BACKGROUND: Cognitive status in females with mutations in the FMR1 (fragile X mental retardation 1) gene is highly variable. A biomarker would be of value for predicting which individuals were liable to develop cognitive impairment and could benefit from early intervention. A detailed analysis of CpG sites bridging exon 1 and intron 1 of FMR1, known as fragile X–related epigenetic element 2 (FREE2), suggests that a simple blood test could identify these individuals.

METHODS: Study participants included 74 control females (<40 CGG repeats), 62 premutation (PM) females (55–200 CGG repeats), and 18 full-mutation (FM) females assessed with Wechsler intelligence quotient (IQ) tests. We used MALDI-TOF mass spectrometry to determine the methylation status of FREE2 CpG sites that best identified low-functioning (IQ <70) FM females (>200 CGG repeats), compared the results with those for Southern blot FMR1 activation ratios, and related these assessments to the level of production of the FMR1 protein product in blood.

RESULTS: A methylation analysis of intron 1 CpG sites 10–12 showed the highest diagnostic sensitivity (100%) and specificity (98%) of all the molecular measures tested for detecting females with a standardized verbal IQ of <70 among the study participants. In the group consisting of only FM females, methylation of these sites was significantly correlated with full-scale IQ, verbal IQ, and performance IQ. Several verbal subtest scores showed strong correlation with the methylation of these sites ($P = 1.2 \times 10^{-5}$) after adjustment for multiple measures.

CONCLUSIONS: The data suggest that hypermethylation of the FMR1 intron 1 sites in blood is predictive of cognitive impairment in FM females, with implications for improved fragile X syndrome diagnostics in young children and screening of the newborn population.

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability. FXS is associated with a number of behavioral, cognitive, and physical problems, and it is a prominent cause of autism spectrum disorder (1, 2). FXS is usually caused by a CGG trinucleotide expansion of >200 repeats [termed “full mutation” (FM)] within the untranslated portion of exon 1 of the X-linked FMR1 (fragile X mental retardation 1) gene. FM-size alleles are usually associated with hypermethylation of the FMR1 CpG island and of the FMR1 promoter, which is located upstream of the CGG expansion (3). Such hypermethylation almost always shuts down FMR1 transcription and results in loss of the FMR1 protein (FMRP), which is essential for normal neural functioning (4). The smaller expansion alleles, which are categorized into “gray zone” (GZ) (45–54 CGGs) and “premutation” (PM) (55–200 CGGs) groups, are highly prevalent in the general population (GZ, 1 in 15 females; PM, 1 in 250 females) (5). PM and GZ alleles have an unmethylated FMR1 pro-
moter and normal or moderately reduced FMRP production (6–9).

FM alleles occur at frequencies of approximately 1 in 4000 males and 1 in 2000 females (10), but only 25% to 50% of all FM females are intellectually impaired (11). With respect to the remainder, 30% to 50% of FM females carry the abnormal allele predominantly on the inactivated X chromosome, and these females typically have a normal intelligence quotient (IQ) (11, 12). Moreover, up to 25% of FM females either have an unmethylated or partially methylated expanded allele or are PM/FM mosaics, and these individuals may have a milder FXS phenotype (7, 11–13). Thus, the skewness of X inactivation, the methylation state of the FMR1 promoter, and FMRP production are more reflective of FMR1 function and patients’ clinical outcomes than CGG size, particularly in females.

Methylation-sensitive Southern blot analysis combined with the PCR, currently the gold standard for the definitive diagnosis of FXS, provides the size of the CGG expansion and the methylation status of the FMR1 promoter (14). For females with expanded alleles, the FMR1 activation ratio (the proportion of active X chromosomes carrying the normal-size allele) can also be evaluated with this approach (11). The methylation status of the expanded alleles and the activation ratio in females have previously been shown to be correlated with FMRP production and cognitive status as assessed with the Wechsler Adult Intelligence Scale (8, 11).

Research has also produced high-throughput tests for FXS, including the use of the PCR to measure expansion size (15) and the use of the PCR and multiplex ligation-dependent probe amplification to measure methylation within the CpG island of FMR1 located within the untranslated portion of exon 1 (16–19). The main limitation of these methylation-based tests is that they do not reliably determine the type and severity of the FXS phenotype in FM females (18).

