Examination of reproductive aging milestones among women who carry the FMR1 premutation

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BACKGROUND: The fragile X premutation is characterized by a large CGG repeat track (55–199 repeats) in the 5′ UTR of the FMR1 gene. This X-linked mutation leads to an increased risk for premature ovarian failure; interestingly, the association of repeat size with risk is non-linear. We hypothesize that the premutation-associated ovarian insufficiency is due to a diminished oocyte pool and examined reproductive aging milestones by repeat size group to determine if the same non-linear association is observed. METHODS: We analyzed cross-sectional reproductive history questionnaire data from 948 women with a wide range of repeat sizes. RESULTS: We have confirmed the non-linear relationship among premutation carriers for ovarian insufficiency. The mid-range repeat size group (80–100 repeats), not the highest group, had an increased risk for: altered cycle traits (shortened cycle length, irregular cycles and skipped cycles), subfertility and dizygotic twinning. Smoking, a modifiable risk, decreased the reproductive lifespan of women with the premutation by about 1 year, similar to its effect on non-carriers. As expected, premutation carriers were found to be at an increased risk for osteoporosis. CONCLUSIONS: Possible molecular mechanisms to explain the non-linear repeat size risk for ovarian insufficiency are discussed.

Keywords: FMRP; infertility; menopause; RNA toxic effect; trinucleotide

Introduction

The CGG repeat sequence located in the 5′ untranslated region (UTR) of the FMR1 gene is now known to lead to three major clinical phenotypes: (i) fragile X syndrome, (ii) late-onset fragile X-related tremor/ataxia syndrome (FXTAS) and (iii) ovarian dysfunction. The expression of each phenotype depends on the size of repeat expansion and the consequent molecular outcome. There are essentially four allelic forms of the gene with respect to the CGG repeat length and stability during transmission. They are referred to as common, intermediate, premutation and full mutation. The full mutation form of the FMR1 gene consists of over 200 repeats and is abnormally hypermethylated. Consequently, no mRNA (or sometimes very small amounts of mRNA) is produced. The lack of the gene product, FMRP, an RNA-binding protein involved in translation suppression, is responsible for fragile X syndrome-related mental retardation (Ashley et al., 1993). Approximately 1/4000 males have fragile X syndrome and by inference, ∼1/8000 females have the allele (for review, see Crawford et al., 2001).

Premutation alleles are defined as such because their long, unmethylated repeat tracks are unstable when transmitted from parent to child and have led to a descendent with fragile X syndrome. Approximately 1/250 females and 1/800 males carry premutation alleles of the range 55–199 repeats. Among women who carry the premutation, ∼16% have premature ovarian failure (POF), or cessation of menses at least 1 year prior to age 40, compared with only 1% in the general population, or a relative risk of 16 (for review, see Sherman et al., 2007). Overall, premutation carriers go through menopause ∼5 years earlier than non-carriers (Hundscheid et al., 2000; Murray et al., 2000; Sullivan et al., 2005) and, among those still cycling, have higher FSH levels (Murray et al., 1999; Hundscheid et al., 2001; Welt et al., 2004; Sullivan et al., 2005). The risk for ovarian dysfunction is not increased among full mutation carriers; thus, the molecular mechanism underlying this premutation-associated disorder is unrelated to the reduction of FMRP.

Interestingly, in previous work, we (Sullivan et al., 2005) and others (Ennis et al., 2006) found that FMR1 repeat size was associated with age of menopause in a non-linear way: the repeat sizes that led to the highest risk for POF and earlier age at menopause appear to be in the mid-range of ∼80–100, not the highest premutation repeat sizes. Here, we have...
investigated this association further and have examined other reproductive aging milestones that may be indicative of ovarian failure.

As reviewed by Nikolaou and Templeton (2004), there is growing evidence that the interval between the critical number of ~25 000 remaining follicles and ~1000, or menopause, is more or less fixed, ~13 years. Assuming the average age at menopause is 51, these data suggest that this critical number of follicles is reached around age 37. It follows that all other reproductive milestones that depend on quantity and quality of follicles are also fixed; i.e. the timing between menstrual cycle alteration and menopause should be fixed. As well, the timing between reduced fertility and menopause should be fixed. The results of Kok et al. (2003) are consistent with this hypothesis. They found that measures of subfertility were correlated with the time of menopause. For example, for every 5-year increase of age at menopause: (i) the probability of reporting menstrual cycle irregularity was reduced by 26%, the probability of ever consulting a physician for fertility problems was reduced by 18%, the probability of staying nulliparous was reduced by 22% and the probability of having a spontaneous abortion was reduced by 11%. In another study, the time interval between the loss of cycle regularity and menopause was found to be ~6 years, irrespective of the age at menopause (Den Tonkelaar et al., 1998). Richardson et al. (1987) directly tied such changes to oocyte reserve. They found that the number of primordial follicles in the ovaries of women who were cycling regularly was 10 times higher than those who had irregular cycles.

