehyde) were also found to be reduced (*71*). A balanced GABA system is required for neuronal activation, network oscillations, neuronal synchrony and facilitation of movement and integration of information in many brain regions (*72*). The imbalance between the GABA and Glutamate systems is believed to contribute to the cognitive impairments, anxiety, hyperarousal, ASD, and epilepsy in children with FXS (*73*).

A novel FMRP target mRNA is the neuronal nitric oxide synthase (*NOS1* or *nNOS*) in mid-fetal human neocortex. FMRP was found to be a positive regulator of *NOS1* translation, controlling *NOS1* protein levels in a dose-dependent manner *in vitro* and *in vivo* (*74*), and the *NOS1* was severely reduced in the fetal and post-natal developing neocortex of FXS patients (*74*). The evidence of the multiple roles of nitric oxide (NO) in multiple neural processes such as synaptic developmental, retrograde signaling and synaptic plasticity (*75-79*) led to the hypothesis that the decrease expression of *NOS1* and secondary depletion of NO in the developing FXS brain may contribute to the neuropathology of FXS (*80*).

The absence of FMRP also affects the Brain Derived Neurotropic Factor (BDNF) levels in early and late development in the murine hippocampus. In early development of the KO mouse brain, hippocampal expression of BDNF is increased compared to wild type (WT) (*81,82*), whereas by age 3-4 months, BDNF expression is reduced compared to the WT (*82,83*). The mechanism of regulation of BDNF remains to be described, but this evidence suggests dual FMRP effects in BDNF expression during brain development. FMRP may also positively regulate many other mRNAs including *SOD1*, *ASCL1*, *Kcnd2*, and *DLG4* (*84-86*). It is estimated that FMRP regulates the translation of about 4% of brain mRNAs (*87,88*). We have discussed the mechanisms of pathogenesis mediated by the absence of FMRP; however, the mechanism that causes the silencing of the *FMR1* gene by the full mutation remains uncertain. There are many targeted treatments that focus on these pathways to reestablish the normal neurobiology in the KO mouse and these have led to clinical trials of targeted treatments in patients with FXS.

2.7. *FMR1 silencing mechanism of the full mutation*

It is intriguing that the premutation can lead to enhanced expression of the gene, whereas the full mutation leads to suppression of transcription. There are mechanisms that could explain the reduced transcription of the *FMR1* gene in the full mutation; these mechanisms can be divided in two groups: DNA-mediated and RNA-mediated (*89*). A model in which hairpin aggregation by the CGG repeats

results in the DeNovo methylation has been suggested because tridimensional CGG-structures can trigger their own methylation by DNA methyltransferases *in vitro* (*90*); another suggested DNA-mediated model involves repeat-binding transcription factors which in turn can aggregate other proteins and prevent transcription. This model was hypothesized from the existing evidence of a similar mechanism in mice where the pericentromic repeats in mice are silenced by Pax3 and Pax9 hybridization and recruitment of H3K9 trimethylase and Suv39h1 (*91*) that finally inactivate these regions. The *FMR1* mRNA products are a variety of transcripts of different sizes and reverted sequences that result from a number of splicing sites and the transcription of both, the sense and anti-sense strands. Colak *et al*. (2014), suggested an RNA mediated mechanism of silencing, in which the *FMR1* gene is silenced through a hybridization of the complementary CGG-repeat track of the *FMR1* mRNA (*92*). Other RNA-mediated mechanisms have been suggested to involve the formation of RNA hairpins subtracts of the enzyme Dicer, RNA-DNA hybrids for chromatin compaction and promoter antisense-transcripts (*89*). The silencing mechanisms of *FMR1* are potential targets for drug therapy. Since the FMRP is a key transcription regulator of many neurobiological pathways, in theory targeted treatments to prevent the inactivation of the *FMR1* gene may lead to more normal FMRP levels and reestablish the function of many neurobiological systems. Therefore silencing gene modifiers could be more efficient, although more difficult to translate into patients than specific-system treatments, such as the mGluR5 antagonist and GABAA agonists.

3. Premutation allele

As previously mentioned in adults the premutation is associated with FXTAS, FXPOI and a variety of other medical/psychiatric problems. Recently the studies of children with the premutation have demonstrated that some carriers can demonstrate limited physical features of FXS in addition to psychological or developmental problems whereas most carriers do not show any symptoms.

3.1. *Physical findings*

Premutation carries can present with facial dysmorphic features and the most common finding is prominent ears (*89,90*). Recently a study of premutation carriers found that 33% of postpubertal carrier males had macroorchidism (*93*). Those with macroorchidism had a lower verbal and full scale IQ and increased *FMR1* mRNA levels compared to those without macroorchidism (*93*). This suggests that about one third of individuals with the premutation have significantly lowered FMRP leading to their macroorchidism and mildly lowered cognitive abilities. Premutation carriers can also have joint-laxity and smooth skin typical of those with FXS (*94,95*).

3.2. *Neurological disorders*

Chonchaiya *et al*. (2011) studied boys with the premutation and found an association between seizures, ASD, and ID. These problems are more common in premutation boys who present clinically compared to those who are identified through cascade testing. FXS children of premutation mothers with autoimmune disorders were found to have increased epilepsy and tics compared to children whose mothers did not have autoimmune problems (*96*).

3.3. *Cognitive and behavioral phenotype*

The cognitive effects of the premutation show variable results depending on the age of the carrier and whether they present as the proband or were identified through cascade testing. Not clinically referred children typically do not show differences compared to controls, particularly in girls (*97*). Probands who presented clinically usually have cognitive deficits compared to controls (*97,98*). ADHD is increased in carriers compared to controls (*97*) and in adulthood these symptoms can persist or present as executive function deficits (*34,99,100*). Myers *et al*. (2001), in a small study of 14 children with the premutation found a trend towards lower performance IQ (*101*). Boys with the premutation have higher rates of ADHD symptoms, shyness, social deficits, autism spectrum disorder (*98,102*) and, less commonly, intellectual disability (ID) compared to controls. Many case reports of premutation involvement and ASD have been published. Clifford *et al*. (2007) reported seven males with the premutation; two were probands, and one of these had ASD (*104*). Goodlin-Jones *et al*. (2004), reported four premutation boys and two girls with ASD, and their levels of FMRP were significantly lower than normal (*103*). In the Farzin *et al*. (2006) study, there were 14 boys with the premutation whose parents sought medical attention for their sons' behavior problems (probands), 13 boys with the premutation diagnosed by cascade testing (non-probands), and 16 boys who were siblings without the premutation (controls). They found that 93% (13 of 14) of probands, 38% (6 of 13) of the non-probands and 13% (2 of 16) of the controls had ADHD. In addition 71% of probands (10 of 14) and 8% of non-probands (1 of 13) had ASD. In a screening study of individuals from families with FXS, about 14% of boys and 5% of girls with the premutation met diagnostic criteria for ASD (*104*). A web questionnaire of more than 1,000 families demonstrated a prevalence of autism or ASD of 13% in boys with the premutation and 1% in girls with the premutation (*105*).

Recently, the Rivera group at the MIND Institute (*106*) using a contrast-detection task found low-level visual processing deficits in infants with deficits in infants with FXS and with the premutation. In both groups of infants the contrast levels needed for detection of motion were significantly greater than those of typically developing infants. They concluded that early in life premutation infants can show visual or perhaps other deficits that are also observed in children with FXS.

Psychiatric problems in adults, including depression and anxiety, occur in about 40% of premutation carriers (*14*). Although initial studies of psychiatric disorders in premutation carriers hypothesized that the mood disorders found were associated with the difficulties of caring for a child with FXS, these problems can occur independently from having an affected child (*17*). In the life-time of individuals with FXTAS, 65% met the clinical criteria for a mood disorder according to the DSM-IV, remarkably for anxiety in 52% of the cases (*17*). It has been found that adult females have more problems with attention, hyperactivity (*105*), sleep problems (*23*), autistic behaviors such as rigidity (*107*), perseverance and aloofness (*108*) and language dysfunction (*109*) compared to controls.

3.4. *Neurobiology*

Hippocampal neurons with the premutation in culture (*in vitro*) showed reduced dendritic maturity with shorter dendritic lengths and fewer branches between 7 and 21 days compared with WT neurons (*110*). The premutation neurons had elevations of stress proteins and their mRNAs, including heat shock proteins (Hsp27 and Hsp70) and αB-crystallin. In addition premutation neuronal cultures die more easily in culture by 21 days compared with WT type neurons (*110,111*). Furthermore, altered embryonic neocortical development in the premutation mouse compared to WT has been reported (*112*). At 12 weeks early deficits in learning were observed in KO mice, the premutation mouse was unable to detect a change in the distance between two objects; and at 48 weeks, they could not detect a transposition of objects (*113*). This suggests that the premutation leads to a clear neuronal susceptibility that in addition to other genetic hits (*93*) or environmental toxicity (*114*) can result in a pathogenic neurobiology. Further studies are necessary to determine the neurobiology of affected individuals with the premutation.

3.5. *Premutation genotypes*

Initially *FMR1* premutation carriers were thought to have normal FMRP levels, however recent research findings suggest that carriers have elevated levels of mRNA due to increased transcription, but decreased level of FMRP because the translation is less efficient (*95,103*). As the premutation increases from 55 to 200, the level of *FMR1* mRNA increases and the levels of FMRP begin to decline (*115,116*). Reduced *FMR1* translation is observed in adult individuals with large size premutation alleles (> 110 CGG repeats) and these individuals can have cognitive deficits. Also recent animal studies of the premutation mouse demonstrate lowered levels of FMRP in addition to elevated *FMR1*-mRNA in many brain areas, particularly the amygdala, hippocampus, and cortex, when compared with controls without the premutation (*117*).

The causative molecular mechanism of cognitive deficits and neurodevelopmental problems were thought to be related to silencing of the *FMR1* gene ("loss of function") and decreased amount of FMRP while the mechanisms involved in FXTAS and FXPOI are thought to be associated with abnormally increased levels of *FMR1* RNA ("gain of function") and RNA-toxicity. However recent evidence supports that both the FMRP deficits and elevated *FMR1* RNA in carriers are associated with amygdala dysfunction, which causes cognitive deficits, anxiety, autism spectrum disorders, social avoidance, and aggressive behavior.

There are at least 3 mechanisms that could explain the elevation of *FMR1* mRNA (*89*). One suggests that the observed increase of acetylated histones at the *FMR1* promoter (*118*) could increase the *FMR1* gene transcription. Second, the long tracts of CGG-repeats have been shown to exclude nucleosomes *in vitro* (*119*) and if this occurs *in vivo* it may increase the accessibility of transcription factors to the promoter. Third, the R-loops formed by the CGG-repeats (*120,121*) may lead to chromatin decondensation (*122*). The mechanism of *FMR1* mRNA-toxicity remains to be established, and there are at least 3 models proposed. The "sequestration" model which proposes that the RNA expanded CGG repeats are pathogenic by sequestrating proteins, including Purα, Rm62, CUGBP1, hnRNP A2/B1, SAM68, and DROSHA-DGCR8 (*123-127*) that in turn alter the transcription of many other proteins. A second model, "RAN translation", represents non-canonical translation that results in expression of toxic polyglycine- and polyalanine-containing products (*128,129*). A third model, "antisense *FMR1* (ASFMR1) toxicity", involves the expression of antisense transcripts products (*130*). Mitochondrial abnormalities have also been found in FXS and premutation carriers. The mechanism of mitochondrial dysfunction is unknown but this mechanism is another cause of premutation and full mutation involvement (*131,132*).

4. Overlapping phenotypes, *FMR1* spectrum disorders

The overlap between premutation disorders and full mutation disorders occurs when the full mutation is partially or completely unmethylated or there is a high level of mosaicism in FXS. This puts those with FXS at

risk for FXTAS and other premutation problems. In fact there have been a handful of individuals with FXS who have developed FXTAS and these individuals are high functioning and have unmethylated alleles or mosaicism (*133-136*). Even in the midrange of CGG repeats in premutation carriers there may be mild deficits of FMRP leading to behavioral problems or psychiatric phenotypes (*137*).

Another area of overlap occurs in the gray zone (45-54 CGG repeats). The rate of *FMR1* gray zone expansions in the general population is variable, but large population studies report rates of 0.8% to 3.0% for repeat sizes between 41 and 54 (*138-140*). In 2006, it was recognized that gray zone expansion carriers can also present with premature ovarian insufficiency at a higher rate that in the general population (*141,142*). In a screening study in 2011 a higher rate of Parkinsonism was found in the gray zone mutation carriers compared to controls without the gray zone. There have also been reports of FXTAS in those with a gray zone (*143,144*) because elevated *FMR1* mRNA can also occur in this range (*145*). Other clinical associations with the gray zone in adults include anxiety (*146*) and cognitive decline (*147*). However other studies did not show this association (*147-151*). Pertinent to children, in 2000, a 5-year survey of boys who required special education showed an excess of gray zone expansions (*152*), however, this result has not been replicated (*153*). Further studies are necessary to study the association of the gray zone mutation and the mechanisms of disease in adults and children.

5. Conclusion

Clinicians need to know that those with an *FMR1* mutation are at risk for a wide range of neurovelopmental and/or psychological disorders/neurological disease, referred as Fragile X Spectrum Disorders (Figure 2). It is also important to have a holistic model of understanding on how the phenotype is related to the number of CGG repeats and/or size-mosaicism, including epigenetic changes or methylation status (partial and full, as well as methylation mosaicism), genetic background (gene modifiers and second genetic hits which can be protective or pathogenic) and environmental exposures (environmental changes, exposures to toxins, and social interactions "socionome" among other factors).

Our understanding of FMRP deficits in the FXSD has been hampered by the limited technology available to assess quantitative FMRP levels. Although the immunocytochemical methodology demonstrated a strong correlation with IQ in those with a fragile X mutation (*35,154*), it was not sufficiently quantitative to show the remarkable variation that exists even in the normal population. This variation has been demonstrated by ELISA technology but the technique is difficult to replicate in subsequent samples (*155*). Newer techniques including the immunoassay utilizing time-resolved Forster's resonance energy transfer (*156*) and also the Luminex immunoassay (*157*). These techniques will lead to a new understanding of FMRP deficits not only in FXSD, but also in other neurodevelopmental/ neuropsychiatric disorders. The recent publication of FMRP deficits in the brains of individuals with bipolar disorder, schizophrenia, depression and autism (*156-158*) has opened our eyes to the importance of FMRP outside of the FXSD population. Even more remarkable is the finding that the age of onset and overall IQ in those with schizophrenia is correlated with FMRP deficits in peripheral blood (*159*). The advances in treatments for FXS may also be helpful for premutation carriers with low FMRP and perhaps in other disorders with low FMRP such as ASD.

An area of overlap that is in need of research is the aging process in FXS because many patients experience cognitive decline and the cause is not known, although occult mosaicism leading to a FXTAS-like picture is possible (*160*). Older patients with FXS also have a high risk for Parkinson's disease and it is uncertain if this is also related to occult mosaicism (*161*). These are important considerations for children with FXS because they are raised by mothers with the premutation who may experience a premutation disorder that could influence the development of their offspring. These intergenerational influences require more study. Certainly the development of effective targeted treatments aim to have a significant effect on the ultimate outcome for those with FXSD.

