Correlation of normal-range *FMR1* repeat length or genotypes and reproductive parameters

Bat-Sheva L. Maslow¹ · Stephanie Davis² · Lawrence Engmann¹ · John C. Nulsen¹ · Claudio A. Benadiva¹

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Abstract

Purpose This study aims to ascertain whether the length of normal-ranged CGG repeats on the *FMR1* gene correlates with abnormal reproductive parameters.

Methods We performed a retrospective, cross-sectional study of all *FMR1* carrier screening performed as part of routine care at a large university-based fertility center from January 2011 to March 2014. Correlations were performed between normal-range *FMR1* length and baseline serum anti-Müllerian hormone (AMH), cycle day 3 follicle stimulating hormone (FSH), ovarian volumes (OV), antral follicle counts (AFC), and incidence of diminished ovarian reserve (DOR), while controlling for the effect of age.

Results Six hundred three *FMR1* screening results were collected. One subject was found to be a pre-mutation carrier and was excluded from the study. Baseline serum AMH, cycle day 3 FSH, OV, and AFC data were collected for the 602 subjects with normal-ranged CGG repeats. No significant difference in

median age was noted amongst any of the *FMR1* repeat genotypes. No significant correlation or association was found between any allele length or genotype, with any of the reproductive parameters or with incidence of DOR at any age ($p > 0.05$). However, subjects who were less than 35 years old with low/low genotype were significantly more likely to have below average AMH levels compared to those with normal/normal genotype (RR 3.82; 95 % CI 1.38–10.56).

Conclusions This large study did not demonstrate any substantial association between normal-range *FMR1* repeat lengths and reproductive parameters.

Keywords Fragile X · *FMR1* · Diminished ovarian reserve

Introduction

The *FMR1* gene, located on the long arm of the X chromosome, is typically associated with Fragile X syndrome, characterized by neurological, psychiatric, and developmental abnormalities. The 5' untranslated region of the gene is comprised of trinucleotide CGG repeats; the length of the CGG repeat region determines the phenotypic expression of the gene. A region with greater than 200 repeats is considered a full mutation of the *FMR1* gene. At these lengths, the *FMR1* gene becomes hyper-methylated and the protein fails to form, resulting in clinical Fragile X syndrome [1–3]. Repeats in the “pre-mutation” range (55–200) are associated with an increased risk of expansion to full mutation lengths in subsequent generations. These individuals are also at risk for two additional phenotypic syndromes: Fragile X-associated tremor and ataxia syndrome (FXTAS) and

Results from this study were presented in an oral presentation at the American Society for Reproductive Medicine Annual Meeting, October 18–22, 2014, Honolulu, HI.

Capsule Normal range *FMR1* CGG repeat length does not reliably correlate with reproductive parameters.

✉ Claudio A. Benadiva
benadiva@uchc.edu

¹ Division of Reproductive Endocrinology and Infertility, University of Connecticut School of Medicine, 2 Batterson Park Rd, Farmington, CT 06032, USA

² Department of Pediatrics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229, USA

premature ovarian failure (POF) in female pre-mutation carriers [2, 4, 5]. These phenotypes are thought to occur as a result of an overproduction of *FMRI* mRNA, rather than a transcriptional abnormality [6, 7].

While repeat lengths between 45 and 54 have been coined “intermediate” and those <35 as “unaffected,” no clinical significance to these designations has been established [8, 9]. Considerable controversy exists over whether variations of CGG repeat length in these normal ranges have any clinical implication with respect to reproductive parameters, particularly the development of diminished ovarian reserve (DOR) or a more rapid decline in ovarian aging. To date, there are studies on either side of the debate. Some studies demonstrated significant associations with either low (<26 repeats) or high (>35 repeats) and decreased ovarian function, while others demonstrate no significant associations between CGG repeat lengths and reproductive outcomes. Previous studies on the subject are predominantly limited by small cohort size. In addition, several of the significant studies utilize inappropriate comparison groups [10], rely on duplicated allele frequencies which can bias statistical analyses [4, 11], are restricted to women already diagnosed with DOR [12], or utilize allele length haplotypes and genotypes that are not biologically significant [13, 14].

The primary objective of this study was to evaluate associations between lengths of *FMRI* CGG repeats both as continuous variables, which allow for more robust statistical inferences, and as categorical genotypes, with a variety of clinically relevant reproductive outcomes in a large, unrestricted, cohort.

