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ARTICLE

The significance of the number of CGG repeats and autoantibodies in premature ovarian failure

Cem Fıçıcıoglu *, Gazi Yildirim, Rukset Attar, Banu Kumbak, Narter Yesildaglar

Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Yeditepe University Hospital, Devlet Yolu, Ankara Cad. No. 102–104, 34752 Kozyatagi, Istanbul, Turkey
* Corresponding author. *E-mail address:* cemficicioglu@gmail.com (C Fıçıcıoglu).

Cem Fıçıcıoglu, Professor, MD, PhD, has been working in Istanbul Yeditepe University Hospital as the Director of the Department of Obstetrics and Gynecology and the Assisted Reproduction Unit. He has PhD degrees in Histology and Embryology. His research efforts are concentrated on ovarian function, premature ovarian ageing and premature ovarian failure as well as the treatment modalities for infertility. He has published several articles and chapters in these areas and also in other areas relating to infertility. Dr Ficicioglu is an active member of the American Society for Reproductive Medicine (ASRM), and the European Society of Human Reproduction and Embryology (ESHRE).

Abstract The objective of this study was to determine whether there was a threshold for the number of CGG repeats in the *FMR1* (fragile X) gene in premature ovarian ageing and premature ovarian failure and to investigate the association of this sequence with serum concentrations of anti-Müllerian hormone (AMH), inhibin B, anti-thyroid and anti-adrenal autoantibodies. In this prospective randomized controlled preliminary study, the number of triple CGG repeats and serum concentrations of FSH, AMH and aforementioned autoantibodies were evaluated in 79 women who were younger than 40 years old. FSH concentrations were between 12 and 50 IU/ml (premature ovarian ageing) in 30 women and were higher than 50 IU/ml (premature ovarian failure) in nine women; FSH concentrations were normal in 40 women. All women whose FSH concentrations were higher than 12 IU/ml had CGG repeats greater than 30. No women whose FSH concentrations were normal had a repeat number above 30. There was no significant relationship between the levels of antibodies and either CGG repeat numbers or FSH concentrations. In conclusion, the number of CGG repeats between 30 and 40 might be used to predict premature ovarian ageing and premature ovarian failure in infertile women.

KEYWORDS: fragile X, CGG repeats, premature ovarian failure, ovarian reserve, AMH, inhibin B, anti-thyroid antibody, anti-adrenal antibody

Introduction

Although it is a natural consequence of the ageing process, some women experience premature ovarian ageing (POA) and premature ovarian failure (POF). POF is defined as cessation of menses before 40 years of age. Many factors are blamed for POF, including previous destructive ovarian surgery, severe and repeated pelvic infections, cytotoxic chemotherapies, ovarian autoimmunity and abnormal karyotype. It was reported that the prevalence of POF is higher in monozygotic and dizygotic twins than in the general population (Gosden et al., 2007). Several rare specific causes have been published, but unfortunately, in most cases, the aetiology of premature ovarian senescence cannot be determined.

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POF is not an uncommon disorder (Anasti, 1998). It has been postulated that the prevalence of POF is approximately 10% in the general population (Nikolaou and Gilling-Smith, 2004), but its actual prevalence is between 1% and 10% according to surveys (Coulam et al., 1986; Luborsky et al., 2003). Recently, the term premature ovarian ageing (POA) has been introduced. POA is different from POF. POA is described as a shift in the normal ovarian ageing curve towards younger ages (Nikolaou and Templeton, 2004). It is important to discriminate between these two entities during infertility treatment. The diagnosis of POF usually means very poor outcomes in assisted reproductive treatment; in contrast, in POA, which might result in POF, there is still a chance of pregnancy after adequate treatment (Gleicher and Barad, 2008).

Unfortunately, no specific test has been described for assessment of the chance of a young woman developing POF in the future. FSH concentrations do not give any indication of the risk of developing POF; in fact, the serum FSH concentration does not necessarily reflect the number of primordial follicles remaining in the ovaries. Non-invasive determination of follicle depletion or dysfunction is not currently possible (Anasti, 1998). Anti-Müllerian hormone (AMH) is a good predictor of the pool of ovarian primordial follicles; however, it shows the actual situation of the ovaries and cannot give any indication about the future perspective. Based on recent data, the number of CGG trinucleotide repeats may be able to give some indication of the risk of developing POA and POF. POF may also be associated with a non-organ-specific autoimmune disorder and testing the parameters related to that sort of disorder in POF patients can start a new debate on future studies. If there is such a relationship, these tests can be used for diagnosis of the risk of developing POF at any age in a woman's life, before having increased FSH concentrations.