Using the Sequenom EpiTYPER system (20), we have recently identified novel epigenetic markers for FXS—fragile X–related epigenetic elements 1 and 2 (FREE1 and FREE2)—that are inversely correlated with FMRP production in males with incompletely methylated FM alleles, as well as in male FM/PM mosaics (9). These regions are located on either side of the CGG expansion (outside of the “classic” 52-bp CpG island), which includes the region previously believed to encompass the FMR1 promoter (9, 21). They are also distinct from the sites routinely analyzed with methylation-sensitive Southern blotting (9, 21) and methylation-specific PCR (18). Although the FMR1 CpG island is located in the 5′ untranslated region of the gene, the FREE2 region is located within the translated portion that follows the ATG within exon 1, which qualifies FREE2 as an intragenic region bridging FMR1 exons 1 and 1. Methylation of intronic sequences has recently been shown to be important in many disorders and biological processes, including prostate cancer (22), modified uterine estrogen response (23), and Parkinson disease (24). Furthermore, the importance of intragenic DNA methylation extending beyond CpG islands has recently been highlighted in the regulation of transcription in the brain (22). Until recently, however, the impact of intragenic and, more specifically, intronic methylation on clinical and molecular outcomes has not been examined in FMR1-related disorders (25). Although our previous studies have shown a significant inverse relationship between FMRP production in blood and the methylation of FMR1 intronic sites in PM and FM females, no quantitative phenotypic data were available for that sample (25).

The primary focus of this study was to evaluate a group of females carrying FM or PM alleles for the relationship between the methylation of specific intronic sites within the FREE2 region found in blood samples and standardized measures of cognitive status [i.e., Wechsler Adult Intelligence Scale III (26) and Wechsler Intelligence Scale for Children III (27) IQ tests]. In this study, we also compared methylation levels of exonic (FREE2 CpG units 1 and 2) and intronic (FREE2 CpG units 6–12) sequences, the FMR1 activation ratios determined with methylation-sensitive Southern blot analysis of the NruI restriction site (within the CpG island), and the results of FMRP immunostaining of blood lymphocytes (28).

Materials and Methods

STUDY PARTICIPANTS

The Australian participants comprised 74 control, 48 PM, and 18 FM females recruited from fragile X families visiting the Victorian Clinical Genetics Services clinic in Melbourne. The US participants comprised 14 PM females recruited from families seen at the Fragile X Treatment and Research Center through a collaborative Genotype–Phenotype National Institute of Child Health and Human Development–funded study. Examiners were blinded to the DNA status of the study participants during the testing period. Standardized assessments of cognitive status were performed with the Wechsler intelligence test appropriate for the chronological age: the Wechsler Adult Intelligence Scale III for individuals older than 16 years (26) and the Wechsler Intelligence Scale for Children III for children between 6 and 16 years of age (27). Cognitive status was assessed at the School of Psychological Science, La Trobe University, Australia, and the Fragile X Treatment and Research Center in the US. IQ results for the 2 sites were
cross-validated with a subsample of study participants and shown to be consistent between the sites, as has been described in our previous joint publications (1). The ages of PM females ranged from 26 to 67 years, and those of the FM females ranged from 6 to 35 years. The controls were between 1 and 59 years of age. All controls had FMR1 allele sizes of <40 CGG repeats but have not been assessed with formal cognitive testing. The control, PM, and FM individuals included in this study were not related.

All adult participants and the parents of underage participants signed an informed-consent form approved by the Royal Children’s Hospital Ethics Committee and by the Institutional Review Board of the University of California at Davis.

