The association of CGG repeats in the FMR1 gene and timing of natural menopause

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**STUDY QUESTION:** Is there an association between the number of CGG repeats in the FMR1 gene in the normal and intermediate range and age at natural menopause?

**SUMMARY ANSWER:** The number of CGG repeats in the normal and intermediate range in the FMR1 gene was not associated with age at natural menopause.

**WHAT IS KNOWN ALREADY:** Excessive triple CGG repeats in the FMR1 gene have been widely associated with primary ovarian insufficiency. Recently, the number of CGG repeats in the normal and intermediate range (up to 55 repeats) was found to be associated with serum levels of anti-Müllerian hormone and follicle-stimulating hormone, as markers for ovarian ageing. This suggests that repeats in the normal and intermediate range could be involved in the rate of exhaustion of the ovarian primordial follicle pool and ultimately the timing of menopause.

**STUDY DESIGN, SIZE:** Cross-sectional study in a population-based sample of 3611 Caucasian women with natural menopause.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** The FMR1 CGG repeat number was determined by PCR amplification in 3611 women with a known age at natural menopause. A possible relation between CGG repeats in the normal and intermediate range (up to 55 repeats) and menopausal age were analysed in various ways, including linear regression analysis and analysis of variance.

**MAIN RESULTS AND THE ROLE OF CHANCE:** The number of CGG repeats in the normal and intermediate range in the FMR1 gene was not associated with age at natural menopause. The mean age at menopause was 50.30 (± 4.2) years for women with <45 repeats and 50.64 (± 3.4) years for women with intermediate-sized repeats (P = 0.37). Linear regression analysis of the number of CGG repeats showed no association with menopausal age (β = 0.019, P = 0.16).

**LIMITATIONS, REASONS FOR CAUTION:** In our cohort, age at menopause was self-reported and determined retrospectively.

**WIDER IMPLICATIONS OF THE FINDINGS:** Earlier observations suggesting that the number of CGG repeats in the normal and intermediate range is associated with the individual variation of the ovarian ageing process could not be confirmed in the current, large sample size study. A relation between the number of CGG repeats in the normal and intermediate range and age at natural menopause appeared to be absent. This finding questions the role of CGG repeat sizes in the ovarian ageing process.

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**TRIAL REGISTRATION NUMBER:** Not applicable.

Key words: age at menopause / FMR1 gene / CGG repeats / fragile X / ovarian ageing
Introduction

Excessive triple CGG repeats in the fragile X mental retardation (FMR1) gene have been associated with early ovarian failure. The FMR1 gene is located in the 3′ untranslated region of the X chromosome and normally contains <55 copies of the CGG repeat. Alleles with <45 repeats are stably inherited. CGG repeats in the intermediate range, 45–55 repeats, however, may become unstable during transmission and have the ability to expand to a premutation (55–200 repeats), which can further expand to a full mutation (>200 repeats) causing the fragile X syndrome in the subsequent generation (Nolin et al., 2003; Kronquist et al., 2008).

CGG repeats in the premutation range have been associated with primary ovarian insufficiency (POI, i.e. menopause before the age of 40 years, also known as POF, premature ovarian failure), referred to as fragile X-associated POI (Allingham-Hawkins et al., 1999; Sherman, 2000; Allen et al., 2007). The prevalence of POI in women carrying the FMR1 premutation is estimated to be between 13 and 26% (Sherman, 2000; Sullivan et al., 2005). The association between the number of repeats in the premutation range and the risk of POI is believed to be non-linear and suggests that carriers of premutations in the mid-size range (80–100 repeats) are at greatest risk for POI (Ennis et al., 2006). Furthermore, premutation carriers experience menopause on average 5 years earlier than non-carriers (Murray et al., 2000; Sullivan et al., 2005). Repeat sizes in the intermediate (45–54 repeats) and higher end of the normal range (35–44 repeats) have also been associated with POI (Bretherick et al., 2005; Bodega et al., 2006). However, this association has not been confirmed in a large group of POI cases (Bennett et al., 2010).