Materials and methods

Study population

We retrospectively collected all *FMRI* carrier-screening tests performed on female patients at a large, university-based, infertility practice from January 2011 to March 2014. FMR-1 carrier screening is routinely offered as part of a general carrier-screening panel at every new patient visit regardless of reason for the visit. This study received approval for exempt status by the university’s Institutional Review Board (#14-182-2).

Only subjects with normal-length (<55 repeats) *FMRI* CGG repeats were included in the study. Carriers of the *FMRI* pre-mutation (>55 repeats) or full mutation (>200 repeats) were excluded from the study. For all included subjects, age at presentation, baseline serum anti-Müllerian hormone (AMH), cycle day 3 follicle stimulating hormone (FSH), ovarian volumes (OV), and antral follicle counts (AFC) were

abstracted from the electronic medical record. These are routinely collected as part of the new patient visit or during the subsequent menstrual cycle, in the case of cycle day 3 FSH.

Laboratory assessments

An outside reference laboratory (Counsyl, San Francisco, CA, USA) performed all routine carrier screening. Polymerase chain reaction followed by capillary electrophoresis was used to determine the number of CGG repeats in the 5′ untranslated region of the *FMRI* gene [15]. Counsyl had no role in the design, funding, or data analysis of this study. Cycle day 3 FSH assays were performed in our in-house endocrine laboratory, utilizing a chemo-luminescent assay (Siemens Immulite 2000). AMH assays were sent to an external reference laboratory (LabCorp, Shelton, CT, USA). The same laboratories and assays were utilized for all subjects.

Reproductive parameters

We defined DOR by both AMH criteria and cycle day 3 FSH criteria. While a range of cutoff values have been suggested for the diagnosis of DOR, based on current recommendations, we selected <1.0 ng/mL for AMH and >10.0 mIU/mL for FSH [16–18]. These values are routinely utilized at our institution, as women with AMH and/or FSH at these levels have been shown to have a diminished response to controlled ovarian stimulation [17]. To account for expected age-related decline in AMH, we also characterized women with below average AMH levels based on age, <2.1 ng/mL for subjects 35 or younger and <1.0 ng/mL for subjects 36–41 years old [19]. Incidence of DOR was determined by calculating percentage of subjects meeting each of the diagnostic criteria for DOR out of the total cohort for which those values were available. Average OV and AFC were calculated for each subject by adding right and left values and dividing by 2. For advanced statistical analyses, serum AMH levels were log transformed in order to allow for normal distribution and to decrease the effect of outliers.

Allele length assessment

Studying the associations of ovarian reserve with *FMRI* repeat lengths poses some challenges. When evaluating studies of allele frequency, the type of analysis that is performed is critical. Each individual inherits two different alleles but X-inactivation ensures that only one allele will be biologically active in any given cell. As a result, *FMRI* carrier status was categorized in several ways for this study. Standard practice for analyzing effects of X chromosome alleles in the genetic literature is to isolate analysis to the longer allele (allele 2) in order to mitigate the effects of X-inactivation. This preference is partially based on the assumption that longer repeats cause

deleterious phenotypes, in which case the estimation of longer allele expression would be more clinically relevant. However, since previous studies suggested that a low number of repeats may also have an impact on reproductive parameters [20, 21], we considered both allele 1 and allele 2 length as separate continuous variables. Some have proposed using the bi-allelic mean, to evaluate the impact of allele length on phenotypic expression, while this is mathematically sound, the product is not biologically relevant, and was not utilized in this study.

Previous studies have utilized haplotypes and genotypes to categorically analyze normal-range allele lengths [13, 14, 22, 23]. Therefore, we also classified each allele length by these previously published repeat length haplotypes, “low” (<26), “normal” (26–34), and “high” (35–55). We further classified each subject into the six genotypes defined by previous studies based on both allele lengths (high/high, high/low, high/normal, normal/normal, normal/low, and low/low).

Statistical analysis

To analyze simple comparisons between cohort demographics and genotypes, we compared median age and reproductive parameters across all six genotypes utilizing Kruskal-Wallis Rank Sum Test. We then calculated the overall rate of DOR by both AMH and FSH criteria across all ages, and for each individual genotype and age group. Each genotype was then compared to the Normal/Normal genotype using Fischer’s Exact Test and stratified Cochran-Mantel-Haenszel Test. Comparisons of DOR and each of the allele lengths as continuous variables were also performed utilizing Student’s *t* test.

