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Increased serum FSH in female fragile X premutation carriers with either regular menstrual cycles or on oral contraceptives

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Fragile X premutations are known to be a risk factor for diminished ovarian function at a relatively young age. We studied endocrine profiles of female fragile X family members ($n = 79$) at risk of premature ovarian failure (POF). Of these 79 women aged <40 years, 45 had menstrual cycles, and 34 were using oral contraceptives. Of the women with menstrual cycles, the premutation carriers had higher serum FSH concentrations than women who were not carrying the premutation. Even premutation carriers with regular cycles showed increased serum FSH concentrations. Moreover, premutation carriers using oral contraceptives also demonstrated increased serum FSH concentrations. Irrespective of whether oral contraceptives were used, a serum FSH concentration of ≥15 IU/l was more common in the premutation carriers than in the other women. One premutation carrier using oral contraceptives had a serum FSH concentration of >40 IU/l, the threshold that defines POF. We confirmed that premutation carriers with menstrual cycles demonstrate premature ovarian dysfunction. However, we also found endocrine signs of unrecognized ovarian dysfunction in premutation carriers using oral contraceptives, despite endocrine alterations by oral contraceptives. Premutation carriers may have a poorer prognosis for future pregnancy, either achieved spontaneously or by assisted reproductive technology. We recommend that premutation carriers should be counselled not to wait too long if they wish to start a family.

Key words: diminished ovarian reserve/fragile X premutation/FSH/inhibin B/premature ovarian failure

Introduction

The fragile X syndrome is an X-linked disorder, which is caused by the absence of the fragile X mental retardation protein (FMRP). Most commonly, this lack of FMRP results from the trinucleotide repeat expansion that occurs in the *FMR1* gene promoter (Verkerk *et al.*, 1991). This expansion eventually results in a process of hyper-methylation, which makes the gene transcriptionally inactive (Imbert *et al.*, 1998). Normally, there are <50 of such (CGG) repeats. Alleles with repeat sizes of 50–200 are classified as premutations, whereas the full mutation contains at least 200 repeats. Female carriers of the full mutation may show signs of the fragile X syndrome. For many years it was thought that female premutation carriers did not show any phenotypical abnormalities, except for the risk of repeat expansion when the mutation was transmitted to their children. This was supported by the fact that FMRP is produced at normal concentrations by alleles in the premutation range (Feng *et al.*, 1995). However, there is now growing evidence that the fragile X premutation is a significant risk factor for premature ovarian failure (POF) (Allingham-Hawkins *et al.*, 1999; Uzielli *et al.*, 1999; Hundscheid *et al.*, 2000; Marozzi *et al.*, 2000). POF is defined as a condition of spontaneous menopause, or secondary hypergonadotrophic hypo-oestrogenic amenorrhoea that occurs before the age of 40 years.

With expanding knowledge and progression in diagnostics and therapeutics, counselling should be given, not only about the genetic risks, but also about preimplantation genetic diagnosis (PGD) and, therefore, IVF. For this reason premutation carriers should be counselled about the risk of fertility problems related to POF.

Presently, little is known about the endocrinology of premenopausal premutation carriers and the spontaneous pregnancy rates or pregnancy rates after IVF procedures. To determine whether young fragile X premutation carriers show endocrine signs of premature ovarian dysfunction, we investigated the endocrine profiles of young women from fragile X families who were using combined oral contraceptives, and those who did not. To our knowledge, only one pilot study (Braat *et al.*, 1999) and one larger study (Murray *et al.*, 1999)

have been conducted on the endocrinology of premenopausal premutation carriers not using combined oral contraceptives. The latter study determined endocrine profiles in blood samples taken on one occasion between days 1 and 10 of the menstrual cycle from premenopausal premutation carriers and women not carrying a premutation aged 17–53 years. The premenopausal fragile X premutation carriers showed higher serum concentrations of FSH than the other women (i.e. full mutations and controls). The present study, however, focused on women aged <40 years, because these women are at risk of developing POF, which occurs, by definition, prior to the age of 40 years. Hence, we studied the endocrine profiles in women with menstrual cycles and in those who were using oral contraceptives.

