The significance of fragile X mental retardation gene 1 (FMR1) CGG repeat sizes in the normal and intermediate range in women with primary ovarian insufficiency

M. Voorhuis1,2,*, N.C. Onland-Moret2, F. Janse1, H.K. Ploos van Amstel3, A.J. Goverde1, C.B. Lambalk4, J.S.E. Laven5, Y.T. van der Schouw2, F.J.M. Broekmans1, and B.C.J.M. Fauser1 on behalf of the Dutch Primary Ovarian Insufficiency Consortium

1Department of Reproductive Medicine and Gynecology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
2Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands
3Department of Medical Genetics, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
4Department of Obstetrics and Gynaecology, VU Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
5Department of Reproductive Medicine, Erasmus Medical Center Rotterdam, ’s Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

*Correspondence address. Department of Reproductive Medicine and Gynecology, University Medical Center, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. E-mail: m.voorhuis@umcutrecht.nl

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STUDY QUESTION: Are fragile X mental retardation gene 1 (FMR1) CGG repeats in the normal and intermediate range (up to 55 repeats) associated with primary ovarian insufficiency (POI) in a large case–control study?

SUMMARY ANSWER: No association was found between CGG repeats of intermediate size and POI compared with controls.

WHAT IS KNOWN ALREADY: CGG repeats in the FMR1 gene in the premutation range (55–200 repeats) have consistently associated with POI. Intermediate range CGG repeats have been considered for a potential association with POI.

STUDY DESIGN, SIZE: A case–control study in 375 well-phenotyped Dutch women diagnosed with POI and 3368 controls with natural menopause ≥40 years of age.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The FMR1 CGG repeat number was determined by PCR amplification in women diagnosed with POI and women with a known age at natural menopause ≥40 years. The prevalence of intermediate sized CGG repeats (45–54 repeats) was compared between POI cases and controls using Fisher’s exact test. Differences in mean CGG repeat lengths on allele 1 and allele 2 between POI cases and controls were tested using analysis of variance.

MAIN RESULTS AND THE ROLE OF CHANCE: The frequency of intermediate sized CGG repeats on the allele with the longest triple repeat number was not statistically significantly different between POI cases and controls (2.7 and 3.8%, respectively, odds ratio 0.72, 95% confidence interval: 0.38–1.39, P = 0.38). In women with POI, linear regression analysis for age at POI diagnosis and CGG repeat size also failed to show any association (β = −0.018, P = 0.74).

LIMITATIONS, REASONS FOR CAUTION: FMR1 CGG repeat lengths in POI cases and controls were genotyped in two different laboratories. The distributions of CGG repeats may vary among the different ethnic populations in our study. Also, in our study women with primary amenorrhea (N = 17) were included in the POI group.

WIDER IMPLICATIONS OF THE FINDINGS: We found no association between intermediate sized CGG repeats and POI compared with controls. Therefore, a role for FMR1 CGG repeat sizes up to 55 repeats in the ovarian ageing process may be questioned. Moreover,
there seems limited value in the evaluation of normal- and intermediate FMR1 repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk of developing POI.

**STUDY FUNDING/COMPETING INTEREST(S):** The Prospect-EPIC study was funded by ‘Europe Against Cancer’ Program of the European Commission (SANCO); the Dutch Ministry of Health; the Dutch Cancer Society; ZonMW the Netherlands Organization for Health Research and Development; World Cancer Research Fund (WCRF) and the Dutch Heart Association.

**Key words:** fragile X mental retardation gene 1 / CGG repeats / menopause / primary ovarian insufficiency / ovarian ageing

### Introduction

Menopause typically occurs ~51 years of age (Treloar, 1981). However, ~1–2% of women experience the cessation of menses before age 40 years (Couplam et al., 1986). This condition—currently referred to as primary ovarian insufficiency (POI) (previously premature ovarian failure)—is characterized by amenorrhea for at least 4 months occurring prior to the age of 40 years, in combination with repeatedly elevated FSH concentrations >40 IU/l and with decreased estradiol levels (Cooper et al., 2010; de Vos et al., 2010).