**MOLECULAR STUDIES**

The aim was to identify which FREE2 CpG sites could be used most effectively as a biomarker to detect low-functioning FM females within a population of control, PM, and high- and low-functioning FM females. For all FM females and 14 PM females, the data on full-scale IQ (FSIQ), verbal IQ (VIQ), and performance IQ (PIQ), as well as DNA extracted from whole blood were already available from our previous study (26). For the remaining PM females, these molecular and cognitive data were freshly collected for this study as part of a new cohort. All DNA samples were retested in this study by means of a PCR assay to determine CGG repeat sizes for control and PM alleles. The Sequenom EpiTYPER system (which uses MALDI-TOF mass spectrometry for quantification) and Southern blotting tools were used to determine the methylation output ratio and the activation ratio, respectively. For the molecular analyses, the participant samples were separated into 4 groups: controls (<40 repeats, n = 74); PM carriers (n = 62) with FSIQ, VIQ, and PIQ values all >70; high-functioning FM carriers, with FSIQ >70 (n = 12), VIQ >70 (n = 13) and PIQ >70 (n = 11); and low-functioning FM females, with FSIQ <70 (n = 6), VIQ <70 (n = 5), and PIQ <70 (n = 7). We have used an IQ of 70 as a cutoff for mental retardation according to the criteria used in earlier relevant studies (11, 12).

We collected 3–10 mL of blood from the participants at the time of phenotypic assessment and extracted DNA according to previously described manual and automated extraction methods (9, 29).

We initially used a fragment analyzer (MegaBace; GE Healthcare) and a validated PCR assay to determine CGG repeat sizes with a higher detection limit of 170 repeats, as previously described (29). For samples with CGG repeat sizes within the PM and FM range, we assessed methylation of the FMR1 CpG island with a fully validated methylation-sensitive Southern blotting procedure with appropriate normal and abnormal controls, as previously described (11). The FMR1 activation ratios for samples from females were calculated on the basis of the ratio of the density (optically scanned) of the 2.8-kb band to the combined densities of the 2.8-kb and 5.2-kb bands, where the 2.8-kb band represents the proportion of normal active X and the 5.2-kb band represents the proportion of normal inactive (methylated) X (11).

Blood smears for FM females were made within 24 h of blood collection, and FMRP immunoreactivity was assessed as previously described (28, 30). FMRP production was expressed as the percentage of lymphocytes that stained positively for the protein. FMRP was not assayed in samples from control females.

The EpiTYPER system was used to measure methylation output ratios for FREE2 sites within exon 1 (CpGs 1 and 2) and intron 1 (CpGs 6–12), as previously described (9). FREE2 CpGs 3–5 were not analyzed, because they had high-mass fragments that fell outside the spectrum window available for analysis with this system. Each sample was analyzed in quadruplicate, giving 4 separate methylation output ratios that reflected the technical variation arising from bisulfite conversion, the PCR, and mass cleave reactions. Ninety-five percent of all samples examined showed reproducibility within 10% of the replicates; the mean of the 4 replicates was used for the epigenotype–phenotype analyses.

**STATISTICAL ANALYSIS**

We used the nonparametric Mann–Whitney 2-sample test to compare medians of the methylation percentage for all FREE2 units for FM samples and control and PM samples, and for FM carriers with FSIQ, VIQ, and PIQ values <70 and FM carriers with FSIQ, VIQ, and PIQ values >70. We then reclassified individuals with FSIQ, VIQ, and PIQ values >70 as negative and FSIQ, VIQ, and PIQ values <70 as positive, and we used the ROC curve to evaluate the ability of the methylation value at each CpG site to assign individuals to the 2 classes. We used the area under the ROC curve as computed with the probabilities predicted from logistic regression as the summary measure of diagnostic accuracy, and we used the Youden index (31) to determine the optimal threshold (cutoff point) for each CpG site’s methylation value. Each diagnostic model was internally validated with a bootstrapping procedure. The relationship between the methylation values of each CpG unit and each cognitive outcome was assessed via simple linear regression analysis. All analyses were conducted with rms (32) and DiagnosisMed (33) in the publicly available R statistical computing package (34).

A best subset of CpG variables that were independently associated with cognitive measures, the percent-
age of blood lymphocytes immunostaining positive for 
FMRP, and the Southern blot activation ratio in blood 
was identified by means of a stepwise forward/back-
ward multiple logistic regression model using the 
Bayesian information criterion (BIC). This method 
calculated BIC each time a CpG variable was included 
or removed. The final model was selected if no further 
reduction in BIC was found. For the comparisons, we 
used a step-up false-discovery rate controlling proce-
dure (35) to adjust P values for multiple testing. We 
used the MMIX package (36) and the multtest package 
in R 2.10.1 to conduct best-subset selection and com-
pute adjusted P values for multiple testing, respectively.

