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Study of telomere length in men who carry a fragile X premutation or full mutation allele

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Abstract

The fragile X premutation is defined by the expansion of the CGG trinucleotide repeat at the 5' UTR of the *FMR1* gene to between 55 and 200 repeats, while repeat tracks longer than 200 are defined as full mutations. Men carrying a premutation are at increased risk for fragile X-associated tremor/ataxia syndrome (FXTAS); those with > 200 repeats have fragile X syndrome, a common genetic form of intellectual disabilities. In our study, we tested the hypothesis that men carrying a fragile X premutation or full mutation are “biologically older”, as suggested by the associated age-related disorder in the presence of the fragile X premutation or the altered cellular pathology that affects both the fragile X premutation and full mutation carriers. Thus, we predicted that both groups would have shorter telomeres than men carrying the normal size repeat allele. Using linear regression models, we found that, on average, premutation carriers had shorter telomeres compared with non-carriers ($n = 69$ vs $n = 36$; $p = 0.02$) and that there was no difference in telomere length between full mutation carriers and non-carriers ($n = 37$ vs $n = 29$; $p > 0.10$). Among premutation carriers only, we also asked whether telomere length was shorter among men with vs without symptoms of FXTAS ($n = 28$ vs $n = 38$ and $n = 27$ vs $n = 41$, depending on criteria) and found no evidence for a difference ($p > 0.10$). Previous studies have shown that the premutation is transcribed whereas the full mutation is not, and the expanded repeat track in *FMR1* transcript is thought to lead to the risk for premutation-associated disorders. Thus, our data suggest that the observed premutation-only telomere shortening may be a consequence of the toxic effect of the premutation transcript and suggest that premutation carriers are “biologically older” than men carrying the normal size allele in the same age group.

Introduction

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability and the most common monogenic cause of autism spectrum disorder. The gene involved in this disorder, *FMR1*, is located at q27.3 on the X chromosome; due to its location on the X chromosome, the prevalence of affected males of $\sim 1/7000$ is higher than affected females, $\sim 1/11,000$ (Hunter et al. 2014). The mutation that leads to FXS involves an expansion of an unstable trinucleotide repeat (CGG) in the 5' UTR of the *FMR1* gene. Normally, the length

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of this repeat motif is between 5 and 54 repeats, the most frequent alleles being between 28 and 32 repeats. Expansions of this repeat motif to lengths of between 55 and 200 are referred to as a premutation. Those with > 200 CGG repeats are known as a full mutation and this expansion leads to silencing of the *FMR1* gene and the loss of its product, FMRP. The loss of FMRP underlies the manifestation of FXS.

FXS is only one of the disorders related to the CGG repeat expansion in *FMR1*. The others are associated with premutation alleles. The carrier frequency of premutation alleles is about 1 in 300 women and 1 in 850 men (Hunter et al. 2014). Some of these disorders affect men predominantly, such as fragile X-associated tremor/ataxia syndrome (FXTAS) (Jacquemont et al. 2004), while others affect only women [i.e., fragile X-associated primary ovarian insufficiency (FXPOI)]. Similarly, neuropsychiatric disorders frequently experienced by both male and female premutation carriers such as anxiety, attention deficit and hyperactivity disorder (ADHD), autism spectrum disorders (ASD) or depression are under what is known as fragile X-associated neuropsychiatric disorder (FXAND) (Hagerman et al. 2018). All are incompletely penetrant and have variable expressivity when present. Some of the variability is explained by the premutation repeat size. At least for FXPOI, the association is non-linear with the risk being highest for those with a premutation in the mid-range (~ 80–100 repeats) (Allen et al. 2007; Ennis et al. 2006; Spath et al. 2011; Sullivan et al. 2005; Tejada et al. 2008).

Whereas the molecular consequence of a full mutation is silencing of the *FMR1* gene, that for a premutation includes an increase in *FMR1* mRNA levels with “toxic” long repeat tracks and a reduction in the translation to FMRP to below normal levels (Tassone et al. 2000; Kenneson et al. 2001; Allen et al. 2004; Primerano et al. 2002; Leehey et al. 2008). One consequence of the large premutation repeat track in the mRNA is the possible formation of secondary structures, such as hairpins (Usdin et al. 2014). Studies show that these altered structures sequester CGG binding proteins and also initiate repeat associated non-AUG (RAN) translation. RAN translation in this region leads to a polyglycine or polyalanine products (Todd et al. 2013). These two mechanisms are most likely involved in the etiology of the premutation-associated disorders.