The high heritability of age at menopause (van Asselt et al., 2004; Morris et al., 2011) and the tendency for POI to run in families (Cramer et al., 1995; van Kasteren et al., 1999) imply a strong genetic component underlying POI. In fact, women were ~5–6 times more likely to reach menopause early if their mother or sister had experienced an early menopause (i.e. menopause before 45 years) (Morris et al., 2011). Furthermore, environmental factors, such as smoking, have also been shown to contribute to the susceptibility to develop POI (Luborsky et al., 2003; Chang et al., 2007). POI is therefore considered a multifactorial heterogeneous condition for which the specific cause remains unknown in the majority of cases (Bachelot et al., 2009; Janse et al., 2010). Genetic causes include numerical or structural chromosomal abnormalities including (full or mosaic) monosomy X (Mattei et al., 1982), or relatively rare monogenic causes such as mutations in the FOXL2, BMP15, FSHR or GDF9 gene (Crisponi et al., 2001; Rossetti et al., 2009; Christin-Maitre and Tachdjian, 2010; Fridovich-Keil et al., 2011). Of all POI cases, 0.8–13% is caused by a premutation of the fragile X mental retardation gene 1 (FMR1), thereby being the most frequent monogenic cause of POI (Wittenberger et al., 2007).

The FMR1 gene is located on the distal long arm of the X chromosome (Xq27.3) and consists of 17 exons (OMIM number 309950). Dysfunction of the FMR1 gene is caused by the amplification of an unstable CGG triplet in the 5′ untranslated region of the first exon (Verkerk et al., 1991). CGG repeat sizes exceeding 200 repeats is considered a full mutation, which leads to hypermethylation and subsequent silencing of the FMR1 gene, and thereby results in loss of the fragile X related mental retardation protein (FMRP) (Oostra and Willemsen, 2003). In these cases the fragile X syndrome develops, which is characterized by mental retardation, characteristic facial features and behavioral problems such as autism and attention deficit disorder (OMIM ID number 300624).

The full FMR1 mutation may result from the expansion of a premutation in maternal transmission from one generation to the next (Nolin et al., 2003). The FMR1 premutation is an amplification of the CGG repeat length to ~55–200 repeats (normal range <45 repeats) (Sherman et al., 2005). CGG repeats in the premutation range have often been associated with POI, referred to as fragile X-associated POI (FXPOI) (Conway et al., 1998; Murray et al., 1998; Allingham-Hawkins et al., 1999; Sherman, 2000; Allen et al., 2007). FXPOI accounts for ~1–8% of all cases of POI (Wittenberger et al., 2007). The severity of ovarian dysfunction depends on the CGG repeat size in a non-linear fashion: women with a mid-range number of repeats (80–100) experience POI earlier and this occurs more frequently than other carrier groups (Ennis et al., 2006; Allen et al., 2007).

The intermediate CGG repeat length (45–54 repeats) (Sherman et al., 2005; Kronquist et al., 2008) may expand to a full mutation after two or more generations (Nolin et al., 2003). Several recent studies investigated the role of intermediate CGG repeat size in idiopathic POI and yielded varying results. Some studies identified an increased frequency of intermediate alleles in (occult) POI (Bretherick et al., 2005; Bodega et al., 2006; Gleicher et al., 2009; Streuli et al., 2009; Ishizuka et al., 2011; Karimov et al., 2011), whereas these findings have not been confirmed in a study with a much larger sample size (Bennett et al., 2010).

A recently published study from our group in 3611 women with known age at natural menopause demonstrated no association between CGG repeats in the normal and intermediate range and age at natural menopause (Voorhuis et al., 2013). However, whether POI cases without an obvious cause (idiopathic POI) may be considered as a separate genetic entity or whether they represent variation within the entire distribution of natural menopause remains unclear. Therefore, the number of CGG repeats may primarily be associated with POI and may play a less important role or even no role at all in determining normal menopausal variation.

In addition, there is no consensus on the definition of intermediate CGG repeat length (Sherman et al., 2005; ACOG Committee on Genetics; 2006; Kronquist et al., 2008), and the lower boundary of FMR1 repeat sizes which alters ovarian function has not yet been defined (Streuli et al., 2009). Therefore, the current study was undertaken to assess whether the risk of POI is associated with CGG repeat length of normal and intermediate FMR1 alleles in a large case-control study of Dutch women with idiopathic POI. Moreover, we aimed to investigate a possible association between number of CGG repeats and age at POI as a parameter of POI severity.

