Novel methylation markers of the dysexecutive-psychiatric phenotype in *FMR1* premutation women

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ABSTRACT

Objective: To examine the epigenetic basis of psychiatric symptoms and dysexecutive impairments in *FMR1* premutation (PM: 55 to 199 CGG repeats) women.

Methods: A total of 35 *FMR1* PM women aged between 22 and 55 years and 35 age- and IQ-matched women controls (CGG <45) participated in this study. All participants completed a range of executive function tests and self-reported symptoms of psychiatric disorders. The molecular measures included DNA methylation of the *FMR1* CpG island in blood, presented as *FMR1* activation ratio (AR), and 9 CpG sites located at the *FMR1* exon1/intron 1 boundary, CGG size, and *FMR1* mRNA levels.

Results: We show that *FMR1* intron 1 methylation levels could be used to dichotomize PM women into greater and lower risk categories ($p = 0.006$ to 0.037 ; odds ratio = 14–24.8), with only *FMR1* intron 1 methylation, and to a lesser extent AR, being significantly correlated with the likelihood of probable dysexecutive or psychiatric symptoms ($p < 0.05$). Furthermore, the significant relationships between methylation and social anxiety were found to be mediated by executive function performance, but only in PM women. *FMR1* exon 1 methylation, CGG size, and *FMR1* mRNA could not predict probable dysexecutive/psychiatric disorders in PM women.

Conclusions: This is the first study supporting presence of specific epigenetic etiology associated with increased risk of developing comorbid dysexecutive and social anxiety symptoms in PM women. These findings could have implications for early intervention and risk estimate recommendations aimed at improving the outcomes for PM women and their families. *Neurology*® 2015;84:1631–1638

GLOSSARY

5-hmC = 5-hydroxymethylcytosine; **ADHD-PI** = attention-deficit/hyperactivity disorder-predominantly inattentive; **AR** = *FMR1* activation ratio; **DASS** = Depression Anxiety Stress Scale; **ELVF** = Excluded Letter Verbal Fluency; **FM** = full mutation; **FMR1** = fragile X mental retardation 1 gene; **FMRP** = fragile X mental retardation protein; **FREE** = fragile X-related epigenetic element; **FXS** = fragile X syndrome; **GEE** = generalized estimating equation; **IQR** = interquartile range; **LNS** = Letter-Number Sequencing; **OR** = odds ratio; **PM** = premutation; **ROC** = receiver operating characteristic.

The common premutation (PM) CGG expansion (55–199 repeats) in the 5' UTR of the *fragile X mental retardation 1* (*FMR1*) gene (~1 in 450 males and ~1 in 150 females)¹ leads to a number of late-onset disorders² and high rates of dysexecutive and psychiatric symptoms, all with incomplete penetrance.³ The RNA gain-of-function toxicity⁴ and deficient *FMR1* protein (FMRP) translation⁵ are primary molecular features of PM-related disorders and are directly related to the CGG size. However, their effects on the phenotype are diluted in PM females through methylation-related silencing as part of X-inactivation.

Editorial, page 1618

Supplemental data
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Methylation of restriction sites that are routinely targeted in fragile X syndrome (FXS) diagnostics in blood have limited prognostic utility in PM women and do not represent well the *FMR1* activation ratio (AR) and FMRP levels in other tissues.^{6,7} Our earlier studies in FXS full mutation (FM: >200 CGGs) females showed that methylation of different CpG sites within *FMR1* intron 1 may be more conserved between tissues, as indicated by a highly significant correlation between their methylation and cognitive status.^{8,9} Interestingly, this region, also known as fragile X-related epigenetic element 2 (FREE2), is the region where RNA:DNA hybrids associated with regulation of FMRP levels in FXS have been reported to form.¹⁰ This study extends our earlier work to investigate the hypothesis that in PM women, FREE2 methylation is significantly related to executive function impairments and psychiatric symptoms. Interrelationships among these parameters, CGG size, *FMR1* mRNA levels, and AR are also investigated.

METHODS **Standard protocol approvals, registrations, and patient consents.** All study participants provided signed informed consent and the study procedures were consistent with the Declaration of Helsinki and approved by the Southern Health Ethics Committee (project 10147B).

Participants. The cohorts of 35 PM and 35 age- and IQ-matched control (CGG <45) women used in this study were recruited previously.¹¹ Further details are provided in note e-1 on the *Neurology*® Web site at Neurology.org. The PM cohort included 6 families that were not large (one family with 3 women, and 5 families with 2 women). The remaining 22 PM women were unrelated.

