PGD for fragile X syndrome: ovarian function is the main determinant of success

Avi Tsafrir1,*,†, Gheona Altarescu2,†, Ehud Margalioth1, Baruch Brooks1, Paul Renbaum2, Ephrat Levy-Lahad2, Ron Rabinowitz3, Irit Varshaver1, and Talia Eldar-Geva1

1IVF Unit, Department of Obstetrics and Gynecology, Shaare-Zedek Medical Center, Hebrew University Medical School, Jerusalem, Israel
2Medical Genetics, Zohar PGD Lab, Shaare-Zedek Medical Center, Hebrew University Medical School, Jerusalem, Israel
3Ultrasound Unit, Department of Obstetrics and Gynecology, Shaare-Zedek Medical Center, Hebrew University Medical School, Jerusalem, Israel

*Correspondence address. E-mail: avits@szmc.org.il

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BACKGROUND: PGD for fragile X syndrome (FRAX) is inefficient, probably owing to fewer oocytes, poor embryo quality and difficulties in genetic analysis. We investigated IVF–PGD in FRAX mutation carriers compared with controls, looking at the effects of oocyte and embryo number/quality on live birth outcome.

METHODS: We performed IVF–PGD in 27 patients with the FRAX mutation and 33 controls with other genetic diseases. Genetic testing was by multiplex PCR.

RESULTS: Seventy-nine and 108 IVF–PGD cycles were started in FRAX mutation carriers and controls, respectively. Twenty-two patients had a premutation (CGG repeat number 60–200) and five had a full mutation (300–2000 CGG repeats). FRAX patients required higher doses of gonadotrophins (6788 + 2379 versus 4360 + 2330, P0.001) but had lower peak serum estradiol levels (8166 + 5880 versus 10 211 + 4673, P = 0.03) and fewer oocytes retrieved (9.8 + 6 versus 14 + 8, P = 0.01). The cancelation rate (unsatisfactory ovarian response) was higher in the FRAX group than in the control group (13 versus 1%, P < 0.001). When embryos were transferred, ongoing pregnancy/live birth rates per transfer were similar (29 versus 36%, P = 0.54).

CONCLUSIONS: Ovarian dysfunction in FRAX carriers is more prevalent and profound than previously appreciated, with a high cancelation rate and reduced efficiency of PGD. The main determinant for successful PGD for FRAX is ovarian dysfunction. When embryo transfer is possible, the results are comparable to PGD for other monogenic diseases.

Key words: fragile X syndrome / PGD / trinucleotide repeats / IVF / ovarian function

Introduction

Fragile X syndrome (FRAX) is the most common inherited form of severe mental retardation. The incidence of the syndrome is 1 in 4000 males and 1 in 8000 females (Wittenberger et al., 2007). Mental retardation is in the moderate to severe range, and autistic range behavior and hyperactivity are also common. Morphological abnormalities include characteristic long face with a prominent jaw and protruding ears, macroorchidism and strabismus.

The molecular genetic defect underlying FRAX is the expansion of a repeated trinucleotide segment of DNA (CGG) in the fragile X mental retardation-I (FMR1) gene located on the X chromosome (Oberle et al., 1991). In the general population, the normal range of CGG repeats is 6–40, with 29–30 being the average. A full fragile X mutation is defined as >200 CGG repeats and will result in FRAX in all males, and in a variable number of females because of X inactivation. The large number of CGG repeats results in hypermethylation and inactivation of the FMR1 gene. Hence, the gene product, fragile X mental retardation protein, is absent or deficient (Jin and Warren, 2000). A premutation is defined by the presence of 55–60 to 200 CGG repeats, while a normal number of repeats is typically transmitted from parents to offspring in a stable manner. Women carrying a premutation do not have FRAX but are at risk of transmitting the disease to their offspring, since the premutation CGG segment is

† These two authors contributed equally to this study.
unstable and may expand and result in a full mutation and an affected child (Visootsak et al., 2005). The distinction between a premutation and an intermediate zone is currently ambiguous; most often a premutation is reported when the number of repeats is $\geq 55$ (Sherman et al., 2005), but other authorities define 41–60 repeats as an intermediate status (ACOG, 2006).