### Methods

#### Study population

**POI cases**

POI cases were recruited from two cohorts. (i) Since 2005, women suspected to suffer from a hypergonadotropic status presenting with either a regular menstrual cycle, oligomenorrhea, or amenorrhea were systematically evaluated in a standardized fashion in the outpatient clinics of 16 fertility clinics in the Netherlands, as has been described in previous studies from our group (COLA study; Knauff et al., 2008; Janse et al., 2010). For the
current study, all consecutive women in this cohort diagnosed with idio-
pathic POI, and in whom data of the FMR1 gene are known, were included
(n = 159). For an additional 165 women with POI, genotyping was per-
determined to determine the FMR1 CGG repeat numbers. POI was defined as
spontaneous cessation of menses for at least 4 months in women younger than
40 years of age, along with repeated FSH concentrations exceeding
40 IU/l (de Vos et al., 2010). This cohort also included women with
primary amenorrhea (n = 17). All women had a normal karyotype. The
study protocol was approved by the institutional review board of the Univer-
sity Medical Center Utrecht, the Netherlands, and all women gave written
informed consent (clinicaltrials.gov identifier: NCT01411644). (ii) In ad-
dition, 55 women with a self-reported natural age at menopause before
40 years, repeat sizes <55 CGG repeats on allele 2, and a normal karyotype
were included from the Prospect-EPIC cohort (the Prospect-EPIC cohort is
described below).

Controls

The Prospect-EPIC cohort is one of two contributions to the European Inves-
tigation 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 women living in Utrecht, The Netherlands, and
surroundings, aged 49–70 years, who were invited to participate in the study
through the national breast cancer screening program between 1993 and
1997. All women filled out detailed questionnaires about dietary, reproduc-
tive and lifestyle factors and about medical history. Furthermore, they under-
gone a physical examination at enrollment. In addition, women donated a
30-ml non-fasting blood sample which was fractionated into serum, citrated
plasma, buffy coat and erythrocyte aliquots. The samples were stored
under liquid nitrogen at −196 °C for future research. All participants gave
written informed consent and the study was approved according to the
guidelines of the Helsinki Declaration by the Medical Ethics Committee of
the Netherlands Organization of Applied Scientific Research.

Natural menopause was defined according to the World Health Organiza-
tion as amenorrhea for at least 12 consecutive months without other obvious
reasons. For the current study we used a selection of 3611 women from the
Prospect-EPIC cohort with a known age at natural menopause and available
data on FMR1 CGG repeat numbers genotyped for a previous study (Voor-
huis et al., 2013). We excluded 105 women because of failed PCR analysis.
Next we excluded 72 women with an unclear homozygous pattern after
PCR amplification (please see the section on laboratory analyses below).
Finally, we excluded 55 women with an age at menopause before 40 years
and 11 cases because the largest allele contained ≥55 CGG repeats
(premutation range). Thus, finally our control group consisted of 3368 women
with a known age at natural menopause ≥40 years and with FMR1 CGG repeat
sizes between 20 and 55 (normal and intermediate range) (Supplementary
data, Fig. S1).

Laboratory analyses

POI cases

For each patient, a blood sample was collected in a 10 ml EDTA tube, and
high molecular weight genomic DNA was extracted using established pro-
dcedures. Amplification was performed by PCR of the CGG-repeat region of
the FMR1 gene using Platinum Pfx DNA polymerase according to the instruc-
tions of the manufacturer (Invitrogen). The forward primer PK2038 (5′-GCTC
AGCTCCGTTCGGTTTCTCATTCCGGT-3′) and reverse primer 5′-FAM
labeled PK2039 (5′-AGCCCGCCACCTTCCACACGACCGTCCCA-3′) were
used. Fragment length determination was performed on an ABI 3130
sequencer (Applied Biosystems) using the GeneMarker genotype analysis
software (SoftGenetics, State College, PA, USA).

Whenever a single allele length was observed in the 159 already genotyped
POI cases, a southern blot analysis was performed on Hind III and EcI XI
restricted DNA to confirm homozygosity for the number of CGG-repeats.
For the additional 165 genotyped women with POI, a total of 46 samples
showed a homoyzogous pattern after PCR amplification. 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. As the patient
characteristics of these 46 samples did not differ from the other POI cases,
we choose to include these samples as homozygous in our analysis. Also sen-
sitivity analysis excluding these 46 samples did not alter any of the results (data
not shown). Because we studied the association between the number of
CGG repeats and POI in the normal and intermediate range (up to 55
repeats), we excluded 4 samples because the largest allele contained ≥55
CGG repeats (premutation range).

