The association between mosaicism type and cognitive and behavioral functioning among males with fragile X syndrome

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Abstract

Mosaicism in fragile X syndrome (FXS) refers to two different *FMR1* allele variations: size mosaicism represents different numbers of CGG repeats between the two alleles, such that in addition to a full mutation allele there is an allele in the normal or premutation range of CGG repeats, while methylation mosaicism indicates whether a full-mutation allele is fully or partially methylated. The present study explored the association between mosaicism type and cognitive and behavioral functioning in a large sample of males 3 years and older ($n = 487$) with FXS, participating in the Fragile X Online Registry with Accessible Research Database. Participants with methylation mosaicism were less severely cognitively affected as indicated by a less severe intellectual disability rating, higher intelligence quotient and adaptive behavior score, and lower social impairment score. In contrast, the presence of size mosaicism was not significantly associated with better cognitive and behavioral outcomes than full mutation. Our

findings suggest that methylation mosaicism is associated with better cognitive functioning and adaptive behavior and less social impairment. Further research could assess to what extent these cognitive and behavioral differences depend on molecular diagnostic methods and the impact of mosaicism on prognosis of individuals with FXS.

1 | INTRODUCTION

Fragile X syndrome (FXS) is the most prevalent inherited form of intellectual disability and is associated with autism spectrum disorder (Hunter et al., 2014). FXS results from an unstable expansion of a cytosine–guanine–guanine (CGG) nucleotide sequence in the promoter region of the *FMR1* gene (Tassanakijpanich et al., 2021). The *FMR1* gene product is FMRP, an RNA-binding protein that regulates protein synthesis at synapses (Bagni & Zukin, 2019) and, therefore, plays a critical role in brain development and synaptic plasticity (Martin & Huntsman, 2012). In the vast majority of cases, deficient or absent FMRP is the basis for the FXS phenotype (Bassell & Warren, 2008). *FMR1* alleles containing <45 CGG repeats are considered normal. Alleles with CGG repeat expansions in the 55–200 range are termed "premutation" alleles and are associated with *FMR1* expression and FMRP synthesis, although mRNA levels may be elevated and FMRP reduced, particularly for larger allele sizes within the premutation range. Premutation alleles are associated with fragile X-associated primary ovarian insufficiency (FXPOI), fragile X-associated tremor/ataxia syndrome (FXTAS), and other less distinctive neurologic phenotypes (Hagerman et al., 2009; Hagerman & Hagerman, 2021). Alleles with CGG repeat expansions of >200 are termed "full mutation" and are associated with atypical *FMR1* methylation and the resulting partial or complete silencing of the gene, which leads to decreased or absent FMRP and the clinical features of FXS (Hagerman et al., 2009; Jin & Warren, 2000).

Because of the instability of the CGG repeat during transmission across generations, individuals can be comprised of a mixture of cells in which a proportion have a normal or premutation *FMR1* allele and the remaining cells have a full-mutation allele. This situation is termed *size mosaicism* and, while FMRP levels in individuals with size mosaicism may be higher than in individuals with only full-mutation alleles, FMRP production only occurs in those cells with the normal or premutation allele (Kumari & Usdin, 2020). Furthermore, *FMR1* mRNA gain-of-function or repeat-associated non-AUG translation-related toxicity can occur, as seen in FXPOI and FXTAS (Glineburg et al., 2018; Rajaratnam et al., 2017). The process of *FMR1* silencing through gene methylation may not occur equally in all cells. This situation is the basis for a second type of *FMR1* mosaicism: *methylation mosaicism*, in which the full-mutation allele escapes methylation and can produce FMRP in a variable proportion of cells (Hagerman et al., 2009; Kumari & Usdin, 2020). Both size and methylation mosaicism can be present, but regardless of mosaicism status, individuals with an *FMR1* full mutation will almost universally show a reduction in FMRP level and are categorized as having FXS.