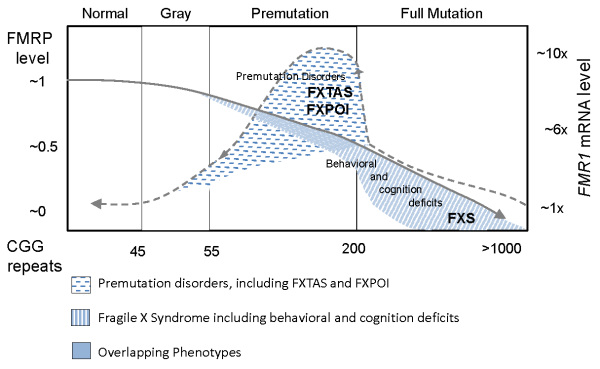

Figure 2. Overlapping phenotypes between FXS and premutation disorders. Dotted-line indicates FMR1 mRNA levels and solid-line indicates FMRP expression levels.

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Original Article

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Participation of underrepresented minority children in clinical trials for Fragile X syndrome and other neurodevelopmental disorders

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Summary **The purpose of this study was to identify demographic data, motivational factors and barriers for participation in clinical trials (CTs) at the University of California Davis, MIND Institute. We conducted a cross-sectional survey in 100 participants (81 females and 19 males). The participants had high education levels (only 2% had not completed high school), a mean age of 44 years (SD ± 9.899) and had at least one child with a neurodevelopmental disorder. The diagnosis of Fragile X syndrome (FXS) had a significant association with past participation in CTs ($p < 0.001$). A statistical significance for age of diagnosis and participation in CTs was also found ($z = –2.01$, $p = 0.045$). The motivating factors were to help find cures/treatments for neurodevelopmental disorders and to relieve symptoms related to child's diagnosis. Factors explaining lack of participation, unwillingness to participate or unsure of participation were: lack of information/knowledge about the trials, time commitment to participation (screening, appointments, assessments, laboratory tests, *etc*.) and low annual household income. These results show that a portion of underrepresented minorities (URM) not participating in CTs are willing to participate and suggests that reducing barriers, particularly lack of knowledge/ information and time commitment to trials are needed to improve recruitment.**

Keywords: Fragile X syndrome, autism, ASD, clinical trials, health disparities, URM, under represent minorities

1. Introduction

More than two decades have passed since the Congress (Revitalization Act of 1993) required that clinical trials, funded by the US National Institutes of Health, include members of underrepresent minorities (URM)(*1*). However, data from the US Census Bureau, National Institute of Health and Tufts Center for the Study of Drug Development (CSDD) demonstrate a clear disparity that exists amongst minority populations in clinical research (US Census Bureau, NIH, and Tufts CSDD, 2010) (*2*). Randomized controlled trials (RCTs) are considered to be the gold standard in evaluating medical interventions. The ability to trust and apply the results of clinical trials, as well as to transfer therapeutic treatments into clinical practice, is related to the type and number of patients enrolled in the studies (National Institute of Cancer, 2002) (*3*). With low minority participation in clinical trials, there is a lost opportunity to discover the effects of a drug-agent amongst URM and increases the existing health disparities within minorities.

Barriers to recruitment, participation, and retention in clinical trials for URM are complex, but can be grouped into 3 categories: *i*) the effect of the disease studied; *ii*) systems factors (*e.g.*, access to clinics, length of appointments or procedures, gap between seeking and receiving care and language barriers (most pharmaceutical companies do not translate the outcome measures into other languages); and *iii*) patient factors (*e.g.*, problems with medication, mental illness, incomplete understanding, race, economical status and mistrust of health care professionals) (*4,5*). Clinical trials in children are often underappreciated even when results have shown major improvements in health care. An illustrious example is the 5-year survival improved from 25% to more than 70% as a result of multicenter trials for acute lymphoblastic leukemia (*6*). However, when compared with adult clinical trials, the number of pediatric clinical trials remains low (*7*) and most of

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them are related to cancer.

The Health Resources and Services Administration (HRSA) and the Center for Diseases Control and Prevention (CDC) found that the prevalence of parent-reported developmental disorders (DDs) in children increased by 17.1% from 1997 to 2008 (*8*). With this rise, a push towards potential targeted treatments for neurodevelopmental disorders has led to multiple Phase II clinical trials for children with neurodevelopmental disorders including Fragile X syndrome (FXS) (*9*) and autism (*10*). Although several studies have proffered reasons for the relative absence of URM among clinical trial participants (*11-13*) to our knowledge none have specifically looked at participation of children with neurodevelopmental disorders. Here we present a small cross-sectional survey of factors associated with participation in clinical trials for children with FXS syndrome and other neurodevelopmental disorders. The intent of this study was to gather general demographic information and attitudes towards CTs amongst the parents of children with neurodevelopmental disorders. We also investigated whether or not URM groups are less likely to participate in CTs than their White counterparts, and whether URM groups were willing to participate in such studies in the future. Factors that impact the decision to participate in CTs were also gathered in order to identify barriers.

2. Methods

2.1. *Participants and procedures*

This study was approved by the Investigational Review Board of the University of California Davis and Touro University California. The research was conducted at the University of California Davis, Medical Center, MIND Institute in Sacramento, California, where about 22 clinical trials were conducted at time of the study. The cross-sectional survey was administered to parents who came to the MIND for their child's treatment and/or to participate in research.

The survey had twenty-nine questions, most of them with a multiple-choice answer and an additional space for free response. Demographic information was collected including: age, sex, race, ethnicity, educational level (if applicable spouse/partner data was also obtained), diagnosis and age of diagnosis, language spoken at home and annual household income. This survey looked at the attitudes of current clinical trials participants based on a four-point Likert scale ranging from "not at all important" to "very important". The questions were validated by a research committee that included multiple members of the MIND staff (physicians, psychologists, social workers, research assistants and volunteers) and patients. US Census's definition of minority was used in classifying the participants into URM and non-URM (*14*). Mean household income was classified according to the US Census (*15*).

2.2. *Data management and analysis*

The data was collected and managed using the REDCap (Research Electronic Data Capture) electronic data capture tools hosted at the UC Davis CTSC. REDCap is a secure, web-based application designed to support data capture for research studies (*16*). The data was analyzed using Statistical Package for the Social Sciences (SPSS) for Windows, Version 21. Descriptive statistics and bivariate statistics included a Chi-square test for independence. Representativeness of sample respondents was assessed using Pearson's or Fisher's exact-test. Nonparametric statistical tests (Kruskall-Wallis tests, Wilcoxon rank-sum/Mann-Whitney U tests) with a significance level of 0.05 were performed for continuous and ordinal variables that were indicated as being a normal based on Shapiro-Wilk Normality test. An α level of 0.05 was used for all statistical tests and all *p*-values were given as two-tailed. A logistic regression analysis was performed for the outcome variable was 'participation in a research trial(s)' (yes/no) and 'future participation in clinical trial(s)' (yes, no, or don't know/unsure).

3. Results

3.1. *Responders demographic*

A total of 100 individuals participated in this survey. All respondents were asked to report their race in addition to ethnicity; 69 were White, 5 Black, 4 Asian, 1 North American Indian/Alaska Native, 18 "Other" and 3 did not respond. When all participants (n = 100) were asked for ethnicity, 21 identified themselves as Hispanic/Latino.

For analysis purposes, due to small sample size of each race and ethnicity the respondents were compared in two broader categories: Non-Hispanic White (69%) *vs*. URM (28%) and Non-Hispanic White (69%) *vs*. Hispanic/Latino (21%).

The majority of the respondents were females (81%), with an average age of 44 years (range 21-69 years, SD 9.8 years). The respondent's mean annual household income was high ($104,972). When controlling for outliers, the mean yearly household income was $91,787 (range $15,000-$200,000, SD 43,925). URM and non-Hispanic Whites reported fairly high annual house income and level of education with no differences observed. The majority of respondents belonged to the "middle economic class" (annual household $104,972), and had at least an Associate's degree. UMR respondents had a similar profile, with the mean annual household income being $108,037.64 and had at least an Associate's degree, or higher.

Twenty-five (25% of all participants) responders indicated that they have had their child participate in clinical trials, 28% of those were URM (n = 7). Descriptive statistics of clinical trial participants and non-clinical trial participants are shown in Table 1.

3.2. *Responders participation in clinical trials*

There were no associations between gender of respondent, race, ethnicity or URM classification and past participation in CTs (Race: χ^2 (1) = 0.795, p = 0.373) (Ethnicity: χ^2 (1) = 0.020, p = 0.887) (URM: χ^2 (1) = 0.718, p = 0.397). A statistically significant association was reported between "other" diagnosis and no participation in CT (χ^2 (1) = 10.68, p = 0.001). The diagnosis of FXS was significantly associated with past participation CTs ($p < 0.001$). A statistical significance for age of diagnosis and participation in CTs was found (z = –2.01, p = 0.045). The level of importance of being assigned to the placebo group (z = –2.27, p = 0.023) and the benefits from the study treatment (z = –2.49, p = 0.013) were associated with no participation (Table 2).

3.3. *Willingness to participate in CTs*

Fifty respondents, of which 30% (n = 15) were URM, indicated that they would be willing to participate in CTs. Twelve respondents, of whom 6 were URM (50%), indicated no willingness to participate in CTs. Thirty-eight of the total respondents were unsure whether they would participate in CT in the future; of those 14 were URM (36.8%).

To evaluate differences among future participation conditions (Yes-would participate in CT, No-would not participate in CT, and Don't know/unsure whether or not to participate in CT) the Kruskal Wallis test was used and revealed a significant effect on future participation in CTs on annual household income (H(2) = 7.24, p = 0.027). A post-hoc test using Mann-Whitney U tests with Bonferroni correction showed the significant differences in household income between those who reported willingness to participate and those who were not willing to participate in future CT ($p < 0.05$, r = 0.24) and between those who reported not participating and those who reported being unsure whether or not to participate in future CT ($p < 0.05$, r = 0.38) (lower income was associated with not willing to participate and unsure to participate). Furthermore, no willingness to participating in CTs and the amount of time involved were also found to be a significant association, (H(2) = 9.92, p = 0.007). The responders did significantly differ in their willingness to participate in future CT when stratified by annual household income, "other" diagnosis, and level of importance for amount of time commitment to CTs (Table 3).

The Wald criterion demonstrated that FXS diagnosis (p = 0.037) and age of diagnosis (p = 0.026) made a significant contribution to prediction. From the analysis, the odds ratio for diagnosis of FXS was 12 times as large and therefore, parents with a child diagnosed with FXS were 12 more times likely to have participated in CT.

Table 1. Description of responders participants and non-participants in clinical trials showing URM, age and annual household income

Group of respondents	All diagnosis	No. of respondents	URM	Age (25 and 75% percentiles)	Annual household income (25 and 75% percentiles)
Clinical trials participants (n = 25)	FXS	10	2	Median 43 years (32-56)	Median $86,000
	ASD	10	5		
	Asperger's	2	0	Mean 42.74 years (+/- 1.449)	Mean $112,801.33
	Learning Disabilities	1	0		
	ADHD	4	0		($27,828-$320,00)
	22q11.2 Deletion	1	0		
	Other*	2	0		
Non-Clinical Trial participants (n = 75)	FXS	4	2	Median 42 years (21-69) **n.s.*	Median $90,000 **n.s.*
	ASD	20	7		
	Asperger's	14	6	Mean 44.97 years (+/- 1.309) **n.s.*	Mean $102,231 ($15,000-$400,000) **n.s.*
	Learning Disabilities	3	1		
	ADHD	10	5		
	22q11.2 Deletion	2	0		
	Down syndrome	1	0		
	Bipolar Disorder	2	1		
	Intellectual disability	1	1		
	Tourette syndrome	1	0		
	Compulsive Disorder	1	0		
	Other*	33	11		

Other* Undiagnosed-going through evaluation, neurotypical, neurotypical with attentional issues, Central Auditory Processing Disorder (CAPD), Dyscalculia-Math Disorder, Expressive/Receptive Language Disorder, depression, Borderline Personality Disorder, Ehlers-Danlos Syndrome, Septo-optic Dysplasia, Phenylketonuria (PKU) birth, schizophrenia, Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS), dyslexia, pulmonary atresia, dwarfism, myotonic dystrophy, Long QT, Neurofibromatosis type I (NFI), Moebius syndrome, Tuberous sclerosis complex. ***n.s.* no significant differences.

Table 2. Associations of participation in clinical trials

Variable	No. of Participants	No. of Non-participants	*p*
Fragile X diagnosis			
Yes	10	4	0.001*
No	15	71	
Other** diagnosis			
Yes	2	33	0.001*
No	23	42	
Level of importance–Child benefits from study treatment			
Not at all important	1	0	
A little important	3	2	
Somewhat important	6	10	
Very important	13	52	0.013*
Level of importance–placebo group			
Not at all important	11	12	
A little important	3	17	
Somewhat important	7	17	
Very important	2	17	0.023*

*Statistical significant at $p < 0.05$. Other**, Undiagnosed-going through evaluation, neurotypical, neurotypical with attentional issues, Central Auditory Processing Disorder (CAPD), Dyscalculia-Math Disorder, Expressive/Receptive Language Disorder, depression, Borderline Personality Disorder, Ehlers-Danlos Syndrome, Septo-optic Dysplasia, Phenylketonuria (PKU) birth, schizophrenia, Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS), dyslexia, pulmonary atresia, dwarfism, myotonic dystrophy, Long QT, Neurofibromatosis type I (NFI), Moebius syndrome, Tuberous sclerosis complex

Table 3. Associations between participation and "other" diagnosis, ethnicity, income and time spent participating

Variable	Will participate (No)	Won't participate (No)	Unsure to participation (No)	*p*
Other diagnosis				0.004*
Yes	12	9	14	
No	38	3	24	
Total	50	12	38	
Ethnicity				0.057
Hispanic/Latino	12	5	4	
Not Hispanic/Latino	38	7	34	
Total	50	12	38	
Annual household income				0.027*
> 250,000	3	0	1	
150,000-249,999	8	0	3	
100,000-149,999	10	0	11	
60,000-99,999	15	4	8	
32,500-59,999	6	2	3	
23,051-32,499	1	2	1	
< 23,051	1	0	2	
Total	44	8	29	
Level of importance–Time				0.007*
Not at all important	6	1	1	
A little important	7	0	2	
Somewhat important	26	1	13	
Very important	9	4	15	
Total	48	6	31	

*Statistical significant at $p < 0.05$

3.4. *Level of satisfaction with participation in clinical trials*

Responders who had children participate in CTs (n = 25) were asked to rate how positive or negative their/ their child's experience was in form of a Likert scale of 'very negative' to 'very positive' (Figure 1). 62% reported a 'very positive' experience. In free text, several respondents amongst neurodevelopmental clinical trials participants reported that they, as well as the child, enjoyed working with the MIND staff and seeing improvements as the primary reason for their positive experience. As a typical example one respondent wrote: "My child enjoyed participating, but there were a lot of forms to complete".