Controls and POI cases from the prospect-EPIC cohort

Genomic DNA was extracted from buffy coat aliquots by KBioscience using
their own in-house silica based systems (http://www.kbioscience.co.uk/
lab%20services/DNA%20extraction/Ext_services_intro.html). Genotyp-
ing 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 Poly-
merase according to the instructions of the manufacturer (Invitrogen).
Primers for the FMR1 gene were: forward 5′-GCTCAGCTCGGTTTCTCATTCCGGT
GGTTTACCTTCCGGT-3′ and reverse 5′-AGCCCGCCACCTTCCACACGACCGTCCCA
CCAGCTCCTCCA-3′. Repeat sizes were measured using an ABI prism
3730 DNA Analyzer (Applied Biosystems) and quantified using ABI prism
Genemapper Analysis Software v3.7 (Applied Biosystems). The PCR
analyses failed in 105 (2.9%) of our 3611 samples. Furthermore, 335 (9.2%)
samples showed a homoyzogous pattern after PCR amplification. For 72 of
these 335 presumed homozygous women, we could not clearly identify 2
alleles. As mentioned above, these samples could be homozygous for the
small allele, or a larger allele (>100 repeats) could not be 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. Next, 11 samples were excluded
because the largest allele contained ≥55 CGG repeats (premutation
range). In total, our control group existed of 3368 women with an age at
natural menopause ≥40 years and <55 CGG repeats (normal and interme-
tiate size range). Additionally, 55 women with a menopausal age ≤40
years and repeat sizes <55 CGG repeats were added to the POI cases.

As quality-control tests, plate-identifying blank wells and blind duplicates
were used. As an additional quality control test, random duplicates were gen-
otyped in both the Genome Diagnostics Section of the Department of
Medical Genetics laboratory and the Department of Medical Genetics,
UMC Utrecht, The Netherlands. We genotyped 18 samples (10 POI cases
and 8 controls) in both laboratories and the repeat sizes of all 18 samples
were an exact match (error rate = 0).

Statistical analyses

Characteristics of the two study populations were described using means
and SD for continuous variables, and frequencies and percentages for catego-
rical variables. The allele with the lowest triple repeat number was referred to as
allele 1. Allele 2 represented the allele with the highest triple repeat number.
A normal CGG repeat count was defined as <45 repeats and the intermedia-
ate allele was defined as 45–54 CGG repeats (Sherman et al., 2005; Kron-
quist et al., 2008). In order to compare our study with a previous study in
which intermediate repeat sizes were defined as 41–58 repeats (Bodega
et al., 2006), we performed an additional analysis on our data before we
excluded all cases and controls with repeat sizes ≥55, and included all
cases and controls with repeat sizes ≤58 repeats. We used analysis of vari-
ance (ANOVA) to test differences in mean CGG repeat lengths on allele 1
and allele 2 between POI cases and controls.
The most frequent repeat sizes on allele 2 ranged from 29 to 34 repeats (78.1% of all POI cases and 75.7% of all controls). 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 Chi square to compare the proportion of women in the three groups, i.e. group 1 <29 repeats, group 2 29–34 repeats (common) and group 3 35–45 repeats, between POI cases and controls.

As stated earlier, the association between CGG repeats in the premutation range and age at menopause is non-linear, where women with a mid-range number of repeats (80–100) are more susceptible to (early) POI in comparison to other carrier groups (Allen et al., 2007; Ennis et al., 2006). To identify a possible non-linear association of number of CGG repeats in the allele with the longest triple repeat number (allele 2) in the normal and intermediate range, and age at menopause in the POI cases, 3-knot restricted cubic splines were used. The restricted cubic splines composed of three polynomial segments in R (version 2.15.2; http://www.r-project.org), did not indicate deviations from linearity (P = 0.153). Next, a linear regression analysis was performed to investigate the association between the number of CGG repeats in allele 2 versus log transformed age at POI (in 344 POI cases with a known age at onset of POI). To adjust for the number of repeats on allele 1 we included the repeat sizes on allele 1 in the multivariate linear regression analysis.

Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS, Inc., Chicago, IL, USA) and R version 2.15.2 (http://www.r-project.org/). A P-value of ≤0.05 was considered statistically significant.

**Results**

A total of 375 POI cases and 3368 controls with natural menopause ≥ 40 years were included for the current study. General population characteristics of both groups are summarized in Table I.

Details for the FMR1 CGG repeat lengths are reported in Table II. No statistically significant difference in the frequency of intermediate sized

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**Table I** Population characteristics of the cases with primary ovarian insufficiency (POI) and controls.