This study determined the frequency of the CGG trinucleotide repeats (fragile X mental retardation 1 gene, *FMR1*) and autoimmune antibodies in patients with POA and POF and compared them with a group of women with normal ovarian function. The first aim was to find a certain threshold of these repeats for premature ovarian senescence and to provoke a new discussion on the early detection of women at risk of developing POF. The second aim was to assess whether adrenal and thyroid autoantibodies are risk factors for POF.

Materials and methods

The study was conducted at the Assisted Reproductive Centre of Yeditepe University Hospital between January 2005 and December 2008 and approved by the institutional review board and ethics committee of the hospital. Thirty-nine randomly chosen infertile patients whose FSH concentrations were higher than 12 mIU/ml signed an informed consent prepared specifically for this trial and these patients were included in the first group of the study; women older than 40 years of age, those who had previously undergone ovarian surgery and those who had a history of cytotoxic chemotherapy or pelvic irradiation were not included in this group. Forty patients with normal ovarian functions who signed an informed consent prepared specifically for this trial were included in the second group; the inclusion criteria for this group were as follows: age below 40 years, normal FSH concentration (below 12 mIU/ml), primary infertility due to a tubal or a mild male factor and infertility duration shorter than 2 years. The exclusion criteria for this group were as follows: previous IVF treatment, ovarian surgery, pelvic irradiations and cytotoxic chemotherapy and the existence of ovarian cysts or endometriomas. The second group was named as the control group, since the patients had normal hormonal profile in this group.

Five of the 39 patients whose FSH concentrations were higher than 12 mIU/ml were not married. They did not receive any treatment. Thirty women whose FSH concentrations were higher than 12 IU/ml were classified as the POA group based on the criteria used in the study carried out by Barad et al. (2007). Nine women whose FSH concentrations were 50 IU/ml or higher were classified as the POF group based on the criteria used in the study carried out by Hoek et al. (1997).

A total of 79 samples were tested for the fragile X gene. Fragile X evaluations were performed in blood samples with a commercially available assay, Fragile X GScan Kit (Gene Link-Hawthorne, NY, USA) using a standard testing procedure published before (Sherman et al., 2005). Fragile X genotyping was performed by direct fluorescent PCR amplification of the CGG trinucleotide repeats region and by DNA sequencer (ABI-310 DNA Sequencer; Applied Biosystems, USA) for fragment analysis. When an amplification/expansion of a region of a DNA sequence was detected by fluorescent PCR, DNA analysis was performed for direct detection of the fragile X mutation, which was based on amplification of a fragment containing the CGG repeat sequence of the *FMR1* gene. This test detected the fragile X mutation from the size of the amplified product. There are two alleles for the fragile X gene: one of them is maternal and the other is paternal. Unfortunately, it is not possible to distinguish between maternal and paternal alleles. For this study, the allele with lower triple repeat number is termed allele 1 and the allele with higher repeat counts is termed allele 2.

The samples were tested for anti-thyroid and anti-adrenal antibodies to determine whether there was any relationship between autoimmunity and early ovarian ageing. Anti-thyroid antibodies were detected with an indirect immunofluorescence antibody test (IMMCO Diagnostic, Buffalo, NY, USA). Anti-adrenal antibodies were detected with an indirect immunofluorescence antibody test (IMMCO Diagnostic).

FSH, oestradiol, AMH, and inhibin B concentrations were assessed by commercial assay (Diagnostic System Laboratories Inc, Texas, USA) using enzyme-linked immunosorbent assay. The coefficients of variation for these three tests were between 2.4% and 8.6%.

