Molecular and imaging correlates of the fragile X–associated tremor/ataxia syndrome

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Abstract—*Objectives:* To assess changes in regional brain volumes associated with the fragile X–associated tremor/ataxia syndrome (FXTAS) and the molecular correlates of these changes. *Methods:* We administered molecular, MRI, and neurocognitive tests to 36 male premutation carriers (ages 51 to 79), 25 affected and 11 unaffected with FXTAS, and to 21 control subjects of similar age and education. *Results:* We found differences among the three groups in whole brain, cerebrum, cerebellum, ventricular volume, and whole-brain white matter hyperintensity, with the affected group showing significantly more pathology than the control and unaffected groups. Brainstem volume was significantly smaller in the unaffected group vs controls but did not differ from the affected group. Within the premutation sample, CGG repeat length correlated with reductions in IQ and cerebellar volume and increased ventricular volume and whole-brain white matter hyperintensity. *Conclusions:* The current findings, coupled with recent evidence linking the degree of neuropathology (numbers of intranuclear inclusions) to the size of the premutation allele, provide evidence that the neurodegenerative phenotype in the fragile X–associated tremor/ataxia syndrome is a consequence of the CGG repeat expansion.

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Fragile X–associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurologic disorder predominately affecting older (>50 years) male carriers of the premutation allele (55 to 200 CGG repeats) in the 5′ untranslated region of the *FMR1* gene.[1,2] Previously reported symptoms of FXTAS include progressive gait ataxia, intention tremor, and cognitive decline.[1-6] Radiologic abnormalities include periventricular, subcortical, and cerebellar bilateral white matter lesions, cerebellar and cerebral atrophy, and enlarged ventricles.[2,7] Postmortem neuropathologic examination has revealed eosinophilic, intranuclear inclusions in both the neurons and the astroglial cells of the cortex, brainstem, and spinal cord.[8,9] Recent evidence indicates that a significant association exists between a higher CGG repeat length and the number of intranuclear inclusions.[9] We have proposed an RNA "toxic gain-of-function" model for FXTAS,[1,4] which suggests that binding and consequent dysregulation of one or more proteins by the elevated *FMR1* mRNA lead to cell stress/toxicity, with subsequent inclusion formation and cell death as downstream consequences of this dysregulation.[10,11]

Previous studies of the relationship between molecular abnormalities and changes in brain structure in premutation carrier males, both with and without FXTAS, found a correlation between higher CGG repeat size and decreased volume of various brain regions, including the cerebellum, brainstem, and amygdala/hippocampal complex, as measured with structural MRI.[12]

In this study, we report a controlled study of adult male premutation carriers, with and without FXTAS, involving molecular analysis of *FMR1* expression, quantitative neuroimaging, and cognitive testing. Additionally, we evaluated the relationship between age and *FMR1* expression associated with decreased brain volume and cognitive ability.

Methods. *Subjects.* We recruited patients and control subjects through families with children affected by FXS and through referrals of patients affected by FXTAS. In many cases, the children with FXS were the index cases through which participants for this study were recruited. As part of the current Fragile X Genotype–Phenotype Project at the MIND Institute, a comprehensive (cascade) testing has been conducted to identify additional family members who may have the full or premutation. This process also

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allows for identification of family-member controls that do not have an *FMR1* mutation. We recruited two additional control subjects of similar age and high education level from university emeriti faculty. All male premutation carriers over age 50 who agreed to participate were included in this study, regardless of presentation of FXTAS symptoms. Following clinical examination, subjects were grouped by presence or absence of tremor or ataxia. Eleven premutation carriers with no significant motor symptoms on clinical neurologic examination were considered unaffected by FXTAS. Twenty-five premutation carriers with tremor or ataxia, according to previously reported diagnostic criteria,[2] were considered affected by FXTAS. There were 21 control subjects of similar age and education levels.

Neuroimaging. Structural MR images were acquired using a 1.5 T GE Signa Horizon LX Echospeed system. The acquisition parameters were as follows: coronal three-dimensional spoiled gradient-recalled echo (inversion recovery prepped SPGR) acquisition, T1-weighted; coronal plane, three-dimensional acquisition, gradient-recalled echo, radiofrequency-spoiled, repetition time (TR): 9.1 milliseconds, spatial resolution: 0.9375 × 0.9375 × 1.5–mm thickness; high-resolution fluid-attenuated inversion recovery (FLAIR) (same orientation as axial spin echo); oblique axial plane, two-dimensional acquisition, inversion recovery spin echo, echo time:144 milliseconds, TR: 11,000 milliseconds, inversion time: 2,250 milliseconds, 14 slices/acquisition, two interleaved acquisitions, resolution 0.9375 × 0.9375 × 3–mm thickness, 0-mm interslice on reconstructed image.

