The effect of CGG repeat number on ovarian response among fragile X premutation carriers undergoing preimplantation genetic diagnosis

Guy Bibi, M.D.,a Mira Malcov, Ph.D.,a Yaron Yuval, M.D.,b Adi Reches, M.D.,b Dalit Ben-Yosef, Ph.D.,a Beni Almog, M.D.,a Ami Amit, M.D.,a and Foad Azem, M.D.a

a Racine IVF Unit; and b Prenatal Diagnosis Unit, Genetic Institute, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Objective: To assess ovarian response among carriers of FMR1 premutation who undergo preimplantation genetic diagnosis (PGD).

Design: Retrospective study.

Setting: Academic IVF unit.

Patient(s): Of 18 carriers of FMR1 premutation referred to PGD, eight had <100 CGG repeats and ten had ≥100 CGG repeats.

Intervention(s): Controlled ovarian stimulation (COH) and PGD.

Main Outcome Measure(s): Correlation between the number of CGG repeats and the level of E2 at day of hCG administration, number of retrieved oocytes, number of two-pronuclear (2PN) zygotes, and dose of recombinant FSH.

Result(s): There was a positive correlation between CGG repeats and the level of E2 at day of hCG administration, number of retrieved oocytes, and number of 2PN zygotes. There was a negative correlation between number of CGG repeats and the total dose of gonadotropins. The E2 level and the number of retrieved oocytes and 2PN zygotes were significantly higher and the dose of gonadotropins significantly lower for premutation patients with ≥100 CGG repeats compared with <100 CGG repeats.

Conclusion(s): There is a positive correlation between E2 level, retrieved oocytes, 2PN zygotes, and number of CGG repeats. Premutation carriers with <100 CGG repeats suffer from impaired ovarian response and decreased fertilization rate.

Key Words: FMR1 premutation, ovarian response, PGD, COH

Fragile X syndrome is the most common cause of inherited mental retardation as well as the most common known genetic cause of autism (1). This disorder is associated with a dynamic triple repeat sequence mutation in the X-linked gene known as fragile X mental retardation 1 (FMR1) (2) and characterized by chromosomal fragility at Xq27.3 (3). The mutation in the FMR1 gene was found to be due to an expanded sequence of CGG trinucleotide repeats in the 5' untranslated region, which was hypermethylated in affected individuals (4, 5). The number of CGG repeats present in normal alleles varies from 6 to 52 (20). The consequence of full mutation, i.e., >200 CGG repeats, is the absence of the fragile X mental retardation protein (FMRP), an RNA-binding protein that leads to the features of fragile X syndrome (6). The mutation tends to expand in size as it is passed from mother to offspring. Premutation alleles range from 55 to 199 CGG trinucleotide repeats. Carriers do not have fragile X–related mental retardation, but they are at risk for expansion from premutation to full mutation over several generations (7, 8). The intermediate range consists of alleles of 45–54 repeats which may be unstable when transmitted from parent to child over several generations (9).

Premature ovarian failure (POF) is a condition in which women develop infertility and cessation of menses associated with high levels of gonadotropins and low levels of estrogen before the age of 40 years (10). Earlier studies demonstrated that the prevalence of POF among fragile X premutation carriers ranges from 13%–26%, compared with 0.4%–14% in the full mutation carriers (11–13).

Premutation carriers have an elevation in the serum FSH level (14–16) and a decrease in the serum inhibin B level, which reflects a decreased follicle number (16). Furthermore, premutation carriers entered menopause approximately 5 years earlier than noncarrier women (13) and typically do not respond well to exogenous gonadotropin stimulation (17). The underlying molecular mechanism of ovarian failure is not clear, but the full mutation females who have reduced FMR1 protein are not affected with POF.
The relationship between the number of CGG repeats and the age of last menstrual cycle is nonlinear (13, 18, 19). The risk of POF appears to increase with increasing premutation repeat size between 59 and 99, after which the risk plateaus or even decreases for women with repeat sizes >100 (19).

Carriers of fragile X premutation are currently offered pre-implantation genetic diagnosis (PGD) as a diagnostic tool for prevention of the subsequent birth of an affected baby (17, 20). Similar to other IVF procedures, the PGD is based upon controlled ovarian hyperstimulation (OH) and ovum pickup (20). The fact that some of these patients may suffer from POF or altered ovarian reserve creates an obstacle for the success of the procedure. Some centers stopped offering PGD for fragile X carriers, and some have suggested other alternatives, such as egg donation (21).

Platteau et al. (17) reported that fragile X patients needed significantly more ampules of FSH to yield the same number of oocytes compared with overall intracytoplasmic sperm injection (ICSI) patients.

The purpose of the present study was to examine the ovarian response in relation to number of CGG trinucleotide repeats in premutation carriers undergoing COH for PGD. The primary end points included: level of $E_2$ at the day of hCG administration, number of retrieved oocytes, number of two-pronuclei pre-embryos (2PN), and gonadotropin dose. The secondary end point was to compare the ovarian response to the administered gonadotrophin dose between two subgroups of premutation carriers using 100 CGG repeats as a cutoff point.