Molecular analyses. CGG sizing and methylation analysis were performed on whole blood DNA. The CGG sizing was performed using the Asuragen (Austin, TX) AmpliX *FMR1* PCR Kit.¹² PCR products were assessed via capillary electrophoresis on an Applied Biosystems (Foster City, CA) 3,130 Genetic Analyzer with electropherogram analysis conducted using GeneMapper software (Applied Biosystems; Life Technologies, Carlsbad, CA). The reverse transcription real-time PCR on a Viia 7 Real-Time PCR System (Life Technologies, Global) was used for the *FMR1* mRNA analysis of peripheral blood mononuclear cell RNA. The relative standard curve method was utilized for *FMR1* 5' and 3' mRNA quantification normalized to mRNA of 3 internal control genes, as previously described.¹³ AR was determined using methylation-sensitive Southern blot targeting *NruI* restriction site in the *FMR1* CpG island, as previously described.¹⁴ FREE2 methylation analysis was performed using the Sequenom EpiTYPER system, consisting of 5 CpG units targeting 9 CpG sites.¹⁵ Methylation analysis for each DNA sample was performed in duplicate bisulfate conversions, with each conversion analyzed twice using the EpiTYPER system. The

mean of the 4 methylation output ratio measurements per sample was used as a summary measure for each CpG unit analyzed. The technical variability between these replicates did not exceed ~5%, as described in figure e-1.

Neurobehavioral measures. Executive function tests were selected on the basis of previously demonstrated sensitivity to impairments in adult PM cohorts: Hayling Sentence Completion Test A and B error scores, Letter-Number Sequencing (LNS) raw score, and Excluded Letter Verbal Fluency (ELVF) performance. The Hayling Test¹⁶ is a sentence completion task designed to test executive function. Participants listen to a series of 15 sentences missing the last word. The task is to complete the sentence verbally by suggesting a word that is in no way connected to the sentence. A response that logically completes the sentence is incorrect and is scored as a Category A error. A response that completes the sentence with a word that does not complete the sentence, yet is semantically related, is considered a Category B error. Thus Category A errors reflect complete failure to inhibit a response and Category B errors reflect partial failure to inhibit response. A greater number of errors indicated worse performance.

Higher LNS indicated better working memory ability, and higher ELVF indicated better verbal fluency performance, as previously described.¹⁷ To measure self-reported psychiatric symptoms, we selected the depression axis from the Depression Anxiety Stress Scale (DASS),¹⁸ the self-report Liebowitz Social Anxiety Scale,¹⁹ and the Brown Attention Deficit Disorder Scales,²⁰ where higher scores suggested more severe psychiatric symptoms.

Statistical analyses. Testing for normality of the distribution of each variable was conducted using the Shapiro-Wilk test at significance level $p < 0.05$. Executive function and psychiatric variables, except LNS and ELVF, were transformed using square root function to achieve normal distribution. Comparison of scores between control and PM women for executive function and psychiatric measures was conducted using the generalized estimating equation (GEE) method, which takes into account correlation within a family (table e-1). This method was also used for PM women to assess associations between each executive function (outcome) and each psychiatric (predictor) variable, as well as between executive/psychiatric variables and each genetic factor (predictor). Given that there were no relatives in the control group, these same relationships were investigated using the method of least square or robust regression for estimation, which downweighted the effect of outlier observations. The moderator/mediator effect of executive function on the association between genetic factors and psychiatric variables was assessed as previously described,²¹ taking the average value for related PM women within one family.

Receiver operating characteristic (ROC) curve analysis was used to evaluate the ability of each molecular variable for classification of positive and negative classes. Area under the ROC curve was computed using predicted probabilities from logistic regression as the summary measure of diagnostic accuracy. Youden Index was then used to determine the optimal threshold (cutoff point) for molecular variables, as described in reference 8. Based on values outside of the interquartile range (IQR) for 35 controls, PM women were classified as a positive class for having poor executive function as determined by Hayling A errors >3, LNS score <12, and ELVF <16.5. PM women were also classified as a positive class for having psychiatric symptoms based on threshold scores within the questionnaire (figure 1). The optimal thresholds were then used to dichotomize molecular variables into a binary