In this study, we evaluated reproductive aging milestones (menstrual cycle characteristics and pregnancy outcomes) in a large sample of women with and without the premutation allele using self-reported data from structured questionnaires. We divided premutation women into three groups: low (59–79 repeats), mid (80–100 repeats) and high (101–199 repeats) repeat sizes. Overall, we found that all reproductive aging milestones with the exception of spontaneous abortion rates were present at a higher rate among premutation carriers with 80–100 repeats compared with all other groups. We discuss the implications of these findings with respect to the etiology of the ovarian dysfunction.

Methods and Materials

Study population

The study population analyzed here extends that reported in Sullivan et al. (2005) and was ascertained using the same protocol. Briefly, we surveyed women in the general population and in families with fragile X syndrome for alleles that fell roughly into the upper fifth percentile of the northern European allele distribution, or those with >40 repeats. This strategy enriched the sample for high repeat allele carriers. For each woman identified, we enrolled a woman with <40 repeats ascertained from the same recruitment site, or family, matched on age and ethnic/racial group. All women were between the ages of 18 and 75 and had English as their primary language. All women completed a reproductive history questionnaire and provided a biological sample to determine repeat size. We also invited mothers of each participant to be in the study. This strategy provided us with an additional group of women who had completed most of their reproductive lifespan. Table 1 shows the frequency of participants in different repeat size categories for women who were younger than 40 years at the time of interview and those that were ≥40 when they were interviewed.

Women became participants through three avenues. A few women (n = 13) came into the study because they had POF; for the analyses outlined here, they were excluded because they would inflate estimates of penetrance and, potentially, severity of reproductive traits. Otherwise, participants were ascertained without prior knowledge of ovarian status. However, they fell into two ‘ascertainment’ groups: (i) those identified without knowledge of their reproductive history (Group 1) and (ii) those identified through an offspring (i.e. mothers of participants or mothers ascertained through a child with fragile X syndrome) (Group 2). Thus, the women in the latter group were known to be reproductively successful.

There were 329 mother/daughter pairs and 357 sister/sister pairs ascertained from 311 families with fragile X syndrome. Seventeen of these women fell into both ascertainment groups described above. That is, they were identified through the fragile X survey without knowledge of their reproductive history. In addition, they were identified as mothers of participants. For Table 1, their demographic information is included in both groups.

The protocols and consent forms for each enrollment strategy were approved by the Institutional Review Board at Emory University.

Data collection

We administered the reproductive history questionnaire in person, over the telephone or through the mail. We obtained demographic information including age at interview, date of birth, ethnic/racial group and education. Information on potential confounders and effect modifiers was collected and included smoking (1, ever smoked on a regular basis; 0, otherwise) and hormone use (1, current hormone use; 0, otherwise).

We obtained menstrual cycle history including age at menarche and age and date of last menstrual period. If the date of last menstrual period was more than 2 months prior to the interview, we identified the cause of menses cessation. We were unable to further assess reproductive traits using hormone levels or ultrasound due to limited resources.

Women completed a pregnancy history, noting dates and outcomes of each pregnancy. We asked specific questions concerning fertility problems along with number of months of unprotected intercourse until pregnancy.

Lastly, women completed a brief medical history concerning disorders associated with ovarian dysfunction and co-morbid disorders related to reproductive aging. Although all questions were started with ‘Has a doctor ever told you that you had…?’ all conditions were based on self-report. Medical records were not obtained to verify fertility problems or medical disorders.

To determine if the method of administration (in person, over the telephone or through the mail) was related to outcome measures, we determined whether age at menarche or age at menopause was associated with ‘Has a doctor ever told you that you had…?’ All conditions were based on self-report. Medical records were not obtained to verify fertility problems or medical disorders.

Study population characteristics

The clinical definition of a premutation allele is defined as 55–199 repeats (Sherman et al., 2005); however, there is no biological foundation for the lower end of this range. We chose to categorize repeat size based on their potential risk for expansion to the full mutation. We hypothesize that the property leading to expansion (e.g. chromatin structure, secondary structure of the repeat sequence)
Table 1: Demographic characteristics of study populations grouped by ascertainment according to known reproductive success

