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Women who carry a fragile X premutation are biologically older than noncarriers as measured by telomere length

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Abstract

Women who carry a fragile X premutation, defined as having 55–200 unmethylated CGG repeats in the 5' UTR of the X-linked *FMR1* gene, have a 20-fold increased risk for primary ovarian insufficiency (FXPOI). We tested the hypothesis that women with a premutation+FXPOI have shorter telomeres than those without FXPOI because they are “biologically older”. Using linear regression, we found that women carrying a premutation (n=172) have shorter telomeres and hence, are “biologically older” than women carrying the normal size allele (n=81). Strikingly, despite having shorter telomeres, age was not statistically associated with their telomere length, in contrast to non-carrier controls. Further, telomere length within premutation carriers was not associated with repeat length but was associated with a diagnosis of FXPOI, although the latter finding may depend on FXPOI age of onset.

Keywords

telomere length; FXPOI; FXTAS; fragile X premutation; *FMR1*

INTRODUCTION

Fragile X syndrome (FXS) is the leading heritable cause of intellectual disability and autism spectrum disorder. The gene involved in this disorder, fragile X mental retardation 1 gene (*FMR1*), is located at q27.3 on the X chromosome. Thus, the presentation is more severe in male than in female carriers, although there is a wide range of impairment for both. The frequency of FXS among males is approximately 1 in 7000 and among females, about 1 in 11000 [Hunter et al 2014]. It is well established that the severity of this phenotype is directly related to the degree of methylation of the *FMR1* promoter region (i.e., the inactivation of the *FMR1*); that is, to the reduction or lack of the *FMR1* protein product, FMRP.

The most typical *FMR1* mutation results from the expansion of an unstable trinucleotide repeat (CGG) in the 5' UTR of the *FMR1* gene. In normal individuals the length of this repeat motif is between 5 and 55 repeats. When it expands to more than 55 repeats but less than 200, it is referred to as premutation. At a cellular level, a premutation is unmethylated and leads to an increase in *FMR1* mRNA levels and slightly below normal levels of FMRP. A premutation is also characterized by its instability and the high risk of expansion to the full mutation (>200 methylated repeats). This expansion from a pre- to full mutation occurs only in the maternal germline. A recent meta-analysis estimated the prevalence of individuals who carry a premutation to be about 1 in 300 women and 1 in 850 men [Hunter et al. 2014].

The carriers of a premutation are at risk for at least two specific disorders that are unrelated to FXS. Women are at risk for fragile-X-associated primary ovarian insufficiency (FXPOI), a disorder that leads to subfertility and early estrogen deficiency. A clinical diagnosis of FXPOI is based on cessation of menses for at least four months prior to age 40. This occurs in about 20% of women carrying the fragile X premutation, although the age at menopause distribution is shifted to younger ages for all carriers compared with noncarriers [Allen et al. 2014; Sullivan et al. 2011].

Fragile X-associated tremor/ataxia syndrome (FXTAS), a neurodegenerative disorder, is 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]. Among premutation carriers, men show a higher risk of developing FXTAS than women [Jacquemont et al. 2004].

Interestingly, the risk for these premutation-associated disorders is dependent on the premutation repeat length: for FXTAS, the association appears to be linear with a significant risk beginning at 70 repeats [Jacquemont et al. 2006]; however for FXPOI, the association is non-linear showing the greatest risk for those with 80–100 repeats, not those with the largest repeat sizes [Sullivan et al. 2005; Ennis et al. 2006; Allen et al. 2007; Tejada et al. 2008; Spath et al. 2011].

The molecular consequences of a premutation are an increase in *FMR1* mRNA levels with “toxic” long repeat tracks and a reduction in 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]

that are a function of increasing premutation size. There is now strong evidence that FXTAS, and most likely FXPOI, is primarily due to effects of the premutation mRNA. These mRNA repeat tracks may lead to sequestration of proteins that bind to the repeat, thus losing their potential to function properly [Berman et al. 2014]. Also, these long repeat tracks may cause repeat associated non-AUG (RAN) translation, leading to a toxic polyglycine or polyalanine product [Todd et al. 2013]. Secondary structures, such as hairpins, that result from the CGG repeat track in the mRNA are part of the mechanisms hypothesized to explain both of these premutation-associated disorders [Usdin et al. 2014].