Pregnant carriers of the FRAX mutation/premutation should be offered prenatal testing for FRAX in the fetus (Sherman et al., 2005). This traditional approach involves the well-known risks of miscarriage related to the invasive prenatal test as well as the medical, ethical and emotional problems of termination of an affected pregnancy, which are unacceptable for some patients. PGD for FRAX is an alternative strategy. The basic advantages of PGD are avoiding the need for invasive prenatal diagnosis and termination of pregnancy in the case of a female carrier.

In addition to the drawbacks inherent in PGD, which include the need for IVF with associated medical and emotional risks, financial cost and risk of misdiagnosis, the procedure of PGD for FRAX is especially challenging.

First, reduced ovarian function is more prevalent among FRAX carriers compared with the general population (Wittenberger et al., 2007; Martin and Arici, 2008). Premature ovarian failure (POF; defined as ovarian failure before the age of 40 years) occurs in 10–28% of women who carry the FMR1 premutation, compared with 1% in the general population (Wittenberger et al., 2007; Martin and Arici, 2008).

Suboptimal response to ovarian stimulation may result in fewer oocytes and embryos available for diagnosis and finally in lower pregnancy and delivery rates.

The next difficulty is the genetic analysis, since abnormally long CGG repeats cannot be reliably amplified by standard PCR. In this case, a false-negative diagnosis of an affected embryo may occur since an expanded CGG repeat will not be amplified in the PCR analysis. Therefore, it is essential to identify normal alleles inherited from both parents in the embryonic genetic material. Initial attempts to perform PGD for FRAX were based on direct amplification of the CGG triplet in the FMR1 gene (Sermon et al., 1999). However, some couples have a similar number of CGG repeats on both alleles, and allele dropout (ADO), i.e. amplification of only one allele present in the cell, cannot be ruled out. ADO is an important possible cause of misdiagnosis in PGD and may result in misinterpretation of an affected embryo as a healthy one (Wilton et al., 2009). In one report, 40% of couples who were FRAX carriers had similar or very close numbers of CGG repeats in both alleles, and were therefore considered ‘non-informative’ for direct analysis of a mutation in FMR1 for PGD (Platteau et al., 2002). Later studies used multiple linked genetic markers, in addition to direct detection of the mutation, in order to reduce the risk of misdiagnosis related to ADO and overcome the problem of ‘uninformativity’ in FRAX families (Burlet et al., 2006; Malcov et al., 2007). In some cases, however, not more than two polymorphic numbers are detected; therefore, the risk for misdiagnosis is not eliminated. Indeed, misdiagnosis in PGD for FRAX using two polymorphic markers leading to a birth of an affected child has been reported (Verlinsky et al., 2002).

Most PGD protocols use blastomere biopsy for diagnosis of the embryos (Sermon et al., 2007). PGD using polar body analysis (PB–PGD) has been shown to be an effective method for maternal autosomal dominant, X-linked and recessive disorders (Verlinsky et al., 2002; Rechitsky et al., 2003; Tomi et al., 2006; Renbaum et al., 2007; Altarescu et al., 2008a,b) and for female carriers of de-novo mutations (Altarescu et al., 2009). PB–PGD has reduced ADO frequency compared with blastomere PGD and reduces the need for embryo biopsy (Rechitsky et al., 1998; Altarescu et al., 2006). For the majority of genes investigated (depending on their position on the chromosome) 60–70% of first PBs (PB1) are shown to be heterozygous (contain two alleles on both chromatids). Detection of two alleles in the PB1 minimizes the possibility of ADO and the diagnosis will depend on analysis of the second PB (PB2) (if the PB2 has the wild-type allele, the oocyte will have the mutant allele and vice versa). Analysis of multiple informative polymorphic markers linked to the gene improves the detection of ADO, further improving the accuracy and efficiency of the PGD diagnosis. Therefore, our policy is to prefer PB biopsy in cases of maternal autosomal dominant or X-linked diseases.

It has been claimed that PGD for FRAX is inefficient owing to both lower numbers of oocytes and embryo quality, and difficulties in genetic analysis (Apessos et al., 2001). We report here our experience in performing IVF–PGD on a relatively large group of FRAX mutation/premutation carriers compared with IVF–PGD for other X-linked or maternal autosomal dominant diseases, with regard to the effect of oocyte and embryo numbers and quality on the final results.

