Diminished ovarian reserve is not observed in infertility patients with high normal CGG repeats on the fragile X mental retardation 1 (FMR1) gene

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STUDY QUESTION: Does an association exist between high normal numbers of CGG trinucleotide repeats on the fragile X mental retardation 1 (FMR1) gene and diminished ovarian reserve (DOR)?

SUMMARY ANSWER: This large data set demonstrated that a high normal number of CGG repeats (35–54 repeats) on the FMR1 gene was not significantly correlated with DOR.

WHAT IS KNOWN ALREADY: The FMR1 premutation (55–200 repeats) is a known cause of primary ovarian insufficiency. However, the relationship between high normal CGG repeat numbers (35–54 repeats) and ovarian reserve has yet to be conclusively demonstrated.

STUDY DESIGN, SIZE, DURATION: This is a retrospective data analysis conducted between January 2012 and February 2014 that included 1287 women. Over 1140 women had complete data.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All women, excluding oocyte donors, who presented to a large private practice specializing in reproductive endocrinology and infertility for treatment and who underwent both fragile X and ovarian reserve testing were included. All fragile X testing was performed using triplet repeat PCR, with confirmation of positives by Southern blot. CGG repeat numbers from both alleles were recorded, and the allele with the higher number of repeats was used for statistical calculations. We did not differentiate between patients with one or two high normal alleles. Women with >54 CGG repeats were excluded from the analysis. For our analysis, we considered both a ‘high normal’ number of CGG repeats (35–44) and an intermediate number of GCC repeats (45–54) as ‘high normal’. Ovarian reserve testing was carried out on Cycle Day 2 or 3 and included measurements of FSH, anti-Müllerian hormone (AMH) and antral follicle count (AFC). A generalized linear regression model assuming gamma distribution and log link function that controlled for age was used to assess correlation between CGG repeat number and FSH, AMH and AFC.

MAIN RESULTS AND THE ROLE OF CHANCE: As expected, there was a significant correlation between increasing age and increasing FSH and decreasing AFC and AMH for the patients in this study. For every 1-year increase in age, FSH increased by a factor of 1.04, AFC decreased by a factor of 0.93 and AMH decreased by a factor of 0.89. After controlling for age, there was no significant correlation between FMR1 CGG trinucleotide repeat number and FSH (P = 0.23), AFC (P = 0.14) or AMH (P = 0.53). Three subgroup analyses were also performed. We found a significant relationship between increasing CGG repeat number and decreasing AMH levels (P = 0.01) in women >44 years old. The second subgroup analysis included only Caucasian patients and found no significant correlation between CGG repeat number and DOR. In a subgroup analysis comparing women with at least one allele <26 repeats, at least one allele >35 and women with both alleles between 29 and 32, there were no significant associations regarding ovarian reserve in any of these groups.
Introduction

The fragile X mental retardation 1 (FMR1) gene in its fully expanded form is responsible for the condition that is the most common cause of inherited mental retardation and autism. The full mutation of this gene, which is on the X chromosome, involves abnormal expansion of a trinucleotide (CGG) repeat sequence in the promoter region, leading to the methylation and halted production of fragile X mental retardation protein (FMRP). The FMR1 premutation causes an increase in mRNA expression, which in turn, causes fragile X-associated tremor/ataxia syndrome and fragile X-associated primary ovarian insufficiency (POI) (Bodega et al., 2006; Wittenberger et al., 2007). Smaller expansions of these CGG repeat sequences generate different clinical manifestations.

Mutations of the FMR1 gene are categorized by the number of CGG trinucleotide repeats present. The full mutation contains >200 repeats, the premutation contains 55–200 repeats and the intermediate zone contains 45–54 repeats (Spath et al., 2011a). The carrier frequency in females is 1 : 178, and the premutation carrier frequency is 1 : 257–1 : 2687 (Hantash et al., 1991). Fu’s landmark work (Fu et al., 1991) defined the upper limit of normal for repeat number to be 32. However, more recent studies have reclassified 35–44 repeats as the high normal range, and most studies examining the relationship of ovarian reserve to trinucleotide repeat number use this range (Fu et al., 1991; Pastore et al., 2012; Barasoain et al., 2013). The extent to which trinucleotide repeat variation impacts ovarian function and contributes to diminished ovarian reserve (DOR) warrants further study.