One of the questions that remains unanswered is why some of the individuals carrying a premutation show symptoms while others do not; repeat size explains only a small part of this risk. Several studies point to other modifying genes and environmental factors to explain this incomplete penetrance [Paul et al. 2010; O'Dwyer et al. 2005; Muzar et al. 2014; Martínez-Cerdeño et al. 2015; Muzar et al. 2015; Allen et al. 2014]. To date, there is no prognostic biomarker for these late-onset disorders.

Telomere length has been shown to be a marker of biological aging [Hastie et al. 1990; Lindsey et al. 1991; Aviv 2008; Martinez et al. 2009] and of environmental exposures of specific types [Lynch et al. 2016].

Jenkins et al [2008; 2012] examined telomere length among pre and full mutation carriers and found that full mutation carriers had the shortest telomere length on average followed by premutation carriers, compared with noncarriers, controlling for age. Thus, the authors suggested that telomere shortening was not associated with the exposure to high levels of *FMR1* mRNA. In their study among premutation men [Jenkins et al. 2008], they did not see an association with symptoms of FXTAS.

To date, there has been no study to evaluate telomere length among women who carry a premutation. We hypothesized that carriers have shorter telomere lengths due to two factors: 1) presence of the fragile X premutation allele as shown by previous studies in men [Jenkins et al. 2008, 2012] and 2) reduced age at menopause. Leukocyte telomere length has been associated with age at menopause and length of reproductive lifespan in some studies [Gray et al. 2014; Aydos et al. 2005], although the strong effects of race/ethnicity may negate this association [Shenassa and Rossen, 2015]. Several studies have also shown effects of estrogen on telomere length in mice [Bayne et al. 2011] and in humans [Lee et al. 2005].

For this study, we had the opportunity to examine leukocyte telomere length in 172 women with a premutation and 81 women without a premutation. For a subset of these premutation carriers we also collected data regarding the size of their CGG trinucleotide repeat, their diagnosis of FXPOI and their hormone intake. This study supports the idea of a relationship between a fragile X premutation and accelerated biological aging 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

Women with a fragile X premutation were identified through families diagnosed with fragile X syndrome (FXS) or through genetic testing due to symptoms of POI. The protocol has been previously described in detail in [Sullivan et al. 2005, Allen et al. 2007]. For families with FXS, the primary family contact was identified and all female relatives were invited to participate in the study. Generalized estimating equation (GEE) regression models [Zeger and Liang, 1986] were used to account for the possible dependent nature of measurements among family members.

Participants were aged between 23 and 59 years old and had English as their primary language. Both cases and controls in our study had a similar race/ethnic background with 95% accounting for white race/ethnicity. All participants were asked to complete a reproductive history questionnaire and provide a biological sample, either buccal or blood, for repeat-length determination. Only those who provided a blood sample were used in this study, as we required that the source of the DNA be consistent. Controls were identified as women who did not carry a premutation and did not have primary ovarian insufficiency. They were ascertained from the general population.

Data Collection and Variable Definitions

We administered the reproductive history questionnaire to obtain demographic information including age at interview, date of birth, ethnic/racial group and education. We obtained menstrual cycle history, including age and date of last menstrual period. If the date of last menstrual period was more than two months prior to the interview, we identified the cause of menses cessation. Women completed a brief medical history concerning disorders associated with ovarian dysfunction and co-morbid disorders related to reproductive aging. Medical records were not obtained to verify fertility problems. For many of the women with a premutation in this study, a reproductive endocrinologist followed up the reproductive history questionnaire with a semi-structured telephone interview as part of another study to better understand care of women with FXPOI [Hipp et al. 2016]. Overall, we found that women provided accurate assessments in the questionnaire of their menstrual cycle traits and timing and cause of cessation of menses when probed in the interview. Thus, we think that misclassification of FXPOI is minimal. We defined age at menopause as the cessation of menses for at least one year.