Although there is some evidence that the premutation may manifest as developmental disorders, the onset of symptoms of FXTAS and FXPOI occurs later in life. We hypothesize that telomere length, a biomarker of aging, may be associated with the onset of these disorders. Here we focus on males who carry a premutation to test this hypothesis, as they are more homogeneous with respect to the X-linked mutation than are females. We pay particular attention to FXTAS, the neurodegenerative disorder associated with onset of tremor or ataxia typically in the sixth decade, accompanied by cognitive decline in later years (Garcia-Arocena and Hagerman 2010; Hagerman and Hagerman 2013). The relationship between the risk of developing FXTAS and the premutation repeat length appears to be linear with a significant risk beginning at 70 repeats (Jacquemont et al. 2006), although more data are needed to confirm this.

Several studies have determined that telomere length can be used as a proxy for biological aging (Hastie et al. 1990; Lindsey et al. 1991; Aviv 2008; Martinez et al. 2009) and for

environmental exposures of specific types (Lynch et al. 2016). Two previous studies have studied telomere length in male individuals who carry a premutation or a full mutation of the *FMR1* gene. In 2008, Jenkins et al. did a stratified comparison of five individuals with symptoms of FXTAS and dementia, two had FXTAS but no dementia and three carried the fragile X premutation without FXTAS or dementia symptoms to the same number of age-matched controls respectively. The authors found that all three groups had shorter telomeres compared with age-matched controls; however, there were no differences among the premutation carriers with respect to symptoms of FXTAS or dementia. In a follow-up study, the authors included a group of individuals carrying the full mutation. Their results showed that the individuals carrying the full mutation had the shortest telomeres of the three groups (Jenkins et al. 2012).

With respect to female premutation carriers, a study in 2017 (Albizua et al. 2017) showed data supporting the hypothesis that the *FMR1* premutation was associated with accelerated biological aging in women. Briefly, authors found that women carrying a premutation ($n = 172$) showed shorter telomeres and hence, were “biologically older” than women carrying the normal size allele ($n = 81$). They also showed preliminary evidence that it was associated with the diagnosis of FXPOI in women.

For our study here, we have compared leukocyte telomere length in 78 men with a premutation, 37 men with a full mutation and 55 noncarriers, the largest sample to date. Among premutation carriers, we also asked whether there was an association of telomere length with repeat length and with symptoms of FXTAS. Our results show a statistically significant difference in telomere length between *FMR1* premutations carriers and controls and support the hypothesis of a relationship between the fragile X premutation and accelerated biological aging in men and could be the basis of future studies aimed to better understand the role of this mutation on cellular senescence.

Materials and methods

Ethics statement

The recruitment and data collection protocols and consent forms for ascertainment were approved by the Emory University Institutional Review Board (IRB).

Study population

Males with a fragile X premutation ($n = 78$) or full mutation ($n = 37$) were identified through families diagnosed with fragile X syndrome (FXS) or through genetic testing due to other fragile X-associated symptoms, e.g., FXTAS. The protocol for recruitment has been previously described in detail in (Sullivan et al. 2005; Allen et al. 2007, 2011). For families with FXS, the primary family contact was identified and relatives were invited to participate in the study. Controls were a convenience sample drawn from the general population who identified as men and did not carry a premutation or full mutation.

Participants were between 7 and 85 years old at time of blood draw. Both carriers of a fragile X mutation and noncarriers in our study had a similar race/ethnic background with 95%

accounting for white race/ethnicity. All participants provided a blood sample from which DNA was extracted to determine *FMR1* repeat length and telomere length.

***FMR1* repeat length determination**

All participants in this study provided a venous blood sample. As previously described in Albizua et al. (2017), DNA was extracted using the Gentra Systems, Puregene Genomic DNA purification kit, quantified and stored at 4 °C. Following Meadows et al. (1996), we used fluorescent-labeled primers in a PCR reaction that provided us accurate repeat lengths estimations of up to 90 repeats. If the length of the trinucleotide repeat was longer than 90, we followed the protocol described in Brown et al. (1993).