**MATERIALS AND METHODS**

**Patients**

Candidates for PGD of fragile X syndrome were recruited from the fragile X screening program or following the birth of an affected individual in the immediate family. Because the risk for CGG expansion to a full mutation is directly related to the number of CGG repeats in the carrier mother (22, 23), only patients with >70 repeats or those with an affected offspring diagnosed before birth or already born were enrolled in the PGD program.

Between January 2006 and July 2008, 18 couples were referred to our IVF unit seeking PGD for fragile X syndrome after genetic consultation. All of the women completed a reproductive history questionnaire. The study was approved by the local ethics committee.

Ovarian stimulation was achieved using a gonadotropin-releasing hormone analogue (Decapeptyl; Ferring, Keil, Germany) from the first day of the cycle followed by recombinant FSH (rFSH; Gonaf F [Serono, Geneva, Switzerland] or Puregon [Organon, Oss, The Netherlands]) from the third day of the cycle. Human chorionic gonadotropin (Ovitrelle 250 μg; Serono) was given when at least three ≥ 17-mm follicles were demonstrated. Oocyte retrieval was scheduled 35–36 hours after administration of hCG. Oocytes were denuded of cumulus cells using hyaluronidase and a fine hand-drawn glass Pasteur pipette. Fertilization was performed by ICSI using a Nikon inverted microscope (Diaphot 300) with Narishige micromanipulators. Oocytes were examined 18–20 hours after ICSI under a dissecting microscope for the presence of pronuclei. Embryo cleavage rate and morphology were evaluated on day 3, before biopsy. The PGD procedure and multiplex nested polymerase chain reaction were as described elsewhere (20). The luteal phase support was supplemented by daily 600 mg micronized progesterone (Utrogestan; Pipette, Brussels, Belgium).

**Statistics**

Correlations between $E_2$ levels, age, number of retrieved oocytes, number of 2PN zygotes, total dose of gonadotropins, and number of CGG repeats were evaluated using Pearson or Spearman correlations as appropriate. The two-sample $t$ test was applied for testing differences between the two study subgroups of premutation carriers. Multiple logistic regressions were applied for testing the difference between the study groups and correlations between the studied parameters adjusted for confounders.

Results are expressed as mean ± SD unless otherwise indicated. All tests applied were two tailed, and a $P$ value of ≤5% was considered to be significant. The data were analyzed using the SAS software (SAS Institute, Cary, NC) (24).

**RESULTS**

The study included 18 fertile women (mean age 32.5 ± 4.4 years, range 27–40 years) with a history of at least one termination of pregnancy due to prenatal diagnosis of a full mutation fragile X embryo in 14 of them. The other four patients were diagnosed as premutation carriers after screening tests. The patients underwent a total of 71 PGD cycles for fragile X syndrome. Their demographic and premutation molecular data are presented in Table 1. All fragile X premutation carriers had an ovulatory menstrual cycle during the study. The mean basal FSH level was 6.8 ± 2.8 mIU/mL (range 4.3–11.3 mIU/mL).

The mean of cycles/couple was 3.9 ± 2.1, and the mean number of CGG repeats was 149.3 ± 63.4.

The correlation between the number of CGG repeats and milestones of ovarian response is presented in Fig 1. We found a positive and significant correlation between the mean number of CGG repeats and the mean number of retrieved oocytes ($P$=.0029), the mean number of 2PN zygotes ($P$=.0034), and the mean $E_2$ serum level at the day of hCG administration ($P$<.0001).

We also found a negative significant correlation between the CGG repeats and the mean dose of rFSH ($P$<.0001).

After adjustments for age, the positive correlation between the number of CGG repeats and the $E_2$ level at the day of hCG administration remained significant ($P$=.0003), as did the
correlation between the number of CGG repeats and the number of retrieved oocytes \( (P = .0074) \) and 2PN zygotes \( (P = .0092; \text{Fig. 1}) \). After adjustments for age, the negative correlation between the number of CGG repeats and the mean dose of administered rFSH remained significant \( (P < .0001) \).

Eight premutation carriers with \(<100\) CGG repeats (group A) underwent 22 PGD cycles, and ten premutation carriers with \(\geq100\) CGG repeats (group B) had 49 PGD cycles. Group A included three women with 70 CGG repeats, three with 80 CGG repeats, and two with 90 repeats, and Group B included one woman with 120 CGG repeats, five with 150 CGG repeats, and four with 200 CGG repeats. Southern blot analysis was carried out to be sure of the allele size and to exclude full mutation carriers. The mean basal FSH level in group A was 6.8 \( \pm \) 2.0 compared with 6.0 \( \pm \) 2.0 in group B.

Table 2 presents a comparison of the ovarian response between premutation carrier groups. The \( E_2 \) levels at the day of hCG administration, the number of retrieved oocytes, and the number of 2PN zygotes were significantly lower in group A compared with group B. Furthermore, the dose of rFSH given during the COH was significantly higher in group A compared with group B.