3.5. *Motivational factors*

During the survey, respondents were asked to select the best reason(s) for their choice to participate or not to participate in CT. Among all respondents who were willing to participate in a clinical trial study, the top two motivating factors were to help find cures/treatments for neurodevelopmental disorders (77.2%) and relieve symptoms related to child's diagnosis (63.6%). Similar results were seen amongst the URM respondents (Figure 2). To consider participation in clinical trials, responders reported it being 'very important' to know more details about the trials; 78% participants indicated knowledge about how much their child/children would benefit from the study; 72% how much other people

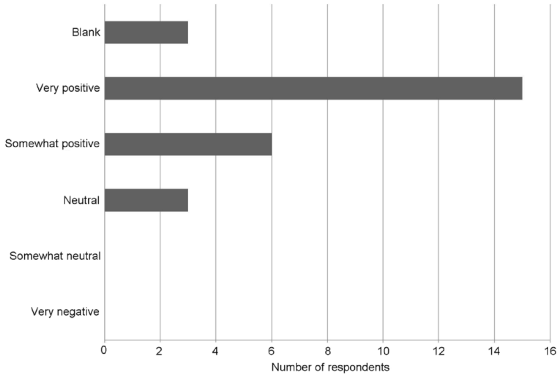

Figure 1. Level of satisfaction in clinical trials partipants.

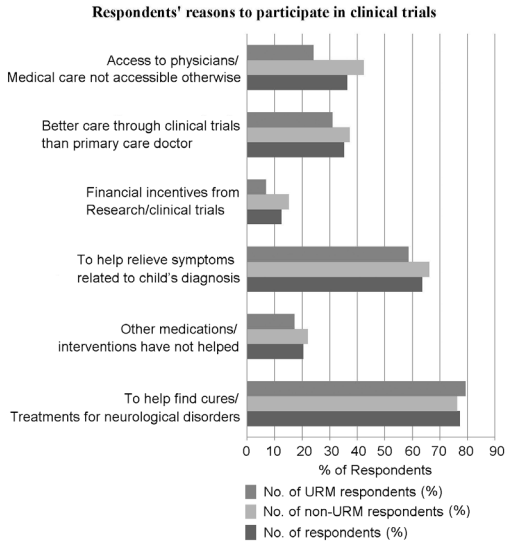

Figure 2. Respondents' reasons to participate in clinical trials.

would benefit from the study; and 70% for information about side effects. 64% of all respondents who were not willing or unsure to participate in CTs reported that lacking general knowledge/information regarding clinical trials was the main reason. In addition, 48% indicated time commitments and 32% indicated "other" reasons. Amongst URM respondents, 75% reported lack of general knowledge/information regarding clinical trials and 60% reported time commitment.

4. Discussion

Findings from this study yielded no association between gender, age, race and level of education (of both respondent and spouse/partner) in CTs participation. In our institute we have one of the only Spanish-speaking clinical trials clinic where personnel involved include coordinators, psychologists and physicians who are bilingual in Spanish. We also have the support of pharmaceutical companies to translate documents and standardized assessments in Spanish for a few of the clinical trials. There was a significant association between annual household income diagnosis and age of the diagnosis and CTs participation or willingness to participate. Low-income families were less likely to participate in CTs. Children who were diagnosed early in life and had diagnosis of FXS where more likely to participate in CTs. Older age at diagnosis and higher levels of importance of being assigned to a placebo group and expected benefits were significantly associated in parents who have not enrolled their children in CTs. This may suggest that education targeted to young parents in regard to diagnosis, benefits for treatment trials and benefits for participating, even when assigned to a placebo group, are necessary.

The observed significant difference in annual household income, between those who were willing and those who were not willing to participate, and between those who have not participated and those who were unsure about future participation, may suggest that the amount of time spent in CTs negatively affects economic status of these families. Therefore, those with lower income are less likely to participate or, consider participation. The work of low income earners may also be less flexible in allowing time off to participate in a clinical trial. Single parents may also find it impossible to participate in such trials. Findings from ASD studies report that URM families with a child with ASD experience more difficulties accessing services than Whites (*17-19*). However, the findings from our study reported otherwise. This could be caused by sampling bias, or the much lower number of respondents identified as URM compared to non-URM; also the URM sample were highly educated and had high household incomes; and finally, the responders were part of a referral center for neurodevelopmental disorders. We also found that a proportion of URM who have not been enrolled in CTs are willing to participate.

This study also highlights that Whites are sharing or facing the same participation barriers as URM, including knowledge and education in study benefits as well as side effects. This study also suggests that educational programs, decreasing time commitment and allowing more flexibility in the CTs schedules will increase participation among all the potential participants. Children who were not diagnosed with FXS and children diagnosed with "other" disorders were less likely to have participated in CTs. This is likely because the MIND Institute is a well-known center for CTs in FXS and exciting translational research has led to targeted treatment trials for this condition (*9*). Many of the responders to our survey included participants of these CTs. Pertinent to FXS, the mothers of FXS children are premutation carriers and may have many medical and psychological problems (*20*) that can be addressed during their children's CTs appointments, creating hybrid trials to help families rather than isolated family members. Efforts to increase minority participation in CTs should focus on ensuring infrastructure, meaningful outreach

and engagement efforts and access to health research for all groups, rather than solely attempting to change URM's attitudes.

There are limitations to performing questionnaire surveys and a greater depth of information could have been obtained by conducting either focus groups or interviewing participants. Thus, enabling the researcher to evaluate respondents' attitudes (negative or positive) and to identify other opinions and recommendations for services. In regards to income, studies have found that families with more than one child with neurodevelopmental disorders have more problems accessing medical care and have lower incomes, regardless of their education (*21*). In this study, the number of children with neurodevelopmental disorders in the families was not collected. In addition, not everyone reporting a low income indicated problems accessing health care as one of the reasons to participate or have participated in CTs. Further studies are necessary to understand and identify barriers for URM clinical trials participation, especially among children with neurodevelopmental disorders.

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Distribution of AGG interruption patterns within nine world populations

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Summary **The CGG trinucleotide repeat within the *FMR1* gene is associated with multiple clinical disorders, including fragile X-associated tremor/ataxia syndrome, fragile X-associated primary ovarian insufficiency, and fragile X syndrome. Differences in the distribution and prevalence of CGG repeat length and of AGG interruption patterns have been reported among different populations and ethnicities. In this study we characterized the AGG interruption patterns within 3,065 normal CGG repeat alleles from nine world populations including Australia, Chile, United Arab Emirates, Guatemala, Indonesia, Italy, Mexico, Spain, and United States. Additionally, we compared these populations with those previously reported, and summarized the similarities and differences. We observed significant differences in AGG interruption patterns. Frequencies of longer alleles, longer uninterrupted CGG repeat segments and alleles with greater than 2 AGG interruptions varied between cohorts. The prevalence of fragile X syndrome and *FMR1* associated disorders in various populations is thought to be affected by the total length of the CGG repeat and may also be influenced by the AGG distribution pattern. Thus, the results of this study may be important in considering the risk of fragile X-related conditions in various populations.**

Keywords: AGG interruptions, *FMR1* allele, CGG repeat, expansion, ethnicity

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1. Introduction

Fragile X syndrome (FXS) and *FMR1* associated disorders are predominantly the result of an expansion of a trinucleotide repeat element located within the 5' UTR of the Fragile X Mental Retardation 1 gene (*FMR1*). In normal individuals the triplet repeat number varies in

length from 5 to 44 CGG repeats. Intermediate alleles are between 45 and 54 repeats, premutation alleles are between 55 and 200 CGG repeats and above 200 CGG repeats are full mutation alleles (*1*). *FMR1* full mutations cause FXS, while premutation alleles lead to fragile X-associated tremor/ataxia syndrome (FXTAS) in an estimated 40% of males and 8-16% of females with the mutation, and fragile X-associated primary ovarian insufficiency (FXPOI) in approximately 20% of female premutation carriers (*2*).

The CGG repeat element, like other trinucleotide repeats, is prone to expansion during transmission from parent to child (*3*). While the mechanism that gives rise to CGG repeat expansion in *FMR1* is not understood, evidence suggests repair of single-strand breaks in the meiotically arrested oocytes form loops, which may be incorporated into the DNA through mismatch repair resulting in an expansion (*4*).

Normal alleles most frequently have 2 AGG interruptions, less frequently they have 1 AGG interruption or 0 AGG interruptions, and rarely greater than 2 AGG interruptions. Within normal alleles the patterns most commonly seen are 9 or 10 CGG repeat segments between interruptions (*5,6*). The 9-A-9-A-9 and 10-A-9-A-9 AGG interruption patterns predominate in all populations that have been studied, evidence that these two patterns were present 200,000 years ago during early divergence of human races or that a strong selection pressure exists at this locus (*7*).

In intermediate and premutation alleles the AGG interruptions tend to occur at the 5' end of the locus and the pure CGG stretch, defined as the longest stretch of uninterrupted CGG repeats, is located at the 3' end (*8,9*). The loss of AGG interruptions appear to have occurred multiple times during human evolution (*10*) but can be a late event in the mutation pathway that leads to expansion (*11*). It is rare for AGG interruptions to be lost during transmission, but observation of its occurrence has been reported (*12-14*).

A normal allele without an AGG interruption has been shown to have an increase mutational rate compared to an allele of similar size containing an AGG interruption (*15-17*). Differences in the distribution of AGG interruption patterns between ethnicities, has been reported, including differences in the frequency of alleles that exceed 35 CGG repeats in length and lack AGG interruptions. These higher frequencies are associated with increased prevalence of FXS (*18*). Conversely, highly interspersed CGG repeat alleles have been observed in the Basque, Native American, and Asian populations, which also have lower estimated FXS prevalence rates (*19,20*).

The presence of AGG interruptions does not seem to affect the transcriptional or translational expression of the *FMR1* gene (*21-24*). However, the presence of AGG interruptions in both intermediate and premutation alleles has been shown to decrease the rate of instability (any change in CGG repeat size) and magnitude of size change in both paternal and maternal transmissions (*12,25,26*).

While the distribution of CGG repeat total length has been reported in a number of populations (*27*), fewer studies have reported the distribution of AGG interruptions within populations. This study reports on the AGG interruption patterns in a total of 3,065 normal alleles (9-40 CGG repeats) from 1,989 participants (males: n = 794; females: n = 1,195) from 9 countries: Australia, Chile, Emirates, Guatemala, Indonesia, Italy, Mexico, Spain, and USA. We compare these results with previous studies that reported AGG interruption patterns in global populations (Figure 1).

Our findings indicate that variations in CGG repeat allele sizes and AGG interruption pattern distributions exist between populations. Two populations (Australia and Indonesia), from the nine newly described, had a higher frequency of long pure CGG repeat stretches (greater than 20 pure CGG repeats), and the USA population had a lower frequency of these long pure stretches. These differences may be important when considering the burden of *FMR1* associated disorders in different populations.

2. Materials and Methods

2.1. *Participants*

Genomic DNA from unrelated individuals with at least one normal *FMR1* allele was included in this study (n = 3,065 alleles). These samples were previously screened to determine the prevalence rates of expanded alleles. Cohorts from Australia (n = 201) (*28*), Chile (n = 77), the United Arab Emirates (n = 263), Guatemala (n = 151) (*29*), Indonesia (n = 312) (*30*), Italy (n = 67), Mexico (n = 277), Spain (n = 358) (*31*), and the United States (n = 1,359) (*32*) were included. Individuals were recruited from the general population for the Italy, Spain, and United States samples. From the USA cohort, participants were from two different geographical areas: Sacramento (California) and Chicago (Illinois). The remaining samples were recruited from high-risk populations including intellectual disabilities, individuals with a family history of FXS and individuals with Parkinsonism. DNA isolation and AGG interruption genotyping were performed at the UC Davis MIND Institute Molecular Laboratory as previously described (*25,32*), except 67 alleles extracted and genotyped in Italy, following IRB approved protocols at the correspondent institutions. Only AGG interruption patterns of unrelated normal alleles less than or equal to 40 CGG repeats in length, therefore within the normal size range (*33-35*), were included in the study.

2.2. *Statistical analysis*

Distributions of categorical variables were compared

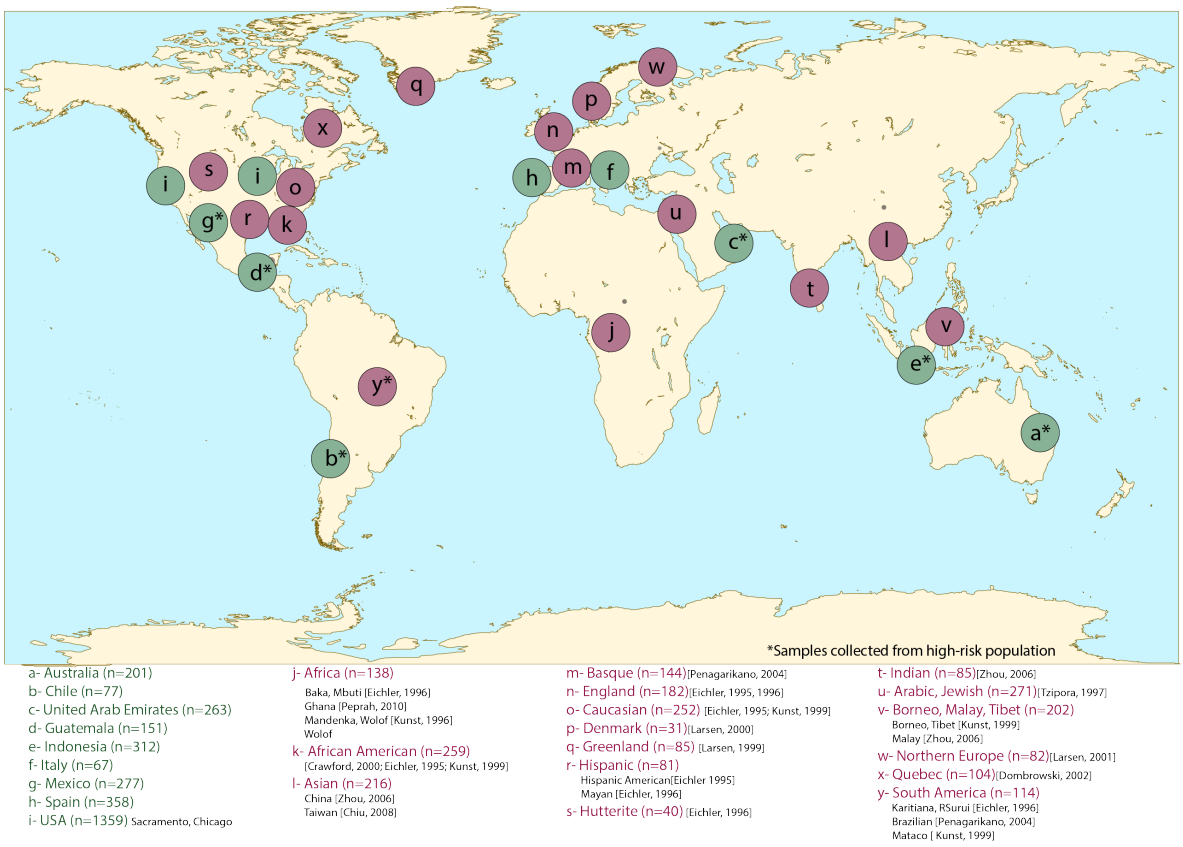

Figure 1. The distribution of 25 global populations with AGG interruption patterns described. AGG interruption patterns were compared between the 9 newly characterized cohorts (a-i, in green) and with previously published studies (j-y, in red). Populations from previous studies were combined if geographical proximity was present to increase sample sizes. Cohorts with samples collected from high-risk populations are denoted with an asterisk, total sample size for each cohort and the studies reporting their AGG patterns are provided next to the cohort's name.

among countries using chi-square tests. Chi-square test *p*-values were obtained by Monte Carlo simulation when the sample size assumptions for use of the chi-square distribution were not met.