Mosaicism is a source of molecular, systemic, and neurobehavioral phenotypical variability that can be observed in both sexes. The reported mosaicism incidence in males with FXS varies widely from 12% to 41%, most likely reflecting the sensitivity of the method used for detecting mosaicism (Nolin et al., 1994). While size or methylation mosaicism is not always incorporated into the assessment of clinical prognosis due to wide inter-individual variation of the impact of mosaicism on the phenotype, it could have consequences on the overall severity of the disorder in terms of physical impairment and neurobehavioral functioning across a large group of individuals with FXS. Literature findings on the relationship between *FMR1* mosaicism and cognitive and behavioral outcomes are mixed. Some publications have reported higher cognitive functioning and more advanced adaptive skills in patients with FXS who have mosaicism compared to nonmosaic FXS (Cohen et al., 1996; Merenstein et al., 1996; Pretto, Yrigollen, et al., 2014; Staley et al., 1993), while other studies did not show differences between males with mosaic and non-mosaic FXS (Backes et al., 2000; de Vries et al., 1993; Harris et al., 2008; Rousseau et al., 1994). Previous studies have also reported the associations of mosaicism type with cognitive and behavioral outcomes. Data consistently show an inverse correlation between methylation level and intellectual functioning, (Basuta et al., 2015; Hagerman et al., 1994; Pandelache et al., 2019; Pretto, Yrigollen, et al., 2014; Wöhrle et al., 1998) with males with FXS having completely or near completely unmethylated full-mutation alleles showing typical intellectual development (Basuta et al., 2015; Hagerman et al., 1994; Wöhrle et al., 1998). On the other hand, studies on size mosaicism have arrived at mixed conclusions. Some studies suggest that the presence of a normal or premutation allele does not compensate for cognitive or behavioral dysfunction associated with *FMR1* full mutation (de Vries et al., 1993; Jiraanont et al., 2017), whereas others suggest that males with FXS who have size mosaicism perform better on tests of intelligence than individuals without this type of mosaicism (Baker et al., 2019; Merenstein et al., 1996). These varied findings are likely dependent on the percentage of cells in the brain that are expressing the normal or premutation allele.

Research on a large sample of individuals with FXS could provide a clearer understanding of the impact of *FMR1* full-mutation mosaicism on cognitive, behavioral, and other types of functioning. Thus, the purpose of the present investigation is to characterize and compare the impact of size mosaicism and methylation mosaicism on cognitive and behavioral outcomes, including intelligence, adaptive behavior, aberrant behaviors, and autism-related social impairment in males with FXS using clinicians' evaluations and standardized assessments. In addition, the present study also investigated whether males with FXS and mosaicism have better cognitive and behavioral outcomes than those without mosaicism.

2 | METHODS

2.1 | Population and procedures

Data analyzed for this study were from Fragile X Online Registry with Accessible Research Database (FORWARD), a registry and longitudinal database funded by the Centers for Disease Control and Prevention. FORWARD includes standardized clinician- and parent-report forms and standardized caregiver-reported instruments (e.g., Aberrant Behavior

Checklist—Community, Social Responsiveness Scale, etc.) submitted by 25 FXS specialty clinics across the United States participating in the Fragile X Clinical and Research Consortium from 2009 through 2018 (Liu et al., 2016). Full details on the creation, enrollment, and data collection for FORWARD are reported in an earlier publication (Sherman et al., 2017).

The analyses for this study utilized baseline data from FORWARD Version 4, obtained from 1471 individuals with FXS (i.e., *FMR1* full-mutation allele) evaluated from 2012 through 2019. The FORWARD Version 4 data are currently housed at the Centers for Disease Control and Prevention and are not available for public use. The study was approved by the institutional review board for each participating FXS clinic where data were collected, and written informed consent was obtained from primary caregivers or adult patients who were their own guardians. Due to the focus of this study, only male participants 3 years and older ($n = 954$) for whom both size and methylation mosaic status were available were included in analyses ($n = 487$).