**Materials and Methods**

**Patients**

From January 2005 until December 2009, 33 FRAX premutation or full mutation female carriers aged $31.4 \pm 4$ (mean $\pm$ SD) years (range 25–40 years) were referred for PGD in our Unit. Twenty-seven patients actually began IVF treatment. The indication for FRAX testing was an affected child (n = 9), affected or carrier other relative (n = 12) or gestation/pregestation screening (n = 6). Three women had two children with FRAX, while six had one such child. In previous pregnancies, 14 women underwent 1–3 invasive prenatal procedures for the diagnosis of FRAX; these included 22 chorionic villous sampling (CVS) exams and three amniocentesis procedures. As a result, nine women had one termination of a FRAX pregnancy, two had two terminations and two women had three pregnancies terminated.

**Control group**

The control group comprised all couples who, during the same time period as the patient group, were found to carry either a maternal autosomal or X-linked disease. During the first 2 years of our work, it was our policy to offer PB biopsy to all such couples. Subsequently, we preferred blastomere biopsy unless PB biopsy was necessary for genetic analysis. Female patients undergoing PGD for maternal myotonic dystrophy were excluded from the control group because of a possible association of reduced ovarian function with this condition (Feyereisen et al., 2006).

**Hormone analysis**

On Day 2–4 of the cycle prior to IVF–PGD treatment, blood was drawn for the measurement of FSH and anti-Mullerian hormone (AMH), and transvaginal ultrasonographic evaluation of the number of 2–10 mm follicles in both ovaries [antral follicle count (AFC)] and ovarian volume was performed.

Serum concentrations of FSH were measured using Dxl 800 (Beckman Coulter Instruments Inc., Fullerton, CA, USA). Assay sensitivities were 0.1 IU/l; inter-assay and intra-assay coefficients of variation were <7%. Serum AMH concentrations were measured using highly sensitive two-site enzyme-linked immunosorbent assays (Diagnostic Systems Laboratories, Webster, TX, USA), with an assay sensitivity of 0.017 ng/ml.
Stimulation protocol

A stimulation protocol and dose of gonadotrophins were chosen based on the patient’s age, basal FSH, AFC and previous ovarian response. In the ‘long’ protocol, pituitary down-regulation consisted of triptorelin acetate 0.1 mg/day s.c. (decapetyl, Ferring Ltd, Herzliya, Israel) for 14 days, then lowered to 0.05 mg/day after the commencement of ovarian stimulation. After pituitary suppression was confirmed, recombinant FSH (Gonal F, Serono, Herzliya, Israel or Puregon, Organon Ltd, Os, the Netherlands) or hMG (Menogon, Ferring) was administered daily. Ovulation was triggered with hCG (Ovitrelle 250 μg, Serono, Switzerland) administered when at least three follicles of >17 mm in diameter developed. In the GnRH antagonist protocol, ovarian stimulation was started on the second day of the cycle. When the largest follicle reached a diameter of 14 mm, GnRH antagonist was administered (Cetrotide 0.25 mg, Serono, Switzerland) daily s.c. When at least three follicles reached 17 mm in diameter, final oocyte maturation was triggered by hCG. For some patients with low ovarian response, we used a GnRH agonist/antagonist protocol: pituitary desensitization was achieved using triptorelin acetate 0.1 mg/day s.c beginning in the mid-luteal phase. This was stopped on the 2nd–3rd day of the cycle, when FSH or hMG was started. GnRH antagonist was given when the largest follicle diameter measured 14 mm, until HCG administration. Unsatisfactory ovarian response was determined when fewer than three large follicles were observed after at least 14 days of FSH treatment or an insufficient rise in serum estradiol (E2) levels was noted. In all cases, oocyte retrieval was performed by the transvaginal approach under general anesthesia 36 h after hCG administration.

