Early Detection of Fragile X Syndrome: Applications of a Novel Approach for Improved Quantitative Methylation Analysis in Venous Blood and Newborn Blood Spots

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BACKGROUND: Standard fragile X syndrome (FXS) diagnostic tests that target methylation of the fragile X mental retardation 1 (FMR1) CpG island 5’ of the CGG expansion can be used to predict severity of the disease in males from birth, but not in females.

METHODS: We describe methylation specific–quantitative melt analysis (MS-QMA) that targets 10 CpG sites, with 9 within FMR1 intron 1, to screen for FXS from birth in both sexes. The novel method combines the qualitative strengths of high-resolution melt and the high-throughput, quantitative real-time PCR standard curve to provide accurate quantification of DNA methylation in a single assay. Its performance was assessed in 312 control (CGG <40), 143 premutation (PM) (CGG 56–170), 197 full mutation (FM) (CGG 200–2000), and 33 CGG size and methylation mosaic samples.

RESULTS: In male and female newborn blood spots, MS-QMA differentiated FM from control alleles, with sensitivity, specificity, and positive and negative predictive values between 92% and 100%. In venous blood of FM females between 6 and 35 years of age, MS-QMA correlated most strongly with verbal IQ impairment (P < 0.002). In the larger cohort of males and females, MS-QMA correlated with reference methods Southern blot and MALDI-TOF mass spectrometry (P < 0.05), but was not significantly correlated with age. Unmethylated alleles in high-functioning FM and PM males determined by both reference methods were also unmethylated by MS-QMA.

CONCLUSIONS: MS-QMA has an immediate application in FXS diagnostics, with a potential use of its quantitative methylation output for prognosis in both sexes.

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Fragile X syndrome (FXS) is a severe neurodevelopmental disorder which is complex and heterogeneous in both clinical phenotype and epigenotype. FXS is one of the major inherited conditions comorbid with autistic behaviors, with an incidence of approximately 1 in 4000 in the general population [reviewed in Hagerman et al. (1)]. The trinucleotide expansion of the CGG repetitive sequence in the fragile X mental retardation 1 (FMR1) gene of more than 200 repeats is termed full mutation (FM), and is usually associated with the “shut down” of the gene expression due to methylation of its promoter and loss of its protein product (FMRP), which is essential for normal neurodevelopment (2–5). The more common small expansions, which are termed grey zone (GZ) alleles (44–54 CGG repeats in 1 in approximately 30 individuals) and permutation (PM) alleles (55–199 CGG repeats in 1 in approxi-
mately 300 individuals) (6–8), have been primarily associated with the late-onset conditions (9–11).

Although real-time PCR-based methylation testing for FXS is available (12) and is specific for FXS males carrying the FM with a methylated FMR1 CpG island located 5' of the expansion, there is currently no real-time PCR-based test that can be used to predict specific cognitive and behavioral impairments in female carriers with expanded FMR1 alleles. Using the MALDI-TOF mass spectrometry (MS)–based Epityper system, we have identified novel epigenetic markers for FXS, fragile X–related epigenetic elements 1 (FREE1) and 2 (FREE2), that are inversely correlated with FMRP expression in males and females with expanded FMR1 alleles (13, 14). Using the MALDI-TOF MS Epityper system, we have shown that methylation analysis of the CpG sites located within FMR1 intron 1 of FREE2 (positioned 3' of the expansion) is superior to methylation-sensitive Southern blot (used in current FXS diagnostics) and FMRP immunostaining in blood as a predictor of cognitive impairment and is related to X-inactivation skewing in FM females (15, 16, 17). To make FREE2 methylation analysis accessible for use in most diagnostic laboratories, we have developed a new real-time PCR–based method named methylation-specific–quantitative melt analysis (MS-QMA), which targets methylation of the same intronic elements. We then performed a large-scale validation of the method in previously clinically described cohorts (15, 17–19) and compared its performance attributes to those of the reference methods.

**Methods**

**Participants and Clinical Assessments**

The patient cohort comprised 258 male and 427 female samples from individuals from birth to 82 years of age collected as part of previous studies (15, 17–19). Of these, formal cognitive assessments were performed on samples from 23 FM females, 21 FM females, and 3 high-functioning unmethylated FM (UFM) males [with full scale IQ (FSIQ) between 71 and 81] determined using the Wechsler intelligence test appropriate for chronological age as described in our previous publications (15, 18, 20). The study was approved by the Royal Children’s Hospital and Southern Health and the Institutional Review Board of the University of California at Davis.