Results

COMPARISON OF CONTROL, PM, AND FM GROUPS
The median methylation output ratio of all FREE2 in-
tronic and exonic units was significantly higher in FM 
females when considered as a single group and in FM 
females separated on the basis of an FSIQ cutoff <70, 
compared with either PM carriers or controls (Fig. 1) 
(P values for intergroup comparisons ranged between 
10^{-3} and 10^{-8}; see Table 1 in the Data Supplement that 
accompanies the online version of this article at http://
www.clinchem.org/content/vol58/issue3). These dif-
fferences could not be accounted for by the age of the 
participants, because age and methylation output ratio 
showed no significant correlation for any of the FREE2 
units in each of the 3 groups (control, PM, or FM; see 
Fig. 1 in the online Data Supplement).

Within the FM group, only intronic CpG units 
(CpG 6/7, 8/9, and 10–12) showed a significantly in-
creased median methylation output ratio in FM fe-
male with VIQs <70 compared with FM females with 
VIQs >70, before and after adjustment for multiple 
measurements (Table 1). In contrast, if the FM group 
was separated according to FSIQ or PIQ (<70 or >70), 
the median methylation output ratio for the low-
functioning FM females was not significantly different 
from that of the high-functioning FM females after ad-
justment for multiple measurements.

SENSITIVITY AND SPECIFICITY ANALYSIS
We found that assay sensitivity and specificity assess-
ments differed, depending on the IQ measure. The as-
say sensitivity and specificity assessments were per-
formed with 154 samples with CGG repeats ranging in 
expansion size between those of normal controls and 
those of FM females—a population analogous to “real-
life” newborn-screening settings. Methylation-positive 
thresholds were determined from the complete cohort 
for each CpG unit. Of all units examined, the methyl-
ation status of CpG 10–12 provided the optimal 
threshold that distinguished the low-functioning indi-
viduals (IQ <70) from high-functioning individuals 
(IQ >70) (Fig. 1, Table 2). The optimal threshold for 
CpG 10–12 varied between 0.35 for FSIQ and PIQ to 
0.435 for VIQ, which provided the highest specificity 
and sensitivity (100% and 98%, respectively). The

![Fig. 1. Comparison of groups for methylation of 
FMR1 intron 1 CpG 10–12 in blood with respect to 
Wechsler IQ scores and CGG expansion size.

The median methylation output ratio (y axis) for CpG 10–12 was significantly increased in FM females with FSIQ and VIQ 
values <70 (x axis) compared with FM females with FSIQ and 
VIQ values >70, but not in FM females with PIQ values <70 
compared with FM with PIQ values >70. The median meth-
ylation output ratio was also significantly increased in FM 
females with FSIQ and VIQ values <70, compared with 
controls with normal CGG repeat sizes and with PM carriers 
with FSIQ, VIQ, and PIQ values >70. The broken line repre-
sents the optimal threshold value (cutoff point) for each IQ 
measure, which was determined with the area under the ROC 
curve and the Youden Index used as summary measures of 
diagnostic accuracy (described in Table 2). FM FSIQ values 
<70 compared with controls: ***, P<0.001. FM IQ values 
<70 compared with PM IQ values >70: #, P<0.05. 
FM IQ values <70 compared with FM IQ values >70: #, P<0.05.
Area-under-the-curve validation of these data with 500 bootstrap replications yielded results similar to those for the original sample (Table 2).

Furthermore, the hypermethylated FM individuals determined to be high functioning according to global IQ assessments, particularly PIQ, were still deficient in arithmetic skills (Fig. 2). Five of 7 of these PIQ false positives had arithmetic subscores below 8, the lower end of the normal range (average scaled normal range is 8–12). Thus, the specificity measures of 91%, 98%, and 97% for FSIQ, VIQ, and PIQ, respectively, at the thresholds of 0.35 and 0.435 presented in Table 2 may be underestimates.