In order to identify specific AGG interspersion patterns, total CGG lengths, pure CGG stretches, or AGG interruptions whose frequency in a given population was significantly higher or lower than would be expected under homogeneity, the adjusted residuals from the chi-square table (*36*) were compared to a standard normal distribution and the resulting *p*-values were adjusted for multiple testing using the Bonferroni correction. All analyses were conducted using R, version 2.13.0 (*37*).

3. Results

In the nine populations we determined the number and position of the AGG interruption within each CGG alleles and thus determined the AGG interruption pattern in 3,065 alleles. We observed 30 different CGG repeat lengths ranging from 9 to 40 CGG repeats and 231 different AGG interruption patterns, each allele contained no AGG interruptions up to 3 AGG interruptions. Consistent with previous population based studies of the CGG repeat locus, 29 and 30 CGG repeats were the most common allele sizes in all 9 populations. Indonesia was the only population with a greater proportion of alleles with 29 (39%) than 30 (28%) CGG repeats. Two AGG interruptions were present in at least 56% of the alleles genotyped for each population; 1 AGG interruption occurred in at least 11% of the alleles genotyped (Figure 2, Table 1).

3.1. *The distribution of total CGG length, pure CGG stretch, and number of AGG interruptions differs between populations*

The mode of total CGG length was 30 in subjects from all countries examined except in Indonesia where it was 29 (Figure 3). The relative proportions of subjects with a total of 29 CGG repeats, 30 CGG repeats, or a value other than 29 or 30 differed significantly by country ($p < 0.001$) (Table 1). Likewise, the relative proportions of subjects with a pure stretch of 9 CGG repeats, 10 CGG repeats, or a value other than 9 or 10 differed significantly by country ($p < 0.001$). Examination of adjusted residuals suggests that significantly more alleles from Australia ($p < 0.001$), Emirates ($p = 0.007$) and Spain ($p < 0.001$) had total CGG lengths other than 29 or 30 and Australia ($p < 0.001$) and Spain ($p < 0.001$) had pure stretch lengths other than 9 or

10 repeats. Further, significantly more alleles from Indonesia ($p < 0.001$) had a total length of 29 CGG repeats, and significantly fewer USA ($p < 0.001$) alleles had total CGG lengths other than 29 and 30. Indonesia had significantly fewer alleles with a pure stretch of 10 CGG repeats ($p < 0.001$), Spain had fewer alleles with a pure stretch of 9 CGG repeats ($p < 0.001$) and USA had more alleles with 10 pure CGG repeats ($p < 0.001$) than was expected under homogeneity, where homogeneity would assume the same allele frequencies are present between populations.

The proportion of alleles with 3 AGG interruptions differs significantly by country ($p < 0.001$) (Table 1); examination of adjusted residuals suggests that significantly more alleles from Indonesia ($p < 0.001$) and Australia ($p < 0.001$), and significantly fewer from USA ($p = 0.005$) had AGG interruptions than was expected under homogeneity.

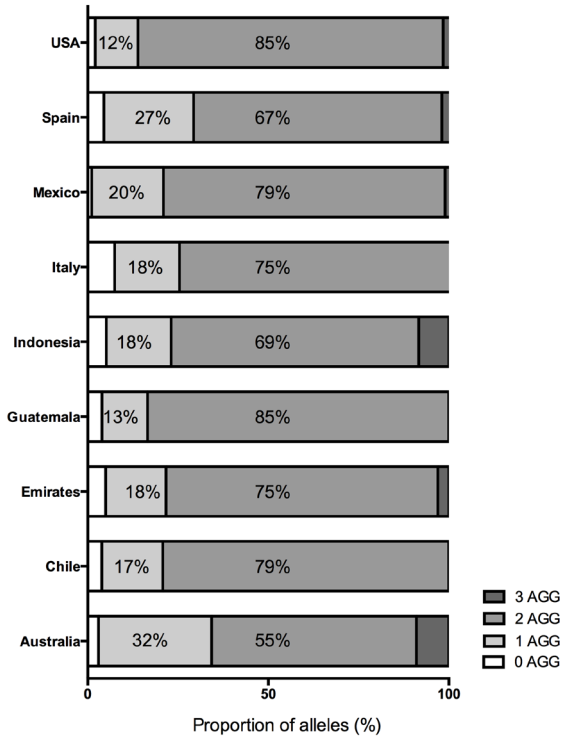

Figure 2. Distribution of number of AGG interruptions. For the nine newly characterized populations the proportion of alleles with 0 to 3 AGG interruptions is graphically represented. Alleles with 2 AGG interruptions were the most common in each cohort, followed by 1 AGG interruption. Four AGG interruptions were observed in Australia, United Arab Emirates, Indonesia, and Spain only. Within the nine populations no alleles were identified with more than 3 AGG interruptions.

3.2. *AGG interspersion patterns by country*

The most common AGG interspersion pattern was 10-A-9-A-9 in all countries except Indonesia. In Indonesia the most common AGG interspersion pattern was 9-A-9-A-9, 10-A-9-A-9 was the second most common pattern. The distribution of AGG interspersion patterns differed significantly by country ($p < 0.001$) and the most common patterns are shown in Supplementary Table 1 (*http://www.irdrjournal.com/docindex.php?year=2014&kanno=4*). Examination of adjusted residuals suggested that significantly more alleles from Australia had the patterns 9-A-9-A-9-A-9 ($p < 0.001$, 8%), 9-A-9-A-19 ($p = 0.012$, 2%), and 10-A-9 ($p < 0.001$, 11%); significantly more alleles from the Emirates had the pattern 10-A-10-A-9 ($p < 0.001$, 6%), 11-A-9-A-9 ($p = 0.022$, 2%), and 9-A-10-A-9 ($p < 0.001$, 7%). Indonesia had significantly more alleles with 30 CGG repeats and no AGG interruptions ($p = 0.010$, 2%), 9-A-13 ($p < 0.001$, 3%), 9-A-22 ($p < 0.001$, 3%), 9-A-9-A-9 ($p = 0.004$, 35%), and 9-A-9-A-6-A-9 ($p < 0.001$, 6%) patterns; significantly more Spanish alleles had the patterns 10-A-9 ($p < 0.001$, 9%), 13-A-9 ($p = 0.013$, 4%) and 9-A-12-A-9 ($p < 0.001$, 4%), and significantly more USA alleles had the pattern 10-A-9-A-9 ($p < 0.001$, 44%) than was expected under homogeneity. Likewise, fewer alleles from Australia and Spain had the pattern 9-A-9-A-9 (both $p < 0.001$, 9% and 14%, respectively), fewer alleles from Indonesia have the pattern 10-A-9-A-9 ($p < 0.001$, 21%), fewer

Table 1. Summary of allele structure in nine populations

Items	Australia	Chile	Emirates	Guatemala	Indonesia	Italy	Mexico	Spain	USA
Total length									
29	23 (11%)	19 (25%)	58 (22%)	47 (31%)	122 (39%)	11 (16%)	96 (35%)	56 (16%)	413 (30%)
30	72 (36%)	41 (53%)	104 (40%)	71 (47%)	86 (28%)	30 (45%)	106 (38%)	143 (40%)	677 (50%)
Other	106 (53%)	17 (22%)	101 (38%)	33 (22%)	104 (33%)	26 (39%)	75 (27%)	159 (44%)	269 (20%)
Pure Stretch									
9	34 (17%)	17 (22%)	57 (22%)	43 (28%)	139 (45%)	11 (16%)	96 (35%)	56 (16%)	422 (31%)
10	100 (50%)	48 (62%)	136 (52%)	75 (50%)	78 (25%)	35 (52%)	127 (46%)	190 (53%)	756 (56%)
Other	67 (33%)	12 (16%)	70 (27%)	33 (22%)	95 (30%)	21 (31%)	54 (19%)	112 (31%)	181 (13%)
Number of AGG									
0	6 (3%)	3 (4%)	13 (5%)	6 (4%)	16 (5%)	5 (7%)	3 (1%)	16 (4%)	28 (2%)
1	63 (31%)	13 (17%)	44 (17%)	19 (13%)	56 (18%)	12 (18%)	55 (20%)	89 (25%)	161 (12%)
2	114 (57%)	61 (79%)	198 (75%)	126 (83%)	214 (69%)	50 (75%)	216 (78%)	246 (69%)	1148 (84%)
3	18 (9%)	0 (0%)	8 (3%)	0 (0%)	26 (8%)	0 (0%)	3 (1%)	7 (2%)	22 (2%)

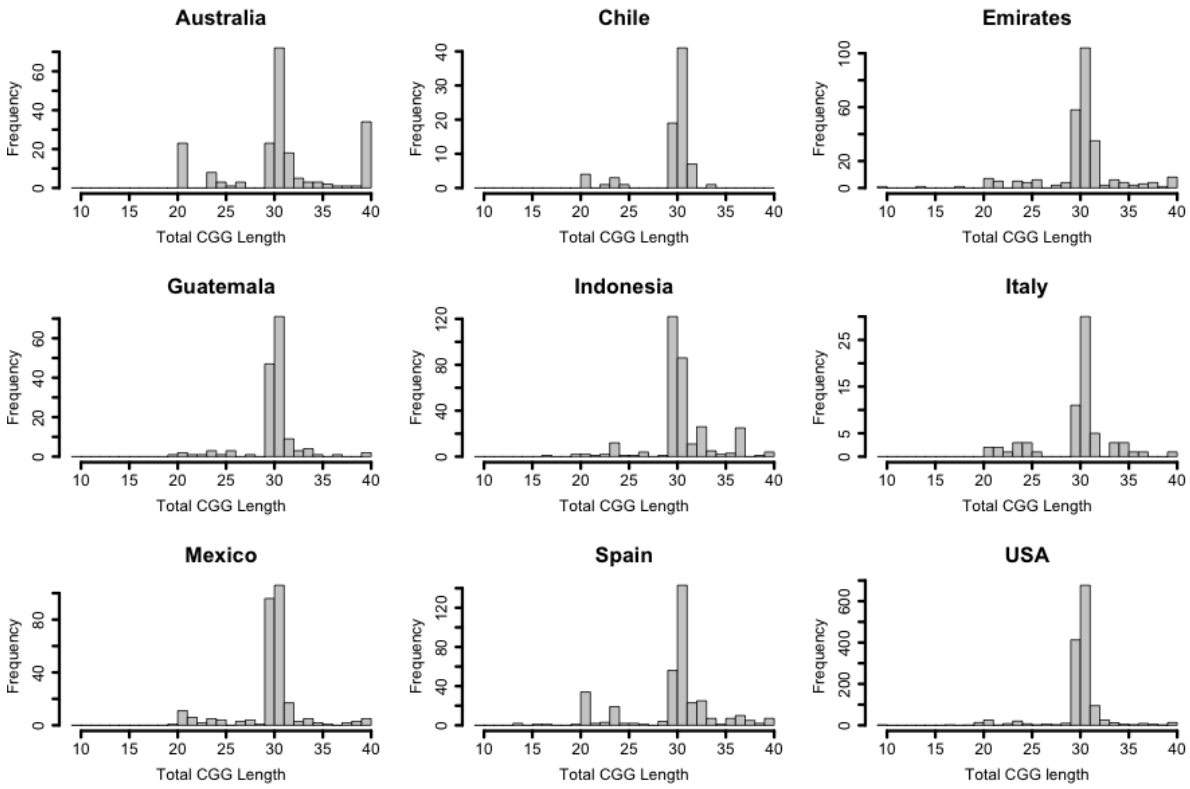

Figure 3. Histogram of total CGG length for 9 populations. The most common total length of CGG repeat sizes for the 9 populations are 30 and 29 CGG repeats, 30 is the most common for every population except Indonesia. Populations show difference in less prominent modes including some previously identified (20 CGG repeats, 23 CGG repeats, and 36 CGG repeats).

alleles from USA had the pattern 10-A-9 ($p < 0.001$, 2%) than was expected under homogeneity.

In 201 normal alleles genotyped from Australia, we observed 74 AGG interruption patterns (Supplementary Table 1; *http://www.irdrjournal.com/docindex.php?year=2014&kanno=4*). A larger proportion of alleles were in the high normal range than observed in the other populations, between 32 and 40 CGG repeats in length, and approximately 9% of the genotyped alleles contained three AGG interruptions. In 77 normal alleles genotyped from Chile, 17 AGG interruption patterns were present. In 151 normal alleles genotyped from Guatemala 39 AGG interruption patterns were observed however no remarkable patterns were observed. In 263 normal alleles genotyped from the United Arab Emirates, 77 different AGG interruption patterns were observed out of which twenty were only observed in the Emirates population. Approximately 1% of the alleles had 3 AGG interruptions. In the 312 normal alleles genotyped from Indonesia, 60 AGG interruption patterns were observed. A large portion of normal alleles with 3 AGG interruptions (8%), with the majority of these alleles having the 9-A-9-A-6-A-9 pattern (6.4% of patterns) was observed in Indonesia. The 9-A-9-A-6-A-9 pattern and CGG length of 36 repeats has been observed in previous studies to occur within Indonesian and Asian cohorts (*30,38,39*). Fifty AGG interruption patterns were observed in 277 *FMR1* alleles genotyped from Mexico. Six distinct AGG interruption patterns were observed only in the Mexico cohort, although these were each observed only once. Eighty-four AGG interruption patterns were observed in 358 normal alleles from Spain. Eleven AGG interruption patterns were only observed in the Spain cohort. Twenty-four AGG interruption patterns were observed in 67 normal CGG repeat alleles from Italy. Three patterns were observed in the Italy cohort only.

3.3. *Regional differences in frequencies of AGG interruption patterns were observed within the USA samples (Chicago and Sacramento area)*

The largest cohort of this study was from the United States, and consisted of samples from a larger collection of newborn blood spots that were collected in both the Sacramento and Chicago area (*32*). The Chicago cohort was comprised of individuals identified as Caucasian (n = 153 alleles), African American (n = 225 alleles), Hispanic (n = 223 alleles), Asian (n = 42 alleles), Southeast Asian (n = 14 alleles), Native American (n = 14 alleles), and other (n = 5 alleles). The Sacramento cohort was comprised of individuals identified as Caucasian (n = 156 alleles), African American (n = 24 alleles), Hispanic (n = 123 alleles), Asian (n = 42 alleles), Pacific Islander (n = 6 alleles), Native American (n = 4 alleles), and other (n = 328 alleles).

There were 105 AGG interruption patterns observed in 1,359 normal alleles. Twenty-five AGG interruption patterns were observed in the USA cohort and were not observed in the other 9 populations.

The most common total CGG length in both Sacramento and Chicago was 30, pure CGG stretch was 10, and number of AGG interruptions was 2 (Supplementary Figure 1; *http://www.irdrjournal.com/docindex.php?year=2014&kanno=4*). Compared to Chicago, Sacramento had a higher frequency of alleles with total CGG lengths of 30 and pure CGG stretches of 10 than would be expected under homogeneity (both, $p < 0.001$). The two cities were similar in the proportion of alleles with a pure stretch that was greater than 20 CGG repeats ($p = 0.2322$), and a total length that was greater than 35 CGG repeats ($p = 0.7471$).