<table>
<thead>
<tr>
<th>Population characteristics</th>
<th>POI N = 320</th>
<th>POI prospect N = 55</th>
<th>Controls prospect N = 3368</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at inclusion (years, mean ± SD)</td>
<td>35.6 ± 7.7</td>
<td>63.4 ± 3.3</td>
<td>63.0 ± 3.4</td>
</tr>
<tr>
<td>Age at POI/natural menopause (years, mean ± SD)</td>
<td>31.5 ± 6.5</td>
<td>35.6 ± 4.5</td>
<td>50.6 ± 3.7</td>
</tr>
<tr>
<td>Range (years)</td>
<td>14–39</td>
<td>18–39</td>
<td>40–64</td>
</tr>
<tr>
<td>Ever pregnant</td>
<td>133/315 (42.2%)</td>
<td>36 (65.5%)</td>
<td>2866 (85.1%)</td>
</tr>
<tr>
<td>Parity (mean ± SD)</td>
<td>0.73 ± 1.0</td>
<td>1.91 ± 2.1</td>
<td>2.67 ± 1.8</td>
</tr>
<tr>
<td>Smoking never</td>
<td>168/293 (57.3%)</td>
<td>31 (56.4%)</td>
<td>1718 (51%)</td>
</tr>
<tr>
<td>BMI (kg/m², mean ± SD)</td>
<td>24.2 ± 4.5</td>
<td>27.4 ± 4.6</td>
<td>26.3 ± 4.0</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>125.5 ± 15.7</td>
<td>141.7 ± 17.6</td>
<td>137.9 ± 20.8</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>79.8 ± 10.48</td>
<td>81.5 ± 10.40</td>
<td>79.4 ± 10.4</td>
</tr>
</tbody>
</table>

**Table II** CGG repeat sizes in POI cases and controls.

<table>
<thead>
<tr>
<th>FMR1 CGG repeat size</th>
<th>POI Cola n = 320</th>
<th>POI Prospect n = 55</th>
<th>POI total n = 375</th>
<th>Controls n = 3368</th>
<th>p^a</th>
<th>p^b</th>
<th>p^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of CGG repeats allele 1</td>
<td>26.90 ± 4.6</td>
<td>27.31 ± 4.2</td>
<td>26.96 ± 4.5</td>
<td>27.11 ± 4.5</td>
<td>0.546</td>
<td>0.431</td>
<td>0.744</td>
</tr>
<tr>
<td>Median (range)</td>
<td>29 (8–40)</td>
<td>30 (12–32)</td>
<td>29 (8–40)</td>
<td>30 (7–47)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of CGG repeats allele 2</td>
<td>31.75 ± 4.6</td>
<td>32.04 ± 4.1</td>
<td>31.79 ± 4.3</td>
<td>32.86 ± 4.9</td>
<td>0.009</td>
<td>0.001</td>
<td>0.241</td>
</tr>
<tr>
<td>Median (range)</td>
<td>30 (19–53)</td>
<td>31 (21–45)</td>
<td>31 (19–53)</td>
<td>31 (20–54)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence of FMR1 allelic form</td>
<td>OR (95% CI)</td>
<td>p^b</td>
<td>p^c</td>
<td>p^d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate (45–54 repeats)</td>
<td>8 (2.5%)</td>
<td>2 (3.6%)</td>
<td>10 (2.7%)</td>
<td>123 (3.7%)</td>
<td>0.72 (0.38–1.39)</td>
<td>0.38</td>
<td>0.33 (0.68–4.10)</td>
</tr>
<tr>
<td>Normal range (&lt;45 repeats)</td>
<td>320 (100%)</td>
<td>53 (96.4%)</td>
<td>365 (97.3%)</td>
<td>3245 (96.3%)</td>
<td>0.72 (0.38–1.39)</td>
<td>0.38</td>
<td>0.33 (0.68–1.40)</td>
</tr>
</tbody>
</table>

FMR1, fragile X mental retardation gene 1; OR, odds ratio; CI, confidence interval. POI Cola: POI cases included systematically evaluated in the outpatient clinics of 16 fertility clinics in the Netherlands. Data shown as mean ± SD, median (range) or n (%).

*a*Analysis of variance (POI total versus controls).

*b*Fisher’s exact test (POI total versus controls).

*c*POI Cola cases versus controls.