While both the American Congress of Obstetricians and Gynecologists and the Accreditation Counsel of Graduate Medical Education have stated that individuals with <45 CGG repeats do not have abnormal phenotypes in regard to POI, DOR is not an overt phenotype and is much more subtle (Pastore and Joshson, 2014). Occult POI is not an easily recognized phenotype and has been defined in the literature as infertility, low anti-Müllerian hormone (AMH) levels, mildly elevated FSH levels and/or a resistance to ovarian stimulation in women <40 years old with either regular or irregular menstrual cycles (Streuli et al., 2009; Karimov et al., 2011). Therefore, the subtle clinical manifestations may have been overlooked in this broad statement regarding phenotype.

The prevalence of POI in premutation carriers (55–200 repeats) is estimated to be between 13 and 26% (Sullivan et al., 2005). Premutation carriers who do not have frank ovarian insufficiency have been reported to have DOR, as evidenced by higher levels of FSH in the third decade of life and an average onset of menopause that is 5 years earlier than in the general population (Loesch and Hagerman, 2012). Additionally, a study by Spath et al. (2011b) concluded that premutation carriers have lower AMH levels at all ages compared with non-carriers. Several small studies have also reported an association between women with high normal and intermediate CGG repeat numbers (35–54 repeats) and DOR (Bretherick et al., 2005; Gleicher, 2009; Gleicher and Weghofer, 2009; Gleicher et al., 2009; Gleicher et al., 2010b; Pastore et al., 2012). However, studies have had conflicting results, and this association has not been conclusively demonstrated.

In 2005, Bretherick et al. conducted a study that included 53 women with POI and 161 population controls (Bretherick et al., 2005). The POI population demonstrated a significantly higher number of FMR1 alleles containing between 35 and 54 CGG repeats (14.2%) compared with controls (6.5%), and the findings indicated that a high normal number of trinucleotide repeats was clinically and statistically significant (P = 0.02). This was supported by Pastore et al. who studied the prevalence of high normal CGG repeats in 62 infertile DOR patients versus the female general population. They found the prevalence of 35–44 CGG repeats to be 14.5% in the DOR patients versus 3.9% in the general population (Pastore et al., 2012).

Gleicher et al. published several studies examining high normal CGG repeats and ovarian reserve with mixed results (Gleicher, 2009; Gleicher and Weghofer, 2009; Gleicher et al., 2009; Gleicher et al., 2010b). In a pilot study in 2009 that included 40 infertile patients, an increasing CGG repeat number was positively correlated with increasing FSH levels (Gleicher, 2009). In contrast, a later study with 158 patients failed to demonstrate a statistically significant correlation between CGG repeat number and FSH levels (Gleicher and Weghofer, 2009). Most recently, a study of 316 patients found that for CGG repeat numbers >34, every increase by five CGG trinucleotide repeats increased the risk of DOR by 50% (Gleicher et al., 2009).

Given the varying results demonstrated by prior smaller studies, the objective of this study was to further investigate the possible correlation between high normal CGG repeat numbers and DOR in a larger population. Due to the high frequency of carriers, accurate information on the ovarian impact of high normal CGG repeat numbers is crucial if we are to...
effectively counsel women on fertility, timing of childbearing and options for fertility preservation.

**Materials and Methods**

**Ethical approval**

Institutional review board (IRB) approval was obtained through the New England IRB. All participants provided written informed consent allowing their data to be used in the analysis.

**Study design**

All women, excluding oocyte donors, who presented to Fertility Centers of Illinois, between January 2012 and February 2014 who underwent both fragile X and ovarian reserve testing were included. The physicians at Fertility Centers of Illinois offer universal genetic screening to all patients who present for treatment regardless of infertility diagnosis and ethnicity. All testing was performed prior to any type of treatment.