**DISCUSSION**

This study is the first to concentrate on the correlation between CGG repeats and ovarian response after COH in patients who underwent PGD. This study differs from earlier ones which looked into the association between fragile X premutation and POF. Ennis et al. (18) and Sullivan et al. (13) demonstrated that the highest risk for ovarian dysfunction, defined by the age at menopause and prevalence of POF, occurred among carriers with a middle range of repeats (80–100). Allen et al. (25) confirmed the nonlinear association of repeat size and ovarian insufficiency: Carriers with 80–99 repeats had increased rates of menstrual dysfunction, infertility, and dizygotic twinning compared with noncarriers. Carriers with 80–99 repeats also had a 7-year reduction in mean age at menopause and consequently an increased prevalence of POF (32% vs. 1%) and increased risk of osteoporosis (25). Finally, carriers of both smaller and larger premutation repeat sizes also suffered from ovarian insufficiency, but not to as great an extent as those with 80–99 repeats.

The association between CGG repeats and ovarian response during COH, however, is less documented. Preimplantation genetic diagnosis has opened a new avenue of looking into the ovarian response. Patients with CGG premutation who undergo PGD need to recruit a sufficient number of follicles to perform the genetic analysis related to difficulties of replication of the mutant gene. In a study by Platteau et al. (17) in which premutation carriers underwent PGD, the authors reported that fragile X patients needed significantly more ampules of FSH to yield the same number of oocytes.
After adjustments for age, positive correlation was found between the mean number of CGG repeats and (A) the E2 level at the day of hCG administration ($P= .0003$), (B) the number of retrieved oocytes ($P=.007$), and (C) the number of two-pronuclear zygotes 2PN ($P=.009$). (D) Negative correlation between the number of CGG repeats and the mean dose of administered recombinant FSH ($P<.0001$).

Some centers have stopped offering PGD for fragile X carriers, and some have suggested other alternatives, such as egg donation (21). In the present study, we found a positive and significant correlation between the mean number of CGG repeats and the number of retrieved oocytes ($r=0.32, p=0.007$).

### TABLE 2

<table>
<thead>
<tr>
<th>Comparison of ovarian response between premutation carrier groups (mean ± SD).</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&lt;100$ CGG repeats (group A)</td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
</tr>
<tr>
<td>No. of retrieved oocytes</td>
</tr>
<tr>
<td>No. of 2PN</td>
</tr>
<tr>
<td>No. of cycles</td>
</tr>
<tr>
<td>Dose of FSH (IU/L)</td>
</tr>
<tr>
<td>Age (yrs)</td>
</tr>
</tbody>
</table>

Note: 2PN = two-pronuclear zygote.
of CGG repeats and the milestones of ovarian response: E\textsubscript{2} levels, retrieved oocytes, and 2PN zygotes. Furthermore, these milestones were significantly higher among patients with \( \geq 100 \) CGG repeats than among patients with \( <100 \) CGG repeats. The findings of a linear association with CGG repeats in the present study are in accordance with those of Allen et al. (25), who found a nonlinear association of menopause age with premutation size, indicating that the age at menopause decreases with increasing repeat number until about 80 repeats, after which increasing repeat number is associated with an increasing age at menopause. The linear correlation in the present study is mainly attributed to the fact that the lowest number of CGG repeats of the fragile X mutations in our patients were in the transition zone of 70–80 CGG repeats, from which the correlation is linear even in the Allen et al. model (25). It is worth noting that the earlier studies cited were concerned with POF rather than ovarian response during COH.

We also found a negative and significant correlation between the number of CGG repeats and the dose of rFSH. These findings are in agreement with those of Platteau et al. (17), who reported that premutation carriers who underwent PGD needed significantly more ampules of FSH to yield the same number of oocytes compared with ICSI patients overall. The present findings show that although the ovarian response was associated with the number of CGG repeats, patients with \( <100 \) CGG repeats had a lower ovarian response compared with those with \( \geq 100 \) CGG repeats. This difference in ovarian response was, however, not associated with basal FSH levels, which were within the normal range.

The present study cohort included fertile women with a history of termination of pregnancies or birth of affected babies, whereas patients in most of the cited studies were infertile. Nonetheless, our observations are in accordance with those of Gleicher et al. (26), who recently reported a strong statistical correlation between the number of triple repeats in FMR1 alleles and ovarian function, as represented by FSH and antimullerian hormone levels. In clinical terms, this means that the number of CGG repeats correlates, in increasing order, with a clinical diagnosis of normal ovarian function, premature ovarian ageing, and POF. Patients with \(<100\) CGG repeats should be considered to be potentially low responders, and, as such, the COH protocol should be adjusted to recruit as many oocytes as possible to enable PGD performance. Furthermore, these patients should be counseled about the risk of POF despite the presence of normal levels of basal FSH.

In summary, the present study demonstrates a positive correlation between the number of CGG repeats and the parameters of ovarian response among patients who undergo COH for PGD. Furthermore, premutation carriers with \(<100\) CGG repeats suffer from impaired ovarian response and a decreased fertilization rate.

REFERENCES