MRI quantification was performed using a custom-written computer program operating on a UNIX Solaris platform (Quanta 6.1). Image evaluation was based on a semiautomatic segmentation analysis involving operator-guided removal of nonbrain elements as previously described.[13] In brief, nonbrain elements were manually removed from the image by operator-guided tracing of the dura mater within the cranial vault, including the middle cranial fossa, posterior fossa, and cerebellum. The resulting measure of the cranial vault was defined as the total cranial volume (TCV) and served as an estimate of head size. All volumes were normalized to TCV.

The TCV was segmented into CSF, brain matter, and white matter hyperintensity volume according to previously published methods.[13-16] The TCV was further subdivided into regions of interest, including cerebrum, cerebellum, brainstem, lateral ventricles, and third ventricle. For the current study, lateral and third ventricular volumes were summed for a total ventricular region of interest. The brainstem–cerebellar region extended from one slice inferior to the anterior commissure to the most inferior slice containing the cerebellar vermis. The inferior border of the cerebellar vermis was used as a tracing cutoff, as it was the most inferior landmark present in all scans. The above measurements were performed on axial FLAIR images.

Hippocampal volumes were quantified by operator-guided tracing as described previously.[15] The sampled hippocampus included the CA1-CA4 fields, dentate gyrus, and subicular complex. Quantification of hippocampal volume was performed on coronal three-dimensional SPGR images.

Intrarater reliability was determined using intraclass correlation coefficients (ICCs) with a minimum score of 0.97 needed for reliability. ICCs were 0.99 for total cranial volume, 0.98 for cerebral volume, 0.98 for right hippocampal volume, 0.97 for left hippocampal volume, and 0.99 for volume of white matter hyperintensity. A single rater performed all of the analyses and was blinded to subjects' experimental condition, molecular status, and demographic information.

Neurocognitive testing. All subjects were administered the Wechsler Adult Intelligence Scale-III,[17] from which Verbal IQ (VIQ), Performance IQ (PIQ), and Full-Scale IQ (FSIQ) were obtained.

Molecular measures. Blood was drawn for all study patients except one who was unavailable. This individual was an obligate carrier by pedigree analysis. For DNA analysis, genomic DNA was isolated from peripheral blood leukocytes (5 mL of whole blood using standard methods; Puregene Kit, Gentra). For southern blot analysis, 5 to 10 μg of isolated DNA was digested with *Eco*R1 and *Nru*I. The probe used in the hybridization was the *FMR1*-specific dig-labeled StB12.3. Details were as previously described.[18] Genomic DNA was also amplified by PCR as previously described.[19] Analysis and calculation of the repeat size for both

Table 1 *Descriptive statistics and comparisons across groups*

	Controls, n = 21		Unaffected, n = 11		Affected, n = 25	
	Median	(Range)	Median	(Range)	Median	(Range)
Age	63	(51–79)	61[a*]	(51–78)	67[u*]	(53–79)
CGG	30[u‡,a‡]	(18–42)	79.5[c‡]	(55–163)	90[c‡]	(62–130)
mRNA	1.43[u‡,a‡]	(0.63–2.00)	3.75[c‡]	(2.09–7.02)	3.44[c‡]	(1.98–9.76)
Whole brain vol	0.85[a‡]	(0.78–0.91)	0.86[a†]	(0.76–0.89)	0.77[c‡,u†]	(0.65–0.88)
Cerebral vol	0.757[a‡]	(0.693–0.795)	0.769[a†]	(0.687–0.79)	0.684[c‡,u†]	(0.569–0.795)
Cerebellar vol	0.075[a‡]	(0.062–0.092)	0.077[a†]	(0.057–0.08)	0.06[c‡,u†]	(0.042–0.078)
Brainstem vol	0.02[u*,a†]	(0.017–0.027)	0.019[c*]	(0.0154–0.022)	0.017[c†]	(0.015–0.022)
Hippocampal vol	0.0026	(0.0022–0.003)	0.0026	(0.002–0.0033)	0.0024	(0.0014–0.0031)
Ventricles	0.009[u*,a‡]	(0.005–0.027)	0.009[c*,a†]	(0.003–0.029)	0.031[c‡,u†]	(0.006–0.076)
Whole-brain wmh	0.0012[a‡]	(0.0007–0.0068)	0.0018[a*]	(0.0009–0.0034)	0.0089[c‡,u*]	(0.0006–0.0372)
Cerebellar wmh	0.0000286[a*]	(1.07E-05–8.72E-05)	2.72E-05	(1.08E-05–5.66E-05)	0.000325[c*]	(1.64E-05–315.2E-05)
FSIQ	119.5[a†]	(90–147)	116	(95–128)	104[c†]	(66–129)
PIQ	114.5[a†]	(89–134)	110.5	(99–124)	100[c†]	(63–121)
VIQ	121[a†]	(84–148)	119[a*]	(84–142)	107[c†,u*]	(74–135)