Figure 1 Xq27.3 sequence organization and associations between biomarker methylation within *FMR1* intron 1, dysexecutive, and psychiatric symptoms in PM women

(A) Specific fragile X-related epigenetic element 2 (FREE2) CpG site locations on GenBank L29074 L38501 in relation to regions targeted by EpiTYPER system-based methylation analysis. Sequences targeted by the primers for FREE2 EpiTYPER system-based methylation analysis are highlighted in green, with Fw next to the black arrow in the 5' to 3' direction indicating forward primer and Rv next to the black arrow in the 3' to 5' direction indicating reverse primer. Locations of methylation sensitive functional motifs—footprints I, II, and I—indicated as FI, FII, and FIII and the Zeste site proximal to the *FMR1* transcription start sites are highlighted by blue arrows. Text highlighted in pink indicates 5' CTCF binding sites from UCSF Chip-Seq that overlap with FREE2 CpG10-12; >> and << symbols indicate forward and reverse primers used in ChIP to show formation of RNA:DNA hybrids denoted as fP (200-400) in Colak et al.¹⁰ (figure 4, figure S16, and table S1). (B-E) Associations between biomarker methylation within *FMR1* intron 1 with (B) executive function assessed using the Hayling A error score and psychiatric symptoms assessed using (C) the self-report Liebowitz Social Anxiety Scale (SR-LSAS). Relationships between FREE2 CpG6/7 methylation with psychiatric symptoms assessed using (D) the Depression axis from the Depression Anxiety Stress Scale (DASS) and (E) the Brown Attention Deficit Disorder subscale (attention-deficit/hyperactivity disorder [ADHD] effort). The horizontal lines represent the normal functioning threshold, which for Hayling A errors represents the score of 3 (defined by the maximum value for Hayling A errors in the third quartile of 35 control women); for SR-LSAS, the score of 54 (the LSAS manual defined scores of 55-65 to indicate moderate social phobia; 65-80 marked social phobia; 80-95 severe social phobia; and >95 as very severe social phobia); DASS the score of 9 (DASS manual defined scores of 0-9 as the normal range; 10-27 mild to severe depression); and ADHD effort the score of 4 (defined by the maximum value for ADHD effort subscale in the third quartile of 35 control women). Vertical broken lines represent the methylation threshold identified from receiver operating characteristic analysis for the maximum specificity and sensitivity for detection of low executive functioning and a probable social anxiety disorder in premutation (PM) women. Dark gray boxes represent the range for the affected PM group (true positives) based on the applied thresholds. Light gray boxes represent the range for the unaffected PM group (true negatives) based on the applied thresholds. β represents standardized coefficients taking into account relatedness between individuals. MOR = methylation output ratio.

value, with values under a threshold treated as the reference category. Logistic regression using the GEE method for estimation was used to estimate odds ratio (OR). All analyses were conducted using STATA, RMS, and DiagnosisMed in the publicly available R statistical computing package (The R Project for Statistical Computing).

RESULTS Relationships between molecular parameters and the dysexecutive/psychiatric phenotype in PM women. Analysis of the intercorrelations between

executive function performance and symptoms of social anxiety, depression, and attention-deficit/hyperactivity disorder—predominantly inattentive (ADHD-PI) revealed many significant relationships within the PM group. Worse performance on the working memory and verbal fluency tasks significantly correlated with increased symptoms of depression, social anxiety, and ADHD-PI. Hayling A errors, which indicate impaired inhibitory control, correlated

significantly with worse symptoms across all psychiatric domains. By contrast, Hayling B errors reflecting impulsivity were correlated with depression and social anxiety symptoms, but were not related with symptoms of ADHD-PI (table e-2). These relationships highlight a dysexecutive/psychiatric phenotype in PM women and were not observed in the age- and IQ-matched control group.

While PM women had significantly higher *FMRI* mRNA levels than controls, *FREE2* methylation and AR were comparable between groups (table e-3). CGG size within the PM group was not significantly correlated with performance on any neurocognitive or psychiatric measure. In contrast, *FMRI* mRNA levels were significantly and positively correlated with better working memory and verbal fluency performance; however, these relationships disappeared when *FMRI* mRNA levels were controlled for AR (table e-4).

Significant correlations were observed between *FMRI* CpG island methylation (represented by AR) and dysexecutive symptoms in PM women. Lower AR was significantly correlated with increased Hayling A errors and worse working memory performance (table 1 and figure e-2). However, there were no relationships between AR and psychiatric symptoms. Methylation of CpG unit 1 located within *FMRI* exon 1 also revealed a significant positive correlation with Hayling B errors (table e-5).