**Molecular Studies**

The MS-QMA protocol was based on a combined real-time PCR standard curve method and high-resolution melt (HRM) analysis performed on bisulfite converted DNA as described in Fig. 1. The input was either one 3-mm dried blood spot or DNA extracted from 0.3–1 mL of venous blood, with DNA or blood spot lysate extracted and CGG repeat sized as previously described (17, 21–24). For 19 FM females, the FMR1 activation ratio has been previously determined using methylation-sensitive Southern blot as described in (25). FMRP immunoreactivity in venous blood smears was previously assessed in 18 of these FM females and was expressed as the percentage of lymphocytes staining positive for the protein (26, 27). FREE2 methylation was also assessed in the same samples using the Sequenom Epityper system, as previously described (13).

**MS-QMA of FREE2 Sequence**

**Real-time PCR standard curve method and HRM analysis.** For newborn blood spots (NBS), 1 or 2 3-mm punches from each spot disk were processed as previously described (17). The 96 bisulfite-converted samples (with 3 controls and 93 unknown samples per plate) were serially diluted 4 times postconversion (Fig. 1A). The four 96-well plates were then transferred into a 384-well format for real-time PCR analysis utilizing MeltDoctor™ HRM reagents in 10-μL reactions per manufacturer instructions (Life Technologies). For real-time PCR, we used a unique primer set that targets specific CpG sites within the FREE2 region that were previously shown to be most significantly associated with cognitive impairment in FM females (15). The annealing temperature for the thermal cycling protocol was 65 °C for 40 cycles. The ViiA™ 7 Real-Time PCR System (Life Technologies) was used to initially measure the rate of dye incorporation into double-stranded DNA to quantify the DNA concentrations of the unknown samples using the relative standard curve method (Fig. 1B). The dynamic linear range (usually between 0.05 and 10 ng/μL) was determined from the standard curve using a series of doubling dilutions of a converted DNA standard from a control lymphoblastoid cell line during each run. To progress to the next stage of the analysis, the unknown samples had to be within this dynamic linear range (Fig. 1, wells on the 384 plate in pink). The HRM step followed the real-time PCR without additional sample handling/ sample transfer. In the temperature range of 74 and 82 °C the products from the methylated FREE2 sequence separated into single strands at higher temperatures than those from unmethylated FREE2. The HRM software module for the ViiA 7 system was then used to plot the rate of PCR product separation to single strands at different temperatures with the difference in fluorescence first converted to aligned fluorescence units (AFU) at the temperature that provided the greatest separation between methylated and unmethylated sequences, and then converted to the methylation ratio (MR) from the methylation curve, as described in Fig. 1.
Testing for normality of the distribution of the MR was conducted using the Shapiro–Wilk test at significance level $P < 0.05$. Depending on results of this test, for the intergroup comparisons the following tests were used: 2-sample $t$-tests, if the data were normally distributed, or nonparametric Mann–Whitney tests, if the data were not normally distributed. We also classified individuals with FSIQ, verbal IQ (VIQ), and performance IQ (PIQ) values of 70 and above (referred to as $\geq 70$) as negative and those with FSIQ, VIQ, and PIQ values below 70 (referred to as $< 70$) as positive.

Fig. 1. Overview of MS-QMA of FREE2.

The protocol is based on a single bisulfite converted DNA plate (1 conversion per sample) (A), being, serially diluted 4 times (B), with these dilutions analyzed using the real-time PCR (RT-PCR) standard curve method (C) and HRM analysis using FREE2-specific primers for dried blood spot (DS) DNA PCR products (D). This system utilizes a custom-designed computer algorithm to simultaneously perform steps (E), (F), and (G), which determines DNA concentrations and quality postconversion for all dilutions from the unknown samples from the RT-PCR standard curve (pink diamonds). The unknown sample dilutions (blue star in the graphs in sections E and F) within the concentration and QC ranges (depicted by pink diamonds) are automatically plotted against the HRM methylation standard curve (section G, right). The sample dilutions outside the QC range (depicted by yellow diamonds in the graph in section F) are not utilized in HRM analysis, and are discarded. (G), The HRM methylation standard curve is also automatically obtained from 100% methylated and completely unmethylated control samples spiked at different ratios. The AFU measure for the methylation standard curve and unknowns is analyzed at the melting temperature for which there is the greatest difference in fluorescence between 100% methylated and completely unmethylated controls, which corresponds to the lowest temperature point for which all double-stranded DNA (DS DNA) from the unmethylated strands has completely melted. AFU measurements for the unknowns are then converted to MR from the HRM standard curve (G).
(ASD) referrals for FXS testing as positive, and all other male samples as negative. ROC curve analysis was used to evaluate the ability of MS-QMA MR to classify the positive and negative classes. Area under the ROC curve (AUC) computed using predicted probabilities from logistic regression was used as the summary measure of diagnostic accuracy, and the Youden Index (28) was used to determine the optimal threshold (cutoff point) for MS-QMA analysis.