Kruskal–Wallis test was used for scale variables such as age, FSH, AMH, inhibin B and CGG repeats in the three groups. These variables were evaluated with Mann–Whitney *U*-test for comparison between two groups. A two-sided chi-squared test or Fisher's exact chi-squared test was performed on the tabulated data. Receiver operating characteristic curve analysis was used to determine cut-off values. Scatterplot graphics was applied where appropriate. Statistical evaluations were performed using the Statistical Package for Social Sciences version 11.5 for Windows (SPSS

Inc, USA). Statistical significance was assumed when a *P*-value was equal to or less than 0.05.

Results

Seventy-nine patients were scanned for fragile X testing. The age range in control patients was between 21 and 39 years while it was between 24 and 40 years in patients with POA and POF. In spite of significantly higher ages in patients with POA, all patients were still under 40 years (**Table 1**). The FSH concentrations in the controls ranged from 6 to 11 mIU/ml (mean ± SD 8.2 ± 1.6), in the patients with POA from 12 to 35 mIU/ml (16.4 ± 5.9) and in the patients with POF from 51 to 90 mIU/ml (60.2 ± 16.0; $P = 0.001$).

The mean ± SD CGG repeats on allele 1 was 26.0 ± 3.4 in the controls, 26.8 ± 3.7 in POA patients and 28.2 ± 4.6 in POF patients. The maximum number of repeats was 35. There were not more than 30 repeats in the control group. Five patients in the POA and one patient in the POF group had over 30 repeats on allele 1. The maximum number of repeats was 36 on allele 2. More than 30 repeats were not observed in the control patients. Eight patients in the POA group and four patients in the POF group had over 30 CGG repeats (**Table 1**). The study did not detect more than 40 repeats in any subject. No patients in the study groups had premutations or full mutations.

All women who had more than 30 repeats were in either POA or POF groups. Controversially, 73.3% of POA patients and 55.6% POF patients had fewer than 30 repeats (**Table 2**). Only 26.7% POA patients and 44.4% POF patients had repeat counts higher than 30. This finding demonstrated that the POF patients had more CGG repeats than the POA and control patients. There were 40 women in the control group and no one in this group had more than 30 CGG repeats.

No relationship was found between autoantibody positivity and POF. There were two patients with positive adrenal autoantibodies in the control group and one in the POF group. There were three positive anti-thyroid antibody test results and all of them were in patients in the POA group. One of these three had less than 30 CGG repeats. The other two had more than 30. All three adrenal autoantibody positive patients had fewer than 30 CGG repeats (**Table 2**). There was no significant relationship between autoimmunity and CGG repeats.

In the controls, AMH concentrations were significantly high (mean ± SEM 2.45 ± 0.26 ng/ml, range 0.32—6.08) and the concentrations of AMH in the POA and POF groups were significantly low (1.03 ± 0.12 ng/ml, range 0.32—2.41 in POA; 0.44 ± 0.07 ng/ml, range 0.29—0.91 in POF; $P = 0.0001$). If AMH concentrations were higher than 2.4 ng/ml, there were not more than 30 repeats (**Figures 1 and 2**). All patients with AMH concentrations lower than 1.0 ng/ml were in the POF group. The meanconcentrations of AMH in the patients who had 30 or fewer repeats and in the patients who had more than 30 repeats were 1.8 ± 0.1 ng/ml and 0.8 ± 0.2 ng/ml, respectively.

Inhibin B is a test for ovarian reserve and it is usually performed during the early follicular phase of a cycle. In the control patients, the blood samples were collected on the third day of the menstrual cycle. Due to irregular periods and amenorrhoea, the blood tests for inhibin B in the patients with POA and POF were performed whenever they agreed to give blood samples. The concentration of inhibin B was very low in the patients with POF (mean 0.9 ± 0.2 pg/ml). The concentration of inhibin B in the patients who had more than 30 repeats was (mean ± SEM) 18.2 ± 6.9 pg/ml.

Discussion

Early ovarian ageing is not an uncommon problem. If women with a high risk for POF can be diagnosed, different

Table 1 Patients' characteristics and the number of triple CGG expansions on the *FMR1* gene.