*d*POI Prospect cases versus controls.
CGG repeats on allele 2 between POI cases and controls was found (2.7 and 3.8%, respectively; odds ratio (OR) 0.72, 95% confidence interval (CI): 0.38–1.39, \( P = 0.38 \)). The distributions of the CGG repeat size on allele 2 and allele 1 for each group are shown in Figs 1 and 2, respectively. When comparing the distributions of normal and intermediate sized CGG repeats (up to 55 repeats) on allele 2, we did observe a statistically significant difference between the two groups (\( P < 0.001 \)), with the most common repeat size for POI cases being 30 (37.3%) and the most common repeat size in controls being 31 CGG repeats (35.7%). The distributions of repeats on allele 1 did not differ between POI cases and controls (\( P = 0.55 \)).

Table III presents the frequencies of women carrying <29 repeats, between 29 and 34 repeats, and over 34 repeats in the POI cases and controls. No statistically significant difference between these three groups was observed (\( P = 0.19 \)).

Linear regression analysis for age at first manifestation of POI versus CGG repeat size of allele 2 did not reveal an association (\( \beta = -0.019 \), \( P = 0.72 \)) and is shown in Fig. 3. Adjustment for the number of repeats on allele 1 did not alter the results.

No differences were found between the number of CGG repeats in the POI cases from the Prospect cohort and in the POI cases from the 16 fertility clinics in the Netherlands (which were performed in the two different laboratories; respectively 32.04 and 31.75, \( P = 0.67 \); Supplementary data, Table SI). Furthermore, exclusion of the primary amenorrhoea cases (\( n = 17 \)) from our sample did not alter any of the results (Supplementary data, Table SII). To assess whether possible associations between CGG repeats and POI were altered by the presence of non-Caucasian women in our POI cohort, a sensitivity analysis excluding non-Caucasian POI cases (\( n = 60 \)) was performed. Exclusion of non-Caucasian POI cases did not alter the results of our performed analyses (Supplementary data, Table SIII), except that the marginal statistically significant finding when comparing intermediate CGG repeats defined as 41–58 repeats between POI cases and controls disappeared (OR 0.7, 95% CI: 0.44–1.11, \( P = 0.15 \)).

**Discussion**

The current study was undertaken to investigate the prevalence of FMR1 intermediate alleles in a large cohort of well-phenotyped Dutch women with idiopathic POI compared with controls. Our data suggest that a CGG repeat length in the intermediate range is not associated with the risk of POI, as the frequency of intermediate sized CGG repeats did not significantly differ between POI cases and controls. Furthermore, an association between the number of CGG repeats and age at first POI manifestation could not be established, even when a nonlinear relationship was considered.

In contrast to the well-known association between FMR1 CGG premutations and POI, the association of intermediate range CGG repeats number and susceptibility to POI is less consistent (Bretherick et al., 2005; Bodega et al., 2006; Bennett et al., 2010; Karimov et al., 2011). The number of women carrying intermediate sized CGG repeats (45–54 repeats) did not differ between the POI cases and controls in our study. Up to now, only one study has used identical definitions to the current study, and observed an increased incidence of intermediate alleles in occult POI (i.e. imminent ovarian failure) compared with controls (Karimov et al., 2011). Most previous publications on POI applied different definitions for intermediate alleles, although not...
Table III Categories of CGG repeat sizes on allele 2 in POI cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>POI cases</th>
<th>POI total</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 375</td>
<td>n = 3368</td>
<td></td>
</tr>
<tr>
<td>POI COLA</td>
<td>n = 320</td>
<td>22 (5.9%)</td>
<td>60 (16%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.9%)</td>
<td>(16%)</td>
</tr>
</tbody>
</table>
| POI Prospect    | n = 55    | 22 (5.9%) | 659 (19.6%)
|                 |           | (5.9%)    | (19.6%)  |
| Group 1*        |           | 19 (5.9%) | 161 (4.8%)
|                 |           | (5.9%)    | (4.8%)   |
| Group 2**       |           | 249 (77.8%) | 2548 (75.7%)
|                 |           | (77.8%)   | (75.7%)  |
| Group 3***      |           | 52 (16.3%) | 60 (16%)
|                 |           | (16.3%)   | (16%)    |

POI COLA: POI cases included systematically evaluated in the outpatient clinics of 16 fertility clinics in the Netherlands.

*Group 1: allele 2 <29 repeats.
**Group 2: allele 2 29–34 repeats.
***Group 3: allele 2 >34 repeats.