The volumetric labels in the first column are normalized to total cranial volume. Significance was evaluated using an age-adjusted nonparametric analysis of variance (Kruskal–Wallis). Post hoc pairwise comparisons of group were performed using age-adjusted Mann–Whitney *U* tests: [c] significant difference vs the control group; [u] significant difference vs the unaffected premutation carrier group; [a] significant difference vs the affected premutation carrier group.

* $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$.

vol = volume; wmh = white matter hyperintensity; FSIQ = Full-Scale IQ; PIQ = Performance IQ; VIQ = Verbal IQ.

southern blot and PCR analysis were carried out using an Alpha Innotech FluorChem 8800 Image Detection System.

FMR1 mRNA levels. All quantifications of *FMR1* mRNA were performed using a 7700 and a 7900 Sequence detector (PE Biosystems), as previously described.[20]

Analysis. Descriptive statistics were computed for the control, unaffected, and affected groups for age, molecular, radiologic, and cognitive measures. Differences between these groups were tested using the Kruskal–Wallis nonparametric one-way analysis of variance by ranks. Post hoc pairwise comparisons of group were performed using Mann–Whitney *U* test. These nonparametric tests were necessary given the small sample sizes for each group and the lack of normality in the distribution of values within the groups.

With selection of only premutation carriers, age-adjusted correlations between molecular, volumetric, and cognitive variables were estimated and tested using Spearman (partial) rho. To assess age interactions, we compared patterns of association between middle-aged (51 to 64 years) and old-age (65 to 79 years) groups of premutation carriers.[21]

Sidak adjustments were used to control for the possible inflation of type I error due to multiple comparisons within each family of hypotheses (e.g., the associations between brain volume measures and cognitive outcomes). These adjustments control for the high degree of correlation between the multiple outcomes.[22] Across all the hypothesis families, the most conservative adjusted p value was 0.02, and this was used as a guide for evaluating the significance.[22]

Results. There was a significant difference in age distributions across the three groups with the post hoc pairwise comparisons indicating that, on average, the affected group was older than the unaffected group ($z = 2.12$, $p = 0.03$), although not older than the control group ($z = 1.67$, $p = 0.10$) (table 1). These differences are also evident descriptively when examining the median ages for the three groups. Because of this age difference, subsequent comparisons across groups, as well as correlations among premutation carriers (described below), used an age-adjusted residual value. Molecular comparisons indicate no difference in CGG repeat length or level of *FMR1* mRNA between unaffected and affected premutation carriers.

Comparison of age-adjusted regional brain volumes across the control, unaffected, and affected groups indicates differences for most areas measured, including whole brain ($\chi^2 = 24.1$, $p < 0.001$), cerebrum ($\chi^2 = 18.8$, $p < 0.001$; figure, A), cerebellum ($\chi^2 = 19.1$, $p < 0.001$), brainstem ($\chi^2 = 9.3$, $p = 0.009$), and ventricles ($\chi^2 = 22.3$, $p < 0.001$), as well as whole-brain white matter hyperintensity volume ($\chi^2 = 14.5$, $p < 0.001$) (table 1). A difference was found for cerebellar white matter hyperintensity ($\chi^2 = 6.3$, $p = 0.04$) between groups. Pairwise comparisons indicate no difference between control and unaffected groups in most of the brain regions measured except for brainstem volume ($z = 2.0$, $p = 0.05$; figure, C) and differences between control and affected groups in all regions measured except for hippocampus ($z = 1.53$, $p = 0.13$; figure, B). Between unaffected and affected groups, differences in regional volumes were found in most areas except for hippocampus, brainstem, and cerebellar white matter hyperintensity (table 1).