Recently, Gleicher et al. reported an association between the number of CGG repeats in both the high end of the normal range and in the intermediate range (35–55 repeats) with anti-Müllerian hormone (AMH) levels in premenopausal women. Furthermore, with increasing numbers of CGG repeats, the risk for early ovarian senescence, defined as elevated levels of follicle-stimulating hormone (FSH), increased (Gleicher et al., 2009b). In addition, women carrying either alleles with <28 repeats or alleles with >33 repeats presented with reduced AMH levels, suggesting diminished ovarian reserve in these CGG repeat ranges (Gleicher et al., 2009a). The results of these and a few other studies (Chatterjee et al., 2009; Gleicher et al., 2010a,b; Karimov et al., 2011) suggest that the number of CGG repeats in the normal and intermediate range might also affect the ovarian ageing process over time and thereby the timing of menopause. Therefore, we aimed to investigate the association between the number of CGG repeats in the normal and intermediate range (up to 55 repeats) and age at natural menopause using a large cohort of Dutch post-menopausal women.

Materials and Methods

Study population

The Prospect-EPIC cohort is one of the two Dutch contributions to the European Prospective Investigation into Cancer and Nutrition (EPIC). The design and rationale of this study has been described previously (Boker et al., 2001). In brief, this cohort consists of 17,357 white women living in Utrecht and surroundings, The Netherlands, aged 49–70 years, who were invited to participate in the study through the national breast cancer screening programme between 1993 and 1997. All women filled out detailed questionnaires about dietary, reproductive and medical history and underwent a physical examination at enrolment. In addition, women donated a 30-ml non-fasting blood sample which was fractioned into serum, citrated plasma, buffy coat and erythrocyte aliquots of 0.5 ml each. The samples were stored under liquid nitrogen at −196°C for future research.

Natural menopause was defined according to the Word Health Organization as amenorrhoea for at least 12 consecutive months without other obvious reasons. A total of 3497 women were premenopausal or perimenopausal at the time of enrolment and therefore excluded. All women who experienced a surgical menopause (n = 4449), used hormones during the menopausal transition (n = 2161) or women with an unknown menopausal status or age (n = 1194) were excluded. Next, all women who were younger than 58 years at inclusion in the Prospect-EPIC cohort were excluded to avoid bias due to differential inclusion of women with an early menopause (n = 2248). Finally, 197 women were excluded because of missing buffy coat samples or failed DNA extraction, leaving a total of 3611 women available for analysis.

DNA extraction and genotyping

Genomic DNA was extracted from buffy coat aliquots by KBioscience using their own in-house silica-based systems for buffy extraction (http://www.kbioscience.co.uk/lab%20services/DNA%20extraction/Ext_services_intro.html). Genotyping was performed at the Department of Medical Genetics, University Medical Center Utrecht, The Netherlands. The FMR1 CGG repeat number was determined by PCR amplification using Platinum PfX DNA Polymerase according to the instruction of the manufacturer (Invitrogen). Primers for the FMR1 gene were: 5′-GCTCGGTTTTCGGTTTCACTTCCGGT-3′ and 5′-AGCCCCGACTTCCACCCACAGCTCCTCCA-3′. PCR conditions are available upon request. Repeat sizes were measured using an ABI prism 3730 DNA Analyzer (Applied Biosystems) and quantified using Genemapper Analysis Software v3.7 (Applied Biosystems).

As quality-control tests, plate-identifying blank wells and blind duplicates were used. Random duplicates analysed in the Genome Diagnostics Section of the Department of Medical Genetics laboratory, UMC Utrecht, The Netherlands, were used as additional quality-control tests. The PCR analyses failed in 105 (2.9%) of our 3611 samples. Furthermore, 335 (9.2%) samples showed a homozygous pattern after PCR amplification. For 72 of these 335 presumed homozygous samples, we could not clearly identify two alleles. This could indicate homozygosity for the small allele, or a larger allele (>100 repeats) may have been present but was not picked up by PCR analyses. Patient characteristics of these 72 samples did not differ from the total cohort. Therefore, we were confident that we could safely remove these samples from the analyses. Because we studied the association between the number of CGG repeats and age at natural menopause in the normal and intermediate range (up to 55 repeats), we excluded 11 samples because the largest allele counted ≥55 CGG repeats (premutation range). Thus, in our final data set, we had a total of 3423 women with repeat sizes between 20 and 55 CGG repeats (normal and intermediate range).

Data analysis

Characteristics of the study population were described using means and standard deviations for normally distributed continuous variables and frequencies and percentages for categorical variables. Alleles with the lower number of CGG repeats were designated as allele 1 and alleles with the higher number as allele 2. Women carrying <45 repeats on allele 2 were considered ‘normal’ and those carrying between 45 and 54 repeats on allele 2 are considered to be within the intermediate range.
We used analysis of variance (ANOVA) to test differences in age at menopause between these two groups.