2.2 | Measures

2.2.1 | Predictor variables

Size and methylation mosaic status: Size mosaicism was determined with two items: (1) "Is the individual a repeat allele mosaic (e.g., pre/full, intermediate/full, normal/full)?" with answers "0 = no," "1 = yes," and "2 = not available;" and (2) "What is the repeat size of the non-full mutation allele?" with a response between 1 and 200. Methylation mosaic status was determined by one clinician-reported item: "What is the methylation status of the full mutation?" with response options "0=fully methylated (without methylation mosaic)," "1 = methylation mosaic," "2=unspecified abnormal methylation," and "3 = not available." Individuals with responses "2" and "3" were omitted from the analysis. Mosaicism in the study was assayed in blood samples, the information for which clinicians gathered from the *FMR1* genetic report produced for FXS diagnostic purposes. When the information was not specified in the test report, mosaicism was indicated as "don't know/not available."

2.2.2 | Outcome variables

Severity of intellectual disability and ASD diagnoses: Clinician-reported severity of intellectual disability (ID) and autism spectrum disorder (ASD) diagnosis, including age at evaluation, were included as outcome variables. Severity of ID was assessed using one item: "Which of these terms best describes the intellectual function of the child currently?" The response options include "1 = no ID," "2 = borderline ID," "3 = mild ID," "4 = moderate ID," "5 = severe ID," and "6 = profound ID." Thus, severity of ID was treated as a continuous outcome variable in analyses. Among 487 participants with reported mosaic status, 428 participants 3 years of age and older with clinician-reported severity of ID were included in the ANCOVA model. ASD diagnosis was performed by the clinician using Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM-IV-TR) or DSM, fifth edition (DSM-V) criteria and reported using one item: "Based on this clinic assessment, does this child currently have a diagnosis of ASD?" with binary answers "0 = no" and "1 = yes." Among 487 participants with reported mosaic status, 448 participants had ASD diagnosis reported.

Intelligence quotient: Intelligence quotient (IQ) scores were obtained from one of two age-appropriate batteries reported by clinicians: the Stanford-Binet Scale—Fifth Edition (SB5) or the Wechsler Intelligence Scales (Groth-Marnat et al., 2000; Roid & Pomplun, 2012). Of the 487 males with FXS, 172 participants had age of testing and full-scale IQ (FSIQ) scores reported. Based on classifications of ID and distribution of IQ scores, the latter were treated in the analyses as a dichotomous outcome variable with two categories: "0=IQ test scores under 55" and "1=IQ test scores above or equal to 55." This IQ level corresponds to the cut-off for mild ID (55–69) on the SB5 (Matthews et al., 2015).

Adaptive skills: Adaptive skills were measured by the Vineland Adaptive Behavior Scales —2nd or 3rd Edition (Vineland-II, Vineland-3; here termed Vineland) published by Pearson (Sparrow et al., 1984). The Vineland changed versions in 2015, and our data are a mix of Vineland-II and Vineland-3 depending on when the assessments were done over the 7-year period. Moderate concordance was observed between Vineland-II and Vineland-3 (Farmer et al., 2020). Composite scores and four domain scores were reported by participants' clinicians. Out of the 487 males with FXS, 173 had their Vineland composite scores reported. Age when an informant, typically caregiver, completed the questionnaire was also documented. The Vineland is a semi-structured interview to evaluate adaptive behavior in four domains: communication, socialization, daily living skills, and motor skills. A higher score indicates a higher level of adaptive behavior. The Vineland composite score was treated as a continuous outcome variable in the analyses.

Problem behaviors: The Aberrant Behavior Checklist—Community Edition (ABC-C), a questionnaire published by Slosson, was used to evaluate a wide range of problem behaviors (Aman & Singh, 1986). Out of the 487 males with FXS, 414 participants had a completed ABC-C questionnaire. Age of the participant when an informant, typically a caregiver, completed the questionnaire was also documented. The ABC-C is the most widely used measure of aberrant behavior in individuals with ID, with higher scores indicating more aberrant behavior (Kaat et al., 2014; Schmidt et al., 2013). While applied in its original version in multiple FXS studies, the ABC-C has been adapted for the disorder using a scoring method based on 55 out of the 58 original items (ABC_{FX}) (Sansone et al., 2012). The original ABC-C consists of five subscales: Irritability, Social Withdrawal, Stereotypic Behavior, Hyperactivity/Noncompliance, and Inappropriate Speech. The ABC_{FX} includes an additional subscale: Social Avoidance. Both total and the 6 ABC_{FX} subscale scores were analyzed and were treated as continuous outcome variables in the analysis.