Characteristic	*Control (FSH <12 IU/ml) (n = 40)*	*POA (FSH ≥12 to <50 IU/ml) (n = 30)*	*POF (FSH ≥50 IU/ml) (n = 9)*	*P-value*
Age (years)	30.4 ± 4.4	35.0 ± 4.5	33.3 ± 4.7	0.001
FSH (IU/ml)	8.2 ± 1.6	16.4 ± 5.9	60.2 ± 16.0	0.001
AMH (ng/ml) (mean ± SEM)	2.45 ± 0.26	1.03 ± 0.12	0.44 ± 0.07	0.0001
Inhibin B (pg/ml) (mean ± SEM)	30.6 ± 4.7	23.2 ± 4.0	0.9 ± 0.2	0.01
Adrenal autoantibody	2	0	1	NS
Thyroid autoantibody	0	3	0	—
CGG repeats				
Allele 1 (min—max 18—35)	26.0 ± 3.4	26.8 ± 3.7	28.2 ± 4.6	NS
Allele 2 (min—max 19—36)	27.2 ± 3.2	27.5 ± 4.4	27.8 ± 3.9	NS
≤30 repeats	40	22	5	0.001
High normal (31—40)	0	8	4	
Intermediate (41—54)	—	—	—	
Premutation (55—200)	—	—	—	
Full mutation (55—200)	—	—	—	

Values are number or mean ± SD, unless otherwise stated.
AMH = anti-Müllerian hormone; FSH = follicle-stimulating hormone; NS = not statistically significant; POA = premature ovarian ageing; POF = premature ovarian failure.

Table 2 The findings based on repeat counts.

	<30 CGG repeats (n = 67)	*≥30 CGG repeats (n = 12)*	P-*value*
Control	40	0	0.001
POA	22	8	
POF	5	4	
Age (years)	32.3 ± 0.5	33.7 ± 1.6	NS
FSH (IU/ml)	15.0 ± 1.8	29.8 ± 6.9	0.005
AMH (ng/ml)	1.8 ± 0.1	0.8 ± 0.2	0.05
Inhibin B (pg/ml)	25.8 ± 3.3	18.2 ± 6.9	NS
Adrenal autoantibody	3	0	NS
Thyroid autoantibody	1	2	NS

Values are number or mean ± SEM.
AMH = anti-Müllerian hormone; FSH = follicle-stimulating hormone; NS = not statistically significant; POA = premature ovarian ageing; POF = premature ovarian failure.

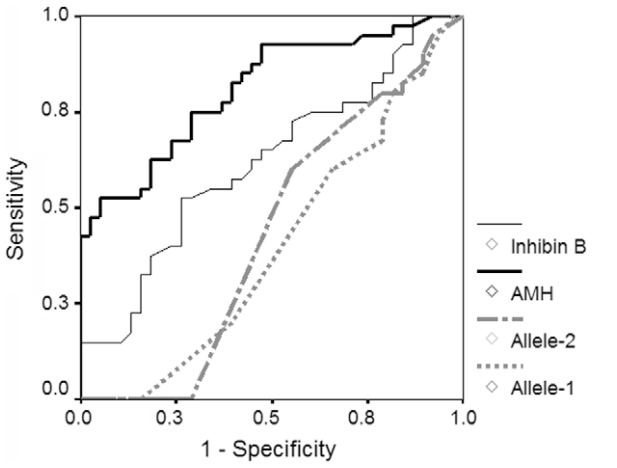

Figure 1 The receiver operating characteristic (ROC) curve for ovarian reserve parameters in premature ovarian senescence (in both premature ovarian ageing and premature ovarian failure). AMH = anti-Müllerian hormone.

treatment modalities can be offered to them, such as an early attempt at childbearing and oocyte or embryo cryopreservation. A predictive test is needed to help these women. This trial took a different approach in the prediction of early ovarian ageing.

There are many articles examining the correlation between fragile X syndrome and premature ovarian senescence (Gleicher et al., 2008; Streuli et al., 2009; Wittenberger et al., 2007). Fragile X syndrome is the most common form of inherited mental retardation. It affects approximately one in 1200 males and one in 2500 females. As suggested by the name, it is an X-linked genetic disorder characterized a triple repeats of CGG in the X chromosome (Hagerman et al., 2009). The number of these repeats is very important. There is a consensus that premutations involve 55—200 repeats and full mutation (so-called fragile X syndrome) over 200 repeats. There is some controversy about the intermediate (or grey) zone. Some researchers consider that the intermediate zone starts with 45 repeats and ends at 54 (Wittenberger et al., 2007). On the other hand, according to the American College of Obstetricians