PB biopsy and IVF

Cumulus–oocyte complexes were identified, washed and transferred to organ culture dishes containing equilibrated culture medium (Medicult, Denmark) and incubated in 5% CO2. Oocytes were denuded with hyaluronidase (Sigma-Aldrich, USA) 2 h after egg collection and were allowed to recover in the incubator for a further 2 h. The PB1s were removed using proteinase K digestion, and inactivation at 94°C for 15 min followed by PCR in a 50 μl multiplex PCR containing 0.2 μM dNTPs, 10% dimethylsulphoxide, 0.1 of μM each primer and 1.25 U of Taq polymerase in a buffer supplied by the manufacturer (JMR801, UK). The reaction was thermocycled for 30 cycles using a program of 20 s at 95°C, 1 min at 62–50°C and 30 s at 72°C. From each multiplex PCR, 1.5 μl was used as a template with a hemi-nested primer 5’ fluorescently labeled with 6-FAM, HEX or TAMRA (Metabion, GmbH), with either the forward or reverse unlabeled primer, for an additional 35-cycle PCR for each individual marker. Reaction products were diluted and run on an ABI Prism 3100 Avant automated sequencer, and analyzed using GeneMapper software (ABI).

Each multiplex PCR protocol included primers for the family-specific markers that can identify the wild-type maternal allele in blastomeres. The battery of markers used was: DXS8028, DXS8045, DXS1200, DXS297, DXS998, DXS548, Frax AC1 (UCSC genome browser assembly March 2006 chrX: 146793985–146794165), Frax AC2 (UCSC genome browser assembly March 2006 chrX: 146813595–146813766), DXS731, DXS1215, DXS6687, DXS8091, DXS1193, DXS8086 and DXS8069. We used a minimum of four microsatellite markers flanking the area of CGG repeats. The amplification, ADO and contamination rates obtained during preclinical testing on unrelated single fibroblasts were within the limits set by the European Society of Human Reproduction and Embryology PGD consortium guidelines (Thornhill et al., 2005).

Embryo transfer

Embryo transfer under ultrasound guidance was performed 3–4 days after oocyte aspiration using a Wallace Catheter. Luteal support consisted of l.m. progesterone in oil (Gestone 50 mg/day, Ferring Ltd) or vaginal micronized progesterone (Utrogestan, Besins International Labs, 900 mg/day, or Endometrin, Ferring, Germany, 200 mg/day) until 12 weeks of gestation. Clinical pregnancy was defined as a viable fetus with a heartbeat demonstrated by transvaginal ultrasonography.

Pregnancy was defined as rising serum hCG levels at 2 and 3 weeks following embryo transfer. Ongoing pregnancy was defined as a pregnancy beyond 12 weeks.

Statistics

The main outcome assessed was live birth/ongoing pregnancy rates. Secondary outcomes were dose of gonadotrophins used, peak serum E2 levels, number of oocytes retrieved, positive hCG test and clinical pregnancy rates.

Kolmogorov–Smirnov test was used to determine a normal distribution of variables. Student’s t-test, Mann–Whitney U-test, χ2 test and Fisher’s exact test were performed for testing for differences between groups, as indicated. Results are presented as mean ± SEM. Statistical significance was considered as P < 0.05.

Results

Of 33 carriers of pre- or full FRAX mutation who attended our PGD clinic, 6 did not begin PGD treatment: two patients (aged 27 and 36 years) already had ovarian failure upon admission, two had questionable clinical relevance because of mosaicism and an intermediate number of CGG repeats and two decided to withdraw from the PGD program. Twenty-seven patients actually began IVF treatment. Twenty-two of those had a FRAX premutation (range 60–200 CGG repeats) and five had a full mutation (300–2000 CGG repeats).

Mean age, the number of previous gestations and deliveries and the prevalence of infertility were similar among the FRAX carriers and the controls (Table 1). Twenty-five of the FRAX women had regular menses, but four of these had high basal FSH levels (>12 IU/ml) at the early follicular phase. When compared with control patients, FRAX patients had a significantly higher basal FSH level (P = 0.03), lower AFC (P < 0.0001) and mean ovarian volume (P < 0.02), and a trend for lower serum AMH levels (P = 0.06).
Table I Baseline reproductive data for carriers of the FRAX mutation and for control women.