Social skills impairment: Social impairment was measured by the Social Responsiveness Scale—Second Edition (SRS-2) published by Western Psychological Services (Constantino & Gruber, 2012). Out of the 487 males with FXS, 352 had completed SRS-2 questionnaires. Age of the participant when the questionnaire was completed was also documented. The SRS-2 provides a continuous measure of social ability, with higher scores indicating more severe social impairment. The SRS-2 is an ASD screening instrument that includes five subscales: Social Awareness, Social Cognition, Social Communication, Social Motivation, and Restricted Interests and Repetitive Behaviors. SRS-2 total scores are highly correlated with those from the Autism Diagnostic Observation Schedule (ADOS) (Morrier et al.,

2017). ADOS is considered a gold standard tool for diagnosing ASD. The present study utilized an SRS-2 scoring method optimized for the FXS population (SRS_{FX}) to provide the highest combination of sensitivity and specificity (Kidd et al., 2020), with a total raw score calculated with 46 out of the 65 original items. The SRS_{FX} total score was treated as a continuous outcome variable in the analysis.

2.3 | Statistical analyses

All statistical analyses were performed using SAS 9.4. In total, data from 487 participants were included in the analyses. Frequencies were calculated for all major study variables. To compare the impact of the two types of *FMR1* mosaicism, a series of 2 by 2 (size mosaicism [with vs without repeat allele] by methylation [full vs mosaic]) analyses of covariance were performed on each of the cognitive and behavioral standardized measures, with age at assessment as the covariate. The exact number of participants included in each ANCOVA analysis varied depending on the available data on the cognitive and behavioral measures. To compare the impact of the two types of mosaicism on the level of ID severity (IQ above/below 55) and ASD status (yes/no), logistic regression models controlling for age were developed. A p-value less than 0.05 was considered statistically significant.

3 | RESULTS

Data from 487 males with FXS and mosaicism status who were 3 years and older were included in this analysis. Seventy-six percent of the participants were White, 11% were Hispanic, 8% were African American, 4% were Asian, and 1% were other races/ethnicities. Participants' age ranged from 3 to 60 years with a mean ($\pm$ SD) age of 13.6 years (± 8.30). Approximately 25% of participants are adults and the median age is 12 years. Based on reported genetic testing, 69% ($n = 338$) had no mosaicism, 11% ($n = 52$) had size mosaicism only, 12% ($n = 57$) had methylation mosaicism only, and 8% ($n = 40$) had both size and methylation mosaicism (Table 1). Table SS1 provides group means by four combinations of the two types of mosaicism.

3.1 | Severity of intellectual disability

Most participants were identified as having moderate ID (59.8%), followed by mild ID (24.5%), severe ID (9.8%), borderline ID (3.3%), no ID (2.1%), and profound ID (0.5%). As presented in Table 2, controlling for age, methylation mosaicism had a significant association with ID severity ($F = 12.74$; $df = 1424$; $p < 0.001$). Individuals with methylation mosaicism had significantly less severe ID ($N = 87$, mean ID severity = 3.41) when compared with individuals who were fully methylated ($N = 341$, mean ID severity = 3.82). In contrast, there was no association between size mosaicism and severity of ID.

3.2 | Intelligence quotient

IQ scores were from either the Stanford-Binet ($N = 131$, range 36–79, mean [$\pm$ SD] score of 45.08 [± 7.35]) or the Wechsler Intelligence Scale ($N = 41$, FSIQ range 34–95, mean [$\pm$ SD] score of 58.10 [± 12.80]). As presented in Table 3, 135 (78%) participants had an IQ score less than 55 (i.e., IQ below mild ID), and 37 (22%) participants had an IQ score above or equal to 55 (i.e., equal or higher level than mild ID). As presented in Table 4, controlling for

age and type of IQ test, methylation mosaicism was a significant predictor of having IQ of 55 or above (odds ratio = 3.93 [95% confidence interval:1.48–10.44], $p < 0.01$). In contrast, size mosaicism was not a significant predictor of IQ.