Information on hormone use was also obtained through self-report. Based on the follow-up interviews described above, we found that it was difficult for a woman to accurately recall the full history of hormone use (number of years used, type of hormone medication, reason for use). However, information about current use of hormones or whether hormones were

ever used was more readily recalled. Thus, for this study, we used a binary variable for ever used or never used (1, hormone use; 0, otherwise).

***FMR1* Repeat Length Determination**

All participants in this study provided a venous blood sample. DNA was extracted using the Gentra Systems, Puregene Genomic DNA purification kit, quantified and stored at 4°C. A fluorescent-sequencer method is used to determine *FMR1* CGG repeat length [Meadows et al. 1996]. Briefly, fluorescent-labeled primers are used to polymerase chain reaction amplify across the repeat region and the resulting product is run on an automated sequencer. Repeat lengths up to 90 can be determined with this method. In the event that a single band was detected in females, indicating either homozygous status or the presence of a larger repeat band from the second allele, an alternative polymerase chain reaction-based, hybridization technique was used [Brown et al. 1994]. For heterozygous females, the CGG repeat length from the largest repeat allele was used to define *FMR1* repeat length.

Determination of Telomere Length

Relative telomere length was measured according to a modified protocol of the method described by [Cawthon et al. 2002]. This method using quantitative PCR proved to be rapid and more importantly, allowed us to obtain reproducible estimates of relative telomere length with the use of only 30ng of genomic DNA. Briefly, two master mixes of PCR reagents were prepared, one with the telomere (T) primer pair and the other with the single copy gene (S) primer pair. Similar to [Cawthon et al. 2002], we used 36B4 gene as a single copy gene, which encodes acidic ribosomal phosphoprotein PO and is located on chromosome 12 [Boulay et al. 1999]. The final concentrations of reagents in the PCR were 0.16× 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 samples (cases) 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. In addition, T/S ratios were repeated for all outliers and found to confirm the original result. Thus they were included in the regression analyses.

RESULTS

Table I provides the general characteristics of the study population, stratified by carrier and FXPOI status. Prior to testing our primary hypothesis that women with a premutation have shortened telomeres, we examined the age distribution in our study sample and its association with telomere length to confirm that we observe the expected age-related decline in telomere length. We confirmed that the age distribution of carriers and noncarriers was similar; thus, our comparison of telomere length was examined among women of the same age span (Figure 1a, Table I). Next we examined the expected negative relationship between age and telomere length in our noncarrier control group. As expected, our results indicated a statistically significant association of relative telomere length (i.e., T/S ratio) and age in controls ($p=0.002$, Table II, Figure 1b). Thus we were confident that our assay accurately identified the expected decline within the age span examined.

To test our hypothesis that relative telomere length was associated with carrier status, we first created a regression model to examine the association of relative telomere length among only premutation carriers. We did not find the expected statistically significant association with age ($p=0.097$, Table II, Figure 1c). To test whether this lack of association differed significantly between carriers and noncarriers, we conducted a regression analysis among all women including age, carrier status and the interaction term as predictors of relative telomere length. Adjusting for age, we found that carrier status and age-by-carrier status

interaction term were statistically significant, ($p=0.007$ and $p=0.046$, respectively, Table II, Figure 1d). This indicates a difference in telomere length between the two groups and more importantly a marked difference in the way that telomeres decline with age for the two groups.