The linearity assumption between CGG repeat sizes on allele 2 was tested using restricted cubic splines composed of three polynomial segments in R (version 2.13.2; http://www.r-project.org), using the following libraries: foreign, Hmisc and Design. This analysis did not indicate deviations from linearity ($P = 0.275$). Next, a linear regression analysis was performed with the number of CGG repeats for allele 2 as the independent and age at menopause as the dependent variable. This analysis was adjusted for the number of repeats on allele 1 by inclusion of the repeat sizes on allele 1 in the multivariate linear regression analysis.

The most frequent repeat sizes on allele 2 range from 29 to 34 repeats (75.7% of all women). For further analysis, we considered these alleles ‘common’. The repeat sizes to the right and left of these common alleles were considered uncommon. We used ANOVA to test differences in age at menopause between the three groups, i.e. Group 1: <29 repeats, Group 2: 29–34 repeats (common) and Group 3: >34 repeats.

To explore whether possible associations between repeat sizes and timing of menopause are driven by both alleles on the FMR1 gene, we performed analysis based on both alleles, organized in six categories. Again, the ‘common’ group was formed by women with both alleles between 29 and 34 repeats. The ‘small’ group consisted of women with both alleles <29 repeats. Women with heterozygous alleles with allele 1 <29 and allele 2 in the ‘common’ range (29 and 34 repeats), or allele 1 <29 and allele 2 >34 repeats, or allele 1 in the ‘common’ range and allele 2 >34 repeats were considered ‘heterozygous S’, ‘heterozygous M’ or ‘heterozygous L’, respectively. Finally, the ‘large’ group consisted of women with both alleles >34 repeats. Association analysis between the six groups and age at menopause was tested using ANOVA.

To assess if possible associations between CGG repeat sizes and age at menopause are driven by the POI cases in our study cohort, we performed sensitivity analyses by excluding women with a menopausal age under 40 years (i.e. POI, $n = 55$). Statistical analyses were performed using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA) and R (version 2.13.2; http://www.r-project.org). A $P$-value of $\leq 0.05$ was considered statistically significant.

## Results

Table I summarizes general characteristics of the women in our study cohort. Out of the total of 3423 women studied, 123 (3.6%) carried CGG repeats within the intermediate range (45–54 repeats) for allele 2. When comparing age at menopause between women carrying intermediate alleles and those carrying <45 CGG repeats, we found no difference in menopausal age. The mean age at menopause was 50.30 ($\pm 4.2$) years for women with <45 repeats and 50.64 ($\pm 4.3$) years for women with intermediate-sized repeats ($P = 0.373$).

Linear regression analysis of the number of CGG repeats (allele 2) showed no association with age at menopause ($\beta = 0.019$, $P = 0.157$). Adjustment for the number of repeats on allele 1 did not alter these results.

There was no difference in the mean age at menopause when comparing menopausal age of women carrying <29 repeats (49.90, $\pm 4.5$ year), between 29 and 34 repeats (50.33, $\pm 4.2$ year) and over 34 repeats (50.34, $\pm 4.1$ year), no difference between these three groups was present (Table II, $P = 0.34$).

Table III presents an analysis of menopausal age of six genotype categories classified by CGG repeat sizes on both FMR1 alleles present in women. Again, no statistical difference in age at menopause was present among the six groups ($P = 0.73$).

Exclusion of POI cases ($n = 55$) from the study cohort did not alter the results of any of the analyses described in the aforementioned.