3.3 | Adaptive behavior skills

Participants' Vineland composite scores ranged from 20 to 92 with a mean (± SD) score of 57.34 (±16.54). As presented in Table 2, controlling for age, methylation mosaicism was significantly associated with Vineland composite score ($F = 10.04$; $df = 1169$; $p = 0.002$). Individuals with methylation mosaicism received significantly higher Vineland composite scores ($N = 43$, mean [± SD] score of 63.84 [±17.76]) compared with individuals who were fully methylated ($N = 130$, mean [± SD] score of 55.19 [±15.60]), indicating higher adaptive skills among participants with methylation mosaicism. In contrast, there was no association between size mosaicism and Vineland composite scores.

3.4 | Problem behaviors

ABC_{FX} total scores ranged from 55 to 193 with a mean (± SD) score of 99.54 (±28.36). As presented in Table 2, controlling for age, neither methylation mosaicism ($p = 0.337$) nor size mosaicism ($p = 0.444$) was significantly associated with ABC_{FX} total scores (Table 2). Similar analyses for the six ABC_{FX} subscales also showed no significant association with mosaicism status.

3.5 | Social skills impairment

SRS_{FX} scores ranged from 14 to 115 with a mean (± SD) score of 67.15 (±20.59). As presented in Table 2, controlling for age, methylation mosaicism was significantly associated with SRS_{FX} total scores ($F = 5.42$; $df = 1348$; $p = 0.020$). Individuals with methylation mosaicism received significantly lower SRS_{FX} scores ($N = 72$, mean SRS_{FX} score = 60.85 [±19.01]) compared with individuals who were fully methylated ($N = 280$, mean [±SD] SRS_{FX} score = 68.77 [±20.70]). In contrast, there was no association between size mosaicism and SRS_{FX} total scores.

3.6 | ASD diagnosis

Among 487 participants with reported mosaic status, 448 participants with information on current ASD diagnosis were included in the logistic regression model. Of these, 225 participants (50.2%) were diagnosed with ASD (Table 3). Table 5 displays the results of the logistic regression. Controlling for age of ASD diagnosis, neither methylation mosaicism nor size mosaicism was a predictor of ASD diagnosis.

4 | DISCUSSION

This study assessed the association between two types of mosaicism and cognitive and behavioral outcomes from a large sample of males age 3 years and older with full-mutation FXS from specialty clinics across the United States. We found that methylation mosaicism had a significant positive association with cognitive and behavioral outcomes among males with FXS. Compared to participants without methylation mosaicism, those with methylation mosaicism had less severe intellectual disability, higher mean intelligence test scores,

and adaptive behavior scores, and better social skills. In contrast, the presence of size mosaicism was not significantly associated with cognitive and behavioral outcomes. The positive association with methylation mosaicism was limited to a similar range of cognitive functioning (e.g., mild–moderate ID). However, the difference in the cognitive functioning may be sufficient to significantly impact life functioning in some individual cases (Basuta et al., 2015; Hagerman et al., 1994; Wang et al., 1996; Wöhrle et al., 1998).

Although our findings suggest that males with FXS and methylation mosaicism have significantly lower scores on the SRS, methylation mosaicism was not associated with a reduced odds of having an ASD diagnosis. The SRS, even when scoring is modified for FXS (SRS$_{FX}$), still might not accurately predict ASD in individuals with FXS (Aldridge et al., 2012). In addition, ASD diagnosis in this analysis is a dichotomous variable, and may not be as sensitive to smaller differences in social functioning that are captured in the SRS$_{FX}$ score (Kidd et al., 2020). Baker et al. (2019) found that the presence of detectable *FMR1* mRNA was associated with increased features of ASD in their cohort. This is not supported by our results; however, it should be noted that increased *FMR1* mRNA could be detected in either size or methylation mosaicism and would not distinguish the two genotypes. Thus, it is difficult to compare the two results. There may also be differences in the two cohorts with respect to the methods for diagnosing ASD that underly the differing findings. Neither methylation mosaicism nor size mosaicism were significantly associated with problem behaviors as measured by the ABC scores. Both ABC$_{FX}$ total scores and six ABC$_{FX}$ sub-domain scores, covering a broad range of behavioral functioning, were not significantly associated with either type of mosaicism. Behavior in FXS may not always correspond to cognitive function and both behavior itself and the rating of the behavior by parents could be more variable than performance-based measures, such as IQ tests. As such, a significant relationship between problem behavior and methylation mosaicism may be more difficult to identify, even though irritable behavior is seen with higher frequency in more severely cognitively impaired individuals with FXS (Eckert et al., 2019). A recent report by the aforementioned research group (Baker et al., 2020) found complex differences in ABC scores between FXS mosaic groups. For instance, increased *FMR1* mRNA levels associated with greater irritability in individuals with incompletely silenced full-mutation alleles. These data are difficult to compare with our findings. Nonetheless, it underscores the potential value of follow-up investigations of the relationship between *FMR1* mosaicism and behavioral abnormalities that include assessments of *FMR1* mRNA levels.