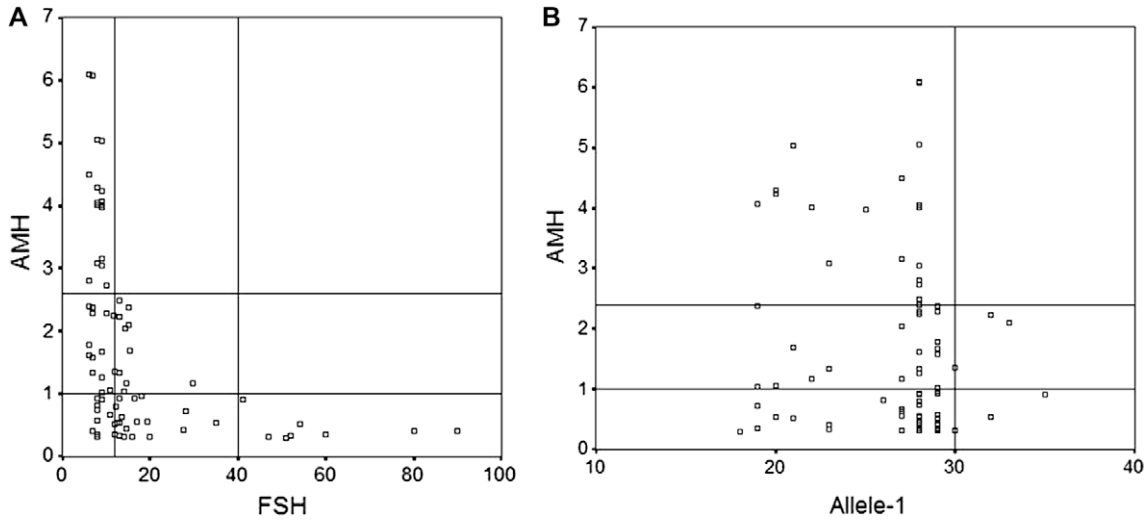

Figure 2 The relationship of anti-Müllerian hormone (AMH) with (**A**) FSH and (**B**) allele 1.

and Gynecologists (ACOG), patients with fewer than 40 trinucleotide repeats are regarded as unaffected, with repeats between 41 and 60 as intermediate, and between 61 and 200 as premutation and over 200 as full mutational patients (American College of Obstetricians and Gynecologists Committee on Genetics, 2006). Carriers of premutation do not show classic symptoms of fragile X syndrome. Completely unaffected persons mostly have 29–30 repeats (Fu et al., 1991).

The lower boundary of *FMR1* repeat sizes that alter ovarian function has not been defined (Streuli et al., 2009). Although the ACOG defines the patients with CGG repeats less than 40 as unaffected, this study's data showed that patients who had repeats between 30 and 40 experienced some problems with regard to ovarian function. It was shown that 30 or more repeats had negative effects on the ovarian reserve (Chen et al., 2003). Gleicher et al. (2008b) fired a debate over their statement that over 30 CGG trinucleotide repeats may be related with poor ovarian reserve. They classified the numbers of CGG repeats as follows: high normal (31–40 repeats), intermediate (41–54 repeats), premutation (55–200 repeats) and full mutation (over 200 repeats): a very comprehensive classification system.

This study did not observe any repeats exceeding 36. A cut-off level for allele 2 can be assumed to be 30 repeats based on the definition given by Chen et al. (2003). None of the patients in the control group had more than 30 CGG repeats. All the patients whose trinucleotide repeats exceeded 30 were in the POA or POF group. This observation was in accordance with the findings reported by Gleicher et al. (2008). The patients with more than 30 repeats had significantly lower AMH concentrations. An AMH concentration above 2.4 ng/ml can predict that CGG repeats were less than 30. Such a relationship was not observed with inhibin B.