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1: women identified without knowledge of reproductive history</th>
<th>Group 2: women identified through a child (known reproductive success)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-carriers</td>
<td>Premutation carriers</td>
</tr>
<tr>
<td></td>
<td>&lt;59</td>
<td>All</td>
</tr>
<tr>
<td>N of women</td>
<td>223</td>
<td>83</td>
</tr>
<tr>
<td>N of women ≥40 years at age of interview</td>
<td>140</td>
<td>128</td>
</tr>
<tr>
<td>Age at interview Mean ± SD (Range)</td>
<td>35.8 ± 13.6 (18–73)</td>
<td>42.7 ± 14.3 (18–75)</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td>African-American</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td>Other/unknown</td>
<td>6.2</td>
</tr>
<tr>
<td>Ever smoked (%)</td>
<td>28.9</td>
<td>38.4</td>
</tr>
<tr>
<td>Body mass index Mean ± SD</td>
<td>26.9 ± 7.3</td>
<td>27.6 ± 6.6</td>
</tr>
<tr>
<td>Education: completed college or more (%)</td>
<td>43.4</td>
<td>42.5</td>
</tr>
<tr>
<td>Current hormone use (%)</td>
<td>34.7</td>
<td>34.1</td>
</tr>
<tr>
<td>Age at first pregnancy Mean ± SD (N)</td>
<td>24.2 ± 5.2 (194)</td>
<td>24.4 ± 5.0 (146)</td>
</tr>
<tr>
<td></td>
<td>24.0 ± 4.2 (147)</td>
<td>24.6 ± 4.8 (217)</td>
</tr>
</tbody>
</table>

Women who were ascertained through both Groups (n = 17, see text) are included twice.
may be involved in the mechanism leading to the risk for ovarian dysfunction. Thus, we defined premutation carriers as those with \( \geq 59 \) repeats based on the observation that alleles in that range can expand to the full mutation in one generation (Nolin et al., 2003). Women with \(< 59\) repeats were designated as non-carriers. We categorized premutation carriers further by the following categories: low (59–79 repeats), mid (80–100 repeats) and high premutation alleles (>100 repeats), these categories being associated with low (>50%), mid (50%–80%) and high risk (>95%) to expand to the full mutation (Nolin et al., 2003). We collapsed women who had alleles with 41–58 repeats (so called intermediate alleles) with non-carriers, because we did not find evidence for an association of intermediate alleles with increased rates of ovarian dysfunction (Sullivan et al., 2005).

Table 1 provides a comparison of the general characteristics of repeat size groups by ascertainment: (i) participants ascertained without knowledge of reproductive success (Group 1) and (ii) those ascertained through offspring (Group 2). Age at interview differed by ascertainment method as well as by repeat size group (\( P < 0.0001 \)). Also, non-carriers and carriers differed significantly with respect to race/ethnicity (\( P < 0.0001 \)). The former represent the ethnic/racial group profile of Metropolitan Atlanta, whereas the premutation group reflects our fragile X clinic population.

### Variable definitions

The goal of this project was to compare reproductive milestones among women grouped by their \( FMR1 \) repeat size using data obtained through retrospective questionnaires. 'Age' was defined relative to the particular characteristic analyzed. Age at interview was used in the fertility analysis, because we did not have an age at the diagnosis of infertility. The age at the time of each pregnancy was used in analyses concerning pregnancy outcomes (i.e. time to first pregnancy, twins and spontaneous abortion rates). For the menstrual cycle analyses, women were asked to describe their cycle traits during the last year that they were cycling naturally and their age at that time was calculated accordingly. Therefore, women who were cycling at the time of interview were asked to describe their cycle characteristics during the year prior to the interview (\( n = 470 \)) and their age was the age at interview. Women who had gone through menopause (defined as cessation of menses for at least 12 months) were asked to describe their cycle characteristic during the last year before cessation of menses (\( n = 206 \)). Women who were still cycling but on hormone medications were asked to describe the year before they went on hormone medications (\( n = 283 \)).

#### Menstrual traits

We first determined whether the continuous menstrual cycle traits were normally distributed. Age at menarche was normally distributed; however, self-reported average cycle length and bleed length were not. Furthermore, self-reported cycle length showed digit preference; e.g. women more often reported 28 or 30-day cycles than 29-day cycles. Thus, we categorized cycle and bleed length based on the top and bottom quartiles of our distribution: >29 days and <27 days for cycle length; >6 days and <5 days for bleed length.

Two questions were utilized to examine cycle regularity. First, we asked if the woman had regular or irregular cycles in the last year that she was naturally cycling. We defined regular as meaning that ‘most cycles were about the same length, plus or minus 2 days’. Next, we asked if the woman ever went 6 weeks or more without a menstrual period. Both were scored as binary variables.

### Infertility traits

Several questions were used to assess fertility problems. Each woman reported whether she ever visited a doctor, clinic or hospital, because she could not get pregnant. In addition, she reported whether she had ever had unprotected intercourse for a year or more without getting pregnant. Finally, each woman reported the number of months of unprotected intercourse that it took to achieve her first pregnancy. For the latter variable, only women who achieved pregnancy were included in the analysis. Time to first pregnancy was dichotomized based on the top quartile of the distribution, i.e. greater than 8 months.

### Pregnancy outcomes

We were primarily interested in the percentage of spontaneous abortion and the percentage of dizygotic twinning, as both traits are known to increase with increasing maternal age (for review, see Niklau and Templeton, 2004). We did not compare the average number of pregnancies, as this measure may differ between premutation and non-carriers for other reasons than infertility. For example, premutation carriers may limit their family size due to the risk of having an offspring with fragile X syndrome.