To explore whether we could identify factors among premutation carriers that led to lack of the association of telomere length and age, we examined: 1) premutation repeat length, 2) hormone medication use and 3) diagnosis of FXPOI. With respect to repeat size, we know that there is a non-linear association with repeat length and other premutation-associated phenotypes such as age at menopause [Allen et al. 2014] or symptoms of depression and anxiety and greater vulnerability to stressful life events [Seltzer et al. 2012]; thus, we included repeat length and the square of repeat length in the regression model to account for the potential non-linear effect. The range of the trinucleotide repeat size in our study was between 56 and 150 repeats and we did not observe a statistically significant association with repeat length alone ($p=0.674$)(Table III) or the square term and the telomere length in women carrying a premutation ($p=0.848$)(Table III).

In order to study the possible relationship between estrogen levels and telomere length, we took advantage of the data collected on hormone medication use (e.g., oral contraceptive, hormone replacement) in this study sample of women carrying the premutation. We created an indicator variable defining women who used ($n=136$) and did not use ($n=16$) any type of hormone medication. We did not observe a statistically significant relationship between the use of any hormone medication and the relative telomere length ($p=0.111$)(Table III).

Lastly, we stratified by diagnosis of FXPOI among premutation carriers to examine the possible effect of early cessation of menses. Women who had cessation of menses for at least 12 months by age of 40 or younger were considered to suffer from FXPOI ($n=41$); those who had their menopause at an age 41 or older were considered not to have FXPOI ($n=66$). We assumed that none of the noncarriers ($n=81$) had POI in this analysis.

First, we studied the relationship between relative telomere length and age for each of the three groups and observed a strong significant association between age and relative telomere length for the control noncarrier group ($p=0.002$, Table IVa, Figure 2a), a significant association for the premutation carriers who suffer from FXPOI ($p=0.001$, Table IVa, Figure 2b) and a non-significant association for premutation carriers with a normal age at menopause ($p=0.648$, Table IVa, Figure 2c).

We noted that the majority of women with FXPOI were younger at blood draw compared with those without FXPOI, due to the fact that the diagnosis of FXPOI is directly related to the woman's age. Because of this, our group of premutation carriers that did not suffer from FXPOI consisted of women older than 36 years old at the time of blood draw. Thus, we restricted our next set of analyses to women older than 36 (Table IVb). We still observed a strong significant relationship between age and relative telomere length among the noncarriers ($p=0.019$, Table IVb, Figure 2d), but not among carriers who suffered from FXPOI ($p=0.583$, Table IVb, Figure 2e) or among carriers without FXPOI ($p=0.648$, Table IVb, Figure 2f).

Lastly, in an analysis among premutation carriers only, we found no statistical evidence for a difference in telomere length or decline when we included the interaction term of age by presence of FXPOI ($p=0.93$) in the regression model, along with the main effects of age and presence of FXPOI.

DISCUSSION

The phenotypes associated with the fragile X premutation genotype have been extensively studied both in women [Sullivan et al. 2011] and men [Hagerman and Hagerman 2013]. Among men, the most common phenotype associated with a premutation is FXTAS, a neurodegenerative disorder with an onset of motor symptoms typically around the sixth decade, many times followed with cognitive decline in later years [Hagerman and Hagerman 2013]. Among women who carry a premutation, fragile X-associated primary ovarian insufficiency (FXPOI) is the most common associated disorder with a frequency of POI in about 20% of carriers [Allen et al. 2014].

In 2008, Jenkins et al. [2008] studied the potential relationship between telomere shortening and the presence of FXTAS and dementia in men carrying the fragile X premutation. There had been a series of studies showing a relationship between telomere length and a wide range of age related human disorders including heart disease [Samani et al. 2001; Benetos et al. 2004] or Alzheimer disease [Panossian et al. 2003]. Jenkins had also reported shorter telomeres in individuals with trisomy 21 who suffered from dementia relative to those without dementia [Jenkins et al. 2006].

For the study on men carrying the fragile X premutation, they measured telomere length in 10 older men carrying a premutation and five age-matched noncarriers using metaphase and interphase preparations from short-term T lymphocyte cultures analyzed using quantitative peptide nucleic acid FISH technology.