The proportion of alleles with 3 interruptions did not differ significantly between Sacramento and Chicago ($p = 0.8271$). The most common pattern in both cities was 10-A-9-A-9. However, the overall distribution of AGG interspersion patterns differed significantly between Sacramento and Chicago ($p < 0.001$). Examination of adjusted residuals reveals that more alleles in Sacramento had the pattern 10-A-9-A-9 ($p < 0.001$) and more alleles in Chicago had the pattern 9-A-9-A-9 ($p = 0.043$) than would be expected under homogeneity.

3.4. *Previously studied populations*

The data from the 9 populations were compared with data from previously published studies including samples collected and sequenced from Quebec (*11*), Taiwan (*40*), Norway, Saami, Nenets (*41*), Greenland (*42*), African American (*43*), Denmark (*16*), Basque (*44*), Caucasian, Mataco, Tibet, Navajo, Borneo, Mandenka, Wolof, African American (*19*), Brazil (*45*), China, Malay, India (*17*), and sub-saharah West Africa (*46*). AGG interruption patterns were determined by *mnl I* digestion for samples collected from England, Hispanic American, African American, and Asian American (*5*), Tunisian Jews, Sephardic Jews, Ashkenazic Jews, and Arabs (*18*), Suriu, Mayan, Karitiana, Baka, Mbuti, and Hutterite (*7*).

Collections were combined as indicated in Figure 1 in order to increase sample sizes; alleles from the Navajo population were excluded because they did not reach a sufficient sample size. The distribution of total CGG repeats length, pure CGG stretch, and number of AGG interruptions was significantly different in the 25 global populations. Seven populations had higher proportions of alleles with more than 35 CGG repeats (Asian, $p < 0.001$; Australia, $p < 0.001$; Caucasian, $p = 0.013$; Denmark, $p = 0.001$; Greenland, $p < 0.001$; India, $p < 0.001$; and Indonesia, $p = 0.007$). Four populations had a larger proportion of alleles with less than 35 CGG repeats (Chile, $p = 0.016$; Guatemala, $p = 0.016$; Hispanic American, $p = 0.044$; and USA, $p < 0.001$). When pure CGG repeat stretch was compared in the 25 populations, 6 populations (Australia, $p < 0.001$; Africa, $p = 0.001$; African American, $p = 0.043$; Basque, $p = 0.043$; Indonesia, $p = 0.001$; and Jewish & Arabic, $p < 0.001$) had higher frequencies of alleles with greater than 20 pure CGG repeats. Seven populations (Asia, $p < 0.036$; Greenland, $p = 0.014$; Hispanic American, $p = 0.006$; Malay, $p = 0.004$; and USA, $p < 0.001$) had higher frequencies of alleles with less than 20 pure CGG repeats. The populations with highly interspersed alleles included Asia ($p < 0.001$), Australia ($p = 0.002$), Greenland ($p < 0.001$), India ($p < 0.001$), Indonesia ($p < 0.001$), and Malay ($p = 0.034$); the USA had significantly less AGG interruptions then expected under homogeneity ($p < 0.001$).

4. Discussion

Differences in the frequency of AGG interruption patterns within the CGG repeat locus of *FMR1* have been previously reported to vary between ethnicities, and suggested that such differences can affect the mutation rate of this locus. We have genotyped 3,065 alleles from 9 global cohorts to investigate how AGG interruption patterns vary between geographic and ethnic populations. The distribution of CGG repeat total length, and AGG interruption patterns were found to be significantly different between populations. Consistent with previous studies two AGG interruption patterns, 10-A-9-A-9 and 9-A-9-A-9, were the most common in all nine populations reported in this study, and in the 14 previously published population studies (Supplementary Table 1; *http://www.irdrjournal.com/docindex.php?year=2014&kanno=4*). 10-A-9-A-9 was the most common allele for all populations except in the African American, Asia, Indonesia, and Malay, Borneo, and Tibet cohort where 9-A-9-A-9 was the most common pattern. The frequency of the 9-A-9-A-9 pattern in Asian ethnic groups was consistent with what has previously been shown (*17,40*), and in the African American group the 9-A-9-A-9 pattern was only 1% higher in frequency than the 10-A-9-A-9 pattern. It is unknown whether these two patterns have a biological advantage, however, CGG repeat length in the normal allele has been shown to alter translational efficiency (*47*) with the highest translational efficiency occurring at 30 CGG repeats. Thus, the common lengths may provide alleles within the optimum size range with the lowest mutation rate.

We combined the AGG interruption pattern results of the 9 population cohorts genotyped for this study to the 16 cohorts from previous published studies. The results showed that six populations had a higher frequency of alleles with a total length greater than 35 repeats, and five populations had a higher frequency of alleles with an uninterrupted stretch greater than 20 repeats. Australia, Denmark, and Quebec had both, suggesting that an increased frequency of expanded

alleles, intermediate, premutation, and full mutation alleles may be present in these populations. It should be noted that as the Australian cohort was part of a high risk screening study, a sample bias affecting these results could be present given that intermediate prevalence rates were found to be increased compared to the general population (*28*). However, only alleles not greater than 40 CGG repeats were included in this study and importantly the distribution of CGG repeat length was not statistically different from the one observed in a group of 3,091 alleles (1,091 male and 2,000 female alleles) derived from Australian newborns from the state of Victoria ($p = 0.3052$). In these two population-based samples the frequencies of GZ alleles were 1.3% (> 40 CGG repeats) and 0.4% (> 44 CGG repeats), in male newborns; and 5.5% (> 40 CGG repeats) and 1.4% (> 44 CGG repeats), in female newborns (unpublished data). The frequency of premutation alleles was 0.3% in both male and female samples. In Canada prevalence estimates for intermediate alleles is 1:86 in females, and for premutation alleles is 1:813 in males and 1:241 in females (Dombrowski *et al.*, 2002). No prevalence estimates are available for the Denmark population. These prevalence rates are not higher than those estimated in other populations and also we do not have any information regarding whether these prevalence rates are increasing or decreasing with generation.

Guatemala, Hispanic American, Mexico, and USA cohorts had a smaller proportion of alleles in the high normal range, and a smaller proportion of alleles with greater than 20 uninterrupted CGG repeats, suggesting increased stability of the normal allele in these populations. However, both Guatemala and Mexico cohorts were collected for high risk screening studies, and sample collection bias may also be present in these two populations. In the USA population prevalence rates for intermediate alleles were estimated to be 1:112 for males and 1:66 for females, and for premutation alleles were estimated to be 1:430 for males and 1:209 for females (*32*). The Hispanic American, Guatamala, and Mexico populations do not have estimated prevalence rates. The prevalence estimates for the USA population are neither in agreement or disagreement with the population having an increased stability compared to the other studied populations.

A comparison of Sacramento and Chicago showed similarities in the distribution of AGG interruption patterns, and the proportion of alleles in the high normal and intermediate range, and with more than 20 uninterrupted CGG repeats (Supplementary Figure 1; *http://www.irdrjournal.com/docindex.php?year=2014&kanno=4*). Interestingly, Sacramento has an increased prevalence of premutation alleles (males, 1:305; females, 1:172) when compared to Chicago (males, 1:308; females, 1:894) (*32*). Both cohorts were collected and genotyped in the same study and were collected as part of a pilot study newborn screening for FXS.

One limitation of this study is the possible sampling bias within the six newly described population cohorts that were collected from high-risk screening studies. A sample bias may also likely be present in the Sacramento and Chicago cohorts that make up the USA population because AGG interruption data was available mainly for samples that were genotyped by the CGG linker PCR assay (*32*) when initial genotyping of females resulted in only one allele. Another limitation of this study, and of the other published studies, is represented by the small sample sizes. The expected variability introduced by sampling error inhibits strong comparisons between prevalence rates of intermediate, premutation, and full mutation alleles and AGG interruption pattern distribution; limitations that could be reduced with increasing cohort sizes.

The AGG interruption patterns within the CGG repeat locus of *FMR1* further characterize the alleles beyond repeat length. The results of the study agree with what is known about the CGG repeat distribution in the nine countries, including increased frequency of the 9-A-9-A-6-A-9 pattern in Asian ethnicities where the 36 CGG repeat length is more frequent. Population structure is important to consider when studying the CGG repeat locus, sub-populations have consistently shown significant differences in the literature, including differences between ethnic and geographic groups. Our results suggest that AGG interruption pattern distributions in populations could be associated with differing prevalence of categorically non-normal alleles, however larger cohort sizes and more prevalence rates will be needed for many ethnicities to confirm these observations.

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Case Report

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Addictive substances may induce a rapid neurological deterioration in fragile X-associated tremor ataxia syndrome: A report of two cases

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Summary **A debilitating late-onset disorder of the premutation in the *FMR1* gene is the neurodegenerative disorder fragile X-associated tremor ataxia syndrome (FXTAS). We report two patients with FXTAS who have a history of substance abuse (opiates, alcohol, and cocaine) which may have exacerbated their rapid neurological deterioration with FXTAS. There has been no case report regarding the role of substance abuse in onset, progression, and severity of FXTAS symptoms. However, research has shown that substance abuse can have a negative impact on several neurodegenerative diseases, and we propose that in these cases, substance abuse contributed to a faster progression of FXTAS as well as exacerbated white matter disease.**

Keywords: Substance abuse, neurological deterioration, FXTAS, premutation, opiates

1. Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) (OMIM: 300623) is a late-onset progressive neurodegenerative disorder with core features of intention tremor and gait ataxia affecting carriers of premutation repeat expansions (55-200 CGG repeats) in the 5' UTR region of the *FMR1* gene. This syndrome affects mainly men, and the mean age of onset is 62 years with incomplete penetrance (*1,2*).

Other conditions such as chronic pain related to neuropathy (*3,4*), fibromyalgia (*5-7*) and migraines (*8*) are commonly reported symptoms by individuals with FXTAS, and in general observation, narcotics are often used to treat the pain symptoms. Moreover, anxiety, depression and other psychiatric disorders are also reported with a higher prevalence in premutation carriers, and self-treatment using addictive substances is increased compared to controls (*9,10*).

While there has been no comprehensive study regarding the role of addictive substances in onset, progression, and severity of neurological symptoms in FXTAS, it is hypothesized that the clinical symptoms can be triggered by environmental factors. General examples of this phenomenon include alpha-1 antitrypsin, in which smoking can exacerbate lung disease (*11*), and hemochromatosis in which alcohol intake results in liver disease (*12*). Some environmental factors, such as chemotherapy, have been reported to exacerbate or induce earlier presentation of clinical symptoms in FXTAS (*9*). In addition previous studies in the general population have shown that chronic use of addictive substances has negative consequences on cognition, memory and other neurological functions. Therefore, we propose that addictive substances particularly opiates, alcohol and cocaine may contribute to a faster progression of neurological symptoms in FXTAS. Here we present two cases of adults with FXTAS with a long history of addictive substance use, including opiates, alcohol and cocaine.

2. Case reports

Case 1, a 79 year-old Caucasian woman who was initially evaluated at the UC Davis MIND Institute

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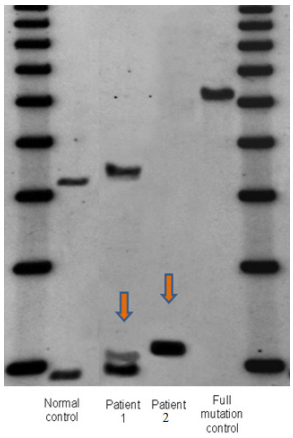

Figure 1. Southern blot results of two patients. 1Kb ladder size marker: **lane 1** and **6**. **Lane 2** and **5**: normal female control and full mutation male, respectively. **Lane 3** and **4** showing the presence of a premutation allele in patient 1 and 2 respectively. CGG repeat sizes were measured by PCR analysis.

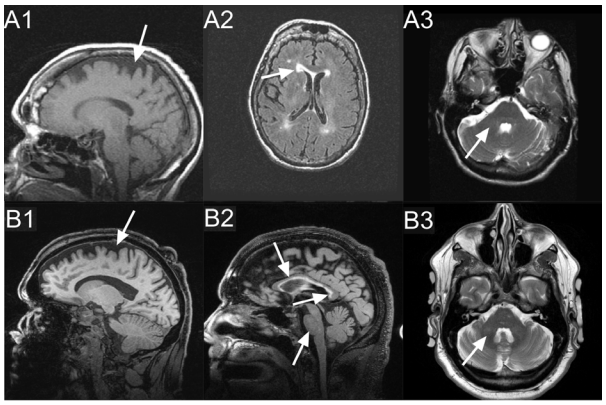

Figure 2. Tesla MRI (1.5): T1 (A1), T2 FLAIR (A2), T2 (A3). 3 Tesla MRI: MPRAGE (B1), T2-FLAIR (B2), T2-TSE (B3); shown by arrows: Case 1: Moderate cerebral (**A1**) and mild cerebellar (not shown) volume loss, periventricular white matter lesions affecting anterior and posterior horns, bilaterally (**A2**). No white matter changes in the middle cerebellar peduncles (**A3**). Case 2: Moderate cerebral (**B1**), mild increased white matter changes in the middle cerebellar peduncles (**B3**) and pons (**B2**), moderately thin truncus of the corpus callosum (**B2**), with severe increased T2 signal intensity in both the truncus and the splenium of the corpus callosum (**B2**).

clinic in 2007, had alleles of 30 and 73 CGG repeats with an X-chromosome activation/inactivation ratio of 0.71 (Figure 1). Her medication history includes codeine with varying doses since age 54. She started on high dose Vicodin (hydrocodone and acetaminophen, 10 mg/300 mg) at age 75, which she was still taking at age 79 (12 tablets a day). She reported the onset of her neurological symptoms at age 54 and she complained of strong intermittent generalized pain which was more severe in her lower extremities and back. She was diagnosed with fibromyalgia at age 58. The patient showed a rapid neurological decline at age 77, when she started to experience handwriting problems, swallowing difficulties, cognitive deficits, frequent falling related

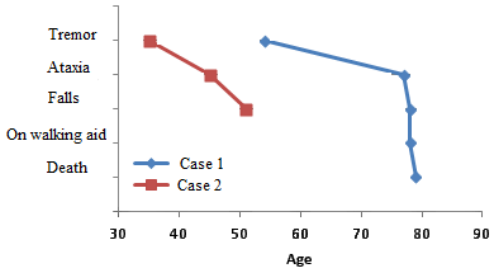

Figure 3. Median onset of pain symptoms, tremor, ataxia, falls, dependence on walking aid and death in two patients with FXTAS and addictive substance abuse.

to lower extremity weakness and the necessity of using a four pronged cane. Her pain intensified and her weakness worsened to the point she needed to use a walker at age 78. The neurological symptoms continued to progress so that she was unable to even sit and finally spent most of her time in bed (FXTAS stage 6). In bed, she was unable to lift her legs against gravity at age 79. She developed respiratory difficulties that were exacerbated by pneumonia and she died at 79 years of age. Her MRI at age 78 shows periventricular white matter lesions affecting the anterior and posterior horns, bilaterally, and moderate cerebral and mild cerebellar volume loss (Figure 2).