#### Additional risk factors for ovarian failure

Women self-reported diagnoses of Lupus, diabetes and/or Graves' disease. Each was considered as an outcome variable and was tested in a model with repeat size group, age at interview, smoking and ethnic/racial group. The effect of smoking on age at menopause was also investigated (see Statistical analysis below).

#### Co-morbid medical conditions

Women self-reported diagnoses of osteoporosis and estrogen-related cancers. Each was considered as an outcome variable and tested in a model with repeat size group, age at interview, smoking and ethnic/racial group.

#### Laboratory methods

DNA was extracted from buccal samples or blood using Qiagen QiAmp DNA Blood Mini Kit. \( FMR1 \) CGG repeat sizes were determined by a fluorescent-sequencer method, as described elsewhere (Meadows et al., 1996), using the ABI Prism 377 DNA Sequencer. For females with only one allele, a second PCR-based, hybridization technique was used to identify a possible high band. The protocol is a modified version of that developed by Brown et al. (1993). For females, if no high repeat allele was identified using this follow-up method, we concluded that the woman was homozygous for the smaller allele.

### Statistical analysis

We examined the age-specific prevalence of POF for women with low, mid and high repeat size groups, as defined above. We calculated the prevalence of POF prior to age 15, 20, 25, 30, 35 and 40. For this analysis, we included in the denominator all women who entered the interval still naturally cycling or had stopped cycling for at least a year prior to that interval. The numerator included women who had self-reported menopause at the specified age or earlier. We excluded from the analysis all women who had chemotherapy or radiation therapy, a hysterectomy or eating disorder. We also used survival analysis to determine the average age at menopause for each group. Hazard ratios were determined using a Cox proportional hazard model adjusting for age at interview, racial/ethnic group and smoking. In addition, we used the same model in frailty analysis to adjust for the dependency of related individuals. We report the \( P \)-values based on frailty analysis.
To account for the potential dependency among women ascertained from the same family, we used generalized-estimating-equation (GEE) methodology (Zeger and Liang, 1986) to determine whether repeat size had an effect on reproductive outcomes. For all analyses, we tested for the potential confounding effects of ethnic/racial group, the appropriate age variable and smoking, an exposure known to affect age at menopause (Cooper et al., 1999). If the variable changed the odds ratio of the repeat size variable by more than 10%, we included these covariates in the model.

Lastly, we examined the effect of smoking on age at menopause among premutation carriers to determine if the effect of this endocrine disruptor was additive or multiplicative (i.e., synergistic). We used the binary variable of ‘ever smoked regularly’ in the presentation of the survival curves for premutation and non-carriers. We used frailty analysis conditioning on family membership to test for significance where age at menopause was defined as the failure event and age at interview was defined as the censored event. Covariates included premutation group, race/ethnicity and age at interview. The interaction term of smoking status and premutation status was used to examine a synergistic effect.

We present odds ratios for premutation repeat size groups adjusted for confounders and 95% confidence interval using non-carriers as the referent group for each analysis. The authors of this manuscript had thorough discussions regarding an appropriate correction for multiple testing within our analyses. A typical multiple-testing correction is a Bonferroni correction that divides the nominal significance level (here alpha = 0.05) by the number of tests performed. However, by using a Bonferroni correction, one implicitly assumes that all tests performed are independent. This assumption is clearly violated in our analyses, because our tested outcomes are functions of ovarian aging. This adjustment, coupled with the fact that all other repeat size groups. At age 40, the cut-off for the clinical definition of POF, the odds ratios for each premutation group compared with non-carriers are: 3.08 (0.89–10.65), 12.57 (5.27–29.98) and 6.77 (1.86–24.69).

Using survival analysis, the unadjusted mean age at menopause for the four groups were 52.3 ± 0.5, 48.5 ± 0.7, 44.9 ± 0.6 and 47.5 ± 1.2, respectively. In a Cox proportional hazards model adjusting for age at interview, racial/ethnic group and smoking, all three premutation groups were significantly different from controls (HR = 2.22 (1.51–3.26), 5.02 (3.52–7.16) and 2.94 (1.69–5.11), respectively; P < 0.0001 based on frailty models.

Reproductive aging milestones

Menstrual cycle characteristics

The mean ± SD age of menarche for controls, low, mid and high premutation groups were 12.44 ± 1.51 (n = 521), 12.18 ± 1.38 (n = 127), 12.35 ± 1.57 (n = 237) and 12.53 ± 1.33 (n = 70), respectively. The difference in age of menarche for women with 59–79 repeats (low premutation) was significantly different from that of non-carriers (P = 0.01) when adjusted for ethnic/racial group and age at interview.