Chi-square, POI total versus controls.
Chi-square, POI COLA versus controls.
Chi-square, POI Prospect versus controls.

repeats of 41–58 were more common in a small study with 53 POI cases compared with 182 controls (OR 2.4, 95% CI: 1.7–7.7, \( P = 0.01 \)) (Bodega et al., 2006), this association could not be replicated in a cohort of 366 POI cases (OR 1.3, 95% CI: 0.8–2.0, \( P = 0.23 \)) (Bennett et al., 2010). Thus, we could not replicate previously reported associations between CGG repeat sizes in the intermediate range and POI.

The second objective of the current study was to examine a possible association between CGG repeat size and age at first manifestation of POI as a measurement for the severity of POI. No significant association could be identified. This finding is in contrast with the results of a study in Asian women, that suggested that having >38 CGG repeats was associated with younger age at onset of amenorrhea (Ishizuka et al., 2011). However, the number of CGG repeats in Asians has a different distribution compared with other ethnicities, with a secondary peak of 34–36 repeats in addition to the most frequent peak of 30 or 31 repeats (Fu et al., 1991; Chen et al., 1997; Otsuka et al., 2010; Seltzer et al., 2012). In the current study, the possibility of a non-linear relationship between CGG repeat length and age at diagnosis was excluded by applying 3-knot restricted cubic spline analysis.

The absence of an association between CGG repeats in the normal and intermediate range and POI in the current study is in accordance with the largest study published to date (Bennett et al., 2010). Non-replication of previous other studies could be explained by a number of reasons. Firstly, previous associations between CGG repeat lengths in the range up to 55 repeats and POI were found in relatively small studies with POI cases varying from 27 to 190 women. In the study by Bennett et al. (2010) in 366 POI cases no association with CGG repeats in this range was found. Possibly, the associations found previously in the small studies could be considered false-positive.

Secondly, ethnic differences in the study populations could also be a cause for the inconsistent findings, since the distribution of CGG repeat lengths seems to differ significantly between ethnic groups (Gleicher et al., 2010; Peprah, 2012). In addition, possible effects of CGG repeat distributions on susceptibility to POI may vary between different ethnic groups (Gleicher et al., 2012) and therefore could not be picked up in our cohort of mainly Caucasian POI cases.

A third explanation may stem from the fact that susceptibility to POI is possibly influenced not by FMR1 CGG repeats distributions alone, but by CGG repeat lengths in interaction with other genes and/or

Figure 3 Linear regression analysis for age at POI versus FMR1 CGG repeat lengths on allele 2.
environmental factors. A recent study suggesting that the earlier found association between BRCA1 carriers and occult POI (Oktay et al., 2010) is actually FMR1-mediated (Weghofer et al., 2012), strengthens this hypothesis.

The current study has some limitations that should be mentioned. First, FMR1 CGG repeat lengths in our POI cases (except the 55 POI cases from the Prospect cohort) and controls were genotyped in two different laboratories, which may lead to divergent results due to technical differences. The observed significant difference in allele 2 size between POI cases and controls (Table II) could be due to two factors: a true difference between POI cases and controls, or a technical (laboratory) difference between genotyping in the two sets of samples (Genome Diagnostics Section of the Department of Medical Genetics laboratory – Department of Medical Genetics). The fact that no differences were found between the number of CGG repeats in the POI cases from the Prospect cohort and in the POI cases from the 16 fertility clinics in the Netherlands (i.e. COLA study; which were performed in the two different laboratories; respectively 32.04 and 31.75, P = 0.67; Supplementary data, Table SII), may suggest that this observed statistical difference in allele 2 between POI cases and controls is due to technical differences. However, the repeat sizes of the 18 samples that were genotyped in both laboratories were 100% concordant. Moreover this difference of a single repeat within the range is generally accepted as reasonable (+4% of the total repeat size; Best practice Fragile X Syndrome, European Molecular Genetics Quality Network, version 2006). In addition, the distributions of allele 1 between the POI cases and controls also showed no differences (Figure 2, P = 0.55), suggesting the observed difference in repeats on allele 2 is in fact a true finding. As far as we are aware, the difference in allele 2 has not been reported earlier. Since, as stated earlier, we cannot exclude the possibility that this difference is due to technical differences between the two platforms used, replication in a separate cohort is needed to clarify this issue. However, even if the difference in repeat lengths on allele 2 between POI cases and controls is genuine, it is not likely that this difference would have clinical implications.