**FMR1 intron 1 methylation in blood is significantly correlated with cognitive scores in FM females**

The level of FREE2 methylation correlated significantly with FSIQ, VIQ, and PIQ within the FM group. Although exonic CpG units 1 and 2 showed a marginally significant correlation (P = 0.049) with FSIQ, VIQ, PIQ, and cognitive subscores (see Table 2 in the online Data Supplement), intronic CpG units 6–12 showed a

<p>| Table 1. Comparisons of median methylation output ratios for FM females reclassified as negative for FSIQ, VIQ, and PIQ values &gt;70 and as positive for FSIQ, VIQ, and PIQ values &lt;70.a |
|---|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Variable</th>
<th>n1,b</th>
<th>n2</th>
<th>med1</th>
<th>med2</th>
<th>IQR1</th>
<th>IQR2</th>
<th>P</th>
<th>Adjusted P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FSIQ &gt;70 (group 1) vs FSIQ &lt;70 (group 2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG 1</td>
<td>12</td>
<td>6</td>
<td>0.42</td>
<td>0.5</td>
<td>0.12</td>
<td>0.07</td>
<td>0.035</td>
<td>0.065</td>
</tr>
<tr>
<td>CpG 2</td>
<td>12</td>
<td>6</td>
<td>0.27</td>
<td>0.29</td>
<td>0.09</td>
<td>0.13</td>
<td>0.06</td>
<td>0.065</td>
</tr>
<tr>
<td>CpG 6/7</td>
<td>12</td>
<td>6</td>
<td>0.47</td>
<td>0.57</td>
<td>0.1</td>
<td>0.1</td>
<td>0.065</td>
<td>0.065</td>
</tr>
<tr>
<td>CpG 8/9</td>
<td>12</td>
<td>6</td>
<td>0.4</td>
<td>0.49</td>
<td>0.08</td>
<td>0.08</td>
<td>0.061</td>
<td>0.065</td>
</tr>
<tr>
<td>CpG 10–12</td>
<td>12</td>
<td>6</td>
<td>0.36</td>
<td>0.47</td>
<td>0.1</td>
<td>0.1</td>
<td>0.035</td>
<td>0.065</td>
</tr>
<tr>
<td><strong>VIQ &gt;70 (group 1) vs VIQ &lt;70 (group 2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG 1</td>
<td>13</td>
<td>5</td>
<td>0.42</td>
<td>0.52</td>
<td>0.1</td>
<td>0.085</td>
<td>0.055</td>
<td>0.055</td>
</tr>
<tr>
<td>CpG 2</td>
<td>13</td>
<td>5</td>
<td>0.27</td>
<td>0.29</td>
<td>0.08</td>
<td>0.16</td>
<td>0.054</td>
<td>0.055</td>
</tr>
<tr>
<td>CpG 6/7</td>
<td>13</td>
<td>5</td>
<td>0.47</td>
<td>0.59</td>
<td>0.09</td>
<td>0.13</td>
<td>0.16</td>
<td>0.027</td>
</tr>
<tr>
<td>CpG 8/9</td>
<td>13</td>
<td>5</td>
<td>0.4</td>
<td>0.49</td>
<td>0.09</td>
<td>0.075</td>
<td>0.009</td>
<td>0.027</td>
</tr>
<tr>
<td>CpG 10–12</td>
<td>13</td>
<td>5</td>
<td>0.35</td>
<td>0.51</td>
<td>0.09</td>
<td>0.11</td>
<td>0.124</td>
<td>0.027</td>
</tr>
<tr>
<td><strong>PIQ &gt;70 (group 1) vs PIQ &lt;70 (group 2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG 1</td>
<td>11</td>
<td>7</td>
<td>0.4</td>
<td>0.49</td>
<td>0.12</td>
<td>0.06</td>
<td>0.046</td>
<td>0.138</td>
</tr>
<tr>
<td>CpG 2</td>
<td>11</td>
<td>7</td>
<td>0.27</td>
<td>0.28</td>
<td>0.08</td>
<td>0.1</td>
<td>0.44</td>
<td>0.440</td>
</tr>
<tr>
<td>CpG 6/7</td>
<td>11</td>
<td>7</td>
<td>0.47</td>
<td>0.56</td>
<td>0.12</td>
<td>0.12</td>
<td>0.09</td>
<td>0.138</td>
</tr>
<tr>
<td>CpG 8/9</td>
<td>11</td>
<td>7</td>
<td>0.4</td>
<td>0.46</td>
<td>0.09</td>
<td>0.1</td>
<td>0.11</td>
<td>0.138</td>
</tr>
<tr>
<td>CpG 10–12</td>
<td>11</td>
<td>7</td>
<td>0.36</td>
<td>0.44</td>
<td>0.11</td>
<td>0.13</td>
<td>0.056</td>
<td>0.138</td>
</tr>
</tbody>
</table>