Low- and mid-premutation carriers were more likely to report short cycle lengths (<27 days) compared with non-carriers, controlling for ethnic/racial group (P = 0.01 and P < 0.001, respectively; Table 2). To determine if premutation carriers differed from non-carriers at both ends of the spectrum of cycle length (shorter or longer), we compared women with short cycles to those with middle 50% and compared those with long cycles (>29 days) to those with the middle 50% of the distribution. There was no association of premutation carriers with long cycles, only with the short cycles (data not shown). There was no association between bleed length and repeat size.

Onset of cycle irregularity is another distinctive reproductive aging milestone that usually follows shortened cycles. Women with mid-size premutation repeats were more likely to report irregular cycles (Table 2; OR = 1.49; 95% CI = 1.04–2.14; P = 0.03).

The next cycle trait in the reproductive timeline is skipped cycles, eventually leading to cessation of cycles. Women with low- and mid-size premutations were more likely to have gone 6 weeks or more without a menstrual period (OR = 1.66; 95% CI = 1.04–2.66; P = 0.03 and OR = 1.80; 95% CI = 1.25–2.58; P = 0.001, respectively).

To ensure that results were robust against recall bias, we stratified the sample into two groups: those who were currently cycling when they reported their cycle characteristics and those who had gone through menopause and reported cycle traits in the last year before that event. We did not include women who were currently on hormones at the time of the interview as this group is more heterogeneous with respect to their reproductive stage. The pattern among mid-premutation women showing shortened cycles, irregular cycles and skipped cycles was similar in menopausal and currently cycling women, although the odds ratio was only significant among menopausal women (data not shown).

Loss of fertility

We assessed fertility with three measures: having unprotected intercourse for a year or more without becoming pregnant,
having consulted a health care provider because of difficulty becoming pregnant and among women who became pregnant, the number of months of unprotected intercourse before becoming pregnant (i.e. ‘time to pregnancy’). By each measure, the results from mid-repeat range premutation carriers indicated that they had lower fertility than the non-carriers or the other permutation carrier groups (Table 2). This difference was most apparent for consultation with a medical professional (OR = 1.27; 95% CI = 1.17–2.59; P = 0.006, compared with non-carriers). When we excluded women with known reproductive success (Group 2), the pattern of odds ratios was similar to that estimated from the entire study sample (data not shown).

**Pregnancy outcomes associated with reproductive aging**

We found a significant increase in the rate of DZ twinning among premutation carriers with 80–100 repeats compared with non-carriers (P = 0.02; Table 2) after adjusting for age of the mother at the time of the birth. The DZ twinning rate was also increased for high-repeat carriers but was not statistically different from non-carriers.

We used spontaneous abortion rates as an indicator of oocyte quality. There were no statistical differences among groups, although the rate among the mid-size repeat group of premutation carriers was increased compared with non-carriers (P = 0.16; Table 2). For each pregnancy, the woman was asked to self-report any associated birth defects. We were particularly interested in those that may indicate a birth defect resulting from chromosome non-disjunction. Out of 2140 pregnancies, only two were reported to involve an extra chromosome. Both women were premutation carriers (65 and 105 CGG repeats) and both were age 28 at the time of their baby’s birth.

**Additional risk factors that may enhance ovarian failure**

We examined two risk factors that are known to be associated with ovarian dysfunction, autoimmune disorders and smoking. These factors may exacerbate the effect of the premutation.

In the structured questionnaire, we asked if women had been diagnosed with Lupus, diabetes and/or Graves disease. There was no association of Lupus, diabetes or Graves’ disease with any of the repeat size groups (Table 3).

In contrast, we found that smoking reduced age at menopause among both premutation groups and non-carriers for smokers. Unadjusted survival curves indicated an additive effect of smoking on age at menopause (Fig. 2). Formally, the interaction term was not significant (data not shown).

Table 4 shows the adjusted hazard ratios for carrier status and smoking. When heavy smoking (defined as ≥10 pack years) was used, the same patterns were seen (data not shown).
Co-morbid medical conditions associated with reproductive aging

Estrogen-deficiency is associated with reproductive aging and can lead to co-morbid conditions. For example, osteoporosis increases with increasing exposure to estrogen deficiency while breast and ovarian cancer decrease with earlier exposure to estrogen-deficiency. Among premutation carriers, women with mid-range repeats reported a significantly increased frequency of osteoporosis (Table 3). When only women who had gone through menopause were analyzed, the same pattern was seen with mid-range repeats having the greatest OR, although the difference was no longer statistically significant. Our sample size was too small to evaluate the frequency of osteoporosis (Table 3).