Women who are using combined oral contraceptives will not show any overt signs of diminished ovarian function. Owing to the administration of oestrogens and the monthly withdrawal bleeding, most women who are using combined oral contraceptives will not know whether they are peri- or post-menopausal. Combined oral contraceptives suppress the hypothalamic–pituitary–ovarian axis (Mishell *et al.*, 1977). Suppression of FSH release seems to be an exclusive effect of 17α-ethinyl oestradiol and inhibin B, whereas the release of LH is modified by both 17α-ethinyl oestradiol and the progestin components. Combined oral contraceptives inhibit normal ovarian function, result in anovulation and cause withdrawal bleeding. During the pill-free interval, the function of the hypothalamic–pituitary–ovarian axis is restored. Serum concentrations of FSH, LH and 17β-oestradiol start to rise, with a parallel increase in inhibin B (Renier *et al.*, 1998). Gonadotrophin concentrations return to the normal early follicular phase concentrations (Cohen and Katz, 1979, 1981), although the 17β-oestradiol concentrations are still lower than those in the early follicular phase (Van der Spuy *et al*., 1990).

We analysed the serum concentrations of FSH and 17β-oestradiol in women aged <40 years. The serum inhibin B concentration was also assessed to evaluate whether inhibin B was decreased in women who were carrying a fragile X premutation. Inhibin B is produced by (pre)antral follicles (Roberts *et al*., 1993). In the early follicular phase, inhibin B has an inhibitory effect on the FSH secretion. As the ovary ages, the number of follicles declines. It has been suggested that a decrease in inhibin B is the earliest marker of reproductive ageing (Welt *et al*., 1999). However, the clinical value of inhibin B testing is doubted by other groups (Corson *et al.*, 1999), and therefore remains controversial.

Studies demonstrating that, with increasing age, FSH starts to rise did not evaluate whether FSH concentrations are, in fact, a measure of ovarian reserve. However, they documented that FSH concentrations increase at the same time that the incidence of diminished ovarian reserve increases (Sharara *et al.*, 1998). Most data concerning the predictive value of FSH are from studies in an assisted reproductive technology setting, in which the predicting value of basal FSH is evaluated for pregnancy rates, cancellation rates, etc., during treatment (Scott *et al.*, 1995; Sharara *et al.*, 1998). Recently, it was demonstrated that the predictive value of FSH in predicting ovarian response was 79%, again in assisted reproductive technology setting (Creus *et al.*, 2000). In such studies, women with an FSH concentration of ⩾15 IU/l have lower spontaneous pregnancy rates as well as lower pregnancy rates after IVF procedures than women with a serum FSH concentration of <15 IU/l (Scott and Hofmann, 1995). We evaluated whether more premutation carriers aged <40 years demonstrated a serum FSH concentration of ⩾15 IU/l than women not carrying a premutation.

As FSH concentrations increase with age in women with regular menstrual cycles (Lee *et al.*, 1988), we evaluated whether premutation carriers with normal cycles had higher FSH concentration than women not carrying the premutation with normal cycles, after correcting for age.

Materials and methods

Patients

In a large ongoing study on previously diagnosed fragile X families (current number of participating families is 71) we approached women who met the following criteria: (i) aged 18–40 years; (ii) previously screened for fragile X mutations; and (iii) with a normal cytogenetic karyotype.

We selected women from our ongoing study who either menstruated at least once every 3 months or were using combined oral contraceptives. All participants gave informed consent for the study, which was approved by the institutional review board. As in a previous paper (Murray *et al*., 1999), the participants were further stratified into premutation carriers and the other women. The latter group comprises women who were not carrying a premutation, i.e. the women with either a normal or a fully-mutated *FMR1* gene (as from here designated as: the other women). As serum FSH concentrations are higher in women who smoke cigarettes (El-Nemr *et al.*, 1998) we stratified the women into two subcategories, i.e. women who were currently smoking and those who were not (ex- and non-smokers).

Study protocol

In this study, normal menstrual cycles were defined as regular cycles with no more than 5 days intercycle time variation and a length of 24–32 days. Cycles that deviated from these criteria were considered to be abnormal. Venous blood samples were taken on the third day of the menstrual cycle for women with menstrual cycles. For the women who were using oral contraceptives, blood samples were taken on the last day of the 7 day pill-free interval. The rationale for these sampling days was day 3 for comparison with previous studies and day 7 as the point at which the basal FSH concentration had returned to that of pretreatment early follicular phase concentrations (Cohen and Katz, 1981). Serum FSH and LH concentrations were measured using the random access analyser AxSym (Abbott Laboratories, Chicago, IL, USA), which is a fully automated analyser system based on microparticle enzyme immunoassay (MEIA) technology. Serum 17β-oestradiol and progesterone concentrations were measured with radioimmunoassays developed in the endocrinology laboratory of this hospital and included preanalytical diethylether extraction of the serum specimens followed by Sephadex-LH20 chromatography and subsequent radioimmunoassay (Thomas *et al.*, 1977). Serum inhibin B assays were performed using the Inhibin B Dimer assay kit (Serotec, Oxford, UK). With this assay, the lowest detection limit of inhibin B is 4 ng/l. In order to perform statistical analyses we considered inhibin B concentrations of <4 ng/l to be 2 ng/l.