There is a continuum of impaired ovarian function in women with the *FMR1* premutation and these patients have significantly elevated FSH concentrations compared with non-carriers (Hundscheid et al., 2001; Welt et al., 2004). It has been reported that some changes that pointed to diminished ovarian reserve occur in women with premutation carriers such as shortened follicular phases, raised FSH concentrations and low AMH concentrations. They claimed that menopause shifted 5 years earlier in these women (Braat et al., 1999; Murray et al., 1999; Rohr et al., 2008; Welt et al., 2004). The main reason for reduced ovarian reserve in premutation carriers has not been determined. Some have claimed that premutation in the *FMR1* gene can interfere with the ovary and reduce the follicular pool *in utero* (Conway et al., 1995); some other researchers explained this situation on the basis of follicular apoptosis induced by the *FMR1* gene (Loesch et al., 2007). Alterations in menstrual cycle abnormalities in women who are premutation carriers have been emphasized as an indicator for subfertility (Allen et al., 2007); the authors claimed that women with premutation have a 13-fold risk of ovarian insufficiency. Expression studies indicate that *FMR1* regulates germ cell progression in the fetal ovaries and it is highly expressed in the germ cells during in-utero life (Bachner et al., 1993). This information makes *FMR1* testing a unique marker for ovarian reserve at any age, even during childhood. But a current study from India pointed out that *FMR1* premutations are rare in sporadic cases of POF who have no family history of fragile X syndrome or mental retardation (Chatterjee et al., 2009).

The main goal of studies to assess the ovarian reserve is to inform young women at high risk that they can lose their ovarian reserve at an early age. According to this study's findings, the best predictor of ovarian reserve is still AMH; however, the number of triple repeats of CGG can predict a diminishing ovarian reserve before the onset of ovarian ageing. Unlike the hormonal parameters, triple CGG nucleotide repeats give a constant result at any age even during childhood and this makes fragile X testing a unique ovarian reserve prediction test.

Autoimmune disorders are the other aetiological factors involved in early ovarian ageing. Although it is controversial, (Gleicher et al., 2007a,b; Part I and Part II) it was proposed that decreased fecundity was related in some way to autoimmune disorders (Nelson et al., 1993; Silman and Black, 1988). This study investigated the existence of anti-thyroid and anti-adrenal antibodies in patients with premature ovarian senescence. Positivity for anti-thyroid antibody was detected in three patients, all of whom were in the POA group. Two of the three patients with positive adrenal antibody were in the control group, while the other was in the POF group. In spite of the small number of patients, this observation was in accordance with the findings reported by Gleicher et al. (2008a). They found low triple repeats and lower FSH concentrations (although still abnormally high) and a higher AMH concentration (although still abnormally low) in patients with positive autoantibody compared with patients with negative autoantibody. They explained that the lower triple repeats in patients with positive autoantibody showed that their risk of ovarian senescence was most likely independent of this genetic mutation and therefore not causally related to the *FMR1* gene. They claimed that abnormal autoimmune function and expansion of triple CGG repeats seem to reflect independent risk of POA. Anti-ovarian antibody testing was not performed because of the poor correlation between the presence of anti-ovarian antibodies and premature ovarian failure (Anasti, 1998).

The most common autoimmune disorder in POF patients was reported to be hypothyroidism (Kim et al., 1997). This study included three patients with thyroid antibody and they were diagnosed with Hashimato's disease. They were given thyroid supplementation by their endocrinologist. Although previous studies have reported the coexistence of some common autoimmune diseases such myasthenia gravis, vitiligo, pernicious anemia, systemic lupus erythematosus, rheumatoid arthritis and autoimmune polyglandular syndromes with premature ovarian failure, this study did not observe such disorders.

In conclusion, despite the small number of patients in the study groups, the results of this preliminary study are in accordance with the current literature. However, these findings should be interpreted with caution. It is widely agreed that any improvement in the early diagnosis of POF can decrease the risk of inappropriate treatment and eventually improve the outcome. The findings indicate that the number of CGG repeats might be used to estimate POA and ovarian reserve. The cut-off level for POF or POA must

be different than the cut-off level for fragile X syndrome and the definition of premutation should be re-examined based on recent studies on POF. AMH is a sensitive test for determining the ovarian primordial follicle pool, but it can only show the current status. Inhibin B, anti-adrenal and anti-thyroid antibodies might be insufficient for predicting POA without other parameters. Larger prospective randomized multi-centric studies are essential to assess parameters and cut-off values for the prediction of POA and POF.

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