Case 2, a 55-year-old Caucasian male, presented at the UC Davis MIND Institute clinic in 2010. He had 100 CGG repeats (Figure 1). He has a long history of significant alcohol and cocaine abuse. He started to smoke marijuana daily in 1998, to help him with chronic pain. He used Vicodin (hydrocodone and acetaminophen, 10 mg/300 mg) three times a day on and off since the age of 35. He has a long psychiatric history including severe anxiety, depression, and post traumatic stress disorder (PTSD), particularly after he was in a vehicle accident at age 36. His neurological symptoms at age 35 included numbness, tingling, and pain in his lower and upper extremities. He had hand writing problems since about age 45. He has a history of intention tremor, beginning at age 51, and occasional resting tremor, as well as balance problems at age 51, and swallowing and choking problems since about age 53. Head tremor was observed at age 54. His MRI at age 55 showed moderate cerebellar and cerebral volume loss, mild increased T2 signal intensity in the middle cerebellar peduncles (MCPs), a moderately thin truncus of the corpus callosum, with severely increased T2 signal intensity in both the truncus and the splenium of the corpus callosum (Figure 2).

3. Discussion

Premutation carries have a susceptibility to develop a variety of symptoms such as neurodevelopmental disorders (*13-16*), psychiatric involvement (*17-19*), immune dysregulation (*5-7,20-23*), and neurological

problems including FXTAS (*1,9*). We recently proposed that the phenotypic variability of premutation carriers could be explained by genetic background effects, including second "genetic hits" and environmental exposures including substance or medication abuse (*9,24*).

In a study of premutation carriers with FXTAS, median onset of tremor was 60 years of age (*25*). Median delay of onset, from the onset of initial motor deficits, for ataxia, falls, dependence upon a walking aid, and death was 2, 6, 15, and 21 years, respectively (*25*). The life expectancy is within the range of 5 to 25 years after the onset of symptoms (*25*). In case 1, the age of onset of pain symptoms was age 54. Tremor, ataxia and falls started at age 77. Dependency on a walker occurred at age 78, with death at age 79. In Case 2, the age of onset of pain symptoms was 35 years. Onset of tremor was age 45. Onset of ataxia and falls was age 51 (Figure 3).

A long history of codeine and hydrocodone use in Case 1 that was intensified at age 75 may be related to a more rapid decline in her late 70s. Her early course was prolonged but this is typical for a female with the premutation and FXTAS. The very early onset of neurological symptoms in Case 2 could have been exacerbated by the long history of alcohol, cocaine, marijuana, and hydrocodone use. Premutation carriers may be more vulnerable to the toxicity of substances of abuse because the premutation neuron dies more easily in culture compared to control neurons (*26*).

Addiction to opiates was associated with decreases in white matter integrity in a diffusion tensor imaging (DTI) study, suggesting widespread axonal injury in the brain (*27*). Another DTI study reported that males with alcohol, cocaine and amphetamine abuse have reduced white matter integrity in the corpus callosum (*28*). Furthermore, reduced white matter integrity in the corpus callosum is an important marker of gait instability in the elderly (*29*), and corpus callosum splenium (CCS) hyperintensity on T2 MRI is a marker of the severity of disease progression in FXTAS (*30*). The above-mentioned MRI findings are in accordance with MRI findings and clinical progressions in our patients.

In our clinical experience, substance abuse by patients with FXTAS occurs either to treat pain symptoms or as a self-treatment of depression and anxiety. Since opiates are associated with white matter disease, in general, we recommend that opiates should be avoided if possible in premutation carriers with neurological symptoms or FXTAS. If opiates are absolutely necessary for pain control, their use should be closely monitored and tapered as soon as possible. Alternative treatments for pain include acupuncture, gabapentin, pregabalin, duloxetine, venlafaxine, tricyclics, omega 3s, and psychological approaches with cognitive behavioral therapy.

In conclusion, the two cases support our previous hypothesis that environmental exposures (in this case addictive substances) may have exacerbated the neurological symptoms of FXTAS, including tremor, ataxia and cognitive decline. We recommend avoiding the overuse of opiates for pain management and also early treatment of depression and anxiety in patients with the fragile X premutation, and especially with FXTAS.

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Case Report

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Parent-delivered touchscreen intervention for children with fragile X syndrome

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Summary **The use of touchscreen applications for the iPad® allows children with disabilities to improve their personal autonomy and quality of life. In light of this emerging literature and our clinical experience with toddlers and children with Fragile X syndrome (FXS), a randomized clinical trial pilot study was conducted of whether an interactive iPad®-based parent training program was efficacious for both individuals with FXS and autism spectrum disorder aged 2-to-12 compared to wait-listed controls. As a second goal, we assessed the difference between direct person-to-person therapy *vs*. online therapy sessions through telehealth. In this case series report it is presented preliminary results of four individuals with FXS enrolled in the study and described the innovative experience including qualitative and quantitative data analysis. Furthermore, we provide professionals with specific guidelines about the use of touchscreen devices as in-home learning tools and parent training strategies to actively involve families in educational treatments in conjunction with clinical guidance.**

Keywords: Touchscreen devices, educational applications, parent training, innovative therapy

1. Introduction

Technology provides an invaluable support for enhancing adaptive skills and learning for individuals with neurodevelopmental disabilities (*1*). Digital technology can improve communication, support social interaction, enhance learning tasks and personal independence and increase leisure time in daily life (*2*). However, there is only very little research on the impact of innovative technology, communication devices, touch-screen tablets, and educational applications (*3*).

In the last ten years, the National Center for Technology Innovation (NCTI) has been following changes in educational and assistive technology (AT), which has shifted into a more portable, networked, customizable, and multitasking approach converging in touch interface devices which, additionally, are widely used by the general population (*4*). Touch screen devices, such as Apple iPad® emerged in 2010, while not specifically designed for education or developmental interventions, have already proven to be suitable for therapeutic and educational benefits in disorders such as autism (*5*) and schizophrenia (*6*). Despite the increased technological research interest in the field of neurodevelopmental disorders and the current application in the clinical practice for education, and promoting communication, there is no research which involves the use of touch-screen devices in children with Fragile X syndrome (FXS). Advances in our understanding of the neurobiological basis of FXS have led to new targeted treatments for the disease (*7*). However, very little progress has been made regarding

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educational interventions mainly consisting of speech-language therapy (SLT), occupational therapy (OT) and behavior management therapy (ABA) (*8*). Many of our families have incidentally found that a variety of learning applications for tablets can be helpful for their children, but there have been no controlled trials or standardized guidelines for their use in FXS so far.

FXS is the most common inherited cause of intellectual disability with a prevalence of 1 in 5,000 males (*9*) and 1 in 8,000 females (*10*). It is the most common genetic disorder associated with ASD (*11-13*). Individuals with FXS show a specific behavioral phenotype of co-occurring conditions including hyperactivity, short attention span, anxiety, social avoidance, difficulty maintaining eye contact, difficulties in sensory processing, lack of reciprocity in relationships, stereotyped behaviors and seizures (*14*). FXS is caused by expansion of a trinucleotide repeat in the *FMR1* gene. The production of FMRP, the *FMR1* gene product, is significantly diminished or absent in FXS because of methylation of the CpG island at the 5' end of *FMR1*, thus silencing the gene (*15*). Studies show that approximately 2 to 5% of people with an ASD carry the fragile X mutation, and 60% of those with FXS have ASD (*16,17*). In general those with FXS plus autism have more anxiety, but more sociability than those with idiopathic autism (*18*).

The combination of intensive educational support and psychopharmacological interventions has remarkable effects on behavior and cognition in children with FXS (*19*). The main purpose of any behavioral interventions in FXS is not only to reduce or eliminate the unwanted behavior, but also to teach children socially appropriate behavior to enhance cognitive and social skills that can be generalized to other settings outside the therapeutic or academic environment (*8*). We believe strongly that technology is increasingly contributing to this generalization in our patient population.

The goal of the present study is to provide information to educate, facilitate, and document the power of touchscreen technology for individuals with FXS, and to describe the best practices in the use of the iPad® for promoting learning and interaction in family settings. This research will provide insights for future professionals (teachers, clinicians, application developers, therapists, researchers, *etc*.) and families hoping to meaningfully use computer tablets to help children with neurodevelopmental disorders. Devices like the iPad®, have an abundance of available educational and recreational applications (*20*) that easily support the Universal Design for Learning(UDL) framework for making a curriculum more inclusive for individuals with special needs. Therefore, specialized digital therapies are essential for addressing developmental challenges in those with FXS and for other neurodevelopmental disorders, although there is little research regarding their efficacy. In addition, interventions that showed efficacy for ASD such as Pivotal Response Treatment (PRT), Applied Behavior Analysis (ABA), Early Start Denver Model (ESDM), *etc*. have not been specifically studied in individuals with FXS.

On the other hand, caring for a child with complex disabilities such as FXS may have negative impact on both the physical and the mental health of the parents and caregivers (*21*). Parental stress could be child-driven (*22*); however, interventions that improve the child's functioning and communication may be expected to decrease the parents' stress level. Therefore, the impact of parent-delivered intervention based on an iPad® intervention could go beyond the educational goals and reduce parental stress through an unknown mechanism. However, to our knowledge, no outcome studies have focused on intervention programs for children with FXS that combine parent-delivered one-on-one behavioral iPad®-based intervention along with learning apps. In this sense, support to parents can also be provided remotely by a telehealth approach, a mechanism that enables individuals to receive professional guidance and effective recommendations from a distance (*23*) and may involve several multimedia platforms from real time video streaming to interactive website and tablet applications that can be effortlessly accessed at any time and location and shared across settings and individuals (*24*). Current studies implementing telehealth have already demonstrated encouraging results in training professionals and family members in ABA behavior management procedures (*25*), and successful outcomes in training parents of children with ASD in specific intervention models such as the ESDM (*26*).

The current series report presents 4 cases, belonging to a larger randomized controlled trial (RCT) cross-over design (n = 18), to describe the challenges and benefits of an innovative in-home iPad®-centered parent-delivered intervention on social interaction skills, language development, and academic gains (early concepts and literacy) in children with FXS. The report also describes qualitative differences between those patients seen at the MIND Institute for weekly outpatient therapy sessions *vs* an online follow-up modality (telehealth). The underlying processes such as motivation, engagement with technology, parent-child interaction, and parent satisfaction will be also reviewed. This is an effort to provide initial information and data to formulate a systematic guideline of what we believe is an innovative promising intervention for children with FXS and their families.

2. Methods

2.1. *Participants*

The 4 cases with a fragile X full mutation were selected from alarger randomized clinical trial study (crossover RCT) (n = 18), MIND APPs, for an iPad®-based

therapeutic parent training for enhancing language development, social interaction skills, and learning in children with FXS and ASD. Of the 4 cases 2 individuals are female and 2 are male, the mean age is 6.2 years old (SD 3.05 years) with a mean IQ of 73.5 (SD 18.8). The current aforementioned RCT (Díez-Juan *et al*. in preparation) was conducted at the Fragile X Research and Treatment Center at the University of California Davis MIND Institute and it was monitored for safety and ethics by the UC Davis Institutional Review Board (IRB). All participants and their caregivers have signed informed consent and were informed about the characteristics of the study with the option of conclude their participation at any point before the end of the study. The subjects had existing molecular results about their genetic status and were administered a series of behavior and cognitive assessments. The four participants' characteristics are shown in Table 1 with information about their genotypic and phenotypic profile and MIND APPs study characteristics. Families were eligible to participate if: *i*) Child was between the ages of 2.0 and 12.0 at the time of enrollment, *ii*) Child had a molecular diagnosis of FXS (with or without ASD) or an ASD diagnosis by a clinical team with the results of the Autism Diagnostic Observation Schedule (ADOS) (*27*), *iii*) Child had a reliable parent or caregiver able to perform a guided iPad® in-home interactive intervention for 32 weeks, *iv*) No serious co-morbid medical condition affecting brain function and/or behavior was present, including uncontrolled seizures, and *v*) Child was not participating in a pharmacological trial simultaneously, although subject could be under stable medication treatment and any kind of behavioral intervention or school condition. Other community care such as behavioral interventions, education program and other therapies are included in Table 1.

Participants were randomly assigned to the active treatment or wait-list group after baseline assessments. The four cases presented in the current report were all assigned to the first active treatment group receiving the training one time per week in 1-hour sessions during 16 weeks. All four families were instructed to continue with their child's usual treatments and to report every in-home iPad® session through Care Circles® application tracking system.

Table 1. Genotypic and Phenotypic features for each patient

Cases	Category	CGG repeats	% Meth.	AR	*FMR1* mRNA levels	Std Error	Ethnicity	IQ/ Level Language	ADOS CSS	VABS II Total COMP	Dysmorphic features	Medications	Behavioral interventions	Treat. Modality	iPad® Total Hours/16w
Case 1♂ (2.9 y)	Full mutation	6,40 1,160 (light UM smear)*	> 98%	–	0.14	0.01	Latino/ Hispanic	55/ Non verbal	4	75	Prominent and large ears	Sertraline Minocycline Melatonin	- ESDM** - Speech Therapy - ABA*** - Preschool aid	Online	24.90
Case 2♀ (5 y)	Full mutation	33, 200, 595, 711, 848	–	0.24	0.24	0.01	White	97/ Verbal	4	72	Prominent yaw and forehead	Sertraline Minocycline Melatonin	-Speech Therapy - OT**** - School support	Online	53.63
Case 3♂ (6.9 y)	Meth. mosaic	360 (light UM smear)*	> 95%	–	1.32	0.08	White	62/ Verbal	2	72	Prominent ears and epicanthal folds	Sertraline Minocycline	-OT - Speech Therapy - School aid	Online	65.15
Case 4♀ (10.1 y)	Full mutation	29, 113, 303, 373, 476, 642	–	0.83	0.68	0.06	White	80/ Verbal	6	70	Hydantoin Syndrome features	Vitamins Allergy pills	- IEP***** at school	Local	50.92

*UM= Unmethylated from normal/pre to full mutation, ** ESDM = Early Start Denver Model, *** ABA = Applied Behavior Analysis, **** OT = Occupational Therapy, ***** IEP = Individualized Education Plan.

2.2. *Genotypic measures*

Genomic DNA was isolated from peripheral blood leukocytes by standard methods (Qiagen, Valencia, CA). CGG size and methylation status were determined using Southern Blot and PCR analyses as previously described in Tassone *et al*. (*28,29*). Total RNA was isolated using Tempus tubes (Applied Biosystems, Foster City, CA). cDNA synthesis and qRT-PCR used to quantify *FMR1* mRNA levels were performed as described in Tassone *et al*. (*30*)

2.3. *Phenotypic measures*

2.3.1. *Baseline measures*

The cognitive baseline assessments described in the current report, depending on age, included standardized IQ tests such as Stanford-Binet Intelligence Scales, 5th edition (SB-5) (*31*); non-verbal IQ and verbal IQ are assessed and combined to a full-scale IQ score (M = 100; SD = 15); or the Mullen Scales of Early Learning (*32*) developmentally integrated scales for toddlers (M = 50; SD = 10). To quantify the severity symptoms of autism also in the fragile X population, we used the Autism Diagnostic Observation Scale, ADOS (*27*), which has been broadly used in other studies of FXS (*16,33*). ADOS autism calibrated severity score (CSS) was determined using the procedures described by Gotham *et al*. (*34*) in which a higher severity score indicates more severe autism features (ADOS classification: 1-3 no symptoms; 4-5 ASD symptoms; 6-10 Autism symptoms). Finally, the Vineland Adaptive Behavior Scales, 2nd edition (VABS-II) (*35*) were used to determine adaptive functioning, like daily life routines, and to identify strengths and weaknesses (M = 100, SD = 15).