Jenkins et al. hypothesized that males with FXTAS and dementia would exhibit shorter telomeres than other comparison groups, including men with FXTAS without dementia, men with a premutation and without FXTAS, or age-matched noncarrier controls. They did find shorter telomeres in premutation carriers ($N=10$, age range 56–73 years) compared with age-matched noncarriers; however this reduced length was irrespective of their diagnosis of FXTAS or FXTAS plus dementia. In 2012, they conducted another study using a similar technology and found the same reduction in telomere length not only in older men, but also younger men with a premutation ($N=9$, age range 9–56 years)[Jenkins et al. 2012].

In our investigation, we focused on telomere length in women with a premutation to determine whether a similar pattern would be observed as that for men. First, we confirmed the expected statistically significant negative association between telomere length and increasing age among noncarriers controls ($N=81$), as pointed out by previous studies [Hastie et al. 1990; Lindsey et al. 1991; Takubo et al. 2002; Ishii et al. 2006; Hoffmann et al. 2009]. Among premutation cases we observed a marginally statistically significant inverse association between telomere length and increasing age ($N=172$, $p=0.097$). We tested the interaction of age and carrier status in our whole sample set and found a statistically

significant association ($p=0.046$). Together, these results are consistent with our hypothesis that women carrying a premutation show a different pattern of decline in telomere length, one suggestive of a “biologically older” age group. With the caveat that our data are cross-sectional, not longitudinal, we found that premutation carriers had shorter telomere lengths in their younger years compared with noncarriers and had less of a decline with increasing age compared with noncarriers (Table II, Figure 1b, 1c and 1d).

In a previous study from our group [Albizua et al. 2015], we found a similar pattern of telomere length among women who had a child with trisomy 21 derived from a nondisjunction error in the oocyte compared with controls. In that study, we suggested that the reduced slope in the age-related pattern reflected a biologically older stage; the telomeres were already shorter at the youngest ages studied and then declined less. We suggest that this, too, may be the explanation for the pattern observed among premutation carriers. Telomere attrition is known to follow a biphasic decline with age [Aubert and Lansdorp 2008] with a steeper decline in the first years of life and the later years of life (after 60 years of age), and a subtler decline in the mid years. Our data fit a model where the telomeres among premutation carriers shorten more rapidly in the younger years and hence their telomere length at the time of measurement is that of a biologically older person compared with noncarrier controls. Studies in children with a premutation would be important to test this hypothesis.

We tested three factors to determine whether we could better understand what may be driving our results among premutation carriers: 1) premutation repeat length, 2) hormone medication use, and 3) diagnosis of FXPOI. First, we did not find a linear or non-linear association of premutation repeat size and telomere length, adjusting for age. Although this suggests a possible threshold effect of repeat size, one greater than 55 repeats, leads to some phenomenon that affects telomere length, a study with larger sample sizes in each premutation repeat group is necessary to better define at-risk repeat alleles. Jenkins et al. [2012] compared telomere length in premutation and full mutation males and found the greatest reduction in telomere length among full mutation carriers, followed by premutation carriers and then noncarrier controls. This result led them to argue that telomere shortening could not be fully explained by the toxic effect of increased mRNA levels found in premutation carriers, but to some other phenomenon also present among full mutation carriers.

We also examined phenotypes associated with estrogen levels, namely hormone medication use and a diagnosis of FXPOI. With respect to hormone medication use, we found that premutation carriers using hormones did not have shorter telomeres compared with those who did not use hormones, adjusting for age. We are cautious about drawing strong conclusions because only 16 of the 152 women with a premutation did not use hormones. Other studies of post-menopausal women from the general population show the opposite effect. For example, Lee et al. [2005] in a study of 130 post-menopausal women from ages 55 to 69 grouped by hormone use (65 on hormone treatment for >5 years and 65 never on hormone treatment) showed that telomere lengths among women using hormones were significantly longer than those in the non-hormone use group. Lin et al. [2011] attempted to tease apart the effects of telomere length and endogenous and exogenous estrogen exposure

in a sample of post-menopausal women (35 with natural and 18 with surgical menopause with hormone treatment). Duration of endogenous exposure was estimated as the difference between age at menopause and age at menarche, and length of time on hormone treatment was used as a measure for duration of exogenous estrogen exposure. They found that longer endogenous estrogen exposure was associated with greater telomere length and with lower telomerase activity. Length of hormone treatment was not associated with either telomere length or telomerase activity. Further, we did not have a precise measure of endogenous and exogenous exposure to estrogen. Thus, a larger study with these added information is needed.