Similar to previous findings, methylation mosaicism was associated with higher cognitive scores when compared to males with a fully methylated full mutation. Positive correlations between methylation status and FMRP levels have been demonstrated previously (De Vries et al., 1996; Pretto, Mendoza-Morales, et al., 2014; Tassone et al., 1999). Methylation is the process by which the *FMR1* gene is silenced, although in males (females methylate the gene as a part of the X-inactivation process) methylation usually does not occur unless the expansion to the full mutation has taken place (Hagerman et al., 1994). The unmethylated full-mutation allele can produce FMRP, most likely at lower levels than the normal allele. Thus, even low levels of FMRP may be important for early fetal brain development and ongoing synaptic plasticity and function throughout life (Abitbol et al., 1993; Hinds et al., 1993). Furthermore, perhaps the lack of methylation of an expansion that would typically

be methylated may be a sign of a general tendency for incomplete methylation in many cells leading to low levels of FMRP in a significant percent of cells and higher functioning (Wang et al., 1996; Wöhrle et al., 1998). This relationship contrasts with size mosaicism, where the fraction of cells making FMRP will be only those with the premutation or normal allele, which may be a very small percent of cells. These differences may help to explain the finding in our study of a relationship between multiple areas of function with methylation but not size mosaicism. Future research could investigate the biological basis of the two different types of mosaicism and examine whether methylation mosaicism is a signal of a more general cellular effect in all or many cells, and whether there are fundamentally different effects on FMRP between the two types of mosaicism that then have an impact on phenotype.

Published studies have reported mixed conclusions about the functioning of individuals with mosaicism, but none have separated mosaicism by type. Most previous studies did not distinguish size mosaicism and methylation mosaicism, which may explain the variation in findings. In our study, we have 40 participants with both methylation and size mosaicism that showed slightly better performance and functioning than the ones with single type of mosaicism or no mosaicism. However, when separating the effects of each type of mosaicism, only methylation mosaicism was significant. Compared with methylation mosaicism, size mosaic alleles in the premutation or normal range can be easily detected by polymerase chain reaction (PCR) even when they only represent a small percent of the cells, because of the relatively small size and preferential amplification relative to a full mutation (Jiraanont et al., 2017). Thus, individuals with size mosaicism may have very small percentages of FMRP-producing cells, which might explain the similarities in functioning between those with size mosaicism and a fully methylated full mutation observed in this study. Until recently, methylation mosaicism had been mainly detected by Southern blotting, a technique that is not as sensitive as PCR and likely requires a larger percent of cells with mosaicism for its detection (Berry-Kravis et al., 2021). Although methylation PCR has recently become available, this method does not allow determination of incomplete methylation unless at least 10%–20% of alleles are unmethylated, a percent likely much higher than the sensitivity for detection of size mosaic alleles (Aliaga et al., 2016; Filipovic-Sadic et al., 2010).