Determination of telomere length

In brief, relative telomere length was measured according to a modified protocol of the method described by Cawthon (2002). The final concentrations of reagents in the PCR were $0.16 \times$ Sybr Green I, 15 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.16 mM each dNTP, 5 mM DTT, 1% DMSO and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems).

The final telomere primer concentrations were: tel 1, 216 nM; tel 2, 720 nM. The final *36B4* (single copy gene) primer concentrations were: 36B4u, 240 nM; 36B4d, 400 nM. The primer sequences were as follows:

TEL 1: 5'—GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT—3'.

TEL 2: 5'—TCCCGACTATCCCTATCCCTATCCCTATCCCTATCC-CTA—3'.

36B4u: 5'—CAGCAAGTGGGAAGGTGTAATCC—3'.

36B4d: 5'—CCCATTCTATCATCAACGGGTACAA—3'.

All PCRs were performed on the Bio-Rad CFX96 real-time PCR detection system. The thermal cycling profile for both PCRs began with a 95 °C incubation for 10 min to activate the AmpliTaq Gold DNA polymerase. For telomere PCR, the protocol used consisted of 26 cycles of 95 °C for 15 s, 54 °C for 2 min. For *36B4* PCR, the protocol was 32 cycles of 95 °C for 15 s, 58 °C for 1 min. BIORAD software was used to generate the standard curve for each plate and to determine the dilution factors of standards corresponding to the T and S amounts in each sample.

Two PCR reactions were performed for each experimental sample: one to measure the single copy gene relative quantity and the other to measure the relative quantity of telomere repeats. A randomly chosen sample (reference sample) was used to generate a standard curve to which the results of the rest of the experimental samples were compared.

The ratio of these two PCR results for each experimental sample was defined as the relative telomere to single copy gene (T/S) ratio. We conducted two replicates on each sample to control for experimental variation: two identical aliquots of the DNA sample were added to plate 1 to assay telomere repeat copy and another two aliquots were added to the same well positions in plate 2 to assay the single copy gene. For each standard curve, one reference

DNA sample was diluted serially to produce five total amounts of DNA: 20 ng, 10 ng, 5 ng, 2.5 ng and 1.25 ng.

For quality control, the efficiency of the PCR reaction and the r-square value of the regression line created by the standard dilutions were examined for each reaction. The efficiency of the PCR reaction measured by the threshold cycles of the standard dilutions used to create the regression line had to be between 90 and 110% and the r-square value of the regression line had to be no less than 0.98. If any of these parameters were not met, the entire PCR for the plate was repeated. We also applied a quality control to each of the duplicates run for each sample. The duplicate values for each sample could not have a threshold cycle value of more than 0.3 cycles apart. If this parameter was not met, both the T PCR and the S PCR for that sample were repeated.

Data collection and variable definitions

Mutation carriers were interviewed to obtain demographic information including age at interview, date of birth, ethnic/racial group and level of education. For a subset of premutation carriers, the presence of FXTAS was determined by a neurologist, as described in Juncos et al. (2011).

Results

Our primary hypothesis was that males with a premutation or full mutation would have shortened telomeres due to accelerated aging. To test this hypothesis, we started by conducting linear regression models to examine the association of telomere length with age, stratified by mutation group: noncarriers, premutation carriers and full mutation carriers. Because of the different time points when mutation carriers are diagnosed, the age distributions for premutation carriers differed from that of full mutation carriers: the majority of males in the premutation group were older than 40 (91.02%), whereas all the full mutation carriers were younger than 47 (Table 1, Fig. 1). Our noncarrier control group was ascertained to span a wide age range, which resulted in a uniform density distribution of individuals between ages 16–85 (Fig. 1).

As expected, among noncarrier controls, we found a statistically significant negative relationship between relative telomere length (i.e., T/S ratio) and age at blood draw ($p = 1.5 \times 10^{-5}$, Table 2, Fig. 2a). Among men carrying a premutation, the slope of the regression line was negative but not statistically significant ($p = 0.1$, Table 2, Fig. 2b). We identified two premutation carriers with T/S ratio values that were considered outliers, as defined by their values being more than two standard deviations away from the mean. When these were excluded from regression analysis to see their influence on the results, both models were essentially the same, although the negative association between relative telomere length and age became statistically significant ($p = 0.02$, Table 2; Fig. 2c). Among the full mutation carriers, there was a strong negative association between relative telomere length and age, similar to the noncarrier controls ($p = 0.01$, Table 2; Fig. 2d).