<table>
<thead>
<tr>
<th></th>
<th>FRAX Controls</th>
<th>Controls</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Full mutation</td>
<td>Premutation</td>
<td>Total (comparing total FRAX with controls)</td>
</tr>
<tr>
<td>Number of patients</td>
<td>5</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>34 ± 4.6</td>
<td>30.6 ± 4</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Number of gestations (mean ± SD, range)</td>
<td>3.6 ± 2</td>
<td>3 ± 3</td>
<td>3.1 ± 2.6</td>
</tr>
<tr>
<td>Number of deliveries (mean ± SD, range)</td>
<td>1.2 ± 0.8</td>
<td>1.7 ± 1.5</td>
<td>1.6 ± 1.4</td>
</tr>
<tr>
<td>History of infertility</td>
<td>1 (20)</td>
<td>7 (32)</td>
<td>8 (30)</td>
</tr>
<tr>
<td>Previous treatment for infertility</td>
<td>1 (20)</td>
<td>5 (23)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>Mean basal FSH (IU/ml)</td>
<td>6.8 ± 4.5</td>
<td>8.5 ± 5</td>
<td>8.4 ± 5</td>
</tr>
<tr>
<td>Basal FSH &gt; 12 (IU/ml), (% of patients)</td>
<td>0 (0)</td>
<td>4 (18)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Serum AMH</td>
<td>1.4 ± 1.6 (n = 4)</td>
<td>1.6 ± 1.4 (n = 11)</td>
<td>1.6 ± 1.4 (n = 15)</td>
</tr>
<tr>
<td>AFC (both ovaries combined)</td>
<td>7.3 ± 5 (n = 3)</td>
<td>9.5 ± 3 (n = 11)</td>
<td>9 ± 5 (n = 14)</td>
</tr>
<tr>
<td>Mean ovarian volume (cm³)</td>
<td>4.3 ± 1.8 (n = 3)</td>
<td>7.5 ± 7 (n = 11)</td>
<td>7.5 ± 5 (n = 14)</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; FRAX: fragile X.
became pregnant naturally and gave birth to a healthy child. Very low ovarian response to high-dose stimulation, but she later conceived spontaneously and delivered a healthy child following CVS. Another 35-year-old (with 85 CGG repeats) had two cycles canceled owing to insufficient ovarian response to hormonal stimulation. PB, polar body; ET, embryo transfer; OR, oocyte retrieval.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>FRAX</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Full mutation</td>
</tr>
<tr>
<td>Number of patients</td>
<td>5</td>
</tr>
<tr>
<td>Number of started cycles (mean per patient)</td>
<td>16 (3.2)</td>
</tr>
<tr>
<td>Dose of FSH IU/cycle</td>
<td>8355 ± 2546</td>
</tr>
<tr>
<td>Maximal estradiol (pmol/l)</td>
<td>6617 ± 5196</td>
</tr>
<tr>
<td>Cancelation before OR* (%)</td>
<td>2 (12.5)</td>
</tr>
<tr>
<td>Total oocytes per OR</td>
<td>8.1 ± 5</td>
</tr>
<tr>
<td>Metaphase II oocytes per OR</td>
<td>7 ± 4.5</td>
</tr>
<tr>
<td>Normal fertilization rate (%)</td>
<td>80</td>
</tr>
<tr>
<td>Genetic analysis of fresh transfer cycles (% of all analyses)</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>1 (14.5)</td>
</tr>
<tr>
<td>Blastomere</td>
<td>5 (71)</td>
</tr>
<tr>
<td>PB and blastomere</td>
<td>1 (14.5)</td>
</tr>
<tr>
<td>Embryo transfer (% of started cycles)</td>
<td>7 (44)</td>
</tr>
<tr>
<td>Transfer cycle of a top quality embryo (% of started cycles)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Number of patients reaching fresh ET after PGD (% of total patients)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Number of embryos per ET</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Clinical pregnancies (% per ET)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Live birth per ET* (%)</td>
<td>3 (43)</td>
</tr>
</tbody>
</table>

A All cancelations were because of insufficient ovarian response to hormonal stimulation. PB, polar body; ET, embryo transfer; OR, oocyte retrieval.

CGG repeats and the number of oocytes retrieved. Comparing two premutation patient subgroups according to the number of CGG repeats (60–99 and ≥100), no significant differences were noted regarding age, FSH dosage, peak E2 levels and the number of oocytes retrieved (data not shown).

Two FRAX carriers also had polycystic ovary syndrome (PCOS), including oligo/anovulation and typical ovarian morphology. Those patients had a normal to high ovarian response to gonadotrophin stimulation and produced nearly 15–25 oocytes per cycle. Both became pregnant; one woman had three pregnancies, including one following the transfer of frozen-thawed supernumerary embryos following IVF–PGD.