A retrospective analysis was performed to evaluate the relationship between CGG repeat numbers and ovarian reserve parameters as measured by Day 3 FSH levels, AMH levels and antral follicle count (AFC). Women with >54 CGG repeats were excluded. All fragile X testing was carried out with triplet repeat PCR, with confirmation of positives by Southern blot analysis (Good Start Genetics®, USA). Less than 35 CGG repeats was considered to be in the normal range, and the high normal range was 35–44 repeats (Pastore et al., 2012; Barasoain et al., 2013). The intermediate range of 45–54 was also considered as ‘high normal’ and included in the analysis. Ovarian reserve testing was carried out on Cycle Day 2 or 3 and included serum levels of FSH, AMH and transvaginal ultrasound-guided measurements of AFC. FSH was measured using a chemiluminescence immunoassay (Siemens®, Immulite 2000XPi, Germany), and AMH was measured using an electrochemiluminescent assay (LabCorp®, USA). All ultrasounds were performed transvaginally on Cycle Days 2–4, and the measured antral follicles were between 2 and 10 mm in mean diameter in the greatest 2D plane.

**Statistical analysis**

CGG repeat numbers from both alleles were recorded, and the allele with the higher number of repeats was used for analysis. Previous publications have indicated that X chromosome inactivation does not impact the clinical manifestation of POI (De Geyter et al., 2013). Therefore, we did not differentiate between patients with one or two high normal alleles. Characteristics of the subjects were summarized with the use of descriptive statistics. Differences across repeat numbers were assessed by one-way analysis of variance for continuous variables and chi-squared tests for categorical variables. To investigate the relationship between CGG repeat number and ovarian reserve parameters, a generalized linear regression model adjusted for age was used. The ovarian reserve parameters are in the form of counts, and their distributions are skewed; thus, in the model, we assume gamma distribution with log link function. First, we used repeat numbers as a continuous variable. To accurately be able to compare our results to other published studies that used discrete groupings categorized by the number of GCC repeats present, we also analyzed the data in this fashion with the four groups defined as <35, 35–39, 40–44 and >44 CGG repeats. Statistical analysis was performed using SAS software, USA. A P-value of <0.05 was considered statistically significant.

As this was a retrospective analysis, the sample size was determined by a defined period of time in which we collected samples. A post hoc power analysis was performed based on FSH measurements and CGG repeat numbers stratified into four groups by repeat numbers: <35, 35–39, 40–44 and >44 repeats. We hypothesized that women with higher repeat numbers would have higher FSH levels, and we calculated the minimum difference (effective size) in FSH that the study was powered to detect. Due to the multiple comparison groups, we used a Bonferroni correction and the significance was set at 0.017. For the repeat group <35 (n = 1079), we had 80% power to detect a 2.3 mIU/ml difference in FSH levels with a significance of 0.017. For the repeat groups 35–39 (n = 110) and 40–44 (n = 65), we had 80% power to detect a 3.5 mIU/ml difference in FSH levels with a significance of 0.017. In the repeat group >44 (n = 33), we had 80% power to detect a difference of 4.8 mIU/ml in FSH levels with a significance of 0.017.

**Results**

A total of 1287 women underwent both FMR1 and ovarian reserve testing. Most women (84%) had <35 repeats (n = 1079). Of these, 208 women (16%) had high normal repeat numbers, defined as 35–54 repeats. Of the 1287 women included in the study, 14 patients (1%) had two high normal alleles, while 195 patients had one high normal allele. The original study population contained 1375 patients before excluding oocyte donors. Of these 1375 patients, the frequency of larger CGG expansions was very rare with only 2 (0.1%) patients possessing alleles with >55 repeats.

Women in this study had a mean age of 35.9 ± 4.4 years, a mean FSH of 9.1 ± 7.3 mIU/ml, a mean AMH of 2.6 ± 3.1 ng/ml, and a mean AFC of 15.2 ± 10.2 (data are presented as mean ± SD). There were no statistical differences in patient characteristics or ovarian reserve testing amongst CGG repeat number groups (Table I). The patients in this study were categorized by infertility diagnosis. Given that the outcomes of the study focus on ovarian reserve, the diagnoses were classified as DOR and all remaining diagnoses such as male factor or uterine anomalies were classified as other. Finally, as seen in Table I, the majority of the patients in this study were Caucasian and non-smokers (67.6 and 63.6%, respectively).