To determine whether the association between telomere length and age differed statistically between groups, we conducted two regression analyses comparing (1) premutation carriers

with noncarriers and (2) full mutation carriers with noncarriers, including age, carrier status and the interaction term as predictors of relative telomere length. We were not able to compare the telomere length of the premutation carriers and the full mutation carriers, because there was little overlap of the respective age ranges (Table 1, Fig. 1).

To compare the relative telomere length between premutation carriers and noncarriers, we used only noncarriers and premutation carriers older than 40 years to make the age distribution of both groups similar (Fig. 3a, c). Adjusting for age, we found that carrier status and age-by-carrier status interaction term were statistically significant both with the outliers included ($p = 0.05$ and $p = 0.04$, respectively, Table 3, Fig. 3b) and with the outliers excluded from the analysis ($p = 0.02$ and $p = 0.02$, respectively, Table 3, Fig. 3d). Examination of Fig. 3 suggests that premutation carriers have shorter telomere lengths, perhaps starting at ages younger than 40. However, the latter speculation is only that, as we have no data points in this younger age range.

In our analyses comparing full mutation carriers and noncarriers, we limited noncarriers to those 50 years of age or younger to maximize the overlap in age range with full mutation carriers (Fig. 4a). Adjusting for age, we found that neither carrier status nor age-by-carrier status interaction terms were significant ($p = 0.16$ and $p = 0.38$, respectively, Table 3, Fig. 4b), indicating a lack of evidence for a difference in telomere length across this age span between these two groups. We note that the slope of the line and where it crosses the y-axis for each group are likely affected by having more samples in the full mutation group with an age younger than 20 years ($n = 14$) than in our noncarrier control group ($n = 3$) (Fig. 4).

Our next set of analysis tested the hypothesis that among premutation carriers, telomere length may differ among those with vs without symptoms of FXTAS, as FXTAS may represent further acceleration of the aging process. A demographics of premutation carriers with respect to presence of tremor or ataxia, the hallmarks of FXTAS, can be found in Table 1. Because of the small number of carriers without tremor, we could not analyze telomere length based on those with and without tremor. For the analyses comparing presence vs absence of ataxia and for presence of both tremor and ataxia vs the lack of either motor symptom, we did not find a significant association with any of the predictor variables, including age, symptom, and the age-by-symptom interaction term (Table 4).

Discussion

Several previous studies motivated us to test the hypothesis that there may be accelerated aging among premutation carriers, as measured by global telomere length. First, studies have shown significantly shorter telomere lengths among those with late-onset disorders compared with controls, including Alzheimer disease (Panossian et al. 2003) and heart disease (Samani et al. 2001; Benetos et al. 2004). As cognitive decline is a feature of FXTAS, we may expect to observe this same pattern. More directly, in a small sample of men with a premutation, Jenkins et al. (2008, 2012) found shorter telomere lengths, but this shortening was not exacerbated by a diagnosis of FXTAS or FXTAS plus dementia (see “Introduction”). Furthermore, in our previous study of 172 women who carry a premutation, we found a similar pattern of shorter telomeres among carriers compared with controls

($n = 81$), suggesting increased biologically aging (Albizua et al. 2017). More specifically, we found that premutation carriers had shorter telomere lengths in their younger years compared with noncarriers and the slope of the line across ages was less pronounced than for controls. Although cross-sectional, these data suggest advanced aging.

Here we accrued the largest sample to date to test the hypothesis that men with a premutation or with a full mutation have shorter telomeres compared with noncarriers. We first confirmed the expected statistically significant negative association between telomere length and increasing age among noncarriers controls ($n = 55$) (Hastie et al. 1990; Lindsey et al. 1991; Takubo et al. 2002; Ishii et al. 2006; Hoffmann et al. 2009) to confirm that our assay produced accurate results and that noncarrier study sample was a valid comparison group.