Discussion

POF was the first premutation-associated disorder identified in families with fragile X syndrome (for review, see Sherman et al., 2007). To identify factors that explain the reduced penetrance of POF, we established a cross-sectional study of premutation carriers and non-carriers. In the study by Sullivan et al. (2005), we first confirmed previous findings that premutation carriers have an increased risk of ovarian insufficiency; they have at least a 13-fold higher frequency of POF and a 5-year earlier age at menopause (by any definition of menopause) compared with non-carriers. Most importantly, the data from Sullivan et al. gave us our first hint that repeat size was strongly associated with ovarian insufficiency, albeit in a non-linear way: the risk for ovarian dysfunction (defined by both prevalence of POF and age at menopause) was highest for mid-range carriers with repeats of ~80–99. These data were recently confirmed by Ennis et al. (2006). In this report, we have now followed up this finding in our updated sample of 948 women and further characterized the repeat association with important measures of reproductive aging as well as the prevalence of POF and age at menopause.

With respect to the clinical disorder defined as POF, we found that the mid-size repeat group has the highest risk and the earliest onset compared with other repeat groups (Fig. 1). The prevalence of POF among low repeat and high repeat carriers is also increased compared with non-carriers, but not to the same extent. Of course, this pattern depends on our a priori definition of repeat size groups. Upon closer examination of POF within the low repeat premutation group, there are 0/17 women who have 59–70 repeats with POF and 5/22 of women with 71–79 repeats report POF. Similarly, among women in the high repeat group, 4/12 women with 101–120 repeats reported POF and 0/3 women with >120 repeats reported POF. Although the sample size in some of these categories is small, this post hoc analysis suggests that at-risk alleles may be better defined as those between 70 and 120 repeats. The upper end of this range is less precise than the lower end due to technical difficulties of accurately defining repeat sizes in the high range. Nevertheless, the mid-size repeat alleles exert the greatest risk for POF. This is also evident based on the mean age at menopause: mid-range repeat size alleles impose a significant 7-year reduction in the overall mean age at menopause.

Table 2: Comparison of reproductive aging milestones by repeat size group

<table>
<thead>
<tr>
<th>Reproductive Trait</th>
<th>Non-carriers</th>
<th>Premutation carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (59–79 repeats)</td>
<td>Mid (80–100 repeats)</td>
</tr>
<tr>
<td>Cycle characteristics</td>
<td>Crude % (N)</td>
<td>Referent group</td>
</tr>
<tr>
<td>Short cycles (&lt;27 days)</td>
<td>22.0 (441)</td>
<td>–</td>
</tr>
<tr>
<td>Irregular cycles (±2 days)</td>
<td>25.8 (469)</td>
<td>–</td>
</tr>
<tr>
<td>Skipped cycles (&gt;6 weeks)</td>
<td>23.0 (469)</td>
<td>–</td>
</tr>
<tr>
<td>Short bleed length (&lt;5 days)</td>
<td>27.9 (469)</td>
<td>–</td>
</tr>
<tr>
<td>Fertility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1 year of intercourse and not pregnant</td>
<td>30.6 (519)</td>
<td>–</td>
</tr>
<tr>
<td>Visit a doctor for fertility problems</td>
<td>12.3 (514)</td>
<td>–</td>
</tr>
<tr>
<td>Time to first pregnancy (&gt;8 months)</td>
<td>18.9 (365)</td>
<td>–</td>
</tr>
<tr>
<td>Pregnancy outcomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous abortion</td>
<td>12.1 (1059)</td>
<td>–</td>
</tr>
<tr>
<td>DZ twinning</td>
<td>0.9 (793)</td>
<td>–</td>
</tr>
</tbody>
</table>

Bold signifies that the repeat size group was significantly different from the referent group (non-carriers) at P ≤ 0.01. Odds ratios were adjusted for the covariates listed in the footnotes. All analyses were done using GEE methodology to adjust for the potential dependency among women ascertained from the same family.

a Adjusted for racial/ethnic group.
b Adjusted for racial/ethnic group and age at interview.
c Adjusted for age at interview.
d Adjusted for age of mother at the time of pregnancy.
e Number of pregnancies.
f Number of live births.

Allen et al.
The increase frequency of POF and the overall reduction in age at menopause may be due to either a smaller ovarian reserve established during the development of oocytes or to an increased rate of follicular atresia. If true, premutation carriers should experience their reproductive milestones earlier than non-carriers. They should experience menstrual cycle alterations, subfertility, increased rates of DZ twinning, increased incidence of aneuploidy and spontaneous abortion (reviewed in Te Velde and Pearson, 2002). Moreover, women with repeat sizes in the mid range should show these traits more frequently based on the above observations. To test this hypothesis, we measured the frequency of such milestones during the last year of natural cycling in a woman’s reproductive life.