As expected, there was a significant correlation between increasing age and increasing FSH and decreasing AFC and AMH for the patients in this study. For every 1-year increase in age, FSH increased by a factor of 1.04, AFC decreased by a factor of 0.93 and AMH decreased by a factor of 0.89 (Table II). After controlling for age, there was no significant correlation between FMR1 CGG trinucleotide repeat number and FSH (P = 0.23), AFC (P = 0.14) or AMH (P = 0.53) (Fig. 1). The estimate results were robust to the multivariate regression model with categorical CGG repeat numbers.

Several subgroup analyses were additionally performed. The first aimed to see if the impact of increasing CGG repeat number on ovarian reserve was more pronounced in older patients. Patients were stratified into groups based on age, which included those <35, 35–37, 38–40, and >40 years old. Interestingly, we found that in women >44 years old, there was a significant relationship between increasing CGG repeat number and decreasing AMH levels (P = 0.01). For the remaining age groups, we found no significant association between increasing CGG repeat number and decreasing AMH levels (P ≥ 0.06). We also performed a second subgroup analysis, which included only Caucasian patients (n = 870). No significant correlation was observed between CGG repeat number and DOR (all P-values were ≥0.1). Finally, we conducted a subgroup analysis comparing women with at least one allele <26, at least one allele >35 and women with both alleles between 29 and 32. There were no significant associations regarding ovarian reserve in any of these groups.
Discussion

This study found that there was no statistically significant correlation between high normal CGG trinucleotide repeat numbers and markers of ovarian reserve in patients with < 55 repeats presenting to an infertility practice. These findings are in line with several other studies that have investigated a potential link between high normal CGG repeats and ovarian reserve. Kline et al. (2014) investigated high normal and

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<th>Table I</th>
<th>Group characteristics of infertility patients in a study of DOR and CGG repeat length in the FMR1 gene.</th>
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<tbody>
<tr>
<td>CGG repeat number</td>
<td>Total</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------</td>
</tr>
<tr>
<td>N</td>
<td>1287</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>35.9 (4.4)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>DOR n (%)</td>
<td>454 (35.3)</td>
</tr>
<tr>
<td>Others n (%)</td>
<td>833 (64.7)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White n (%)</td>
<td>870 (67.6)</td>
</tr>
<tr>
<td>Hispanic n (%)</td>
<td>88 (6.8)</td>
</tr>
<tr>
<td>Black n (%)</td>
<td>84 (6.5)</td>
</tr>
<tr>
<td>Asian n (%)</td>
<td>150 (11.7)</td>
</tr>
<tr>
<td>Others/unknown n (%)</td>
<td>95 (7.4)</td>
</tr>
<tr>
<td>BMI (kg/m²) Mean (SD)</td>
<td>25.3 (6.3)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Y n (%)</td>
<td>161 (12.5)</td>
</tr>
<tr>
<td>N n (%)</td>
<td>818 (63.6)</td>
</tr>
<tr>
<td>Unknown n (%)</td>
<td>308 (23.9)</td>
</tr>
<tr>
<td>FSH (mIU/ml) Mean (SD)</td>
<td>21.2 (7.5)</td>
</tr>
<tr>
<td>AFC (n) Mean (SD)</td>
<td>15.2 (10.2)</td>
</tr>
<tr>
<td>AMH (ng/ml) Mean (SD)</td>
<td>2.6 (1.5)</td>
</tr>
</tbody>
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*p-values for age, BMI, FSH, AFC and AMH were calculated by analysis of variance, and smoking, race and diagnosis were calculated by chi-squared test. DOR, diminished ovarian reserve.

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<tr>
<th>Table II</th>
<th>Results of generalized linear regression models with two different types of CGG repeat numbers; continuous (Model 1) and four categories (Model 2).</th>
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<tbody>
<tr>
<td>FSH Estimate</td>
<td>95% CI</td>
</tr>
<tr>
<td>Model 1</td>
<td>Intercept</td>
</tr>
<tr>
<td>CGG repeat</td>
<td>0.99</td>
</tr>
<tr>
<td>Age</td>
<td>1.04</td>
</tr>
<tr>
<td>Model 2</td>
<td>Intercept</td>
</tr>
<tr>
<td>CGG repeat</td>
<td>&lt;35 (Ref.)</td>
</tr>
<tr>
<td></td>
<td>35–39</td>
</tr>
<tr>
<td></td>
<td>40–44</td>
</tr>
<tr>
<td></td>
<td>&gt;44</td>
</tr>
</tbody>
</table>