The relationships between MS-QMA MR and each outcome variable, including cognitive scores and other molecular measures, were assessed using simple linear regression analysis. All analyses were conducted using RMS, DiagnosisMed, and the publicly available R statistical computing package (15, 29).

Results

DEVELOPMENT AND TECHNICAL VALIDATION OF THE FREE2 MS-QMA ASSAY
To assess the intrarun variation and the ability of the assay to predict the expected MR, we performed 16 different spiking experiments (see Fig. 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/supplement). We identified 78 °C as the lowest temperature at which all unmethylated alleles are completely melted, at which point no further fluorescence is emitted. At this temperature, the 100% methylated alleles are actively melting and emitting fluorescence. We found that the most reliable method with the lowest inter- and intrarun variation (2 SDs) and the lowest detection limit (limit of detection of 0.02 MR) used the AFU from the aligned fluorescence curves at 78 °C. This produced a correlation coefficient of 0.998 for the HRM standard curve, representing the relationship between AFU at 78 °C and the expected MR in the spiked samples.

SENSITIVITY AND SPECIFICITY OF MS-QMA ANALYSIS OF VENOUS BLOOD DNA AND CORRELATION OF COGNITIVE IMPAIRMENT IN FEMALES WITH FMR1 EXPANSIONS AND IN FM MALES
We performed MS-QMA analysis in 138 females (Fig. 2) previously examined for the relationship between FREE2 methylation and cognitive impairment using the MALDI-TOF MS-based EpiTYPER system (15) (Fig. 2 and online Supplemental Table 1) and 288 females (Fig. 3), the majority of whom had no formal cognitive assessment performed (Fig. 3). We found that assay sensitivity and specificity for FM females with IQ <70 in the subgroup of 138 participants varied depending on the type of IQ measure used. The optimal threshold of 0.39 MR that provided sensitivity of approximately 95% and specificity of 100% was for detection of FM females with IQ <70 (Table 1A). The threshold of 0.37 MR provided the highest sensitivity and specificity, of approximately 92% and 100%, respectively, for both the FSIQ and PIQ assessments in FM females.

Notably, there was also some overlap between controls and PM and low-functioning FM females at the lower threshold of 0.37 for FSIQ and PIQ (Fig. 2), suggesting that the VIQ threshold of 0.39 should be used instead. At 0.39 MR there were no female controls (CGG <40) with MR values above this threshold. When applied to a group of 288 females (Fig. 3), approximately 50% of all FM females had an MR above 0.39 MR. In contrast, only 1 PM female had an MR above the 0.39 threshold, and only 2 PM females had an MR of 0.39. This equates to 0% of controls and approximately 3% of PM females at or slightly above the VIQ <70 threshold. These 3% of PM females and 19% of FM females had MS-QMA output within a borderline range of 0.39–0.41 MR (Fig. 2 and 3), suggesting that CGG sizing should be used on all samples in this range to resolve any potential overlap between allele classes.

It is also important to note that in this borderline range (0.39–0.41 MR) there was overlap between VIQ <70 and >70 for a proportion of FM females. However, for all PM and FM samples above and below this borderline range, MS-QMA VIQ <70 sensitivity, specificity, and positive and negative predictive values were 100% (Fig. 2 and online Supplemental Fig. 2). Each FM female within the borderline range (0.39–0.41 MR) had 86% probability for VIQ >70 (high functioning). Furthermore, all PM females within this range had 100% probability for VIQ >70, suggesting that in relation to verbal cognitive impairment, the MS-QMA results should be reported as risk or probability depending on where the MR values fall.