Our findings related to the age at diagnosis of FXPOI/menopause also differ from those in the general population. Most studies have shown an inverse association with telomere length and natural age at menopause [e.g., Aydos et al. 2005; Gray et al. 2014; Dalgard et al. 2015], although there are complicating factors. For example, in a longitudinal study of 405 women, Dalgard et al. [2015] found that telomere attrition rate was fastest among women within the pre-menopausal stage compared with those measured after menopause. They suggested that the faster rate of attrition in pre-menopausal women might be due to differences in estrogen levels during cycles. In a study of 799 post-menopausal women, Shenassa and Rossen [2015] found that the association of telomere length and menopause age depended on a woman's racial/ethnic background. Gray et al. [2014] restricted their study to only white post-menopausal women (N=486; 65 years of age) and found that longer telomere lengths were associated with later age at menopause, after adjusting for covariates. When they excluded women from their study with POI (N=26), the predicted magnitude of the telomere length attrition by increasing age was reduced. This suggests that women with POI had longer telomeres than women going through menopause at later years. This finding of longer telomeres among women with POI is similar to that of Hanna et al. [2009]. These authors speculated that this longer than expected telomere length may be due to abnormal hormone levels, to slow cellular division rates, or to autoimmunity.

In our study, we hypothesized that the group of premutation carrier women who had been diagnosed with FXPOI (N=41) would have shorter telomere length due to a two hit model. We hypothesized the first hit is related to being a premutation carrier, or those with statistically shorter telomeres than noncarriers, and the second hit related to fewer years of estrogen protection due to cessation of menses prior to age 40. Thus, such women with FXPOI would be predicted to have further loss of the telomeric sequence compared with those who did not have FXPOI. We found a significant association of reduced telomere length with increasing age among women who suffered from FXPOI. This association was driven by younger women, as exclusion of those younger women lead to a nonsignificant association of telomere length and age. That is, we did not find a statistically significant difference in telomere length or attrition based on FXPOI diagnosis for premutation carriers >36 years of age at blood draw. Thus, it may be that women with very early onset of FXPOI have a different rate of telomere attrition compared with other premutation carriers. For women >36 years old with or without FXPOI, the difference in their exposure to estrogen deficiency is not large enough to affect telomere length or the effect of a premutation itself masks any effect related to years of estrogen exposure. The former would suggest that simply grouping women by a diagnosis of FXPOI is not sensitive enough to identify any

effect. Indeed, we have shown previously that women with a premutation who do not have a diagnosis of FXPOI have indicators of ovarian dysfunction (e.g., lower AMH levels and higher FSH levels, altered cycles, earlier age at menopause compared with noncarriers) [Sullivan et al. 2005; Allen et al. 2007; Rohr et al. 2008; Allen et al. 2014].

Study Limitations

Our study is the largest study to date on the relationship between telomere length and the fragile X premutation in women. However, as we have noted above, there are some limitations that future studies should overcome. A larger sample size of women with accurate measures of hormone use (dosage and type) would help to confirm and extend our current findings. Also, 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 women at several different time points in their lifetime to study true loss of telomere length. We understand the difficulty of this approach but it would provide the most accurate estimates of telomere length from pre- to post-menopausal stages among both premutation and noncarriers.

Conclusions

Telomere length is considered a biomarker of biological age and our results support the hypothesis that women carrying a fragile X premutation allele have shorter telomeres than age-matched controls. Thus, based on our T/S ratios telomere measurements, they appear to be “biologically older” than women carrying the normal size alleles in the same age group. We did not find an association of telomere length with repeat size of the premutation but we did find an association with having a diagnosis of FXPOI, especially among younger women.