CI, confidence interval.
intermediate range CGG repeats (35–54) and failed to show a correlation between these repeats and ovarian aging as measured by FSH and AMH in fertile women. Another study published by Lledo et al. (2012) looked at oocyte donors and found that donors with high normal CGG repeats (>35) had similar clinical outcomes, specifically oocyte yield and days of stimulation, when compared with controls. Additionally, Voorhuis et al. has produced two studies that found no correlation between high normal CGG repeats and POI. The first study involved 3611 women and revealed no association between high normal and intermediate-sized CGG repeats and the age of natural menopause (Voorhuis et al., 2013). The second study found no statistically significant difference in the frequency of intermediate-sized CGG repeats on the allele with the longest triplet repeat number between POI women and controls who underwent natural menopause at age ≥ 40 years (Voorhuis et al., 2014). Finally, Murray et al. performed a study involving over 2000 women who underwent menopause before the age of 46 years. They found that the presence of intermediate-sized CGG repeats was a risk factor for neither early menopause nor POI (Murray et al., 2014).

Two of the biggest strengths of the present study are that there was complete data on over 1140 women and the results can be generalized to an infertility population. In addition, the study was performed at a single site, and all FSH and AMH assays were performed in a single laboratory. A limitation of this study is that it involves a heterogeneous infertility population with mixed diagnoses. Patient characteristics not accounted for that may have independently contributed to DOR include medical comorbidities, family history, genetic mutations, endometriosis, prior ovarian surgery, and prior chemotherapy and/or radiation. Finally, there was a lack of racial diversity, with 67.8% of the patients being Caucasian. One group has demonstrated that Caucasians have more FMR1 allele abnormalities than African-American and Asian females (Gleicher et al., 2010a).

This study found no correlation between high normal CGG repeat numbers on the FMR1 gene and ovarian reserve parameters. One possible explanation for this finding may lie in the biologic function of the FMR1 gene. Though it is well established that the fully expanded FMR1 mutation causes POI, the exact mechanism by which this occurs is not well understood (Wittenberger et al., 2007). The gene behaves differently when the trinucleotide repeat numbers exceed 200 than with fewer repeat numbers. The fully expanded mutation leads to methylation and impaired expression of FMRP. In contrast, premutations lead to increased mRNA expression. These gain of functions are hypothesized to cause a reduction in the available oocyte follicular pool by either direct damage to the ovaries or by interfering with signaling mechanisms at the level of the hypothalamus (Loesch and Hagerman, 2012). It can be further hypothesized that a critical level of mRNA must be present to cause ovarian damage and that the high normal range mutations do not reach the critical threshold needed to reduce ovarian reserve.

DOR is a serious problem that impacts many women seeking fertility treatment. Unfortunately, its genetic causes remain elusive. Identification of genetic markers that predict POI might help to identify women at an earlier age so that they could be advised to conceive sooner or be offered oocyte vitrification or other methods of fertility preservation. Women who are found to have high normal CGG repeat numbers can be counseled that this is not causative for DOR and that the number of repeats will not contribute to increased risk of infertility in female offspring. Further studies are needed to investigate whether increasing CGG repeat numbers are associated with altered responsiveness to gonadotrophin stimulation, increased need for donor oocytes, higher spontaneous abortion rates or worse IVF outcomes when compared with women with CGG repeats in the normal range.

Figure 1 Relationship between FMR1 CCG repeat number and FSH, AFC and AMH.
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Authors’ roles

A.S. participated in data gathering and interpretation, manuscript preparation, revision, submission as well as approval of the final draft. D.B.M. participated in data analysis and interpretation, manuscript revision and approval of the final draft. S.M.L. participated in data analysis as well as revision of the Materials and Methods and Results sections of the manuscript. Finally, she approved the final version for publication. R.A. participated in data acquisition. M.L.U. participated in study design, data acquisition, manuscript revision and approval of the final manuscript draft. J.D. participated in data acquisition. E.C.F. participated in study conception and design, data acquisition, manuscript revision and final approval of the draft for publication.

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Conflict of interest

The authors have no conflict of interest to declare except for J.D., who was an employee of Good Start Genetics® during the period of data acquisition.

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