Statistical analysis

Means and SD were compared using independent sample *t*-tests. We also used multiple linear regression analyses to compare the endocrine

Table I. Age, FSH and inhibin B characteristics in premutation carriers and in the other women (i.e. women with either a normal or a fully mutated *FMR1* gene) with menstrual cycles or on combined oral contraceptives (COC). Values in parentheses are SD

	Cycle or COC	Cycle	No. of women	Age (years)	FSH (IU/l)	Inhibin B (ng/l)
Premutation carriers						
	Cycle	Normal	12	36 (2.4)	11.0 (7.89)	66.7 (40.2)
		Abnormal	4	35 (1.3)	27.0 (14.1)	11.1 (7.60)
		All	16	36 (2.2)	15.0 (11.7)	52.8 (42.6)
	COC	NA	13	34 (5.3)	20.4 (13.5)	78.6 (68.2)
The others						
	Cycle	Normal	25	34 (4.9)	6.92 (2.71)	69.6 (36.6)
		Abnormal	3	36 (2.1)	4.40 (2.16)	79.7 (55.0)
		All	28	34 (4.7)	6.65 (2.74)	70.7 (37.8)
	COC	NA	21	31 (4.5)	8.35 (3.48)	84.0 (45.8)

NA = not applicable.

profiles of the premutation carriers with those of the other women, while adjusting for the effects of age and smoking. To stabilize the variance, we first transformed our data by taking the natural logarithm (ln) of the serum concentrations. Sometimes more than one carrier from the same family was participating in this study. To avoid familial influences on FSH concentrations, we reanalysed the data while including only one randomly selected premutation carrier per family. In case of more than one woman not carrying the premutation (the other women), the analysis was conducted in an identical manner, thus only including one randomly selected other woman. A χ^2 test was used to compare the significance of differences in the number of premutation carriers with a serum FSH of $\geqslant$15 IU/l to the number of the other women with a serum FSH concentration of $\geqslant$15 IU/l. All analyses were performed with the Statistical Package for Social Sciences software (SPSS), version 9.0. $P < 0.05$ was considered to be statistically significant.

Results

Our study group comprised 79 women and was divided into two groups of women: those who had menstrual cycles, and those who were using oral contraceptives. For the sake of clarity, each group will be discussed separately.

Women with menstrual cycles (n = 45)

A total of 17 women were carriers of the fragile X premutation, while 28 were not (other women). The latter group comprised 16 women with a normal *FMR1* gene and 12 who had a full mutation. One premutation carrier had a serum progesterone concentration of 16 nmol/l on day 3 of the menstrual cycle. This concentration probably reflected the luteal phase concentration of the previous cycle, so this woman was excluded from further analysis. The mean age of the premutation carriers and the other women was 36 ± 2.2 and 34 ±4.7 years respectively (Table I). Six (37.5%) premutation carriers and six (21.4%) of the other women were currently smoking. The serum FSH in the premutation carrier group was in the range 5.4–39.1 IU/l, while that in the other women was 1.8–12.9 IU/l (Figure 1).

Analysing the data from all women irrespective of their menstrual cycle pattern, the mean serum FSH concentration of the premutation carriers was 15.0 ± 11.7 IU/l, while that