Intergroup comparison of MS-QMA in venous blood DNA from 124 males showed that the median MR for FM males was significantly higher for FM males (identified through investigation of developmental delay/ASD), FM methylation mosaics, and PM/FM size mosaics than for male controls, PM males, and high-functioning UFM males with FSIQ, VIQ, and PIQ of >70 (Fig. 3). A threshold of 0.1 MR provided sensitivity and specificity approaching 100% for FM males and FM methylation mosaics, whereas for PM/FM size mosaics the sensitivity was 88% and specificity was 100% at this threshold (Table 1).

CORRELATION OF FREE2 MS-QMA ANALYSIS WITH COGNITIVE SCORES AND OTHER MOLECULAR MEASURES
The level of FREE2 methylation in venous blood DNA determined using MS-QMA correlated significantly with the Wechsler Adult Intelligence Scale (WAIS) (30) FSIQ, VIQ and PIQ, and most WAIS subtest scores (Table 2). The epigenotype–phenotype correlations were most evident for the relationships of MS-
Fig. 2. Comparison between groups of FREE2 MS-QMA MR in venous blood based on Wechsler IQ scores and CGG expansion size in 138 females.

(A), The median MS-QMA MR was significantly increased in FM females with scores < 70 for FSIQ compared to FM females with scores > 70, PM females and controls. This was also the case for VIQ (B), PIQ (C). The broken line represents the optimal threshold value (cutoff point) for each IQ measure determined using AUC and Youden Index as summary measures of diagnostic accuracy described in Table 1. The red broken line represents threshold determined to provide optimal separation for FM females based on VIQ of 70. The purple broken line represents the upper limit of the borderline range where for VIQ there is overlap between FM females with VIQ < 70 and > 70. The blue broken line represents threshold determined to provide optimal separation for FM females based on FSIQ and PIQ of 70, and is equivalent to the maximum value of the female control sample. Note: FM IQ < 70 compared to controls: ***P < 0.001; **P < 0.01; *P < 0.05; FM IQ > 70 compared to PM IQ > 70: ◆◆◆◆P < 0.001; ◆◆P < 0.01; ◆P < 0.05; and FM IQ < 70 compared to FM IQ > 70: ####P < 0.001; ####P < 0.01; #P < 0.05.
QMA MR with VIQ, and the Arithmetic and Information subtests ($P < 0.01$). However, in PM females these subscale and subtest scores did not show significant correlations. In these samples, MS-QMA MR was also significantly correlated with FREE2 CpG sites examined using the EpiTYPER system (see online Supplemental Table 3), with $P < 0.0001$ for all of these sites. Of the other molecular parameters available through our previous studies for these samples (13, 14), FREE2 MS-QMA also showed a significant correlation ($P = 0.018$) with the $FMR1$ activation ratio determined using methylation-sensitive Southern blot, which represents the methylation status of the $FMR1$ CpG island 5’ of the expansion on normal-size alleles in these females. Correlation with FMRP levels was of borderline significance ($P = 0.058$), with no significant correlation observed with CGG size in the FM range of these females (see online Supplemental Table 2).

**MS-QMA DETECTION OF FXS ALLELES IN NBS**

In NBS at the threshold of 0.1 for males and 0.39 for females, the sensitivity, specificity, and positive and negative predictive values were determined.
negative predictive values for the presence of an FM allele approached 100% (Fig. 4 and online Supplemental Fig. 2). For male NBS, the exception was an FM spot which had an MR value identical to those of UFM high-functioning individuals. This was not an artifact because it was shown to be unmethylated using EpiTYPER system analysis of FREE2 \(^{(17)}\). Unfortunately, there was no phenotypic information with this deidentified sample.

Unexpectedly, in NBS, MS-QMA identified more than 90% of all FM females above the 0.39 threshold, whereas in an earlier study \(^{(17)}\) MALDI-TOF MS analysis of CpG10–12 identified only 50% above the affected threshold (which for CpG10–12 was 0.435). In an attempt to explain the NBS discrepancy, we performed MALDI-TOF MS analysis on the same NBS for FREE2 CpG10–12 and CpG6–12 (Fig. 4, C and D). Consistent with the previous study \(^{(17)}\), MALDI-TOF MS analysis of CpG10–12 identified only 50% above the affected threshold. However, inclusion of additional CpG sites for MALDI-TOF MS analysis (present in the MS-QMA amplicon) increased the methylation output ratio of both control and FM groups, and altered the proportion of FM females above the affected threshold (Fig. 4 D). This suggests that differences in the number and the way the CpG sites were analyzed is the most likely the reason why in NBS MS-QMA identified more than 90% of all FM females above the 0.39 threshold, whereas in this and earlier studies \(^{(17)}\) MALDI-TOF MS analysis of CpG10–12 identified only 50% above the affected threshold. Furthermore, although the EpiTYPER approach targets CpG 3, 4, and 5