Two of five women who demonstrated no ovarian response to gonadotrophins conceived naturally shortly after medication was stopped. One 33-year-old (a mother of four children) with 73 CGG repeats had minimal E2 elevation following 10 days of high-dose hMG and the treatment was canceled. Soon afterward she conceived spontaneously and delivered a healthy child following CVS. Another 35-year-old (with one child) with 85 CGG repeats had two cycles canceled owing to very low ovarian response to high-dose stimulation, but she later became pregnant naturally and gave birth to a healthy child.

**Discussion**

We report our experience of using PGD for patients who carry the FRAX mutation, summarizing data of 79 IVF cycles in 27 women. To the best of our knowledge, this is the largest detailed report on assisted reproduction treatment in FRAX patients so far. Our main finding is that ovarian dysfunction in FRAX carriers is more prevalent and profound than previously appreciated, and this led to a high cancelation rate of embryo transfer and overall reduced efficiency of PGD for this condition. However, with a reasonable ovarian response, oocyte and embryo quality were comparable to PGD in other monogenic diseases (i.e. the control group), accurate genetic analysis was achieved in all cases and, when embryo transfer was possible, pregnancy and delivery rates were also comparable to PGD in controls.

Two out of 33 young FRAX carriers in our group had POF upon admission. The prevalence of POF in women who carry the FMR1 premutation is 10–26% (Wittenberger et al., 2007; Martin and Arici, 2008) compared with 1% in the general population. In general, menopause occurs 6–8 years earlier in FRAX carriers (Parvington et al. 1996). The factors that determine ovarian function and the risk of POF in FRAX premutation carriers have been only partially identified. Controversies exist regarding the effect of the parent of origin of the premutation, the ratio of the inactivated X chromosomes and interruption of the CGG repeat by AGG on the relative risk of POF. For research purposes, while menopause is well defined and easy to detect, less is known about infertility in FRAX carriers. In the general population, it is estimated that age-related infertility occurs about 10 years prior to menopause (te Velde and Pearson, 2002). This figure may not necessarily be comparable in women with POF, which differs in several aspects from simply an early menopause.
(Nelson et al., 2005). Similar to our results (Table II), interviewing nearly 350 FRAX premutation carriers, Allen et al. (2007) reported that infertility was not more prevalent compared with controls although some indirect measures associated with ovarian function, such as irregular and skipped cycles, were more common in FRAX premutation carriers. However, we are not aware of another study describing infertility or its management in FRAX carriers. Most of our patients were young with proven fertility, and underwent IVF for the purpose of PGD. However, as a group they required higher doses of gonadotrophins for ovarian stimulation, with fewer oocytes and lower maximal E2 levels, compared with other PGD patients. With regard to more advanced tests of ovarian reserve, AFC and ovarian volume were significantly lower in FRAX carriers than in controls, and a similar trend was observed in serum AMH levels. It is of note that some patients who were completely refractory to ovarian stimulation were young, had regular cycles, normal reproductive history and normal basal FSH levels. Therefore, although not uncommon, severely reduced or absent ovarian response to hormonal stimulation may not be predictable in FRAX mutation carriers using basic tests alone. Therefore, the more advanced tests of ovarian reserve, such as AFC and serum AMH, may be particularly useful in this group of patients.

The relationship between the number of CGG repeats and the risk of POF was reported to be nonlinear. Several studies described a higher risk for POF in women having 59–99 CGG repeats (Tejada et al., 2008) or 80–100 CGG repeats (Sullivan et al., 2005; Ennis et al., 2006; Allen et al., 2007), and thereafter the risk plateaus, or even decreases, for women with CGG repeat numbers over 100. In a group of FRAX premutation carriers undergoing IVF for PGD, 10 carriers with >100 CGG repeats performed better than 8 carriers with <100 repeats, with regard to ovarian response (Bibi et al., 2009). Our results do not support this view as we could not detect a correlation between the number of CGG repeats and ovarian response within the premutation group of patients. The results from our small group of patients with full mutation do not support the claim that those carriers are not at risk of ovarian dysfunction (Allen et al., 2007); our full mutation carriers required more gonadotrophins for ovarian stimulation when compared with premutation carriers. The present cohort is obviously too small to allow definite conclusions regarding the association between the number of CGG repeats and ovarian performance. However, it seems that women with either the premutation or the full mutation are at risk of ovarian dysfunction.