Most interestingly, methylation of all 3 CpG units (representing 7 CpG sites) located within *FMRI* intron 1 was significantly correlated with at least one executive function or psychiatric measure (table 1, figure e-2, and figure e-3). Methylation of CpG 10-12 showed the strongest correlation with the executive function and psychiatric measures, followed by methylation of CpG 6/7 and CpG 8/9 (figure 1 and table 1).

Prognostic potential for identifying the dysexecutive/psychiatric phenotype. PM women were classified into groups called affected or unaffected based on probable dysexecutive/psychiatric disorder or IQR (see statistical analysis) threshold scores. Of all molecular measures, methylation of *FREE2* intronic CpG10-12 showed the highest sensitivity for correctly identifying PM participants in the executive function and social anxiety affected groups. Specifically, Hayling A errors (100% sensitivity) and social anxiety (~92% sensitivity) were ascertained for methylation thresholds of 0.31 and 0.32, respectively (figure 1 and table e-6). Methylation of *FREE2* intronic CpG6/7 showed the highest specificity for correctly identifying 2 of the unaffected groups derived from psychiatric measures. These were the depression scale, which showed 92.6% specificity, and the ADHD-PI effort subscale, which had ~88% specificity, determined at methylation thresholds of 0.46 and 0.44, respectively (figure 1 and table e-6). When the PM group was dichotomized based on these CpG10-12 and CpG6/7 optimal methylation thresholds, PM women with *FREE2* methylation analysis positive results were significantly more likely to be in the affected groups defined by at least one dysexecutive or psychiatric measure. For CpG10-12, this was indicated by Hayling A errors (OR 14; $p = 0.006$), and for CpG6/7 this was indicated by both symptoms of depression on the DASS (OR 17.4, $p = 0.037$) and low effort on the ADHD-PI measure (OR 14.4; $p = 0.020$) (table 2). In contrast, *FMRI* mRNA-positive results defined by optimal molecular thresholds were not significantly more likely to be in an affected group for dysexecutive symptoms, although AR-positive results were significantly more likely to be in an affected group defined by Hayling A errors (OR 7.53; $p = 0.014$) (table 2).

Table 1 Relationship between molecular variables and dysexecutive or psychiatric outcome variables for the PM group

Outcome variables	AR	<i>FMRI</i> mRNA	CpG1	CpG2	CpG6/7	CpG8/9	CpG10-12
Dysexecutive symptoms							
Hayling A errors ^a	-0.38 (0.019) ^b	0.05 (0.787)	0.04 (0.810)	0.11 (0.508)	0.55 (<0.001) ^b	0.46 (0.004) ^b	0.73 (<0.001) ^b
LNS	0.40 (0.009) ^b	0.30 (0.014) ^b	-0.16 (0.369)	0.07 (0.486)	-0.24 (0.097)	-0.17 (0.315)	-0.42 (0.007) ^b
ELVF	-0.06 (0.726)	0.31 (0.046) ^b	-0.14 (0.428)	0.20 (0.268)	-0.08 (0.651)	-0.29 (0.081)	-0.52 (0.001) ^b
Neuropsychiatric symptoms							
Anxiety (LSAS) ^a	0.07 (0.717)	-0.20 (0.264)	0.10 (0.563)	-0.15 (0.366)	0.29 (0.084)	0.17 (0.321)	0.46 (0.003) ^b
ADHD-effort ^a	0.01 (0.981)	-0.12 (0.409)	0.23 (0.128)	-0.04 (0.830)	0.38 (0.011) ^b	-0.52 (0.001) ^b	0.13 (0.431)
ADHD-affect ^a	-0.02 (0.916)	-0.11 (0.543)	-0.01 (0.974)	-0.11 (0.554)	0.33 (0.051)	0.02 (0.916)	0.37 (0.026) ^b

Abbreviations: ADHD = attention-deficit/hyperactivity disorder; AR = *FMRI* activation ratio; LNS = Letter Number Sequencing; ELVF = Excluded Letter Verbal Fluency; LSAS = Liebowitz Social Anxiety Scale; PM = premutation.

Standardized regression coefficients and p values were estimated using generalized estimating equations method.

^a Square root transformed data.

^b Significant values.