FSIQ, PIQ, and VIQ scores were lower in the affected group than the control group (FSIQ: $z = 3.24$, $p = 0.001$; PIQ: $z = 3.46$, $p = 0.001$; VIQ: $z = 2.62$, $p = 0.009$); they were also lower (though not significantly) vs the unaffected group (table 1). There was no difference in these measures between the unaffected and control groups.

Age-adjusted correlations among premutation carriers indicate that level of CGG repeat length was associated

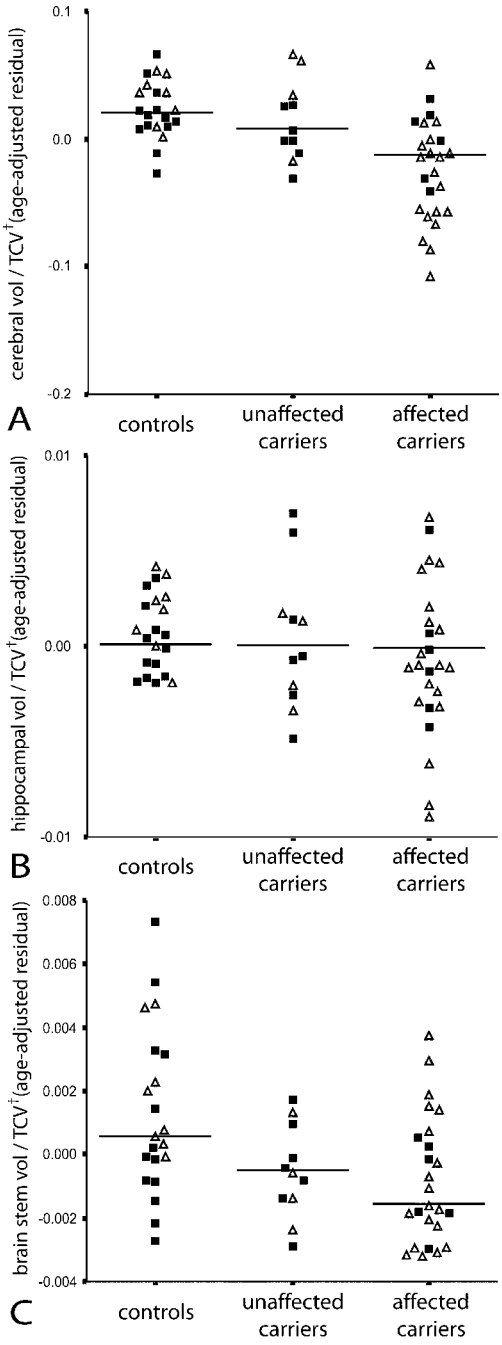

Figure. Comparison of age-adjusted cerebral volume (A), hippocampal volume (B), and brainstem volume (C) between controls, unaffected premutation carriers, and premutation carriers affected with fragile X–associated tremor/ataxia syndrome (FXTAS). The younger age group (age 51 to 64) is represented by filled squares. The older age group (age 65 to 79) is represented by open triangles. Nonparametric analysis of variance showed differences between groups for cerebral volume ($\chi^2 = 18.79$, $p < 0.001$) and brainstem volume ($\chi^2 = 9.35$, $p < 0.009$). Post hoc tests showed differences between controls and carriers with FXTAS in cerebral volume ($z = 4.13$, $p < 0.001$) and brainstem volume ($z = 2.88$, $p = 0.004$). There was also a difference between unaffected carriers and carriers with FXTAS in cerebral volume ($z = 2.59$, $p = 0.006$) but not in brainstem volume. There were no significant differences between groups in hippocampal volume. †Total cranial volume.