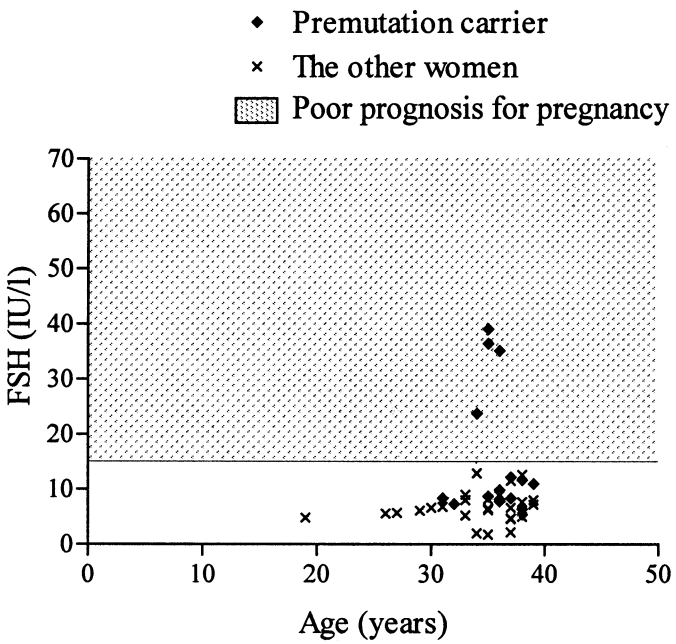

Figure 1. Serum FSH concentrations as a function of age for women with menstrual cycles.

of the other women was 6.65 ± 2.74 IU/l (Table I). Linear regression analysis demonstrated that after adjustment for age and smoking behaviour, the premutation carriers still had significantly higher serum FSH concentrations than the other women ($P = 0.002$). A serum FSH of $\geqslant$15 IU/l was observed in four out of 16 premutation carriers aged <40 years (Figure 1), but not in any of the 28 other women (two-tailed χ^2 test; $P = 0.013$).

Since our analyses sometimes included more than one premutation carrier or other women from the same family, we reanalysed our data as described in above. It was demonstrated that after correction for possible familial influences on FSH, the premutation carriers had significantly higher FSH concentrations than the other women ($P = 0.002$).

In addition, we assessed serum inhibin B concentrations. In the premutation carrier group, they were in the range 2.0–148 ng/l, while in the other women they were 2.0– 169 ng/l. The

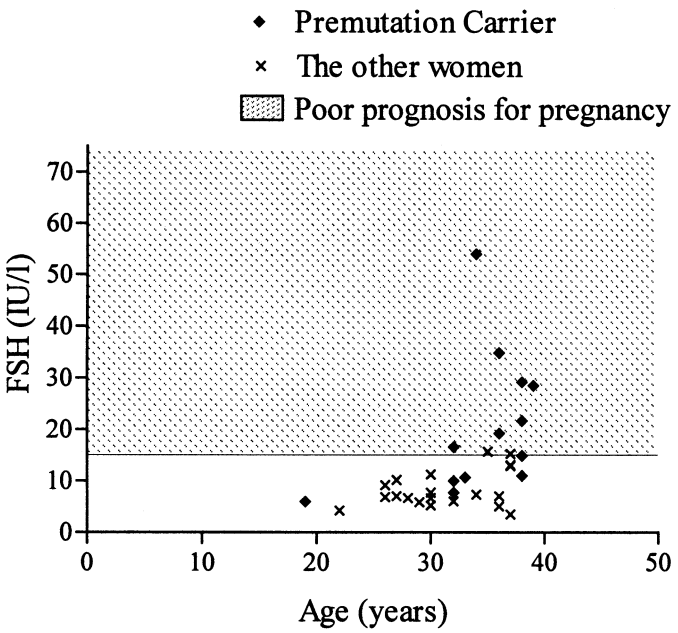

Figure 2. Serum FSH concentrations as a function of age for women who were using combined oral contraceptives.

mean serum inhibin B concentration was 52.8 ± 42.6 ng/l in the premutation carriers, and 70.7 ± 37.8 ng/l in the other women (Table I), which was not significantly different. The concentrations of 17β-oestradiol were evenly distributed over the two groups.

To evaluate whether the premutation carriers with normal cycles had endocrine signs of diminished ovarian function, we first excluded the data from the women with abnormal menstrual cycles (n = 7). We subsequently analysed the data from the women with normal cycles (n = 37). The mean serum FSH concentration was 11.0 ± 7.89 IU/l in the regularly menstruating premutation carriers, and 6.92 ± 2.71 IU/l in the other women (see Table I). Multiple linear regression analyses demonstrated that these premutation carriers had significantly higher serum FSH concentrations than the other women with a normal cycle (P = 0.029). A serum FSH of ⩾15 IU/l was observed in one out of 12 premutation carriers, but in none of the 25 other women ($P > 0.05$). There were no significant differences in serum inhibin B and 17β-oestradiol.