---

a Comparisons were carried out with the nonparametric Mann–Whitney 2-sample test. P values <0.05 are highlighted in boldface.

b n1, number of negative results (IQ >70); n2, number of positive results (IQ <70); med1, median of group 1; med2, median of group 2; IQR1, interquartile range of group 1; IQR2, interquartile range of group 2.

---

<p>| Table 2. Sensitivity and specificity for FMR1 intronic FREE2 CpG unit 10–12 for detection of FM females with FSIQ, VIQ, and PIQ values &lt;70.a |
|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Variable</th>
<th>AUC,b</th>
<th>95% CI</th>
<th>AUC2</th>
<th>Optimal cutoff value (Youden index)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSIQ, CpG 10–12</td>
<td>0.979</td>
<td>0.953–1.000</td>
<td>0.978</td>
<td>0.350</td>
<td>1.000</td>
<td>0.912</td>
</tr>
<tr>
<td>VIQ, CpG 10–12</td>
<td>0.991</td>
<td>0.978–1.000</td>
<td>0.991</td>
<td>0.435</td>
<td>1.000</td>
<td>0.980</td>
</tr>
<tr>
<td>PIQ, CpG 10–12</td>
<td>0.972</td>
<td>0.948–0.997</td>
<td>0.972</td>
<td>0.350</td>
<td>1.000</td>
<td>0.972</td>
</tr>
</tbody>
</table>

---

a Results of ROC curve analysis for a sample of 74 control, 62 PM, and 18 FM females. Sensitivity and specificity were calculated according to the Youden index optimal cutoff value (0.35 for FSIQ and PIQ, and 0.435 for VIQ).

b AUC2, area under the ROC curve; AUC2, AUC after 500 bootstrap replications.
much stronger correlation with all of these measures ($P \leq 0.014$) (Table 3). Furthermore, of all FREE2 units examined, CpG 10–12 showed significant correlation with most cognitive subscore measures and showed the strongest magnitude of correlation with these measures. These findings were most evident for the relationship with arithmetic skill and with picture arrangement when the results were adjusted for multiple measures ($P = 0.8; P < 0.0001; n = 18$), neither measure was significantly correlated with any of the IQ scores or subtest scores that showed strong correlation with methylation of CpG unit 10–12 when adjusted for multiple measures (Table 3).

**Discussion**

This pilot study has shown that methylation of *FMR1* intronic CpG unit 10–12 (and to a lesser extent units 6/7 and 8/9) is the most significant predictor of cognitive impairment in FM females. This measure differentiates low-functioning females from high-functioning FM, PM, and normal control females with high sensitivity and specificity. The positive methylation thresholds for CpG 10–12 were different for FSIQ, PIQ, and VIQ measures and were determined in this study on the basis of an affected status, which was considered an IQ $< 70$. The threshold of 0.35 was optimal for FSIQ and PIQ assessments and was consistent with the threshold that we have previously reported for an independent cohort of PM and FM females, which was determined with PM upper-range values [see Table 1 in Godler et al. (25)]. When the affected status was based on VIQ results (37), the optimal threshold was higher (0.455), and the specificity was also higher (approaching 100%) than that determined for FSIQ and PIQ measures.

Given that the VIQ is a combination of scores representing different aspects of verbal skills and memory, we considered the set of subtest scores of the VIQ in a correlation analysis with methylation outputs and FMRP production in FM females. We found that most subscores, as well as working memory and organization indices, were significantly correlated with methylation of intronic CpG unit 10–12.