To identify significant correlation between allele length and reproductive parameters, Pearson’s correlation was performed utilizing allele 1 and allele 2 length with each of the reproductive parameters (AMH, logAMH, FSH, Avg OV, and Avg AFC) as continuous variables. Given the expected influence of age on reproductive parameters, most notably serum AMH, we controlled for age by performing partial correlation analyses with allele length and logAMH. To assess the overall impact of genotype on AMH levels, we performed an analysis of covariance (ANCOVA), while controlling for the effect of age. All statistical analyses were performed utilizing IBM SPSS Version 21. A two-tailed *p* value of <0.05 was considered significant.

Results

A total of 603 *FMRI* carrier-screening results were reviewed. One subject was identified as an *FMRI* pre-mutation carrier and was excluded from the study. Six hundred two subjects met inclusion criteria for the study. Subject demographics for the entire cohort are listed in Table 1. Baseline AMH levels

Table 1 Cohort demographics

Demographic (<i>N</i>)	Median ± IQR (range)
Age (<i>n</i> = 602)	33.5 ± 6.9 (20.2–46.5)
AMH (<i>n</i> = 602)	2.2 ± 3.5 (0.03–39.95)
Day 3 FSH (<i>n</i> = 536)	6.6 ± 2.9 (1.4–94.2)
Average ovarian volume ^a (<i>n</i> = 477)	8.0 ± 5.0 (1.75–60.3)
Average antral follicle count (<i>n</i> = 474)	7.5 ± 7.0 (1.0–55.5)
<i>FMRI</i> allele 1 length (<i>n</i> = 602)	29 ± 7 (9–48)
<i>FMRI</i> allele 2 length ^b (<i>n</i> = 602)	30 ± 2 (20–50)

^a Average ovarian volumes and antral follicle counts were calculated by adding the right and left values and dividing by 2 for each subject

^b Allele 2 represents the longer of the two *FMRI* alleles for each subject

were available for all participants. Cycle day 3 FSH levels were available for 89 % (536/602) of the participants. Similar to previous studies, the median allele length for the longer *FMRI* allele (allele 2) was 30 [1]. There were no differences in age or reproductive parameters amongst subjects when divided by *FMRI* genotype, as demonstrated in Table 2.

The overall incidence of DOR was 26.6 % (160/602) by AMH <1.0 ng/mL and 11.6 % (62/536) by Day 3 FSH >10.0 mIU/mL. Table 3 demonstrates differences in incidence of DOR between the normal/normal genotype and each of the other genotypes. A significant difference was noted in the incidence of below average AMH levels (<2.1 ng/mL) in subjects less than 35 between the normal/normal genotype and the low/low genotype (61/181 vs. 11/16, RR 3.82 95 % CI 1.38–10.56, *p* = 0.01). None of the other reproductive parameters in this analysis demonstrated statically significant differences with any other genotypes or any of the haplotypes for both allele 1 and allele 2. Furthermore, when allele 1 and allele 2 were analyzed as continuous variables in this age group, there was no significant association between allele length and the incidence of below average AMH level (allele 1 *p* = 0.37; allele 2 *p* = 0.35).

Across all age groups, there were no significant correlations between allele 1 or allele 2 with either AMH levels, log-transformed AMH (logAMH) levels, day 3 FSH levels, average OV, or average AFC (*p* > 0.05). There was no significant correlation between allele 1 length (*R* = −0.14, *p* = 0.72) or allele 2 length (*R* = 0.05, *p* = 0.18) with logAMH, even when controlling for age. Furthermore, when utilizing an ANCOVA, we found no significant effect of genotype on logAMH levels after controlling for effect of age (*F* = 1.84, *p* = 0.10).

Discussion

In this large study of women with normal-ranged *FMRI* CGG repeat lengths, we failed to find substantial correlations

Table 2 Cohort demographics subdivided by FMR1 genotypes.