Among premutation carriers, we observed a statistically significant inverse association between telomere length and increasing age after removing two outliers ($n = 76$, $p = 0.02$). We then compared premutation carriers with noncarriers. We had to focus on those older than 40 years of age due to the later age at diagnosis of male carriers, and therefore, the later age at blood draw. We found a similar pattern as we did for female carriers (Albizua et al. 2017). Male carriers had shorter telomeres at younger years and a regression slope that was less than noncarriers, suggesting a different pattern of decline of telomere length with age (Table 3). This statistically significant difference was observed, despite the fact that the age span was only 40–60 years in our sample.

Next, we asked whether the presence of symptoms of FXTAS exacerbated telomere shortening. Comparing premutation with and without ataxia or those with both ataxia and tremor to those with neither motor symptom, we found no statistically significant difference (Table 4). Our results, based on a larger sample size, are similar to previous results from Jenkins et al. in 2008, where they also found no evidence for a difference in telomere length when symptoms of FXTAS were present.

Lastly, we compared males with a full mutation to noncarriers. We had a younger sample of full mutation carriers due to the opportunistic use of blood that was drawn for another study; all were younger than 50. Nonetheless, we found no statistically significant difference in the pattern of telomere length with age between noncarriers and full mutation carriers (Table 3, Fig. 4). This finding differs from the only other study of full mutation carriers by Jenkins et al. (2012). They found that full mutation carriers had shorter telomeres than age matched noncarriers and than premutation carriers. This led the authors to suggest that telomere shortening could not be solely caused by the increased levels of *FMR1* RNA that are present only in the premutation carriers. A limitation of their study was the sample size compared with ours: 9 premutation carriers and 6 full mutation carriers compared with 78 and 37 males, respectively. Thus, our findings suggest that the different pattern of telomere length with age observed among only premutation carriers may be due to the toxic effect of the high levels of *FMR1* premutation transcripts.

Study limitations

Our study is the largest study to date on the relationship between telomere length and the *FMR1* premutation and full mutation in men. However, the sample was limited by the age range among premutation and full mutation carriers. Also, the fact that those age ranges overlapped little among the two mutation carrier groups limited our ability to statistically compare these two groups directly. Also, a larger sample size of men with fragile X-associated tremor/ataxia syndrome (FXTAS) would increase the power to detect smaller differences in telomere length, if they exist, as well as help to confirm and extend our current findings.

We suggest that the explanation for why we do not observe a difference among premutation carriers with and without FXTAS when we did observe a difference among carriers with and without FXPOI (Albizua et al. 2017) is based on power. First, our sample size was almost double for the FXPOI study compared with the FXTAS comparison. Second, because the age of onset of FXPOI is much younger than that for FXTAS, we are able to define the disease states for FXPOI at an earlier age. Thus, groups for comparison are better classified across a wider age span for FXPOI compared with FXTAS. At least at this point in our understanding of premutation-associated disorders, it is difficult to assign a biological explanation for the observed differences.

Lastly, our data are cross-sectional and thus the interpretations about the decline in telomere length by age are meant to be taken only speculatively. Future studies should sample males at several different time points in their lifetime to study true loss of telomere length.

Conclusions

Both male and female premutation carriers show the same pattern of shorter telomere lengths and a smaller regression slope with age compared with noncarriers. Although our sample of males was limited to 40 years and older, we expect the pattern to hold if younger males were added to the sample based on our previous findings in female carriers. However, this suggestion needs to be confirmed. No difference in telomere shortening with age was observed for males with a full mutation compared with noncarriers. Thus, we speculate that *FMR1* premutation transcripts may affect telomere length, most likely mediated by a process of accelerated biological aging. Further studies aimed to better understand the role of this mutation on cellular senescence are needed.