Arithmetic skills, which largely rely on working memory and attention, stood out as the subtest score with the strongest correlation with methylation status of all intronic units, with the correlation again being most pronounced for CpG 10–12. Arithmetic skill was...
also the only phenotypic measure that showed a highly significant relationship with methylation of the exonic units (see Table 2 in the online Data Supplement). Some earlier studies found arithmetic skills to be impaired in FM females, even in the absence of other cognitive impairments (38, 39). Consistent with these data, we found that even in high-functioning FM females (FSIQ, VIQ, and PIQ values >70), analysis of CpG unit 10–12 can identify individuals with specific impairments in arithmetic skills. Thus, the use of methylation analysis of these sites for FXS diagnosis and prognosis has the potential to minimize the number of these FM females who are misclassified as “unaffected” on the basis of an overall assessment of cognitive ability but who may still have impairments in specific cognitive skills, such as working memory and attention.

Importantly, neither the Southern blot FMRI activation ratio nor FMRP production were significantly correlated with any of the IQ measures in this study. Although Southern blot analysis combined with the PCR is the current gold standard in FXS diagnostics, published data concerning relationships of cognitive status with the FMRI activation ratio obtained with Southern blotting are inconsistent, perhaps owing to differences in sample selection. Kaufmann et al. (8) have reported that the Southern blot activation ratio was significantly associated with FSIQ in FM females. de Vries et al. (11) confirmed these findings and reported that PIQ, but not VIQ, was significantly correlated with the activation ratio. On the other hand, Taylor et al. (12) found no correlation between any of these cognitive measures and the Southern blot activation ratio. Given that methylation from random X inactivation varies among different tissues and organs, this finding may also explain why we (and others) have reported the absence of a correlation between the activa-

<table>
<thead>
<tr>
<th>Outcome variable</th>
<th>n</th>
<th>Estimate coefficient</th>
<th>SE</th>
<th>P</th>
<th>Adjusted P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSIQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18</td>
<td>−136.90</td>
<td>39.08</td>
<td>0.003</td>
<td>0.014</td>
</tr>
<tr>
<td>VIQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18</td>
<td>−134.42</td>
<td>39.68</td>
<td>0.004</td>
<td>0.014</td>
</tr>
<tr>
<td>PIQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18</td>
<td>−112.92</td>
<td>34.76</td>
<td>0.005</td>
<td>0.014</td>
</tr>
<tr>
<td>Verbal Comprehension Index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>−68.71</td>
<td>43.53</td>
<td>0.138</td>
<td>0.138</td>
</tr>
<tr>
<td>Vocabulary&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td>−22.17</td>
<td>9.59</td>
<td>0.037</td>
<td>0.039</td>
</tr>
<tr>
<td>Similarity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−43.14</td>
<td>13.21</td>
<td>0.006</td>
<td>0.014</td>
</tr>
<tr>
<td>Information&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−23.53</td>
<td>7.11</td>
<td>0.005</td>
<td>0.014</td>
</tr>
<tr>
<td>Comprehension&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−23.66</td>
<td>10.30</td>
<td>0.036</td>
<td>0.039</td>
</tr>
<tr>
<td>Working Memory Index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>−125.63</td>
<td>43.23</td>
<td>0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>Arithmetic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−28.65</td>
<td>5.92</td>
<td>1.2 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Digit Span&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16</td>
<td>−17.22</td>
<td>6.33</td>
<td>0.017</td>
<td>0.025</td>
</tr>
<tr>
<td>Perceptual Organization Index&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>−225.81</td>
<td>77.00</td>
<td>0.013</td>
<td>0.022</td>
</tr>
<tr>
<td>Block Design&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−19.62</td>
<td>6.83</td>
<td>0.012</td>
<td>0.023</td>
</tr>
<tr>
<td>Picture Completion&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−23.49</td>
<td>8.75</td>
<td>0.017</td>
<td>0.025</td>
</tr>
<tr>
<td>Picture Arrangement&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>−48.69</td>
<td>10.07</td>
<td>1.3 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Block Design&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17</td>
<td>−32.76</td>
<td>14.23</td>
<td>0.037</td>
<td>0.039</td>
</tr>
<tr>
<td>Object Assembly&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
<td>−35.14</td>
<td>13.12</td>
<td>0.034</td>
<td>0.039</td>
</tr>
<tr>
<td>FMRP, % positive&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18</td>
<td>−87.14</td>
<td>37.56</td>
<td>0.034</td>
<td>0.039</td>
</tr>
<tr>
<td>Southern blot activation ratio&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18</td>
<td>−0.89</td>
<td>0.27</td>
<td>0.005</td>
<td>0.0136</td>
</tr>
</tbody>
</table>