Table 2 Estimation of whether the probability of being in the dysexecutive and psychiatric symptoms subgroup of PM women is significantly different when separated based on the optimal molecular predictor determined using ROC analysis

Outcome variables	Threshold	OR	p Value	95% CI
Hayling A errors ^a	0.31 ^b	24.8	0.006 ^f	2.53
	0.59 ^c	7.53	0.014 ^f	1.49
LNS	0.32 ^b	0.78	0.697	0.23
	0.58 ^c	0.77	0.655	0.25
	1.29 ^d	0.54	0.183	0.22
ELVF	0.32 ^b	1.19	0.817	0.26
	1.26 ^d	1.55	0.509	0.42
Social anxiety ^a	0.32 ^b	2.33	0.258	0.54-10.1
Depression ^a	0.46 ^e	17.4	0.037 ^f	1.19-254
ADHD-effort ^a	0.44 ^e	14.4	0.020 ^f	1.51-137

Abbreviations: ADHD = attention-deficit/hyperactivity disorder; CI = confidence interval; ELVF = Excluded Letter Verbal Fluency; LNS = Letter Number Sequencing; OR = odds ratio; PM = premutation; ROC = receiver operating characteristic.

OR, p value, and 95% CI estimated by logistic regression using the generalized estimating equations method. The outcome variables were dichotomized using values from ROC curve analysis (table e-6), while the molecular predictors were dichotomized below (including) or above optimal cutoff threshold as described in table e-6, with the below cutoff score treated as reference category.

^aSquare root transformed data.

Molecular thresholds are marked by ^bfor CpG10-12, ^cfor activation ratio, ^dfor *FMR1* mRNA, and ^efor CpG6/7.

^fSignificant values.

Relationships between *FMR1* intron 1 methylation and the dysexecutive/psychiatric phenotype are unique to PM women. In PM women, *FMR1* intron 1 CpG10-12 methylation was significantly correlated with performance on 3 out of 4 executive function measures ($p < 0.001$ to $p = 0.007$) and psychiatric symptoms of both social anxiety ($p = 0.003$) and ADHD-related affective (or emotional) regulation ($p = 0.026$) (table 3). Mediation analysis in the PM group further revealed that the observed relationship between *FREE2* intronic CpG10-12 methylation and social anxiety symptoms was indirect and mediated by executive function performance (table 4). These relationships were not observed in the age- and IQ-matched control group.

DISCUSSION Previous investigations of psychiatric symptoms and cognitive status in PM women have focused on AR or *FMR1* mRNA for almost a decade. None have demonstrated relationships with the dysexecutive and psychiatric symptoms in PM women of comparable significance to that reported in this study for methylation of *FREE2* intronic units 10–12 and 6/7 (and, to a lesser extent, 8/9). Analysis of methylation for these intronic CpG units enabled differentiation of probable dysexecutive/psychiatric disorders with high sensitivity (CpG10-12: 92%–100% for

Table 3 Relationship between *FREE2* CpG10-12 methylation levels (predictor) and dysexecutive or psychiatric outcome variables using least square or robust regression for control data and generalized estimating equations for the PM group

Outcome variables	Controls			PM		
	Coefficient	SE	p Value	Coefficient	SE	p Value
Dysexecutive symptoms						
Hayling A errors ^a	0.105	0.180	0.565	0.732	0.118	<0.001 ^b
Hayling B errors ^a	0.198	0.189	0.302	0.045	0.176	0.796
LNS	−0.268	0.169	0.122	−0.419	0.156	0.007 ^b
ELVF	0.025	0.180	0.892	−0.515	0.151	0.001 ^b
Psychiatric symptoms						
Depression ^a	−0.079	0.178	0.662	0.268	0.167	0.108
Anxiety (LSAS) ^a	0.022	0.198	0.910	0.458	0.157	0.003 ^b
ADHD-Brown ADD ^a	−0.103	0.185	0.583	0.212	0.165	0.199
ADHD-activation ^a	−0.059	0.184	0.751	0.004	0.146	0.979
ADHD-attention ^a	0.010	0.191	0.959	0.246	0.165	0.136
ADHD-effort ^a	−0.165	0.191	0.394	0.130	0.165	0.431
ADHD-affect ^a	−0.139	0.185	0.459	0.372	0.168	0.026 ^b
ADHD-memory ^a	−0.034	0.214	0.876	0.271	0.159	0.089

Abbreviations: ADD = attention-deficit disorder; ADHD = attention-deficit/hyperactivity disorder; ELVF = Excluded Letter Verbal Fluency; *FREE2* = fragile X-related epigenetic element; LNS = Letter Number Sequencing; LSAS = Liebowitz Social Anxiety Scale; PM = premutation.