Table 2 *Correlations of CGG repeat length and age with brain volumes and IQ in premutation carriers*

	CGG				Age	
	Spearman rho (unadjusted)		Spearman rho partial (age-adjusted)		Spearman rho (unadjusted)	
	r_s	p	r_s	p	r_s	p
Whole brain vol	−0.041	0.82	−0.353	0.04	−0.715	<0.001*
Cerebral vol	0.016	0.93	−0.3	0.08	−0.736	<0.001*
Cerebellar vol	−0.235	0.17	−0.499	0.002*	−0.431	0.009*
Brainstem vol	0.154	0.38	−0.013	0.94	−0.525	0.001*
Hippocampal vol	−0.213	0.22	−0.32	0.099	−0.23	0.178
Ventricles	0.245	0.16	0.635	<0.001*	0.568	<0.001*
Whole-brain wmh	0.375	0.03	0.476	0.004*	0.417	0.011*
Cerebellar wmh	0.003	0.99	0.201	0.25	0.479	0.003*
FSIQ	−0.423	0.01*	−0.544	0.001*	−0.254	0.14
PIQ	−0.38	0.03	−0.485	0.004*	−0.163	0.35
VIQ	−0.385	0.02*	−0.48	0.004**	−0.229	0.179

* $p \leq 0.02$.

vol = volume; wmh = white matter hyperintensity; FSIQ = Full-Scale IQ; PIQ = Performance IQ; VIQ = Verbal IQ.

with the volume of many of the brain regions measured, including cerebellum ($r = -0.3$, $p = 0.002$), ventricles ($r = 0.635$, $p < 0.001$), and whole-brain white matter hyperintensity ($r = 0.476$, $p = 0.004$) (table 2). Whole-brain ($r = -0.353$, $p = 0.04$) and cerebral ($r = -0.3$, $p = 0.08$) volumes exhibited a trend toward significant association with CGG repeat length. Volumes of brainstem, hippocampus, and cerebellar white matter hyperintensity did not show association with CGG repeat length (table 2). When the younger premutation carrier group (ages 51 to 64) was analyzed separately, no association was found between CGG repeat and any volumetric measures. In contrast, among the older group of premutation carriers (ages 65 to 79), CGG repeat was associated with volume of whole brain ($r = -0.553$, $p = 0.006$), cerebrum ($r = -0.498$, $p = 0.02$), cerebellum ($r = -0.532$, $p = 0.009$; table 2), ventricles ($r = 0.761$, $p < 0.001$), and whole-brain white matter hyperintensity ($r = 0.673$, $p < 0.001$). In the older group, hippocampal volume nearly reached significant association with CGG repeat length ($r = -0.453$, $p = 0.03$).

Levels of *FMR1* mRNA were associated with ventricular volume ($r = 0.664$, $p = 0.02$) within the younger age group only. The lack of association in the older group may be a matter of restricted range in *FMR1* mRNA in the older sample.

When considering the associations between cognitive level and regional brain volume, we analyzed all premutation carriers together because we did not hypothesize differential patterns of association across age groups. FSIQ and PIQ scores were significantly associated with the volumes of many brain regions including whole brain, cerebrum, cerebellum, hippocampus, ventricles, and whole-brain white matter hyperintensity (table 3). The magnitude of associations between VIQ scores and regional brain volumes was not as strong as FSIQ and PIQ, with the exception of whole-brain white matter hyperin-

Table 3 *Correlation between brain volumes and IQ in premutation carriers (Spearman rho, age-adjusted)*

	FSIQ		PIQ		VIQ	
	r_s	p	r_s	p	r_s	p
Whole brain vol	0.464	0.005*	0.465	0.005*	0.323	0.05
Cerebral vol	0.474	0.004*	0.438	0.009*	0.355	0.03
Cerebellar vol	0.392	0.02*	0.461	0.005*	0.201	0.24
Brainstem vol	0.043	0.80	0.188	0.28	−0.087	0.62
Hippocampal vol	0.476	0.004*	0.461	0.005*	0.316	0.06
Ventricles	−0.519	0.002*	−0.564	0.001*	−0.37	0.03
Whole-brain wmh	−0.636	<0.001*	−0.531	0.001*	−0.613	<0.001*
Cerebellar wmh	−0.261	0.13	−0.184	0.29	−0.308	0.07

* $p \leq 0.02$.

vol = volume; wmh = white matter hyperintensity; FSIQ = Full-Scale IQ; PIQ = Performance IQ; VIQ = Verbal IQ.

tensity ($r = -0.613$, $p < 0.001$). Cerebral volume ($r = 0.355$, $p = 0.03$) and ventricular volume ($r = -0.37$, $p = 0.03$) showed near significant associations with VIQ. Cerebellar volume was not associated with VIQ (table 3).