2.3.2. *Outcome Measures*

The outcome measures consisted of a battery of standardized assessments administered at three time points across the duration of the study (baseline, follow up 1 after 16 weeks, and follow up 2 after 32 weeks). The measures included the Expressive Vocabulary Test, Second Edition (EVT2) (*36*), measuring expressive vocabulary and word retrieval (M = 100, SD = 15), Preschool Language Scales, Fifth Edition (PLS-5) (*37*), an interactive assessment of developmental language skills based on auditory comprehension and expressive communication standard scores (M = 100, SD = 15), and the Process Assessment of the Learner, Second Edition (PAL-II) (*38*), measuring a variety of reading and writing processes for children in Kindergarten to Grade 6 (K-6). PAL-II subtests and composite scaled scores are derived from normative data and have a mean of 10 and a standard deviation of 3.

A Likert-scaled Parent Satisfaction Survey (scores 1-5) specifically designed for the purposes of the study was used to measure the level of parent satisfaction towards particular components of the iPad®-based training program at the end of the intervention. Higher scores mean better satisfaction levels referring to eight particular treatment domains: *i*) *STeachIntervention*, the study helps you to better teach your child using the iPad® for interaction with you, *ii*) *SProgInter*, level of progress in shared interactions you observed, *iii*) *SComm*, level of progress in communication, *iv*) *SLang*, progress in language (expressive and receptive), *v*) *SAcadem*, progress in academic learning, *vi*) *SPConfident*, parent's confidence about helping the child with the iPad® for educational purpose and interaction, *vii*) *SSClinical*, satisfaction with the clinical guidance, and *viii*) *SProgApps*, satisfaction with the program of educational applications provided. Figure 1 summarizes the parent's satisfaction scores for each scale after the active treatment.

2.4. *Procedures and timeline*

The intervention program for the individuals in the present case series consisted of 2 periods of an iPad®-based intervention program. The first period is guided intervention with a therapist and the second is a maintenance period without the therapist. The initial active period consisted of a 16-week long, low-intensity (1-hour/week of therapist/clinical guidance and parent-delivered intervention (3-hour/week in-home sessions) with an estimated average of 64 hours of iPad® intervention during the 16 weeks. During the maintenance period no clinical guidance was provided and only the parent-delivered 3-hour/week of in-home intervention was administered by following the guidelines learned in the previous period.

The clinical guidance and supervision across the 16 weeks was provided on-line or on-site according to participants' preferences and consisted of:

i) General iPad® management orientation to parents and child (depending on age): common terms of use, multitasking gestures, accessibility, guided access, Apple Store® operation, code redeeming, applications downloading, updating and deleting (Week 1-4).

ii) Care Circles platform application installation and creation of family profile to track in-home iPad® sessions and initiate daily parents-professional interactive journal (Week 1-4).

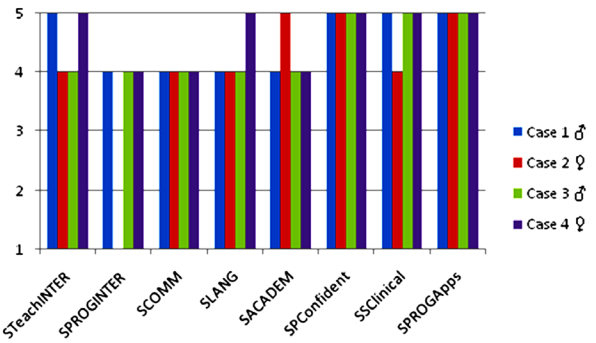

Figure 1. Parent Satisfaction Survey (Likert Scale 1–5). *STeachIntervention* = the study helps you to better teach your child using the iPad® for interaction with you; *SProgInter* = level of progress in shared interactions you observed; *SComm* = level of progress in communication; *Slang* = progress in language (expressive and receptive); *SAcadem* = progress in academic learning; *SPConfident* = parent's confidence about helping the child with the iPad® for educational purpose and interaction; *SSClinical* = satisfaction with the clinical guidance; *SProgApps* = satisfaction with the program of educational applications provided.

iii) Weekly review and explanation of educational applications and parent-child customized guidance for interactive, communicational and learning purposes (Week 1-16).

iv) Establishment of individual family goals regarding communication, social interaction, learning and behavior through iPad® use (Guidance and supervision of goals from week 1-16).

v) Share progress and handle behavior or learning difficulties during iPad® sessions at home (Week 1-16).

vi) Closure and review of training principles and applications, thus parents could continue intervention during maintenance period afterwards (Week 14-16).

2.5. *iPad®-based training program*

The iPad® pilot study's primary aim was to evaluate the effects of a comprehensive educative program in which parents are receiving individual coaching about the use of the iPad® as a learning device and as an interactive therapeutic tool for their children.

The iPad®-program was based on: *i*) A comprehensive selection of Apple store educational applications, previously reviewed and analyzed by experts in the field, which were distributed according to three developmental domains (language, social interaction and academic learning); *ii*) Individually-tailored treatment objectives to the child's individual learning profile, dominant interests and family educational values; *iii*) A set of psychoeducational strategies substantiated on the principles of broad spectrum applied behavior analysis (ABA), cognitive techniques about theory of mind and emotions management programs, naturalistic learning through interpersonal interaction, and meaningful teaching approaches. Figure 2 includes the main applications that were used during the intervention periods according to developmental stages and skill domains.

2.6. *Adherence to iPad® intervention*

Care Circles® application from the Apple Store® was implemented as a digital platform to follow on adherence to intervention and to measure the time of in-home applied intervention, level of motivation, attention and frustration during the parent-child interaction at home. An interactive journal was used for the family to professional everyday communication.

3. Results

3.1. *Case 1 (FXS, boy 2. 9 y)*

3.1.1. *Personal background*

Case 1 is an almost 3-year-old boy diagnosed with FXS shortly after birth through cord blood due to positive family history (mother, maternal aunt and maternal

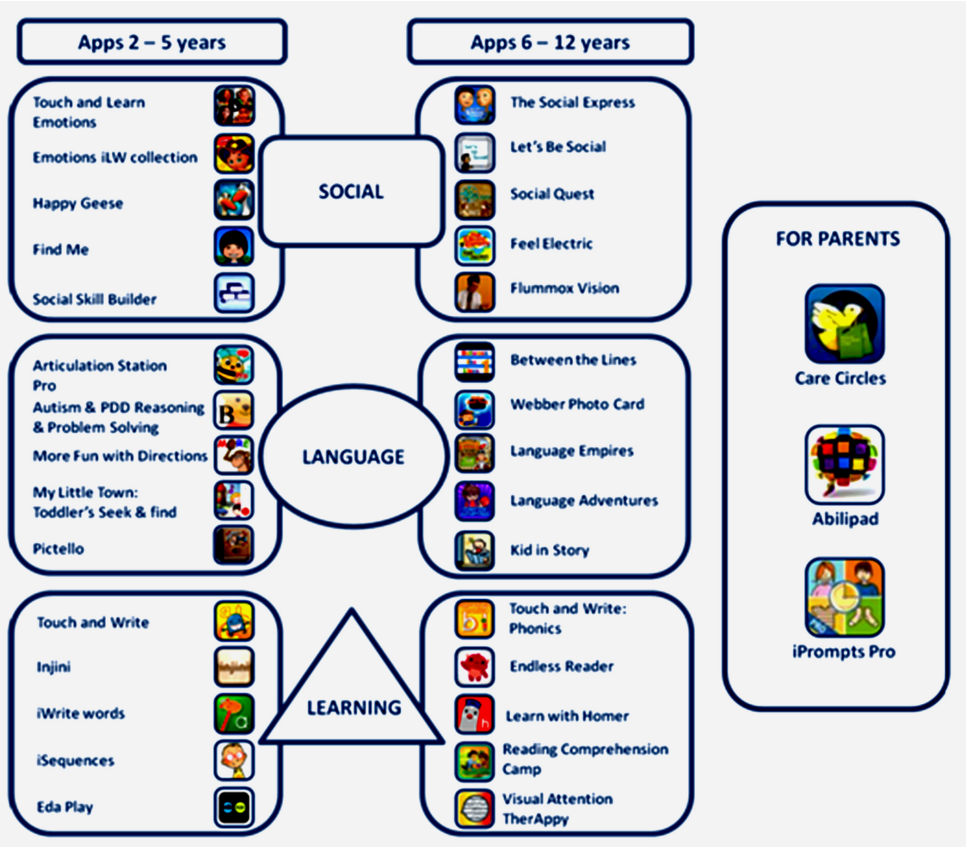

Figure 2. MIND APPs applications program.

grand-father with the premutation). He did not present with hypotonia, had fairly good eye contact, and a high energy level, which eventually led to meltdowns involving throwing himself to the floor to demonstrate frustration. He also presented with attentional problems and perseverative behaviors, such as spinning and flipping through book pages impulsively. He did not have staring spells or seizures, although he showed shivering episodes once or twice a day. He had prominent ears with ear pinnae cupping bilaterally, epicanthal folds and flat feet with mild degree of pronation. He showed psychomotor delay (Baseline MSEL- Early Learning Composite 55) started walking after 15 months, and his overall Adaptive Behavior Composite was 75 on the Vineland (VABS-II). He presented with a number of autistic behaviors including lack of joint attention and language difficulties. At the time of the study he also met criteria for developmental speech and language disorder (no words at the age of 35 months) in addition to the ASD features (ADOS CSS 4).

3.1.2. *MIND APPs study involvement, outcomes and challenges*

At the in-take interview for the iPad® study he had developed a sleep disturbance where he would awaken two to three times a night. Melatonin was used to treat these symptoms together with applied behavior analysis techniques (ABA). Parents were applying Early Start Denver Model (ESDM) (*39*) principles at home for developmental purposes learned through a telehealth study about early intervention in children with FXS (*40*), and he was receiving speech therapy at preschool where he also had special support personnel. He was on a stable treatment with sertraline and minocycline before and throughout the study.

Parents recently purchased an iPad®, and they had limited experience with it before the study. The patient was completely unable to actively use the iPad®, and the parents mainly used the touch-screen device for playing games and entertainment. Family had not used educational apps before the study, nor had they received any training in using iPad® for promoting parent-child interaction and learning. The child and parents were highly motivated to participate in the study. Case 1 was trained online through telehealth. The family successfully completed the 16-week training, and after-treatment assessment, nevertheless it was impossible to obtain the last follow up due to travelling distance to the MIND Institute, and family issues at that time point. Right after the active treatment period, parents felt better prepared to use the iPad® as an educational tool and observed specific areas of improvement such as: increased vocabulary, improved language (expressive and receptive) and also more precise fine motor skills. The patient improved his abilities to sort objects, trace lines and solve puzzles. He also enjoyed and learned letters, basic counting, shapes and colors. Parents reported that "clinical guidance was of key importance" in the sense of receiving individualized professional guidelines when introducing a new app, and handling behavioral challenges during the interaction as reflected in the satisfaction survey.

Numerous behavioral challenges needed to be addressed during the iPad® training for Case 1. He presented with low flexibility and concurrent repetitive behaviors toward the device and it was not his preference to share during the activities with his caregivers. Establishing the iPad® time routine together was an elaborate process; although once it was part of his schedule he accepted it and looked forward to it with appropriate requesting of the tasks from the parents. Apps of his interest were related to matching numbers, letters, shapes, colorful images, music, and later on tracing letters and numbers. Caregivers felt highly discouraged during the first 4 weeks of the training given that Case 1 did not want to collaborate and share the iPad® time together. Clinical guidance was critical in order to support parents and enhance their skills and confidence as essential providers and supporters of their child's progress.

Figure 3 demonstrates an example for a high quality parent-child iPad® interactive situation in which joint attention, positive social reinforcement and shared learning experience can be observed.

3.2. *Case 2 (FXS, girl 5 y)*

3.2.1. *Personal background*

Case 2 is a 5-year-old girl with the full mutation who has participated in targeted treatments since early in life, including sertraline and minocycline trials. She showed significant improvement with minocycline and sertraline and a good response to early interventions, such as occupational therapy, physical therapy and language intervention in an enriched in-home

Figure 3. Case 1♂- Parent-child interactive iPad® time.

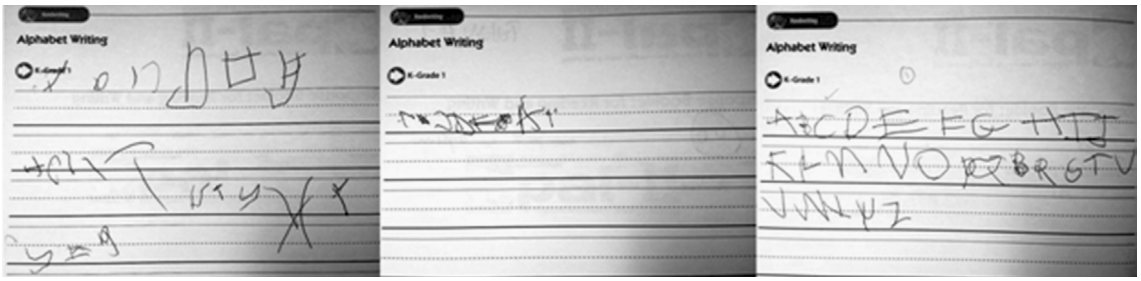

Figure 4. Case 2 ♀- PAL-II Alphabet Writing Task (Baseline, Follow-up 1 & Follow-up 2).

environment since the mother has an educational background in special needs. She showed adequate developmental milestones, walking at 13 months, saying first words at 17 months and simple sentences after 20 months with a mild delay in later language development. She had a very short attention span, and it was difficult for her to sit through a whole story and follow verbal prompts. She had a sleep disturbance, picky eating and anxious behavior towards new situations and animals. On early examinations, she had a mildly prominent forehead, epicanthal folds, hyperextensiblefinger joints and overall normal motor tone. She was hyperactive and inattentive throughout the study and she met criteria for ADHD. She was taking sertraline, minocycline, folic acid and melatonin during her participation in the iPad® clinical trial. At school she is receiving speech and occupational therapy (OT), but no assistive technology was implemented in her individualized education plan (IEP).

3.2.2. *MIND APPs study involvement, outcomes and challenges*

Baseline assessments confirmed intellectual ability in the normal range (IQ 97), no ASD diagnosis (ADOS CSS 4), and low adaptive skills (VABS II 72). She had been using the iPad® since she was eighteen months old when the first device emerged on the market. She used it mainly by herself for watching cartoons, playing fun educational games, and reading stories. She knew how to handle the device and she could even create folders in the screen herself, which is an advanced skill. Parents played together with her about 3 to 4 times a week and they knew a great variety of educational apps, nevertheless, they never received a parent-based training in the use of technology for interaction targeting language, literacy and social development. Parents were motivated to complete the 16-week study, and they reported all their iPad® sessions through Care Circles® app and completed all the follow-up assessments. The patient went through several reactive behaviors when the caregiver was trying to share the screen together and it took some weeks for her to get used to the new "we-work-iPad® -together" routine. Once the digital task was part of her daily schedule, she became smoothly involved in the interactive dynamic with parents and even started to ask them to play together. Parents reported moderate satisfaction to the psychoeducational program since they also expressed concern about the new routine being time consuming and overwhelming at particular points. Tracking sessions and reporting data was not always convenient for them and they wish it could have been addressed through a more practical modality. Otherwise they identified progress in their child mainly related to an enhancement of speech fluency, fine motor skills and tracing letters ability.