### Table I Population characteristics ($n = 3423$)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Association analysis $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at inclusion (years, mean $\pm$ SD)</td>
<td>63.0 $\pm$ 3.41</td>
<td>60.3 $\pm$ 3.41</td>
<td>58.0 $\pm$ 3.41</td>
<td>0.93</td>
</tr>
<tr>
<td>Age at natural menopause (years, mean $\pm$ SD)</td>
<td>50.31 $\pm$ 4.21</td>
<td>50.33 $\pm$ 4.2</td>
<td>50.34 $\pm$ 4.1</td>
<td>0.34</td>
</tr>
<tr>
<td>Range (years)</td>
<td>18–64</td>
<td>18–64</td>
<td>18–64</td>
<td>0.33</td>
</tr>
<tr>
<td>No. of women who were ever pregnant</td>
<td>2923 (85.4%)</td>
<td>2682 (78.2%)</td>
<td>2538 (74.4%)</td>
<td>0.275</td>
</tr>
<tr>
<td>Parity (mean $\pm$ SD)</td>
<td>2.65 $\pm$ 1.84</td>
<td>2.71 $\pm$ 1.84</td>
<td>2.67 $\pm$ 1.84</td>
<td>0.66</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>521 (15.2%)</td>
<td>494 (14.5%)</td>
<td>480 (13.8%)</td>
<td>0.157</td>
</tr>
<tr>
<td>Parous</td>
<td>2902 (84.8%)</td>
<td>2188 (65.5%)</td>
<td>2057 (60.2%)</td>
<td>0.019</td>
</tr>
<tr>
<td>No. of women who ever used oral contraception</td>
<td>1180 (34.5%)</td>
<td>1158 (32.9%)</td>
<td>1145 (32.9%)</td>
<td>0.66</td>
</tr>
<tr>
<td>No. of women who ever used HRT*</td>
<td>226 (6.6%)</td>
<td>234 (6.7%)</td>
<td>232 (6.8%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Smoking never</td>
<td>1749 (51.1%)</td>
<td>1740 (51.1%)</td>
<td>1741 (51.1%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Current or past</td>
<td>1674 (48.9%)</td>
<td>1655 (48.9%)</td>
<td>1644 (48.9%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Body mass index (kg/m², mean $\pm$ SD)</td>
<td>26 $\pm$ 4</td>
<td>26 $\pm$ 4</td>
<td>26 $\pm$ 4</td>
<td>0.33</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>85 $\pm$ 10</td>
<td>85 $\pm$ 10</td>
<td>85 $\pm$ 10</td>
<td>0.33</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>138 $\pm$ 21</td>
<td>138 $\pm$ 21</td>
<td>138 $\pm$ 21</td>
<td>0.93</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 $\pm$ 10</td>
<td>80 $\pm$ 10</td>
<td>80 $\pm$ 10</td>
<td>0.93</td>
</tr>
<tr>
<td>No. of CGG repeats allele 1 (mean $\pm$ SD)</td>
<td>27 $\pm$ 4.5</td>
<td>27 $\pm$ 4.5</td>
<td>27 $\pm$ 4.5</td>
<td>0.93</td>
</tr>
<tr>
<td>Range</td>
<td>7–47</td>
<td>7–47</td>
<td>7–47</td>
<td>0.93</td>
</tr>
<tr>
<td>No. of CGG repeats allele 2 (mean $\pm$ SD)</td>
<td>33 $\pm$ 4.9</td>
<td>33 $\pm$ 4.9</td>
<td>33 $\pm$ 4.9</td>
<td>0.93</td>
</tr>
<tr>
<td>Range</td>
<td>20–54</td>
<td>20–54</td>
<td>20–54</td>
<td>0.93</td>
</tr>
</tbody>
</table>

*HRT, hormone replacement therapy.

### Table II CGG repeat sizes and age at menopause

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of women (%)</th>
<th>Age at natural menopause (years, mean $\pm$ SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>164 (48.8)</td>
<td>49.90 $\pm$ 4.5</td>
</tr>
<tr>
<td>Group 2</td>
<td>2592 (75.7)</td>
<td>50.33 $\pm$ 4.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>667 (19.5)</td>
<td>50.34 $\pm$ 4.1</td>
</tr>
</tbody>
</table>

*aGroup 1: allele 2 <29 repeats.
*bGroup 2: allele 2 29–34 repeats (common).
*cGroup 3: allele 2 >34 repeats.
*dANOVA.*
Discussion

Our study investigated the possible association between the number of CGG repeats in the FMR1 gene and timing of natural menopause in a large cohort of Dutch post-menopausal women. We found that the number of CGG repeats in the normal and intermediate range (up to 55 repeats), analysed in various ways, was not associated with age at natural menopause.