* Indicates methylation values for CpG 10–12.
* Indicates methylation values for CpG 2. The FMRP unit of measure is the percentage of positively staining lymphocytes.
* Indicates methylation values for CpG 6/7.
tion ratio (determined via Southern blotting) and cognitive outcome.

In contrast, the significant correlation between the methylation status of intronic CpG unit 10–12 on the one hand, and FSIQ, VIQ, and PIQ suggests that methylation of these sites is more conserved in the blood and brain than methylation of the Nrl site targeted by Southern blotting. Although that result is potentially explained by the difference in genomic location targeted by Southern blot and FREE2 analyses, the possible mechanism that would account for this phenomenon is beyond the scope of the current study.

This study shows that the FREE2 methylation test may have clinical utility in screening newborns for FXS and may be especially attractive for screening female newborns. Identification of FM carriers likely to develop cognitive impairment would allow early therapeutic interventions to be targeted to the girls who are most likely to benefit. In contrast, the test will not detect FM or FM/PM mosaic female infants who will not develop verbal cognitive impairment, and that is also important in the context of newborn screening. Furthermore, the FREE2 methylation test will not identify individuals with PM or GZ mutations that would be identified with CGG-based tests. Detection of these small-sized expansion alleles has been considered inappropriate for newborn screening because they are markers of late-onset nonpreventable diseases of incomplete penetrance (40–42).

In summary, our data show that use of MALDI-TOF mass spectrometry for FREE2 methylation analysis of FMR1 intron 1 sequences is superior to methylation-sensitive Southern blotting and FMRP immunostaining in blood as a predictor of cognitive impairment in female carriers of expanded FMR1 alleles. Because previously developed PCR-based tests for FMR1 CpG island methylation analysis (18) are clinically meaningful only in males, our demonstrated identification of affected females, as well as males with high sensitivity, places the MALDI-TOF mass spectrometry test in a unique position for newborn screening. Considering the test’s high throughput and specificity for pathogenic FM alleles and its minimal DNA requirements, further validation of this test in larger independent cohorts and its implementation into diagnostic settings may offer an ethically acceptable, simple, accurate, and inexpensive assay for FXS screening of the newborn population, as well as targeted screening in both males and females.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: H.R. Slater, Murdoch Childrens Research Institute; D.J. Amor, Victorian Clinical Genetics Services.

Consultant or Advisory Role: None declared.

Stock Ownership: None declared.


Research Funding: Victorian Government’s Operational Infrastructure Support Program; D.E. Godler, E.W. Al Thrasher Award and National Health and Medical Research Council of Australia development grant (no. 1017263); H.R. Slater, National Health and Medical Research Council of Australia development grant (no. 1017263) and E.W. Al Thrasher Award; E. Storey, National Health and Medical Research Council of Australia project grant (no. 330400); R.J. Hagerman, National Institute of Child Health and Human Development grant (no. HD36071) and E.W. Al Thrasher Award; D.Z. Loesch, National Institute of Child Health and Human Development grant (no. HD36071), National Health and Medical Research Council of Australia project grant (no. 330400), and E.W. Al Thrasher Award.

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank the study participants for their contribution and Dr. Benjamin Ong from the Sequenom Platform Facility (Murdoch Childrens Research Institute) and Dr. Annette Taylor and Debbie Hennerich (Kimball Genetics, a division of LabCorp) for methylation analysis, fragile X CGG sizing, and FMRP testing for a proportion of the FM and PM cases. This work is dedicated to the memory of Sasha Godler.

References

9. Godler DE, Tassone F, Loesch DZ, Taylor AK,