Although we utilized a large clinic-based sample from a national registry, participation in FORWARD is completely voluntary and only half of participants had complete data and, thus, selection bias is possible, and the sample may not be representative of all individuals with FXS. Similar to previously published studies of mosaicism in FXS, our study was limited by lack of data on the exact percent methylation of full-mutation alleles, percent of size mosaicism, FMRP production, and *FMR1* mRNA levels. Potential inaccuracy in clinician interpretation of the DNA report could be a potential limitation; however, FXS DNA reports from standard molecular diagnostic labs are most likely quite accurate. The type of mosaicism is usually specified and, in the case of size mosaicism the allele sizes are specified, so there is not extensive interpretation needed. Also, if the report did not specify methylation or size (premutation/normal allele) mosaicism, the clinicians could just report that mosaicism status was unknown. Furthermore, many of the FXS clinicians at the clinics participating in FORWARD are geneticists or work with a genetic counselor or a geneticist

to read the FXS DNA report. So, it is unlikely that inaccuracy in genetic result reporting occurs with sufficient frequency to affect the findings. Future studies with direct molecular analyses could further expand and contribute to an explanation of our findings, since *FMR1* mRNA levels cannot be estimated from diagnostic reports of mosaicism. In addition, our study focused exclusively on comparing the two types of mosaicisms separately in the models; future studies could also examine the interaction between mosaicism types and the effect of age on cognitive and behavioral outcomes.

The ASD diagnosis we collected is based on clinician report and there can be variability in assessment and interpretation. Future investigations could also explore how *FMR1* mosaicism affects physical/systemic phenotypes of FXS. In addition, the present study used two measures for IQ and two versions of the Vineland scales (Vineland-II and Vineland-3) to maximize sample size for analysis. The IQ tests employed in this study may not be sensitive to the full range of IQ functioning in FXS due to floor effects; thus, z-deviation methods are needed to accurately characterize IQ in FXS (Sansone et al., 2014). Future studies expanding on the biology of mosaicism could evaluate larger cohorts with z-deviation IQ scores on a single measure, such as the SB5, and Vineland scores collected with a single version of the measure. In theory, clinicians (and caregivers) reporting on the outcome measures would not have been necessarily blinded to the methylation/mosaic status of the participants, and this could have influenced their reporting, introducing a potential bias. However, for IQ, the bias is not likely to be significant, as this is a direct performance-based measure, and it is unlikely that the participants with FXS who are being tested on the measure would have the faintest idea what their mosaicism status is or what it means. The psychologists performing the IQ test would not have known the DNA result either. For the Vineland assessment, as this is in most cases a clinician interview, the interviewed families would have not been biased to mosaicism status if the interview was performed correctly. Furthermore, only a small proportion of caregivers knows and understands their methylation status. They may have known the participant was mosaic, but most would not know the difference between size and methylation mosaicism. Thus, it is unlikely that this and other outcomes were significantly biased by the lack of blinding.

In conclusion, methylation mosaicism, but not size mosaicism, seems to be associated with cognitive/adaptive/social functioning in FXS based on multiple measures. This study suggests that methylation mosaicism may be important as a marker for population stratification in clinical studies and a factor in clinical trial design that could impact response to interventions. Determination of mosaicism and methylation status may be important in establishing ways of managing the disorder, for anticipatory guidance in clinic, and future research could assess to what extent these cognitive and behavioral differences due to mosaicism affect the prognosis of individuals with FXS. Thus, this study identifies a key genetic parameter in FXS and it provides directions for future studies with more stringent designs to examine the effect of methylation mosaicism on phenotype and prognosis of individuals with FXS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding information

DATA AVAILABILITY STATEMENT

The analyses for this study utilized baseline data from FORWARD Version 4, obtained from 1471 individuals with FXS (i.e., *FMR1* full mutation allele) evaluated from 2012 through 2019. The FORWARD Version 4 data are currently housed at the Centers for Disease Control and Prevention and are not available for public use.