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Figure 1.

1a. Frequency of age at blood draw in cases and controls

1b. T/S ratio by age at blood draw in noncarrier controls

1c. T/S ratio by age at blood draw in premutation cases

1d. Frequency of T/S ratios in cases and controls

Figure 2.

- 2a. T/S ratio by age at blood draw in noncarrier controls only
- 2b. T/S ratio by age at blood draw in premutation cases with FXPOI only
- 2c. T/S ratio by age at blood draw in premutation cases without FXPOI only
- 2d. T/S ratio by age at blood draw in noncarrier controls only older than 36
- 2e. T/S ratio by age at blood draw in premutation cases with FXPOI only older than 36
- 2f. T/S ratio by age at blood draw in premutation cases without FXPOI only older than 36

Table 1

Characteristics of the study population, stratified by carrier and FXPOI status.

	n	Age at blood draw (mean±SD (range))	Age at onset of FXPOI/menopause (mean±SD (range))	Repeat size (mean±SD (range))	% ever used hormones
Non-carriers	81	38.3±8.0 (23–55)	n/a	n/a	n/a
Premutation carriers	172	40.0±8.1 (23–59)	n/a	88.9±17.7 (56–150)	89.5
With FXPOI	41	40.7±7.9 (26–59)	32.2±4.8 (20–40)	89.5±15.2 (61–130)	94.1
Without FXPOI	66	46.3±4.9 (37–58)	48.2±4.5 (41–56)	89.1±17.5 (56–140)	82.7

Results for the linear regression models examining the association of T/S ratio with age at blood draw and premutation carrier status.

Table II

	Noncarriers (n=81)		Premutation carriers (n=172)		All (n=253)	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Intercept	2.99(0.37)	<0.000	1.88(0.18)	<0.000	2.99(0.37)	<0.000
Age	-0.03(0.01)	0.002	-0.01(0.004)	0.097	-0.03(0.01)	0.002
Carrier status	n/a	n/a	n/a	n/a	1.11(0.41)	0.007
Age x carrier status	n/a	n/a	n/a	n/a	-0.02(0.01)	0.046

Table III

Results from the linear regression models examining the association of T/S ratio with repeat size and with ever using hormones, adjusting for age at blood draw, among premutation carriers only.

	Repeat size (n=172)		Hormone use (n=152)	
	β (SE)	p-value	β (SE)	p-value
Intercept	1.38 (0.86)	0.109	2.186(0.26)	<0.000
Age	-0.007 (0.004)	0.082	-0.008(0.005)	0.088
Repeat length	0.007 (0.017)	0.674	n/a	n/a
Repeat length squared	0.000 (0.000)	0.848	n/a	n/a
Hormone use (referent group is no hormone use)	n/a	n/a	-0.273(0.17)	0.111

Results for the linear regression models examining the association of T/S ratio with age at blood draw stratified by premutation carrier status and the diagnosis of FXPOI

Table IVa

	Total Noncarriers (n=81)		Total Premutation with FXPOI (n=41)		Total Premutation without FXPOI (n=66)	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Intercept	2.99(0.37)	<0.000	2.3 (0.025)	<0.000	1.80(0.53)	0.000
Age	-0.027(0.009)	0.002	-0.016(0.005)	0.001	-0.005(0.011)	0.648

Results for the linear regression models examining the association of T/S ratio with age at blood draw stratified by premutation carrier status and the diagnosis of FXPOI for women older than 36 years old

Table IVb

	Total Noncarriers (n=45)		Total Premutation with FXPOI (n=28)		Total Premutation without FXPOI (n=66)	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Intercept	3.34(0.06)	<0.000	1.79 (0.51)	0.000	1.80(0.53)	0.000
Age	-0.035(0.015)	0.019	-0.006(0.011)	0.583	-0.005(0.01)	0.648