We found that premutation carriers and, most significantly, mid-size repeat premutation carriers reported menstrual cycle alterations (short, irregular and skipped cycles) more often than non-carriers. These alterations were perhaps indicators of subfertility: only the mid-size group with earlier onset of POF, the 7-year reduction of age at menopause and altered cycle characteristics had significant infertility problems. We also found that DZ twinning was increased among the mid-size repeat group, perhaps indicating reduced ovarian feedback to the increased secretion of pituitary gonadotrophic hormone (for review, see Lambalk et al., 1998). Interestingly, studies in the past have been inconsistent with respect to observing an increased rate of DZ twinning among premutation carriers; some finding a significant increase (Fryns, 1986; Turner et al., 1994; Vianna-Morgante, 1999), whereas others did not (Sherman et al., 1996; Murray et al., 2000; Hundscheid et al., 2003). We suggest that the inconsistent data are due to the difference in the study population with respect to repeat size.

We did not find an increased rate of spontaneous abortions among premutation carriers. The finding is consistent with other studies of premutation-associated ovarian dysfunction (Murray et al., 2000; Hundscheid et al., 2003). An increased rate of spontaneous abortion is strongly associated with maternal aging and primarily the result of chromosome non-disjunction. Although rate of spontaneous abortion is only a crude surrogate for rate of non-disjunction, the lack of an association among premutation carriers suggests that the quality of the oocyte is not compromised.

Applications to the clinical realm
Many surveys have been conducted to determine the frequency of premutation carriers among women with idiopathic POF.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Non-carriers</th>
<th>Premutation carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude % (N)</td>
<td>Referent group</td>
</tr>
<tr>
<td><strong>Autoimmune disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lupusa</td>
<td>0.4 (520) –</td>
<td>0.8 (126) 1.78 (0.17–18.90)</td>
</tr>
<tr>
<td>Diabetesa</td>
<td>5.2 (519) –</td>
<td>8.7 (127) 1.49 (0.63–3.50)</td>
</tr>
<tr>
<td>Graves diseaseb</td>
<td>10.1 (516) –</td>
<td>18.1 (127) 1.57 (0.87–2.82)</td>
</tr>
<tr>
<td><strong>Co-morbid medical conditions associated with reproductive aging</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoporosisa</td>
<td>6.6 (517) –</td>
<td>11.5 (122) 1.24 (0.60–2.53)</td>
</tr>
<tr>
<td>Breast cancera</td>
<td>1.7 (520) –</td>
<td>1.6 (124) 0.77 (0.16–3.68)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>0.6 (520) –</td>
<td>0 (124)</td>
</tr>
<tr>
<td>Uterus removeda</td>
<td>17.0 (519) –</td>
<td>22.8 (127) 0.91 (0.55–1.52)</td>
</tr>
</tbody>
</table>

Odds ratios compare the frequency of the disorder in the carrier group to non-carriers adjusted for confounders indicated in footnotes. Odds ratios in bold indicate a significant difference from non-carriers at \( P \leq 0.01 \).

aAdjusted for age at interview.

bAdjusted for age at interview and ethnic/racial group.

cAdjusted for age at interview and menopause status.

dAdjusted for age at interview and smoking status.
Since the original survey by Conway et al. (1995) that showed an increased frequency of premutation carriers among POF women, others have examined various series of women with POF ascertained through infertility clinics, obstetrics and gynecology clinics, genetic laboratories and general surveys (for review, see Sherman et al., 2007). Using studies that identified women primarily through a reproductive endocrinology clinic and that clearly distinguished familial from sporadic POF (Murray et al., 1998; Marozzi et al., 2000; Mallolas et al., 2001; Bussani et al., 2004), the estimated percentage of women who are premutation carriers is 11.5% (familial POF) and 3.2% (sporadic POF). Based on the data presented here, we predict that the majority of women identified in such clinics would have mid-size repeat alleles. Such repeats have a high risk to expand to the full mutation when transmitted from the mother to her offspring. Importantly, only a subset of premutation carriers experience fertility loss. Any environmental exposure that reduces age at menopause can lead to increased subfertility in this group of women who are at risk for a shortened reproductive lifespan.

Smoking is a well-known endocrine disruptor and leads to decreased age at menopause (Cooper et al., 1999). We hypothesized that smoking may significantly reduce the age at menopause among premutation carriers and, potentially, have a synergistic effect. Although we did not see a synergistic effect, smoking reduced age at menopause by 1 year, a significant reduction if a woman’s reproductive years are already compromised.

Earlier menopause leads to an earlier deficiency in estrogen, which is associated with several other co-morbid conditions. First, all premutation carriers showed an increase in risk for osteoporosis, and for mid-premutation carriers, this increase was significant. Although the numbers are very small, premutation carriers also show a lower frequency of breast and ovarian cancers.

Implications for molecular etiology

We have confirmed the non-linear association of premutation allele size and risk for ovarian insufficiency as measured by early cessation of menses as well as other reproductive aging milestones. Thus, any hypothesis proposed to explain the molecular etiology of the premutation-associated disorder must be put into this context.