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TABLE 1

Distribution of mosaicism types among male participants, Fragile X Online Registry with Accessible Research Database, 2012–2019

Methylation mosaicism	Size mosaicism		
	No	**Yes**	**Total**
No	338 (69.4%)	52 (10.7%)	390 (80.1%)
Yes	57 (11.7%)	40 (8.2%)	97 (19.9%)
Total	395 (81.1%)	92 (18.9%)	487 (100.0%)

TABLE 2

Two by two ANCOVA model (controlling for age) analysis results for outcome variable: Clinician reported intellectual disability (ID) severity, Vineland Adaptive Behavior Scales (VABS) composite score, aberrant behavior checklist adapted for fragile X (ABC$_{FX}$) total score, and Social Responsiveness Scale adapted for Fragile X (SRS$_{FX}$) total score, Fragile X Online Registry with Accessible Research Database, 2012–2019

	No methylation mosaicism		With methylation mosaicism			No size mosaicism		With size mosaicism		
	N	Mean (± SD)	N	Mean (± SD)	p	N	Mean (± SD)	N	Mean (± SD)	p
Severity of ID[a]	341	3.82 (±0.74)	87	3.41 (±0.87)	<0.001	344	3.78 (±0.76)	84	3.52 (±0.84)	0.140
VABS[b]	130	55.19 (±15.60)	43	63.84 (±17.76)	0.002	139	55.88 (±16.21)	34	63.29 (±16.79)	0.481
ABC$_{FX}$[c]	330	100.33 (±28.19)	84	96.43 (±28.98)	0.337	334	100.13 (±28.63)	80	97.06 (±27.22)	0.444
SRS$_{FX}$[d]	280	68.77 (±20.70)	72	60.85 (±19.01)	0.020	279	68.49 (±20.44)	73	62.04 (±20.49)	0.120

[a] Severity of ID was categorized based on clinician report as 1 = no ID, 2 = borderline ID, 3 = mild ID, 4 = moderate ID, 5 = severe ID, and 6 = profound ID.

[b] VABS measure adaptive skills, with higher scores indicating higher adaptive skills.

[c] ABC$_{FX}$ measures 6 problem behaviors: irritability, social withdrawal, stereotypic behavior, hyperactivity/noncompliance, inappropriate speech, and social avoidance, with higher scores indicating more aberrant behavior.

[d] SRS$_{FX}$ measures social ability, with higher scores indicating more severe social impairment.

TABLE 3

The number of participants in intelligence quotient (IQ) score categories and autism spectrum disorder (ASD)[a] diagnosis by types of mosaicism, Fragile X Online Registry with Accessible Research Database, 2012–2019

	Total N (%)	No size mosaic		With size mosaic	
		No methylation mosaic N	With methylation mosaic N	No methylation mosaic N	With methylation mosaic N
IQ test					
IQ lower than 55	135 (78%)	98	17	14	6
IQ higher than 55	37 (22%)	15	10	6	6
ASD diagnosis					
No ASD diagnosis	223 (50%)	149	28	28	18
With ASD diagnosis	225 (50%)	165	22	21	17

[a] ASD diagnosis was documented based on clinician report.

TABLE 4

Logistic regression model (controlling for age and type of intelligence quotient (IQ) test) analysis results for outcome variable, IQ level[a] (55 vs. <55), Fragile X Online Registry with Accessible Research Database, 2012–2019

Variable	Df	Odds ratio	SE	p
Intercept	1	—	0.7597	0.0097
Methylation mosaicism	1	3.928	0.4988	0.0061
Size mosaicism	1	0.465	0.5207	0.1411
Type of IQ test[b]	1	0.869	0.0549	0.0105
Age (in years)	1	0.082	0.4755	<0.0001

[a]N = 172 participants with IQ scores were included in the logistic regression model.

[b]Two types of IQ tests were included: (1) the Stanford-Binet Scale – Fifth Edition (SB5) and (2) the Wechsler Intelligence Scales.

TABLE 5

Logistic regression model (controlling for age) analysis results for outcome variable, autism spectrum disorder (ASD) diagnosis[a], Fragile X Online Registry with Accessible Research Database, 2012–2019

Variable	Df	Odds ratio	SE	p
Intercept	1	—	0.2815	0.4414
Methylation mosaicism	1	1.170	0.2517	0.5326
Size mosaicism	1	0.825	0.2534	0.4475
Age (in years)	1	0.993	0.0112	0.5197

[a]ASD diagnosis was documented based on clinician report, and $N = 448$ participants with ASD diagnosis were included in the logistic regression model.