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Fig. 1.
Density of age distribution for the 3 sample study groups

Fig. 2.
a Linear regression plot of control samples. **b** Linear regression plot of premutation samples including outliers. **c** Linear regression plot of premutation samples excluding outliers. **d** Linear regression plot of full mutation samples

Fig. 3.

a Density plot of age at blood draw flow for permutation carriers and controls older than 40 years. **b** Linear regression plot of permutation ($n = 71$) and controls ($n = 36$) older than 40 years including outliers. **c** Density plot of age at blood draw flow for permutation carriers and controls older than 40 years. **d** Linear regression plot of permutation ($n = 69$) and controls ($n = 36$) older than 40 years without outliers

Fig. 4.
a Density plot of age at blood draw flow for full mutation carriers and controls younger than 50 years. **b** Linear regression plot of full mutation ($n = 37$) and controls ($n = 29$) younger than 50 years

Table 1

Characteristics of the study population

	Size (n)	Age at blood draw mean $\pm$ SD (range)	Repeat size mean $\pm$ SD (range)
Non-carriers	55	50.03 $\pm$ 21.62 (16–85)	n/a ^a
Premutation carriers	78	64.28 $\pm$ 14.29 (21–84)	85.14 $\pm$ 19.5 (55–150)
Ataxia	28	69.67 $\pm$ 8.18 (50–84)	90.71 $\pm$ 18.82 (60–150)
No ataxia	38	67.97 $\pm$ 9.29 (47–84)	78.23 $\pm$ 16.99 (55–150)
Tremors	52	66.85 $\pm$ 10.27 (23–84)	86.33 $\pm$ 19.86 (55–150)
No tremors	16	71.69 $\pm$ 9.78 (47–84)	75.31 $\pm$ 9.13 (59–95)
Ataxia and tremors	27	69.18 $\pm$ 7.89 (50–84)	91.14 $\pm$ 19.04 (60–150)
No ataxia and tremors	41	67.19 $\pm$ 11.64 (23–84)	78.85 $\pm$ 16.56 (55–150)
Full mutation	37	25.4 $\pm$ 10.39 (7–47)	> 200

^aWe did not measure the repeat size due to the low frequency of *FMR1* mutations in males in the general population

Table 2

Results for the linear regression models examining the association of relative telomere length (T/S ratio) with age at blood draw for non-carrier controls, premutation carriers and full mutation carriers

	Noncarriers (<i>n</i> = 55)		Premutation carriers (<i>n</i> = 78 with 2 outliers)		Premutation carriers (<i>n</i> = 76 without 2 outliers)		Full mutation carriers (<i>n</i> = 37)	
	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value
Intercept	1.95 (0.11)	< 0.000	1.63 (0.18)	< 0.000	1.64 (0.15)	< 0.000	2.15 (0.14)	< 0.000
Age	-0.01 (0.002)	1.5×10^{-5}	-0.004 (0.002)	0.1	-0.005 (0.002)	0.02	-0.01 (0.005)	0.01

Table 3

Results for the linear regression models examining the association of relative telomere length (T/S ratio) with age at blood draw for controls, premutation carriers and full mutation carriers

	Noncarriers (<i>n</i> = 36) vs Premutation with outliers (<i>n</i> = 71)		Noncarriers (<i>n</i> = 36) vs Premutation without outliers (<i>n</i> = 69)		Noncarriers (<i>n</i> = 29) vs Full mutation (<i>n</i> = 37)	
	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value
Intercept	2.46 (0.29)	< 0.000	2.46 (0.24)	< 0.000	1.81 (0.18)	< 0.000
Age	- 0.017 (0.004)	0.0001	- 0.017 (0.004)	0.00001	- 0.005 (0.005)	0.281
Status	- 0.78 (0.4)	0.05	- 0.81 (0.34)	0.02	0.33 (0.24)	0.164
Age $\times$ status	0.01	0.04	0.01 (0.005)	0.02	- 0.006 (0.007)	0.384

Table 4

Results for the linear regression models examining the association of relative telomere length (T/S ratio) of premutation carriers, grouped by presence/absence of symptoms of FXTAS

	Ataxia (<i>n</i> = 28) vs no ataxia (<i>n</i> = 38)		Ataxia + Tremors (<i>n</i> = 27) vs not having both symptoms (<i>n</i> = 41)	
	β (SE)	<i>p</i> value	β (SE)	<i>p</i> value
Intercept	1.31 (0.43)	0.0034	1.50 (0.33)	< 0.000
Age	0.0006 (0.006)	0.91	− 0.002 (0.004)	0.65
Symptom	0.34 (0.73)	0.64	0.30 (0.69)	0.67
Age × symptom	− 0.007 (0.01)	0.52	− 0.006 (0.01)	0.54