To our knowledge, our study is the first to investigate a possible relation between normal and intermediate CGG repeat sizes in the FMR1 gene and age at natural menopause in a large population-based cohort. This work basically builds on the studies where variations in the FMR1 CGG repeat sizes within the normal and intermediate range were associated with the level of basal FSH and serum AMH, suggesting a further role of the FMR1 gene in the mechanisms of ovarian ageing (Chatterjee et al., 2009; Gleicher et al., 2009a, 2010a; Karimov et al., 2011). In case these normal range variations would indeed affect follicle wastage, the end-point of this wastage process (i.e. menopause), could well be influenced by these variations. This would also be in line with studies on non-normal variation in the CGG repeats sizes (i.e. premutation carriers; 55–200 repeats), where large effect sizes have been reported regarding earlier age at menopause (Murray et al., 2000; Sullivan et al., 2005, Ennis et al., 2006).

In exploring the relation between normal ranged CGG repeats and markers for ovarian reserve, an association was previously demonstrated between the number of CGG repeats in the range of 35–55 repeats and AMH levels in a small pilot study of 40 premenopausal women (Gleicher et al., 2009b). Furthermore, they found that with increasing numbers of CGG repeats, FSH concentrations increased (Gleicher et al., 2009b). In addition, a study in 316 infertility patients reported that both alleles with $<28$ repeats and alleles with $>33$ repeats correlated with lower AMH levels, suggesting diminished ovarian reserve in these specific CGG repeat ranges (Gleicher et al., 2009a). In another study, the frequency of intermediate alleles (45–55 repeats) was higher in 535 women with elevated FSH levels or with poor response to gonadotrophin stimulation (i.e. occult POI or imminent ovarian failure, IOF) compared with women in the control group ($n = 521$) composed of oocyte donors and infertile women with no evidence of occult POI ($P = 0.046$) (Karimov et al., 2011). FSH concentrations were also found to be significantly raised ($P < 0.0001$) in women carrying 31–40 repeats compared with those carrying fewer than 30 repeats in a study population composed of 80 Indian POI cases and 70 Indian controls (Chatterjee et al., 2009).

The observed associations between CGG repeats in the normal and intermediate range and proxies for (early) ovarian senescence in the above-mentioned studies suggest a role for the FMR1 gene in ovarian ageing. However, we were not able to detect a relationship between FMR1 CGG repeats and the ultimate long-term outcome of the ovarian ageing process, i.e. age at the onset of menopause. Following the analytical steps from these earlier studies did not yield any association with timing of menopause in our large study of post-menopausal women. Several explanations can be proposed to substantiate this seemingly inconsistent finding.

First, age at natural menopause may not accurately represent the ovarian ageing process taking place in the decades prior to menopause. Possibly, FMR1-related ovarian ageing is not due to an inherently smaller follicle pool, but to a diminished recruitment rate from the
primordial follicle pool. It was suggested that this diminished recruit-
ment rate reflects lower circulating AMH levels in young women,
but not an early menopause due to preservation of ovarian reserve
into older age (Gleicher et al., 2010a). Thus, it could be that the
numbers of CGG repeats in the normal and intermediate range
have an effect on the rate of decline of ovarian reserve and not the
end-point of ovarian ageing (i.e. menopause) (Gleicher et al.,
2010a). However, studies showing that AMH is highly predictive
for timing of menopause would contradict this hypothesis (van
Rooij et al., 2004; van Disseldorp et al., 2008; Broer et al., 2011;
Tehrani et al., 2011). The same holds true for poor response to gonadotrophin
stimulation and prediction of menopausal age. Since it has been found
that women with poor response to gonadotrophin stimulation have an
increased chance of early natural menopause (de Boer et al., 2003),
the possible increased frequency of intermediate CGG repeat sizes
in these women would also be reflected in an earlier age at natural
menopause. In fact, in a recently published study by Lledo et al.
(2012), no relationship was found between normal and intermediate-
sized CGG repeats and ovarian stimulation in 204 oocyte donors, sug-
gesting no negative effect of CGG repeat in these ranges and ovarian
reserve (Lledo et al., 2012).

Secondly, the previous associations were found in relatively small
studies, varying from 40 to 535 women, whereas we investigated a
population-based sample of 3423 post-menopausal women. We
cannot exclude the possibility that the earlier found associations
between normal ranged repeat sizes and proxies for ovarian ageing
(i.e. FSH and AMH) were in fact false-positive findings. Confirmation
of the associations with FSH and AMH levels in large cohorts is there-
fore urgently needed.