Molecular correlations among the premutation carriers indicate that higher CGG repeats are associated with lower FSIQ ($r = -0.544$, $p = 0.001$), PIQ ($r = -0.485$, $p = 0.004$), and VIQ ($r = -0.48$, $p = 0.004$). The younger age group of premutation carriers shows a similar pattern of association to the older premutation carriers in terms of magnitude and direction; however, only the association between FSIQ and CGG repeat length reached significance ($r = -0.678$, $p = 0.015$; table 2). *FMR1* mRNA was not significantly associated with any of the IQ measures.

Discussion. We report significant volume loss in the whole brain, cerebrum, and cerebellum, as well as increases in whole-brain white matter hyperintensity volume associated with FXTAS. These changes correlate with CGG repeat number and become more severe with age. We also see subtle differences between unaffected carriers and controls with a smaller brainstem and a trend toward increased ventricle volume in unaffected carriers. It is possible that these relatively subtle brain differences are early indicators of later-developing FXTAS. However, longitudinal studies are needed to confirm this hypothesis. Alternatively, these brain changes could represent a developmental effect of the premutation caused by high mRNA levels or perhaps subtle FMRP deficits. Hippocampal volume does not appear to be significantly affected by FXTAS, although we know that the hippocampus has the highest rate of inclusions[8] relative to other regions measured. In a previously published study, larger hippocampi were found in unselected older premutation carriers compared with controls,[6] perhaps related to a developmental effect of the premutation. However, increased CGG repeat length has been associated with reduced hippocampal volume[23] and reduced gray matter density in the amygdalohippocampal complex in unaffected male premutation carriers.[12] Further research should clarify the relationship between molecular variables and hippocampal size in premutation carriers.

When considering the associations of molecular abnormalities of the *FMR1* premutation with FXTAS, it is clear that increased CGG repeat length adversely affects many brain regions. CGG repeat size in blood and brain are typically consistent in patients assessed in postmortem studies[8,18]; however, *FMR1* mRNA levels are different between blood and brain and also vary in each region of the brain with the highest levels observed in the hippocampus.[18] Therefore, it is not surprising that the mRNA levels of blood do not correlate with brain volumes as do the CGG repeats.

We compared regional brain volumes with the general measures of cognition provided by FSIQ, PIQ, and VIQ. An expected finding was the positive association between cerebellar volume and PIQ, coupled with the lack of association between this same region and VIQ. Central to the PIQ measure are motor-based tasks, such as object manipulation, so we would expect this measure to be sensitive to involvement in FXTAS. VIQ, which measures verbal comprehension and fluency, is relatively well preserved early on in FXTAS, which helps to differentiate the cognitive decline in FXTAS from Alzheimer disease.[24]

The association between CGG repeat length and cognitive ability in the premutation carriers, including the younger age sample, suggests that molecular abnormalities may contribute to cognitive decline prior to manifestation of obvious structural abnormalities. In an additional study, which includes detailed neuropsychological studies in FXTAS, the early cognitive problems involve memory and executive function deficits.[25] This suggests hippocampal and frontal effects initially from the elevated CGG repeats and is consistent with previously reported data in premutation males without FXTAS.[26] Alternatively, premutation males with a higher CGG repeat number may show a decrease in FMRP, which can lead to intellectual deficits beginning in childhood.[27,28]

The influences of the CGG repeat, coupled with increasing age, on cognition and brain volumetric changes have important prognostic implications. Additional studies have found that the CGG repeat length correlates with age at onset of tremor,[29] and in our study, there is greater brain atrophy and white matter changes in those who carry *FMR1* premutation alleles with a higher repeat number. CGG repeat number has been found to correlate with reduced total brain and cerebral volumes in a study of eight premutation carriers unselected for neurologic symptoms.[6] Our study extends this finding to more regions and to the total volume of white matter disease. We also know that a higher CGG repeat number in 11 men who died with FXTAS correlated with the percentage of inclusions in multiple regions of the brain and with age at death.[9] Further studies with larger sample sizes are needed to better understand the relationship of *FMR1* molecular variables to the onset and severity of disease. Finally, studies are needed to examine environmental and other genetic factors contributing to the trajectory of neurodegeneration in FXTAS.

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