	High/high	High/low	High/normal	Normal/normal	Normal/low	Low/low	<i>p</i> value
Age	<i>N</i> =4 31.6±7.1 (27.5–35.8)	<i>N</i> =17 31.1±4.3 (25.9–44.6)	<i>N</i> =79 34.2±6.2 (25.1–44.3)	<i>n</i> =320 33.5±7.4 (20.2–46.5)	<i>N</i> =158 33.7±7.0 (23.6–45.1)	<i>N</i> =24 33.5±8.5 (24.0–44.0)	0.12
AMH	<i>N</i> =4 1.5±4.9 (0.7–6.9)	<i>N</i> =17 2.3±3.8 (0.1–27.5)	<i>N</i> =79 2.2±3.9 (0.03–18.8)	<i>N</i> =320 2.2±3.5 (0.03–34.8)	<i>N</i> =158 2.4±3.1 (0.03–25.8)	<i>N</i> =24 1.5±1.6 (0.16–40.0)	0.25
D3 FSH	<i>N</i> =4 5.0±5.4 (4.1–11.2)	<i>N</i> =15 6.7±3.7 (3.8–15.0)	<i>N</i> =71 6.9±3.3 (3.8–90.4)	<i>N</i> =287 6.5±2.8 (1.8–27.4)	<i>N</i> =139 6.8±2.9 (1.4–67.2)	<i>N</i> =20 7.3±3.1 (4.1–11.6)	0.55
Avg OV	<i>N</i> =3 9.5 (5.5–12.5)	<i>N</i> =15 8.5±6.1 (3.9–14.1)	<i>N</i> =67 7.9±4.1 (2.6–23.0)	<i>N</i> =247 8.1±5.1 (1.75–56.6)	<i>N</i> =124 8.1±4.8 (2.0–60.3)	<i>N</i> =21 7.4±3.7 (2.2–12.2)	0.81
Avg AFC	<i>N</i> =3 6.5 (4.5–10.0)	<i>N</i> =15 8.0±9.5 (2.0–25.0)	<i>N</i> =70 7.8±7.1 (1.0–30.0)	<i>N</i> =240 7.8±7.4 (1.0–55.5)	<i>N</i> =126 7.5±7.0 (1.0–30.0)	<i>N</i> =20 7.3±4.5 (2.5–30.0)	0.99

Associations determined using Kruskal-Wallis Rank Sum Test. Average ovarian volumes (Avg OV) and antral follicle counts (AFC) were calculated by adding the right and left values and dividing by two for each subject

between number of repeats and reproductive parameters such as serum AMH levels, cycle day 3 FSH levels, ovarian volumes, antral follicle counts, or incidence of diminished ovarian reserve. When analyzed as discreet genotypes, we were only able to demonstrate a significant increase in women less than 35 with below average AMH levels in the low/low genotype compared to the normal/normal genotype. We also failed to find an association between any of the six *FMR1* genotypes and the other aforementioned reproductive parameters.

Previous studies have led to conflicting results regarding the association of normal-ranged *FMR1* CGG repeat

lengths and reproductive parameters (Table 4). Gleicher et al. found a correlation between lower AMH levels in 41 infertile women with ≥35 repeats compared to 122 with <35. However, the absolute reduction in AMH levels was not significant. This study also failed to find a difference in cycle day 3 FSH levels [24]. Pastore et al. found a higher incidence of 35–44 repeats in women diagnosed with DOR compared to previously published comparison groups [12]. From this, the authors conclude that the incidence of high-normal *FMR1* repeats is increased in women with DOR compared to those with normal ovarian reserve. However, this study utilized an external comparison group and

Table 3 Comparison of diminished ovarian reserve (DOR) and below average AMH levels in Normal/Normal genotype compared to others and subdivided by age group. Associations determined using Fischer's Exact Test