All variables were standardized to have a mean of 0 and SD of 1.

^aSquare root transformed data.

^bSignificant values.

Table 4 Mediation analysis for total, direct, and indirect effect of FREE2 intronic CpG10-12 methylation on social anxiety (LSAS) mediated by executive function performance (Hayling A errors, LNS and ELVF) in the PM group

	Coefficient	SE	p Value
Total effect	0.567	0.180	0.005
Univariate mediation analysis			
Hayling A errors ^a (mediator)			
Direct effect	−0.026	0.191	0.892
Indirect effect	0.593	0.179	0.001 ^b
LNS (mediator)			
Direct effect	0.227	0.168	0.175
Indirect effect	0.340	0.138	0.014 ^b
ELVF (mediator)			
Direct effect	0.238	0.182	0.192
Indirect effect	0.329	0.140	0.019 ^b
Multivariate mediation analysis			
Direct effect	−0.254	0.152	0.094
Hayling A errors ^a indirect effect	0.528	0.145	<0.001 ^b
LNS indirect effect	0.293	0.110	0.008 ^b
Total indirect effect	0.822	0.183	<0.001 ^b

Abbreviations: ELVF = Excluded Letter Verbal Fluency; FREE = fragile X-related epigenetic element; LNS = Letter Number Sequencing; LSAS = Liebowitz Social Anxiety Scale; PM = premutation.

All variables were standardized to have mean 0 and SD of 1.

^a Square root transformed data.

^b Significant values.

probable dysexecutive and social anxiety disorders) and specificity (CpG6/7: ~90% for problems with ADHD-related effort and depression). By contrast, when the PM group was dichotomized based on optimal molecular thresholds for CGG size, exon 1 methylation, and *FMRI* mRNA level, positive results could not be used to predict probable dysexecutive/psychiatric disorders. Interestingly, methylation analysis of the *FMRI* CpG island represented by AR also significantly correlated with dysexecutive symptoms, but to a lesser extent.

The severity of PM-related neurologic disorders is likely to be directly related to the proportion of cells with the normal size allele on the active X chromosome—presumably the higher AR, the more cells carry silent methylated PM alleles on the inactive X chromosome, and the less severe the phenotype. However, evidence for the prognostic potential of AR has been limited in PM-related disorders. While in PM women one study showed that CGG repeat size, when controlled for AR, was significantly associated with ataxia score,⁷ the prognostic utility of AR in blood, when examined alone, has not been demonstrated previously.^{7,22} In contrast, decrease in FMRP levels in hair root samples was significantly

correlated with cognitive status of PM women.²² Subsequently, it has been suggested that methylation of the CpG sites used to determine AR is not well conserved between blood and other tissues, and thus provides limited representation of FMRP activity in the brain.²²

Our previous studies suggest that methylation of *FMRI* intron 1 CpG sites is well-conserved between different tissue types including adult and newborn blood, lymphoblasts, and amniocytes, and is stable over time in PM females.^{8,15,23,24} This could explain why *FMRI* intron 1 methylation was more likely than AR to correlate with severity of dysexecutive/psychiatric symptoms in the present study of PM women. Increased methylation of these *FMRI* intron 1 CpG sites in FM females was also significantly correlated with both FMRP levels in blood and cognitive impairment.^{8,15,23} Avitzour et al.²⁵ have independently shown that FXS embryonic stem cells also have increased methylation of these same intron 1 sites, and that this was associated with abnormal histone modification and *FMRI* regulation.

Importantly, Colak et al.¹⁰ have recently described formation of RNA:DNA hybrids within the same intronic region (figure 1A). When these hybrids were disrupted, the *FMRI* gene was reactivated in FM neurons derived from FXS human embryonic stem cells. RNA:DNA hybrids in FXS have been linked to repressive epigenetic changes associated with abnormal methylation,²⁶ and have been also shown to form in PM fibroblasts.²⁷ Of potential functional relevance is also the location of the most informative FREE2 intronic CpG10-12 site being at the 5' end of a CCCTC binding factor (CTCF) binding site (figure 1A). CTCF is a chromatin boundary factor that has been associated with pathogenesis of trinucleotide repeat disorders,²⁸ involved in gene regulation²⁹ and X chromosome inactivation.^{28,30}