There are at least two possible mechanisms that could be proposed. First, we hypothesize that the premutation effect occurs during the prenatal development of the oocyte pool, reducing their numbers in the store. Expression studies indicate that FMRP may be important at this stage, since it is highly expressed in the germ cells of the fetal ovary (Bachner et al., 1993; Rife et al., 2004). We know that FMRP regulates translation of a subset of mRNAs through a suppression mechanism (Jin et al., 2004). Perhaps, increased levels of FMRP at specific times during development can lead to haploinsufficiency of the proteins needed in oocyte development. Analyses of X chromosome alterations identified in women with POF provide support for this model (Bione et al., 2004; Rossetti et al., 2004; Rizzolio et al., 2006). As Rizzolio et al. (2006) have shown that the most plausible explanation for POF identified in women with balanced X-autosome translocations is a position effect of the breakpoints on flanking genes, causing them to be either silenced or down-regulated. In order to fit the non-linear association between severity of ovarian insufficiency and repeat size, we propose that translation inefficiency of FMRP is restricted to alleles with >~100 repeats. Below that repeat length, increasing mRNA levels produce higher FMRP levels, leading to haploinsufficiency of FMRP-suppressed transcripts. Model systems provide some suggestions for candidate genes that may interact with FMRP. For example, in Drosophila, two genes involved in oogenesis have been shown to be associated with dFmr1: lgl and FMRP proteins form a complex in both flies and mice (Zarnescu et al., 2005). Orb is negatively regulated by dFmr1 (Costa et al., 2005). In a saturating screen with dfmr1, Zarnescu et al. (2005) also identified two genes that have been linked to POF, diaphanous and dachshund. Alternatively, we hypothesize that the large repeat track in the mRNA produced by the premutation allele causes a toxic effect over time, leading to an increased rate of follicular atresia later in a woman’s reproductive life. This dominant gain-of-function mechanism is well supported for the other known premutation-associated disorder, FXTAS (Hagerman and Hagerman, 2002; Jin et al., 2003; Willemsen et al., 2003). FMR1 expression studies identified transcripts in granulosa cells of ovarian follicles (Hinds et al., 1993; Hergersberg et al., 1995). Interestingly, transcripts were present in maturing follicles only, not those in the early stages (Hergersberg et al., 1995). One study did fail to identify expression in the older ovary of a 28-week-old mouse, but that may have been due to the lack of maturing follicles (Bachner et al., 1993). To explain the non-linear association of repeat size, we posit that rCGG repeats take on a different conformation or behavior when above 100 repeats (Sullivan et al., 2005).

Although we suggested time points for these particular mechanisms, they could have their impact at other stages of development. Nevertheless, these suggested mechanisms can be used as a platform for asking specific questions. If the ovarian pool is indeed diminished during fetal development, we expect that reproductive aging milestones will occur at the same fixed intervals relative to the start of menopause, but they will occur earlier than in non-carriers (Fig. 3). Also, we would expect to observe diminished ovarian reserve compared with non-carriers at all ages. Alternatively, if the premutation effect is due to an accumulation of toxic mRNA, as is the case in FXTAS, we expect that the interval between the typical reproductive aging milestones and menopause may be reduced (Fig. 3). Moreover, we expect that young premutation carriers should have measures of ovarian reserve that are similar to controls.

Limitations

This analysis was based on the largest number of premutation carriers and non-carrier controls collected to date. However, the data are limited in that they are based on self-report through a structured questionnaire. Also, not all women were ascertained at the same time in their reproductive lifespan. Thus, some reported characteristics of their cycle traits that were occurring at the time of interview, whereas others were
Figure 3: Hypotheses for loss of ovarian function among premutation carriers based on number of oocytes and reproductive stages

reporting on those in the past. Resources were not available to assess cycle or fertility traits using more sophisticated measures such as ultrasound imaging or hormone levels. Lastly, we were unable to abstract medical records to corroborate medical diagnoses. These limitations were not restricted to any one repeat size group. Thus, the ‘noise’ or misclassification will bias results toward the null hypothesis, i.e. it will reduce or dampen observed differences between the groups.

Summary
In conclusion, we have confirmed the non-linear association of repeat size and ovarian insufficiency: carriers with 80–99 repeats compared with non-carriers have increased rates of menstrual dysfunction, infertility and dizygotic twinning. They also have a 7-year reduction in mean age at menopause, and consequently, an increased prevalence of POF (32% versus 1%) and an increased risk of osteoporosis. Carriers of both smaller and larger premutation repeat sizes also suffer from ovarian insufficiency, but not to as great an extent.

Although repeat size can account for much of the variation in the severity of ovarian insufficiency, most likely other genetic factors as well as the environment also plays a role. Thus in the clinical setting, modifiable risk factors such as environmental exposures (e.g. smoking) should be identified and addressed. Also, patient education for premutation carriers should be provided not only on reproductive issues, but also their increased risk for co-morbidities associated with reproductive aging, such as osteoporosis.

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