	All genotypes	Normal/normal	High/high	High/low	High/normal	Normal/low	Low/low
Overall							
AMH <1.0	160/602 (26.6 %)	88/320 (27.5 %)	2/4 (50.0 %) <i>p</i> =0.31	6/17 (35.3 %) <i>p</i> =0.58	21/79 (26.6 %) <i>p</i> =1.00	35/158 (22.2 %) <i>p</i> =0.22	8/24 (33.3 %) <i>p</i> =0.64
FSH >10.0	62/536 (11.5 %)	31/287 (10.8 %)	1/4 (25 %) <i>p</i> =0.37	2/15 (13.3 %) <i>p</i> =0.67	10/71 (14.1 %) <i>p</i> =0.41	16/139 (11.5 %) <i>p</i> =0.90	2/20 (10.0 %) <i>p</i> =1.00
<35							
AMH <1.0	52/348 (15.2 %)	28/181 (15.5 %)	1/3 (33.3 %) <i>p</i> =0.41	5/15 (33.3 %) <i>p</i> =0.14	6/43 (14.0 %) <i>p</i> =1.00	8/90 (8.9 %) <i>p</i> =0.18	3/16 (18.8 %) <i>p</i> =0.72
AMH <2.1	123/348 (35.3 %)	61/181 (33.7 %)	2/3 (66.7 %) <i>p</i> =0.27	6/15 (40.0 %) 0.78	16/43 (37.2 %) 0.72	27/90 (30.0 %) <i>p</i> =0.58	11/16 (68.8 %) <i>p</i> =0.01 ^a
FSH >10.0	20/306 (6.5 %)	8/158 (5.1 %)	1/3 (33.3 %) <i>p</i> =0.16	2/14 (14.3 %) <i>p</i> =0.19	3/37 (8.1 %) <i>p</i> =0.44	5/81 (6.2 %) <i>p</i> =0.77	1/13 (7.7 %) <i>p</i> =0.54
36–41							
AMH <1.0	108/254 (42.5)	60/139 (43.2 %)	0/1 (0 %) <i>p</i> =1.00	1/2 (50 %) <i>p</i> =1.00	15/36 (41.7 %) <i>p</i> =1.00	27/68 (39.7 %) <i>p</i> =0.66	5/8 (62.5 %) <i>p</i> =0.47
FSH >10.0	42/230 (18.3 %)	23/129 (17.8 %)	0/1 (0 %) <i>p</i> =1.00	0/1 (0 %) <i>p</i> =1.00	7/34 (20.6 %) <i>p</i> =0.80	11/58 (18.9 %) <i>p</i> =0.84	1/7 (14.3 %) <i>p</i> =1.00

^a Statistically significant

Table 4 Summary of previous studies of normal range CGG repeat length and reproduction

Study	Design	Population	Analysis	Results	Association
Gleicher et al. 2009 [24]	Cross-sectional	158 infertility patients	Regression model with number of CGG repeats on allele 2	AMH levels lower in with ≥ 35 repeats compared to < 35 repeats. No difference in FSH	Yes
Lledo et al. 2012 [25]	Prospective cohort	204 oocyte donors	Categorical associations by allele 2 repeat length (< 35 , 35–39, 40–45, > 45)	No significant difference in IVF outcomes based on allele length group	No
Pastore et al. 2012 [12]	Prospective cohort	62 patients with DOR	Categorical association by haplotype	Incidence of CGG 35–44 higher in DOR compared to previously published comparison groups	Yes
Choe et al. 2013 [20]	Retrospective cohort	228 Asian IVF patients	Regression and categorical with bi-allelic mean and allele-2 repeats	No association between AMH or FSH and CGG repeat overall In woman older than 35 ($n = 80$), CGG repeat associated with MoM AMH	No Yes
De Geyter et al. 2013 [26]	Prospective cohort	620 women (200 fertile + 372 infertile + 48 POI)	Categorical and regression by allele 2 repeats	No correlation between CGG repeat number and FSH or AMH in any group	No
Kline et al. 2014 [27]	Cross-sectional	258 women with SAB, 325 women with live birth	Categorical and regression by allele 2 repeats	No significant difference in AMH/FSH between CGG 30 and CGG 35–54	No
Pastore et al. 2014 [28]	Cross-sectional	79 women with DOR	Regression model by allele 2 repeats	Steeper decline of AMH in women with ≥ 35 repeats compared to < 35 repeats	Yes
Schuffeider et al. 2015 [29]	Retrospective cohort	1287 women seeking care at an infertility center	Regression models using allele 2 repeats as continuous variable or 4 categorical variables	A difference in AMH, FSH, or AFC in 208 women with 35–54 repeat in allele 2 compared to 1079 women with < 35 repeats. In older women (> 44), increasing repeats were associated with lower AMH	No Yes
Gustin et al. 2016 [30]	Retrospective cohort	566 women seeking care at an infertility center	Regression model using allele 2 repeat length as continuous variable	Increase in AMH as CGG repeats increased until age 40, after which increasing CGG repeat length was associated with decreasing AMH	Yes

limited their study sample to women already diagnosed with DOR, which limits the generalizability and clinical relevance of their findings. This same group also found a steeper age-related decline of AMH in 9 women with DOR and ≥ 35 repeats compared to 70 women with DOR and < 35 repeats [28]. However, these findings rely on a small sample size and again are isolated to those with a predetermined diagnosis of DOR, which does not necessarily support the use of *FMRI* allele length as a predictor of DOR.