Finally, the observed relationship between FREE2 intronic CpG10-12 methylation and the social anxiety (but not depressive) phenotype in PM women was indirect and mediated by executive function performance. Social anxiety is the fear and avoidance of social situations and one of the most common and longstanding psychiatric complaints for PM women.^{31,32} The relationship with executive function is intriguing as it suggests that deficits in core neurocognitive systems, such as executive function and attentional distribution, might contribute toward threat-related bias in PM women,³³ and over time, this may increase their risk for developing social anxiety. Thus future studies could investigate whether early intervention with cognitive training to improve executive function leads to remediation effects for social anxiety, particularly in PM women within the higher risk group.

Limitations of this study are a relatively small sample size, the cross-sectional design, and the molecular analyses being performed only in blood while the phenotypes examined are largely CNS-based. For these reasons, this study is not equipped to fully explain the prognostic nature of the relationships between methylation of different intronic sites with different clinical outcome measures, or the potential changes in these relationships over time. Moreover, for most outcomes the molecular measures examined in this study were not 100% predictive. This suggests a phenotypic influence from other factors previously described to contribute to PM-related disorders including deficient *FMR1* protein (FMRP) translation,⁵ abnormal expression of long noncoding RNA genes, *ASFMR1/FMR4*,¹³ *FMR5*, and *FMR6*,³⁴ as well as repeat associated non-ATG translation leading to neuronal toxicity³⁵ and mitochondrial dysfunction.¹³ Environmental factors are also likely to contribute, as in other settings these have been shown to have a direct effect upon epigenetic status, gene expression, and the expressed phenotype.^{36,37}

It is also important to note that all assays utilized in this study examined total methylation, which is converted to 5-hydroxymethylcytosine (5-hmC), and subsequently 5-formylcytosine and 5-carboxylcytosine.³⁸ Thus, while total methylation may reflect to a degree 5-hmC levels, the likely differences among the 3 converted forms could not be identified in this study. Future studies should examine 5-hmC, especially for the most informative *FMR1* intron 1 sites described here, in light of recent studies showing that (1) 5-hmC levels are increased in repetitive regions and brain specific enhancer regions in a PM mouse model of fragile X–associated tremor/ataxia syndrome³⁹; (2) 5-hmC has been found to be differentially methylated in regions important for neurodevelopment, including CpG island shores⁴⁰; and (3) 5-hmC has been suggested to function as an intermediate step for environmental regulation specific to the brain genome.⁴⁰

This study showed that specific methylation thresholds for *FMR1* intron 1 and to a lesser extent AR could be potentially used to dichotomize PM women into greater and lower risk categories for dys-executive and psychiatric symptoms. Importantly, this was not possible using CGG size, *FMR1* mRNA level, or *FMR1* exon 1 methylation. If validated in future studies, these findings could have important implications for early intervention recommendations aimed at improving the outcomes for affected PM women and their families.

AUTHOR CONTRIBUTIONS

K.M.C., C.M.K., and D.E.G. conceptualized and designed the study, provided intellectual input into the interpretation of the data, and

co-wrote the first draft of the manuscript. Q.M.B. conducted statistical analyses and provided intellectual input into drafts of the manuscript. D.E.G. performed and supervised sample processing, *FMR1* mRNA, Southern blot, and MALDI-TOF MS–based molecular data analyses. X.L. and Y.I. performed *FMR1* mRNA, Southern blot, and MALDI-TOF MS–based molecular data analyses, and were involved in drafting of the manuscript. H.R.S. cosupervised Southern blot–based analyses and was involved in drafting of the manuscript. S.M., A.A., and J.C. contributed to study design, assisted with recruitment, and provided intellectual input into drafts of the manuscript. M.A.B., D.R.H., and J.N.T. provided intellectual input into the drafting of the manuscript.

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DISCLOSURE

K. Cornish, C. Kraan, Q. Bui, M. Bellgrove, S. Metcalfe, J. Trollor, D. Hocking, and H. Slater report no disclosures relevant to the manuscript. Y. Inaba is an inventor on patents related to the technology described in this article. X. Li, A. Archibald, E. Turbitt, and J. Cohen report no disclosures relevant to the manuscript. D. Godler is an inventor on patents related to the technology described in this article. Go to Neurology.org for full disclosures.

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