Case 2 presented an impressive advance in tracing and basic writing skills, being able to actually trace alphabet letter and copy short full words both on screen and on paper at the end of the second follow-up after the maintenance period. Her main progress turned up clearly after 32 weeks of low intensity iPad®-based intervention. See Figure 4 regarding the progress at baseline, follow-up 1 and follow-up 2 assessments through PAL-II, Alphabet Writing task in which the child is asked to print the alphabet in lowercase in alphabetic order as quickly and accurately as possible. Case 2 was only 5 years old at the beginning of the study so her scores fell out of PAL-II normative data even at the last follow-up assessment (5y 8m). Although her writing is performed in uppercase, it is possible to observe the improvement of her copy skills regarding legibility and accuracy. The main applications implemented to practice literacy abilities are listed in Figure 2 (Learning apps 2-5 years).

3.3. *Case 3 (FXS, boy 6 y)*

3.3.1. *Personal background*

Case 3 is a 6 years and 10 months old boy with FXS and high levels of anxiety in crowded situations. He also presents global developmental delay (IQ 62), is highly inattentive and hyperactive especially in academic settings or in a larger group, and his adaptive skills are below average (VABS-II 72). He receives OT and SLT at school and has a special aid in class. Just before enrolling in the study he stopped ABA therapy. He was on minocycline and sertraline before and throughout the study. Family owned a device for less than a year before the study enrollment and usually used it as a

reward tool after successfully completing homework or chores. Usually he used it to watch cartoons or play games. No educational applications were used before the trial and he used to play by himself. His mother was highly motivated to conduct the treatment at home and they properly completed the whole sequence of clinical sessions and follow-ups.

Case 3 presented with a history of motor and speech delay; he walked at 19 months and said first words at15 months and combined words around 30 months. On previous exam he had a slightly long and narrow face, mildly prominent ears, hyperextensible finger joints and flat feet bilaterally. Behaviorally he did not meet an autism diagnosis (ADOS CSS 2) but he had intermittent poor eye contact and hand flapping. Under pressure he can show some self-injurious behaviors, like biting, and has calluses on his right hand, he rocks his whole body at times and sucks his thumb when stressed. Eventually he can get aggressive and may kick or push others.

3.3.2. *MIND APPs study involvement, outcomes and challenges*

During the study he demonstrated progress in language fluency, being able to narrate tales, and also creating social stories about his daily life activities and social events. The family demonstrated progress in parent-child interactions, and implementing the iPad®-time rules, and the parents saw moderate improvement in tracing letters and spelling short words. They commented that clinical guidance was "extremely valuable during the study as they were able to use the acquired skills in everyday activities outside of the iPad® too".

Primary caregiver in the training was the mother, a premutation carrier who was also implementing the iPad® program with her daughter, a full mutation 5-year-old girl, in the second active treatment period. Because the burden of active intervention maintained over 8 months, the mother experienced high levels of stress, expressing the push to complete the study as it involved a great family and educational effort. However, parents reported the program contained extremely suitable applications and that clinical guidelines were useful and even went beyond the interactive iPad®-time itself. Figure 1 shows the results in the Parent's Satisfaction Survey (Likert Scale 0-5), in which higher scores relate to higher levels of satisfaction. Case 3 showed the highest scores (*5*) in Parent's Self-Confidence, Satisfaction to Clinical Guidance and Satisfaction to Program of Applications.

3.4. *Case 4 (FXS, girl 10 y)*

3.4.1. *Personal background*

Case 4 is an almost 11-year-old girl who has the full mutation of FXS that was diagnosed in utero. Family pedigree reveals her great-grandfather died from fragile X-associated tremor ataxia syndrome (FXTAS). Her mother has FXS with normal intellectual abilities, but she took phenytoin during pregnancy due to a seizure disorder. Thus Case 4 is not only affected by FXS, but shows additional features of fetal hydantoin syndrome, as a second hit, identified by mild bowing of the upper lip in addition to the broad and low nasal bridge. Her early development included sitting at 8 months, crawling at 1 year, walking at 18 months, and delays in receptive and expressive language. Her behavior included hand flapping, finger biting and inconsistent eye contact. She had appropriate join attention and good social skills, although she had severe shyness, social anxiety and learning difficulties. She also underwent developmental testing in childhood with adaptive behavior problems and mild motor delay. She met criteria for selective mutism, anxiety disorder and borderline intellectual functioning prior to the beginning of our study.

3.4.2. *MIND APPs study involvement, outcomes and challenges*

When she joined the RCT she was 10 years 6 months old and parents already owned an iPad®, which was used mainly for entertainment. At that time she was receiving neither psychosocial nor medical treatment, but had an Individualized Educational Plan (IEP) at school (5th grade) where she used the computer for learning purposes. She could properly manage the iPad® and parents played with her by sharing games and educational applications. They had never received an iPad®-based training program and were highly motivated to be involved in the therapy. Case 4 fully completed the three timeline assessments. During the study she was not taking any medications apart from allergy pills and inhalant for asthma symptoms. We assessed autistic behavior as part of the baseline measures and she met criteria for moderate ASD (ADOS CSS 6). She had a low average cognitive level (IQ 80) and below average adaptive skills (VABS-II 70).

Case 4 completed the 16-week iPad®-centered training together with her parents and they noted mild progress in academic learning and moderate improvement in expressive language and social comprehension. Her program followed educational apps with a particular emphasis in applications for enhancing literacy, expressive language and social skills, such as The Social Express®. She was followed locally so the family came to the MIND Institute clinic once a week and also tracked the online data through the Care Circles® platform application.

Her mother needed to stop being the primary therapist in the pilot study due to overwhelming feelings of anxiety and a high level of stress. Case 4's father and grandmother needed to step in for the iPad®-

based sessions at home one month before the end of the active period.

Figure 5 shows Case 4's PAL-II Reading and Writing Profile in which literacy tasks progress can be seen across the study timeline. The Receptive Coding task consisted of identifying single letters from a word. The patient presents maximum improvement in this task, and dramatically decreases the scores during the maintenance period. Alphabet Writing, in which the child is asked to print the alphabet in lowercase as quickly and accurately as possible presents a mild improvement, nevertheless a loss can be seen in the follow-up 2. Finally, Copying Task A, in which the child is asked to copy a sentence as quickly and accurately as possible shows a flat scoring across the 3 time points. We believe this is due to the complexity of the assignment where the low-intensity iPad®-based intervention cannot impact on complex literacy performance. Overall Figure 5 indicates that the active intervention was positively affecting learning in simple literacy tasks while during the maintenance period performance decreases, maybe caused by the lack of practice.

4. Discussion

The search for touchscreen-based intervention procedures that are efficient, family and socially relevant and therapeutically viable is essential to the improvement of the services provided to children with FXS and their families. However, the present case series report is the first of its kind, and indicates that there is still a need for more controlled studies, with a larger number of participants, involving school setting and a multidisciplinary team, and more appropriate standardized tools to assess the outcomes of technology-based educational treatments.

The great majority of existing literature reveals that touch-screen devices can be successfully utilized within educational programs targeting academic skills, communication, employment, and recreational activities for individuals with developmental and intellectual disabilities (*3*). Success relies on the use of well-established instructional procedures based on the principles of ABA, early intervention models, psychosocial approaches, or other specific models integrated in the community, as well as the school and in-home setting. Therefore, ownership of a tablet alone does not guarantee parental engagement in supporting their child for using this technology for learning (*41*) and that the presence of these mainstream devices does not automatically lead to a meaningful implementation for therapeutic interventions.

The current case series explored an innovative psychoeducational intervention for children with FXS and their families to help them to acquire new skills regarding touchscreen technology and its use for learning purposes. By the end of the iPad®-based training parents reported having a better understanding and appreciation for assisting their child on managing the iPad® for interaction, communication and learning at home. Parents felt more confident in providing their child with educational guidelines and sharing social time together using technology as a learning tool. They also described weekly clinical interaction, both locally and on-line, as the most valuable resource for supporting their progress in the apps comprehension and behavioral strategies acquisition and administration. The telehealth modality was rated as effective as traditional one-on-one guidance sessions and parents attending the on-line training did not feel the need to see the therapist since clinical orientations followed the same structure, but based on a multimedia platform (video conference). Video conferencing with the therapist was highly important to understand how to apply the iPad®-based program in their family routine. However, the delivery of the intervention in a different format could affect the effectiveness of the treatment; so further research on a larger sample is needed out.

The iPad®, as well as other touchscreen devices, have the capacity to be used with learners of different ability levels and ages if educational applications are selected appropriately, and subjects are given equal teaching opportunity to access this type of technology for communication and/or learning purposes. As we described before, even 2-year-old toddlers with FXS are not too young or low-functioning (review Case 1) to start a comprehensive parent-delivered iPad® intervention at home. However, it is important to follow an age-appropriate structured program, based on available applications at the Apple Store, such as Injini® (Child Development Game Suite's) which provides excellent and engaging learning opportunities to young children with developmental delays. When parents are provided with behavior management techniques and a previous explanation of the app, they can perform high quality teaching sessions facilitating learning through a social and interactive parent-child exchange.

In addition to these caveats, it is difficult to quantitatively show improvement on standardized measures. Case 1 displayed a relevant improvement in his iPad® management and learning skills, such as fine motor abilities, audio-visual processing, matching,

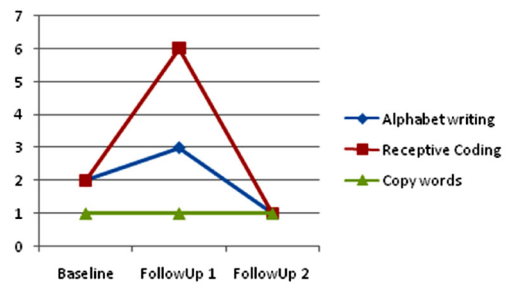

Figure 5. Case 4 ♀- PAL-II Writing Profile.

Table 2. Principal clinical outcomes (Parent report)

Cases	Previous iPad® Knowledge/ Interactive use	LANGUAGE GAINS	SOCIAL SKILLS ACQUISITION	ACADEMIC LEARNING PROGRESS	BEHAVIORAL OUTCOMES	PARENT SATISFACTION
Case 1♂ (2.9 y)	Low/Low	Vocabulary acquisition	Turn taking and waiting skills	Fine motor skills and early concepts	Proper use of the device for waiting time periods	Very Satisfied
Case 2♀ (5 y)	High/Medium	Language fluency	Sharing the screen and accepting others while playing	Tracing letters and words	Increase of self-regulation towards the interactive games	Moderately Satisfied
Case 3♂ (6.9 y)	Medium/Low	Increase of utterances in sentences	Accept losing in cooperative games (*apps) with adult and siblings	Motivation for tracing letters and initial reading stage	Acceptance of iPad® time as a reward for a particular amount of time	Very satisfied
Case 4♀ (10.1 y)	High/Medium	Expressive language fluency	Communication and social reciprocal skills	Tracing and written expression improvement	Use of the iPad® as a coping tool when she is upset and anxious	Very satisfied

*Applications

sorting and tracing but our outcome measures did not document significant gains after the 16-week active treatment period. Table 2 shows a summary of the principal outcomes of the participants in 6 clinical categories.

On the other hand, a more high-intensive intervention approach focused on a specific developmental skill may be more likely to show a significant improvement using more reliable objective data collection in an ongoing touchscreen-based therapy.

Enjoyment when using the iPad® across the 16-week period was highly regarded according to parent report, and overall, this type of technology was perceived to have the potential to promote more engagement in the learning process at home.

The interactive technology intervention was well accepted by the children and their parents. However, families also reported increased levels of stress at the end of the active treatment period. In the last weeks of the intervention training, some caregivers were exhausted by the iPad® tasks at home and they needed a reduction of the training rhythm and even a break from their educative duties. In Case 4 we described how the primary study iPad® caregiver, a mother with the full mutation and significant anxiety herself, needed to be exchanged with another family member because of the anxiety of performing the sustained interactive sessions at home in addition to other daily life routines. Clinicians must be sensitive to the parent's needs and careful about not to further increase the stress personal levels and family burden.

In general, in the presented study from the 3 hours/ week of recommended usage by families, iPad® time was lowered to an average of 1.5 hours/week during a 4-month period which is minimal input for therapy purposes (See Table 1). We highly recommend longer treatment duration and intense periods facilitated by greater professional involvement and incorporation to school setting by educators so that the burden on the families remains manageable.

We believe better standardized outcomes measures need to be designed since the ones implemented in the pilot study were not sensitive enough to quantify improvements over time, for example including video analysis tools for follow-ups could improve the progress tracking throughout treatment. Additionally, the study's design included a wide spectrum of applications targeting different skill domains with a low intensity and specificity in various areas proves to be difficult to measure improvement. We believe a more targeted approach to a particular domain and more intensive iPad® intervention duration will lead to more successful intervention results. Also, newer combinations of treatments will be needed, particularly those that tie this innovative intervention with pharmacological treatments and other educational and social approaches from a multidisciplinary point of view.

Optimal efficacy on a group level was not statistically documented in the preliminary analysis; nevertheless we can qualitatively describe a better performance in the 2 girls in the present report rather than for the boys, probably due to the higher IQ and expressive language levels in girls with FXS. Figure 6 presents the 2 girls' and 2 boys' expressive language profile measured by EVT2 and PLS-EC, depending on the individuals' age, in which we can observe a clear higher trend in girls than in boys. In general, all the participants decrease scores during the maintenance period with no clinical guidance. The hyperactivity was much more severe in the boys than girls interfering with the behavior management and learning progress. A combination of ADHD medication with iPad®-based interventions should be considered in the future.

Touchscreen tablets and educational application

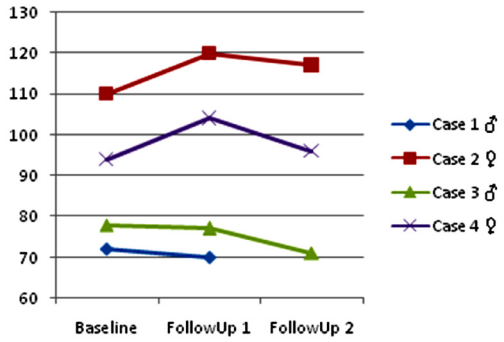

Figure 6. Expressive Language EVT2/PLS-EC Profile for all cases.

programs can be modified to fit particular needs and goals of each individual with neurodevelopmental disorders, particularly with FXS, and are designed to facilitate a more natural use of technology and diminish stigmatization. The emerging research and clinical experience described in these four cases offer a promising vision of the use of technology in children with FXS, particularly in a convenient in-home setting, and a deep understanding of how therapists can implement an individualized touchscreen-based program, and assist families in the best use of computer tablets for support and interaction.

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Author Index (2014)

Subject Index (2014)

Editorial

Reviews

Original Articles

Case Reports

Commentaries

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