Gleicher et al. in several small studies also demonstrated associations between “low” alleles (< 26 repeats) and decreased pregnancy rates in women undergoing in vitro fertilization [13], decreased oocyte yield in older women [22], lower AMH levels in oocyte donors [10], and more rapid decline in AMH amongst young oocyte donors [21]. Here, the authors compare young oocyte donors to older women with a diagnosis of infertility, which one may argue was an inappropriate comparison group. Alternatively, Gustin et al. recently demonstrated that increasing CGG repeat length correlate with increasing AMH levels through age 40 but after age 40 it is associated with a decline in AMH level [30].

In contrast, Lledo et al. found no difference amongst 204 young oocyte donors’ response to ovarian stimulation based on allele 2 sub-genotypes [25]. De Geyter et al. did not find higher incidence of “high” *FMRI* alleles in 372 infertile women compared to 200 fertile women [26]. Kline et al. also failed to find an association between CGG repeats of 35–54 compared to 30 repeats with decreased AMH or FSH levels amongst over 500 fertile women. In fact, they demonstrated an increase in AMH with higher repeat counts, although this was not adjusted for age [27]. Recently, Schufreider et al. failed to find a difference in AMH, FSH, or AFC in 208 women with 35–54 repeat in allele 2 compared to 1079 women with < 35 repeats [29].

Perhaps most importantly, a plausible biologic mechanism for impaired reproductive capacity with normal-ranged *FMRI* repeat length has yet to be determined. No studies have identified alterations in mRNA or protein production with less than 55 *FMRI* CGG repeats [2, 8, 31, 32].

Studying the associations of *FMRI* repeat lengths with reproductive parameters has several inherent challenges. Most notably, robust statistical inferences are best made by utilizing continuous variables without creating non-biologic categories. However, in this circumstance, not only are there several continuous variables that need to be considered (*FMRI* repeat length, AMH levels, FSH levels), but the primary outcome is significantly influenced by age [33], requiring the use of sophisticated analyses.

The aim of this study was to assess the correlation between *FMRI* repeat length and several reproductive variables of clinical importance, utilizing the more biologically relevant continuous variables whenever possible. The lack of correlation

demonstrated by these analyses in a large cohort is this study’s greatest strength. Given the utilization of genotypes in previous studies, we also categorized the cohort into the six previously defined genotypes, where we found a weak association in young women between low/low genotype and below average AMH levels compared to those with normal/normal genotype. Since more than half the subjects (320/602, 53 %) were normal/normal, these analyses were limited by small subgroup sample sizes and are likely statistically underpowered. For example, there were only four subjects in the high/high group, which critically limited the ability to draw conclusions about this subgroup. However, of note, this study represented the largest to date analyzing all six *FMRI* genotypes separately.

Other limitations include the lack of data related to ethnicity, which has been shown to be associated with *FMRI* repeat length [14]. Additionally, since the data was collected from new patient visits, data related to diagnosis or ultimate treatment and pregnancy outcomes were not included in the analyses. However, unlike other studies, we did not restrict our analyses to subjects already diagnosed with infertility or DOR.

Lastly, with a large, unrestricted cohort, we were unable to reliably correlate normal-ranged *FMRI* CGG repeat lengths with several clinically relevant reproductive parameters, while also correcting for the effect of age on the reproductive parameters. Some have recommended that utilization of normal-ranged *FMRI* repeat lengths be incorporated into clinical decision making [34]. Having a tool with which to predict the advent of infertility or DOR would be clinically useful, as physicians could counsel these women to seek treatment or consider oocyte cryopreservation prior to their reproductive decline. The significant increase in below average AMH levels amongst young subjects with low/low genotypes compared to normal/normal genotypes may support the suggestion that low repeat counts are associated with a more rapid ovarian senescence [21]. However, the overall findings of this study indicate that there may not be enough evidence at this time to support the use of normal-range *FMRI* repeat lengths as a reproductive tool.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Disclosure of funding None

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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