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### Table 1. Sensitivity and specificity for MS-QMA MR in venous blood DNA.\(^{a}\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>AUC</th>
<th>SE</th>
<th>95% CI</th>
<th>Optimal cutoff value (Youden index) for MS-QMA MR</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females(^{b})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSIQ</td>
<td>0.983</td>
<td>0.013</td>
<td>0.946–0.998</td>
<td>0.37</td>
<td>1.000</td>
<td>0.911</td>
</tr>
<tr>
<td>VIQ</td>
<td>0.993</td>
<td>0.007</td>
<td>0.958–0.999</td>
<td>0.39</td>
<td>1.000</td>
<td>0.943</td>
</tr>
<tr>
<td>PIQ</td>
<td>0.983</td>
<td>0.011</td>
<td>0.946–0.998</td>
<td>0.37</td>
<td>1.000</td>
<td>0.918</td>
</tr>
<tr>
<td>Males(^{c})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FM males with ASD</td>
<td>1.000</td>
<td>0.000</td>
<td>0.965–1.000</td>
<td>0.10</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>FM methylation mosaics</td>
<td>1.000</td>
<td>0.000</td>
<td>0.935–1.000</td>
<td>0.10</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>PM/FM size mosaics</td>
<td>0.959</td>
<td>0.033</td>
<td>0.876–0.991</td>
<td>0.10</td>
<td>0.8824</td>
<td>1.000</td>
</tr>
</tbody>
</table>

\(^{a}\) Specificity and methylation cutoff values were calculated at 100% sensitivity. Sensitivity and specificity were calculated based on the Youden index optimal cutoff value.

\(^{b}\) FM females with FSIQ, VIQ, and PIQ <70 determined using the ROC analysis in a sample of 94 controls and 23 PM and 21 FM females. Sensitivity and specificity were calculated based on the Youden index optimal cutoff value, which for FSIQ and PIQ was 0.37, and for VIQ was 0.39.

\(^{c}\) Fifty-two FM males identified through investigation of developmental delay/ASD, 4 FM methylation mosaics, and 17 PM/FM size mosaics in a sample of 124 males, including 20 male control and 28 PM and 3 high-functioning FM males with FSIQ, VIQ, and PIQ >70, who were determined to be unmethylated using methylation-sensitive Southern blot identified through cascade testing.

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### Table 2. Relationships between FREE2 MS-QMA MR (predictor) with outcome variables WAIS FSIQ, subcales, and subtests using simple linear regression.

<table>
<thead>
<tr>
<th>Outcome variable</th>
<th>n</th>
<th>Estimated coefficient</th>
<th>SE</th>
<th>P*</th>
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<tbody>
<tr>
<td>FSIQ</td>
<td>20</td>
<td>-236.7</td>
<td>64.99</td>
<td>0.002**</td>
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<tr>
<td>VIQ</td>
<td>19</td>
<td>-239.0</td>
<td>64.64</td>
<td>0.002**</td>
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<td>PIQ</td>
<td>19</td>
<td>-184.5</td>
<td>66.59</td>
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<td>Verbal comprehension index</td>
<td>15</td>
<td>-197.9</td>
<td>72.95</td>
<td>0.018*</td>
</tr>
<tr>
<td>Perceptual organization index</td>
<td>15</td>
<td>-161.5</td>
<td>80.34</td>
<td>0.066</td>
</tr>
<tr>
<td>Working memory index</td>
<td>9</td>
<td>-201.5</td>
<td>72.57</td>
<td>0.027*</td>
</tr>
<tr>
<td>Processing speed index</td>
<td>15</td>
<td>-115.0</td>
<td>52.42</td>
<td>0.047*</td>
</tr>
<tr>
<td>Picture completion</td>
<td>17</td>
<td>-40.43</td>
<td>14.19</td>
<td>0.012*</td>
</tr>
<tr>
<td>Vocabulary</td>
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<td>-44.63</td>
<td>15.75</td>
<td>0.013*</td>
</tr>
<tr>
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<td>-28.87</td>
<td>11.91</td>
<td>0.031*</td>
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<td>13.72</td>
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<td>-41.15</td>
<td>10.13</td>
<td>0.001**</td>
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<td>18.34</td>
<td>0.265</td>
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<tr>
<td>Digit span</td>
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<td>-23.48</td>
<td>12.61</td>
<td>0.084</td>
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<tr>
<td>Information</td>
<td>17</td>
<td>-42.85</td>
<td>12.19</td>
<td>0.003**</td>
</tr>
<tr>
<td>Picture arrangement</td>
<td>15</td>
<td>-31.67</td>
<td>12.11</td>
<td>0.021*</td>
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<tr>
<td>Comprehension</td>
<td>17</td>
<td>-42.24</td>
<td>17.54</td>
<td>0.029*</td>
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<td>0.086</td>
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<td>Letter number sequencing</td>
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<td>14.49</td>
<td>0.024*</td>
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<td>Object assembly</td>
<td>17</td>
<td>-21.27</td>
<td>13.90</td>
<td>0.147</td>
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</table>