Second, as mentioned above, distributions of CGG repeats may vary among ethnic populations. In the current study, POI cases consisted of women with different ethnicities (although 84% of the POI cases were Caucasian), whereas our controls were all Caucasian. To assess whether possible associations between CGG repeats and POI were altered by the presence of non-Caucasian women in our POI cohort, a sensitivity analysis excluding non-Caucasian POI cases (n = 60) was performed. Exclusion of non-Caucasian POI cases did not alter the results of our performed analyses (Supplementary data, Table SII), except that the marginal statistically significant finding when comparing intermediate CGG repeats defined as 41–58 repeats between POI cases and controls disappeared (OR 0.7, 95% CI: 0.44–1.11, P = 0.15).

Third, in our study women with primary amenorrhea (n = 17) were included in the POI group. It is not clear yet whether idiopathic primary amenorrhea has a different etiology than idiopathic POI. Moreover, it has not been investigated yet whether primary amenorrhea has an association with FMR1 premutations. Exclusion of the primary amenorrhea cases (n = 17) did not alter any of the results (Supplementary data, Table SIII).

Carrying the FMR1 premutation has been consistently associated with POI during the last decade (Bodega et al., 2006; Ennis et al., 2006; Allen et al., 2007; Bennett et al., 2010; Karimov et al., 2011). In FMR1 premutation carriers gene transcription is significantly increased and the resulting increased FMR1 mRNA levels probably contribute to the pathogenesis of FXTAS through toxic gain-of-function effect (Garcia-Arocena and Hagerman, 2010). To date, a similar contribution of the FMR1 premutation or intermediate repeat size to the pathogenesis of FXPOI has only been suggested (Willemsen et al., 2011). However, FMR1 mRNA transcript levels are highly variable in CGG repeat numbers at the lower end of the premutation range (Allen et al., 2004), and this variability does not correlate to the variance of CGG triplet numbers in women with POI (Schuettler et al., 2011). Destabilization of the intermediate FMR1 stability in POI resulting from CGG repeats without AGG interruptions could be another potential biological pathway (Bodega et al., 2006). However, the finding that AGG interruptions do not seem to decrease FMR1 mRNA levels argues against this mechanism (Peprah et al., 2010). Clearly, the possible biological mechanism of intermediate (if any) and premutation sized CGG repeats in the pathogenesis of POI needs further study.

In conclusion, we observed no association between intermediate sized CGG repeats and POI compared with controls with an age at natural menopause above 40 years. Next, no statistically significant association between CGG repeat size and age at first manifestation of POI could be identified. Our results are in line with a previous study in 366 POI cases that found no association between CGG repeats up to 55 repeats and POI. Therefore, there seems limited value in the evaluation of normal and intermediate FMR1 repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk for developing POI. Together with our earlier published data in which no association was found between normal and intermediate sized CGG repeats and age at natural menopause in a large cohort of Caucasian women, the role of FMR1 CGG repeat sizes up to 55 repeats in the ovarian ageing process could be questioned.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/. Acknowledgements

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Authors’ roles


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Appendix

Collaborators of the Dutch POI Consortium (10):

Y.M. van Kasteren, MCA, Alkmaar, The Netherlands; Email: y.m.van.kasteren@mca.nl

F.M. Helmerhorst, LUMC, Leiden, The Netherlands; Email: f.m.helmerhorst@lumc.nl

P.J.Q. van der Linden, DZ, Deventer, The Netherlands; Email: P.vanderLinden@dznl.

B.J. Cohlen, Isala Kliniek Zwolle, The Netherlands; Email: b.j.cohlen@isala.nl

H.R. Franke, MST, Enschede, The Netherlands; Email: h franke@ms tl.

A. Hoek, UMCG, Groningen, The Netherlands; Email: a.hoek@ og.umcg.nl

J.L. Evers, MUMC, Maastricht, The Netherlands; Email: Jlh.evers@ mumc.nl

C.C. Beerendonk, UMC St Radboud; Email: C.C.eerendonk@ obgyn.umcn.nl

P.A. van Dop, Catharine Ziekenhuis, Eindhoven, The Netherlands; Email: p.vandop@onsnetieenhoven.nl

M.J. Ten Kate Boooi, Erasmus MC, Rotterdam, The Netherlands; Email: m.tenkate-booij@erasmusmc.nl