Women on oral contraceptives (n = 34)

All these women were using low-dose (<50 μg 17α-ethinyl oestradiol) combined oral contraceptives. A total of 13 women were premutation carriers, while the 21 other women were not. In the latter group, 11 women had a normal *FMR1* gene and 10 had a full mutation.

The mean age of the premutation carriers and other women was 34 ± 5.3 years and 31 ± 4.5 years respectively (Table I). Six (46%) premutation carriers and five (24%) of the other women were currently smoking.

Endocrine profiles were assessed in the blood samples obtained on the last day of the 7-day pill-free interval. Serum FSH ranged from 6.0 to 54 IU/l in the premutation carrier group and from 3.5 to 16 IU/l in the other women (Figure 2). The mean serum FSH concentration was 20.4 ± 13.5 IU/l in the premutation carriers and 8.35 ± 3.5 IU/l in the other women (Table I). After adjusting for age and smoking behaviour, linear regression analysis demonstrated that the premutation carriers still had significantly higher serum FSH concentrations (P = 0.001). We observed a serum FSH of ⩾15 IU/l in significantly more of the premutation carriers than in the other women (χ^2 test; P = 0.013). Overall, this was the case in seven (54%) out of the 13 premutation carriers, and in two (9.5%) out of the 21 other women (Figure 2). We observed one 34 year old premutation carrier who had an FSH concentration of 54 IU/l. After correcting for possible familial influences in FSH, it was shown that the carriers had significantly higher FSH concentrations than the other women (P = 0.001).

Serum inhibin B ranged from 2.0 to 207 ng/l in the premutation carrier group and from 2.0 to 202 ng/l in the other women. Linear regression analyses did not demonstrate any significant difference in serum inhibin B and in 17β-oestradiol between the premutation carriers and the other women.

Discussion

Since fragile X premutations are known to be a risk factor for diminished ovarian function at a relatively young age, the aim of this study was to investigate the endocrine profiles of premenopausal women out of fragile X families. To date, only two studies have been conducted on the endocrinology of premenopausal carriers of the fragile X premutation. These studies found an increased serum FSH concentration in premutation carriers (Braat *et al.*, 1999; Murray *et al.*, 1999). The present study confirms the finding that the premutation carriers had higher serum concentrations of FSH than the other women, irrespective of menstrual cycle pattern. Even in women with normal menstrual cycles, the premutation carriers had higher serum FSH concentrations than the other women. This may indicate that, even if the premutation carriers have normal cycles, they may already be suffering from ovarian ageing, which would be in line with the high occurrence of POF in premutation carriers (Allingham-Hawkins *et al.*, 1999). Furthermore we found that, of the women who were using combined oral contraceptives, the premutation carriers also had significantly higher serum FSH concentrations than the other women.

A serum FSH concentration of ⩾15 IU/l was more frequently observed in premutation carriers (both oral contraceptive users, and non-users) aged <40 years than in other women, which suggests that these premutation carriers have a poorer prognosis for future pregnancy. However, most data concerning the predictive value of FSH are from studies in an assisted reproductive technology setting. It remains questionable whether these findings also apply to women who are not being treated for infertility problems.

An item of concern was that sometimes more than one female per family participated in our study. We investigated whether familial bias factors influencing FSH concentrations were present in our data. It was found that after correction for multiple familial participation premutation carriers had higher FSH concentrations than the other women. This was the case

in both carriers who were using oral contraceptives and in those who were not.

Although inhibin B was assumed to be the first marker of ovarian ageing (Welt *et al.*, 1999), clinical relevance is still elusive. Furthermore, to our knowledge, there are no reports on the interpretation of serum inhibin B concentrations in the pill-free interval. Nevertheless, we measured inhibin B and found no statistically lower concentrations in premutation carriers than in the other women; neither in the contraceptive users, nor in the women with menstrual cycle. This is in line with a recent study (among a comparable study population) that did not find a significant association either, although the authors consider their inhibin data to be inconclusive (Murray *et al.*, 2000). Therefore, it remains questionable whether the assessment of inhibin B has any clinical relevance.