\(^* P < 0.05; ** P < 0.01.\)
within FREE2, these cluster as 1 fragment which is too big in size (Da) to be captured within the mass spectrum. For this reason, CpGs 3–5 cannot be analyzed by the EpiTYPER approach.

**Discussion**

In this study we present a novel method, MS-QMA, which is much simpler and more cost-effective than the Sequenom EpiTYPER approach for methylation analysis of the FREE2 region if used in FXS diagnostics and population screening. In females the FMR1 activation ratio determined using the Southern blot was significantly correlated with FREE2 methylation assessed using MS-QMA and the EpiTYPER system (14). In males, 2 PM/FM mosaics and 3 high-functioning FM males (IQ > 70) unmethylated in the FMR1 CpG island 5’ of the expansion by Southern blot (13), were below the 0.1 MR threshold within FREE2 as determined using MS-QMA and the EpiTYPER system (Fig. 4A). Therefore, the ability of MS-QMA to differentiate the high-functioning FM males from the typical FXS males is likely to be of prognostic value when used for early detection of FXS in males.

A somewhat surprising finding was that in NBS at a 0.39 threshold MS-QMA identified not only most FM males, but also almost all FM females, with sensitivity, specificity, and positive and negative predictive values
for the presence of an FM allele between 92% and 100%. For this reason, we suggest that the MS-QMA test may have applications for early detection of all FXS FM in females as well as males, particularly if used within the first year of life.

However, in the age range from 6 to 35 years at the same threshold, in venous blood MS-QMA identified only 50% of all FM females. This FM group had VIQ of <70, with the test showing sensitivity of 100% and specificity approximately 95% or VIQ <70. Rather than a technical issue or bias of ascertainment, the likely explanation for this is that the age range of the participants was different between venous blood and NBS cohorts. Whereas in the venous blood for FM, PM, and control females there was no significant relationship between age and MS-QMA output (see online Supplemental Fig. 3), the venous blood cohort had only a few FM females in the age range below 5 years, and no FM females in the first year of life.

If there is stochastic loss of methylation at 1 or more of the CpG sites within the FREE2 amplicon in FM females in the early years of life, as suggested by this study, it would be consistent with our earlier cross-sectional age vs methylation comparisons in FM females (16). One of the mechanisms that may explain this possibility is that such changes in methylation over time may be due to gradual selection for cells expressing FMRP in FM females (16). So the small proportion of cells that express FMRP at birth may increase over time because of selection advantage, and this would be accompanied by proportional decreases in methylation of CpG sites negatively correlated with FMRP expression in blood. In fact, using MALDI-TOF MS, we have determined that the levels of methylation of at least 3 CpGs within the FREE2 amplicon increase at birth and early childhood, and the decreases with age until puberty in FM females but not in FM males (16). MS-QMA provides combined methylation output from 10 CpG sites (CpG2 to -12), including those with methylation reported to change with age (16), those that have stable methylation over time, and 3 additional CpG sites for which the methylation status could not be analyzed by MALDI-TOF MS owing to their fragments being too large. Together this may explain the twin and related issues of the MS-QMA and MALDI-TOF MS differences for NBS (Fig. 4) and similarities for older venous blood.