The inconsistent findings could also be due to differences in ethnicity
in the studied populations. A recent study demonstrated differences
in CGG repeat distribution amongst various racial/ethnic groups
(Gleicher et al., 2010b). Moreover, it could be possible that the
association between CGG repeats and (markers for) ovarian ageing
varies between ethnicities (Gleicher et al., 2012). So the observed discrepancy with other studies could be explained by the fact that the current study was performed on a Caucasian population,
leaving the possibility open that in other ethnic groups, the relation
between CGG repeat distribution and the ovarian ageing process is
fundamentally different.

Another explanation may stem from the fact that the populations in
the previous studies consisted, at least partly, of infertile women and/or
women with (occult) POI, whereas we investigated a population-
based sample. Possibly, the numbers of CGG repeats are in fact asso-
ciated with timing of menopause in an infertile population experiencing
early ovarian senescence, rather than women in the general popula-
tion who experience normal menopause (i.e. menopause between
45 and 55 years). However, subgroup analysis of nulliparous women
(n = 521) and infertile women (women who were ever diagnosed with infertility by a medical doctor; n = 80) within our population
also lacked an association between the number of CGG repeats and
age at menopause (data not shown).

Next to markers for ovarian ageing, CGG repeats in the normal and
intermediate range have been also associated with susceptibility to
POI, albeit not consistent. An association with POI has been demon-
strated in three studies comprising 53, 190 and 128 POI cases (Breth-
erick et al., 2005; Bodega et al., 2006; Ishizuka et al., 2011).

Importantly, the findings could not be replicated in the largest study
performed so far, which studied 366 women with POI (Bennett et al., 2010). Whether POI cases with no obvious cause (idiopathic
POI) may be considered as a separate genetic entity or to be part
of the variation towards the entire distribution of natural menopause
remains unclear. Therefore, it could be that the number of CGG
repeats is primarily associated with early menopause (POI) and
possibly has a less important or even no role in determining normal
menopausal variation.

When interpreting our results, some strengths and limitations
should be kept in mind. First, we conducted a study in a large, well
phenotyped cohort of natural menopausal women, which provides
power to detect possible small effect sizes. Next, in studies associating
CGG repeat lengths with ovarian ageing, no standard method is con-
sistently used. Most studies focus on the allele with the higher number
of CGG repeats (allele 2), although a solid biological rationale is
lacking. Linear regression analysis of the number of CGG repeats
on allele 1 or alleles 1 and 2 combined, however, did not change our
results ($p_{\text{allele }1} = 0.026$, $P = 0.122$ and $p_{\text{allele }1 + \text{allele }2} = 0.029$,
$P = 0.092$). Furthermore, it has been previously suggested that
associations between CGG repeat sizes and ovarian reserve are
dependent on heterozygous or homozygous expression of the
 genetic abnormality (Gleicher et al., 2010a). Again, as showed in
Table III, no association with ANM was found in any of the categories.
A limitation might be that in the Prospect-EPIC cohort, as in most
studies conducted in timing of menopause, age at menopause was self-
reported and determined retrospectively. Self-reported menopausal
age could be susceptible to bias (den Tonkelaar, 1997; Hahn et al.,
1997). However, the reproducibility of self-reported menopause is
high (Rödström et al., 2005). Also, it is unlikely that misclassification
due to recall bias differs across genotypes. However, we cannot
fully exclude the possibility that the non-differential bias precluded us
from picking up a very small association.

In conclusion, in our comprehensive genetic study on the relation
between the FMR1 gene and ovarian ageing, we observed no associ-
ation between the numbers of CGG repeats in the normal to inter-
mediate range and age at natural menopause. The evidence from
recent studies for involvement of CGG repeat sizes up to 55
repeats in ovarian ageing process could thereby not be confirmed
by the current study.

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Authors’ roles

and F.J.B.: manuscript drafting. M.V., N.C.O.-M., B.C.J.M.F.,
H.K.P.A., Y.T.S. and F.J.B.: critically revision and final approval of the
manuscript.
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**Conflict of interest**

M.V., N.C.O.-M., Y.T.S. and H.K.P.A. have nothing to declare. B.C.J.M.F. has received fees and grant support from the following companies (in alphabetic order): Andromed, Ardana, Ferring, Genovum, Merck Serono, MSD, Organon, Pantharei Bioscience, PregLem, Schering, Schering Plough, Serono and Wyeth. F.J.B. is a member of the external advisory board for Merck Serono, The Netherlands, and does consultancy work for MSD.

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