Young premutation carriers who are using combined oral contraceptives will not be able to recognize peri-menopausal signs, due to the administration of exogenous oestrogens. In the present study, we demonstrated that unrecognized ovarian dysfunction, or even worse, POF (as was the case in the premutation carrier with a serum FSH of 54 IU/l) was present in female carriers of the fragile X premutation using combined oral contraceptives. To our knowledge, this is the first report on unrecognized diminished ovarian function in women who are using combined oral contraceptives.

In women who have just started using oral contraceptives, the suppressed FSH concentrations return to the normal baseline concentration (of the control cycle) during the pill-free period (Cohen and Katz, 1979; Cohen and Katz, 1981). In peri- or post-menopausal women who have just started using oral contraceptives, FSH is not restored to the previously established control baseline concentration during the short pill-free interval (Creinin, 1996). All these reports compared the baseline concentration of FSH with the hormone concentration at the end of the pill-free interval. Thus, it seems reasonable to conclude that endocrine profiles assessed on the last day of the pill-free interval do not necessarily reflect the (non-suppressed) baseline concentrations. As the present study lacked data on baseline FSH, it was not possible to determine whether the serum FSH values returned to baseline. It is possible that women with high serum FSH concentrations may have even higher baseline concentrations of FSH. In the present study, the premutation carriers had higher serum FSH concentrations and so we expect that especially the premutation carriers will have even higher baseline FSH concentrations.

Four women were using combined oral contraceptives to regulate their irregular cycles: one premutation carrier (36 years, FSH 34.9 IU/l) and three other women (aged 25, 28 and 28 years, with serum FSH concentrations of 9.2, 6.7 and 7.1 IU/l respectively). It is possible that, at that time, these irregular cycles were caused by diminished ovarian function. In order to minimize the risk of potential selection bias (by including women who were taking combined oral contraceptives for diminished ovarian function), we also performed a multiple regression analysis which excluded these four women from the analysis. The FSH concentrations remained significantly higher in the premutation carriers ($P = 0.006$).

In order to analyse the endocrinology of normally menstruating women, we had to exclude seven women from this analysis because they reported having abnormal menstrual cycles (four premutation carriers and two other women). In the women with abnormal cycles, the premutation carriers only differed from the other women in having significantly higher serum FSH concentrations (t-test; $P = 0.043$). In the premutation carriers the mean serum FSH was 27 IU/l, while the mean serum inhibin B was 11.1 ng/l. In the other women, these concentrations were 4.40 IU/l and 79.7 ng/l respectively (Table I). In our view, it seems that especially the premutation carriers with abnormal cycles demonstrated signs of ovarian ageing, which may have caused these cycle abnormalities. However, the numbers were small; further research may help to elucidate this issue.

Figures 1 and 2 depict a few outlying observations, which may represent a subset of women who had ovarian dysfunction. All these outlying observations are accounted for by premutation carriers. However, not all premutation carriers had endocrine signs of ovarian reserve diminution, and therefore it is possible that the group of premutation carriers could have been divided in at least two subgroups with different phenotypes and, possibly, genotypes. Recently, we found that women with a paternally-inherited premutation (PIP) have a high risk for developing POF, whereas women with a maternally-inherited premutation (MIP) do not (Hundscheid *et al.*, 2000). We evaluated whether a parent-of-origin effect could be detected in the endocrine profiles. Therefore, we analysed the data on all the premutation carriers in whom the parental origin of the premutation could be determined. We found no associations between ovarian dysfunction and the parental origin of the premutation. The numbers, however, were too small to draw any firm conclusion. Further research is warranted to determine whether women with a PIP have a different endocrine profile from other women.

In conclusion, premutation carriers demonstrated increased serum FSH concentrations in comparison with the other women. This applies to premutation carriers with regular cycles as well as to those who were using oral contraceptives. Moreover, serum FSH concentrations of $\geqslant$15 IU/l were more frequently observed in premutation carriers, irrespective of the use of oral contraceptives. Both findings suggest that premutation carriers show endocrine signs of diminished ovarian function, which is in line with the observation that premutation carriers have a high risk of developing POF. Women with an FSH of $\geqslant$15 IU/l have lower spontaneous pregnancy rates as well as lower pregnancy rates after IVF than women with a serum FSH of $<$15 IU/l (Scott and Hofmann, 1995). Hence, premutation carriers need to be informed about this risk, especially if they are considering PGD, which implies IVF. In the case of mild FSH elevations, it might be possible to achieve pregnancy with assisted reproductive technology. At present, we can only advise young carriers of the fragile X premutation not to postpone starting a family for too long.

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