METHYLATION ANALYSIS FOR NEWBORN SCREENING AND IN THE DIAGNOSTIC CONTEXT IN VENOUS BLOOD

This study confirmed our previous finding in venous blood, using a different method, of significant correlations between FMR1 intron 1 methylation and measures of cognitive function (15). In the subgroup of 20 FM females for whom we had a set of IQ subscales, subtest scores, and indices available, we found that MS-QMA MR strongly correlated with subtest scores representing different aspects of VIQ. Arithmetic subtest scores (Working Memory Index), which largely rely on working memory and attention, and the Information subtest scores (Verbal Comprehension Index), which examine general knowledge, stood out as the subtest scores most strongly correlated with MS-QMA MR (P < 0.01). It is also of interest that most FM and all PM females who were within the MS-QMA borderline range 0.39–0.41, were high-functioning (VIQ >70). This, however, does not rule out other forms of FXS-related impairment that may be identified using more subtle measures of cognitive function, such as the subscores and indexes of IQ, or measures of behavioral impairment such as the Autism Diagnostic Observation Schedule—Generic.

The current study also suggests that an increase in the MS-QMA threshold to 0.47 at birth in venous blood and NBS of FM females may predict the high sensitivity and specificity for verbal cognitive impairment in FM females at 6 years of age and older. This threshold separates NBS from 15 FM females into 2 almost equal parts in the same way that the lower 0.39 threshold separates the 20 venous blood samples in older FM females and is strongly related to cognitive impairment. Validation studies are now underway to address the sample size and age range limitations by increasing the venous blood and NBS sample sizes, especially for FM females in the age range of <5 years. These studies will also examine longitudinally the changes in MS-QMA MR during this period and the value in the first year of life of the MS-QMA analysis at the 0.47 threshold to predict cognitive impairment at >2 years of age in FM females.

Based on the pilot data presented in this study, the MS-QMA methylation assay has important potential for use in newborn FXS screening because there has been no test available that is suitably sensitive in males and females and has high throughput and low cost. The benefits of identifying most male and female probands early are improved clinical management, identification of other carriers through cascade testing, and the provision of this information for reproductive planning. As part of the first NBS screen, the lower 0.39 threshold could be used, followed by second-line testing that would involve CGG sizing, and this would confirm that all positives carry an FM allele. This may present a better alternative to using CGG sizing as a first-line test in newborns or very young children, because detection of PM alleles that have been associated with late-onset disorders (30, 31) would raise the ethical issue of presymptomatic testing for currently untreatable and nonpreventable disorders with incomplete penetrance (32). Furthermore, detection of the relatively common
GZ and PM alleles which do not cause FXS at a population-wide level, would require large-scale genetic counseling follow-up, which would have significant add-on cost–benefit implications.

In the diagnostic context, MS-QMA can be easily combined with several PCR-based approaches recently developed to reliably amplify PM and small FM alleles (23, 33, 34). MS-QMA methylation values could be used to accurately separate most high-end unmethylated PM from low-end methylated FM alleles, and may in the future provide prognostic information from quantitative methylation data in both males and females. This could remove the need for the cumbersome Southern blot and identify all categories of expanded alleles.

In summary, the intended applications for MS-QMA methylation analysis in venous blood or NBS are to identify (a) those individuals likely to be cognitively impaired, as reflected by low VIQ (<70) at >2 years of age, with analyses performed any time after birth, and (b) most FM males and females through NBS or venous blood samples, with analyses performed in the first year of life. The main difference between the 2 applications (which are not mutually exclusive) is in the thresholds used to distinguish between the positive and negative cases. In this study, the utility of the borderline range between 0.39 and 0.41 MR in venous blood could effectively split the FM female group at >5 years of age into 2 equal parts and differentiate low-functioning FM females (VIQ <70), from high-functioning FM and PM females and from females that do not have an expanded FMRI allele. In NBS, however, the cutoff threshold had to be increased to 0.47 MR to achieve the same 50/50 split for FM females analyzed at birth. We suggest that this may be due to stochastic loss of methylation at 1 or more of Cpg sites within the FREE2 amplicon, and that future longitudinal studies are required to validate the prognostic potential of this assay if tests are performed at birth or the first year of life to predict cognitive impairment at >2 years of age. This would maximize the period when the therapeutic intervention may be most effective. However, if the lower 0.39 threshold is used in NBS, we show that it can identify more than 90% of all FM males and females. This is superior to the comparator EpiTYPER system, which targets the same sites as MS-QMA but cannot analyze some of these because their fragments are too large for MALDI-TOF MS–based assessment (35).

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