Interestingly, two FRAX carriers also had PCOS. Both had good ovarian response, and became pregnant (one of them three times and one twice) and delivered following IVF–PGD. It therefore appears that the endocrine profile which is characteristic of PCOS can help to ‘overcome’ the presumed ovarian defect associated with FRAX.

One lesson learned from our results is that an increased awareness of fertility problems in FRAX carriers is essential. Our patients were frequently unaware of this aspect of the Fragile X carrier state when referred for PGD. Indeed, the sister of one of our FRAX patients, although she had been diagnosed as a premutation carrier, was found to have suffered ovarian failure at the age of 35 on initial presentation to our Unit. This is even more frustrating because these women had many previous interactions with medical professionals, including geneticists, pediatricians and gynecologists, when the potential risks might have been discussed.

Low ovarian response was a major contributor to the risk for cycle cancelation or no transfer of embryos after PGD in our study. Since current strategies, including some used in this study, for improving IVF results in poor ovarian responders in IVF are generally unsuccessful (Tarlatzis et al., 2003), ovarian dysfunction is expected to remain the major obstacle to effective PGD for FRAX. When fewer than three oocytes were collected, embryos were frozen on Day 1, prior to zona splitting and biopsy. This strategy allows the accumulation of a reasonable number of both fresh and frozen–thawed embryos for genetic analysis on the same day, thus reducing costs and laboratory time required for the analysis of a small number of embryos. Given the current low survival rate of biopsied embryos in conventional slow freezing (Joris et al., 1999; Magli et al., 1999; Ciotti et al., 2000; Jericho et al., 2003), pre-biopsy cryopreservation may be useful when the estimated chances for success are small in a fresh cycle, owing to a small number of embryos. In such cases, we analyzed blastomeres only using at least three informative markers flanking the FMR1 gene. These ‘accumulated’ embryos may have contributed to two births in our study.

Surprisingly, two of five women who demonstrated no ovarian response to gonadotrophins conceived naturally shortly after medication was stopped. A similar case in a FRAX carrier, totally refractive for hormonal stimulation who later became pregnant naturally, was described in the first report on PGD for FRAX (Sermon et al., 1999). These sporadic cases are too few in number to allow us to determine whether this is a coincidence or a biological phenomenon which is found in young women with ovarian dysfunction in the general population or in FRAX carriers specifically, and requires further attention. In contrast to IVF patients who have low ovarian response because of advanced age, young FRAX carriers perhaps face a more optimistic prognosis.

PGD in general has inherent drawbacks, such as the need for IVF, cost and the risk for misdiagnosis. It is of note that in our program no misdiagnosis occurred in PGD of FRAX, as well as for all other monogenic diseases examined in controls. We relate these good results to a strict policy, including examination of at least three polymorphic markers in addition to the specific mutation examined, or four markers overlapping the mutation. PGD is clearly less efficient in carriers of FRAX compared with other monogenic diseases. One may claim that the risks, cost and efforts involved are not worthwhile, considering a nearly 50% chance of no embryo transfer per started cycle. On the other hand, the conventional approach of prenatal diagnosis has significant drawbacks. For example, more than half of the carriers in our group underwent invasive prenatal diagnosis in a previous pregnancy, and had undergone termination of 1–3 pregnancies with an affected fetus. Therefore, the ‘conventional’ route may carry medical risks and significant psychological consequences. Hence, the decision in favor of PGD for FRAX should be preceded by a thorough discussion on the risks and benefits of each strategy, including the financial, psychological and ethical aspects.

In conclusion, the main determinant for successful PGD for FRAX is ovarian dysfunction. Difficulties in genetic analysis are tractable after the identification of a suitable number of polymorphic markers has been achieved. Thereafter, PGD can be safely performed with a negligible risk of misdiagnosis using a strict strategy for genetic analysis. When embryo transfer was possible, the outcomes were good. Education of both the caregiver and patient regarding ovarian function in
FRAX carriers is of great importance in order to prevent any delay in reproductive planning and referral for PGD.

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