Anti-Mullerian hormone indicates early ovarian decline in fragile X mental retardation (\textit{FMR1}) premutation carriers: a preliminary study

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\textbf{BACKGROUND:} Women who carry the fragile X mental retardation (\textit{FMR1}) premutation are at risk for fragile X-associated primary ovarian insufficiency. Past studies have shown that carriers who are still cycling have increased levels FSH compared with non-carriers. As anti-Mullerian hormone (AMH) has been shown as an excellent marker of ovarian decline, we examined AMH levels among premutation carriers to characterize their ovarian function.

METHODS: We determined the level of FSH and AMH in serum samples collected during early follicular phase of ovarian decline, we examined AMH levels among premutation carriers to characterize their ovarian function.

RESULTS: For all age groups, AMH levels were significantly lower among longer repeat allele carriers compared with shorter repeat alleles (\textit{n} = 70 repeats, \textit{n} = 75), identified by DNA analysis. Comparisons were made stratified by age and carrier status. In contrast, increased FSH indicative of early ovarian decline was only evident for longer repeat allele carriers aged 31–40 years (\textit{P} = 0.099, 0.001 and 0.261 for women ages 18–30, 31–40 and 41–50 years, respectively). CONCLUSIONS: These preliminary data suggest that AMH levels indicate early ovarian decline among women with longer \textit{FMR1} repeat alleles; moreover, AMH appears to be a better marker than FSH in identifying this early decline.

\textbf{Keywords:} Mullerian inhibiting substance; fragile X; premature ovarian failure; FSH; menopause

\section*{Introduction}

The fragile X mental retardation (\textit{FMR1}) premutation is now a well-established cause of premature ovarian failure (POF), or cessation of menses prior to age 40 years, and the leading known cause of inherited POF. The premutation is clinically defined as having 55–199 unmethylated CGG repeats in the 5\textsuperscript{\prime} untranslated region (5\textsuperscript{\prime} UTR) of the \textit{FMR1} gene (Sherman \textit{et al}., 2005). When further expanded to over 200 repeats during transmission from mother to offspring and consequently hypermethylated, this allele is referred to as the full mutation and leads to fragile X syndrome, an inherited form of X-linked mental retardation. The prevalence of POF is only 1\% in the general population, but reaches to over 20\% among women carrying the premutation. Full mutation carriers do not have an increased risk for POF, thus ovarian insufficiency is restricted to premutation carriers (Sherman \textit{et al}., 2007). Menopausal age is shifted to a younger age by ~5 years compared with non-carriers (Murray \textit{et al}., 2000; Sullivan \textit{et al}., 2005). Other measures of ovarian insufficiency have also been documented at higher frequencies among premutation carriers who are still cycling, including increased gonadotropins, altered menstrual cycle characteristics and infertility (Murray \textit{et al}., 1999, 2000; Hundscheid \textit{et al}., 2001; Welt \textit{et al}., 2004; Sullivan \textit{et al}., 2005; Allen \textit{et al}., 2007). To capture this spectrum of traits associated with ovarian insufficiency among premutation carriers, the term fragile X-associated primary ovarian insufficiency (FXPOI) has been suggested (Welt, 2007; Abrams, 2007).

The significance of FXPOI among women with ovarian insufficiency has also been established. Studies which surveyed women with POF identified primarily through reproductive endocrinology clinics, (Murray \textit{et al}., 1998; Marozzi \textit{et al}., 2000; Mallolas \textit{et al}., 2001; Bussani \textit{et al}., 2004), estimate the frequency of women who are premutation carriers to be 11.5\% in those with a family history of ovarian failure (familial POF) and 3.2\% in those without a family history (sporadic POF). This frequency compares to the 1/250 frequency of premutation carriers present in the general population.

As yet there is no clinical marker that is prognostic of early ovarian decline among premutation carriers. We and others have shown that the severity of FXPOI is clearly associated...
with repeat size in a non-linear fashion (Sullivan et al., 2005; Ennis et al., 2006; Allen et al., 2007). Interestingly, the repeat size alleles that carry the highest risk of FXPOI are those in the mid-range of ~80 to ~99, not in those carrying the longest premutation repeat sizes (i.e. 100–200 repeats). Women who carry the 80–99 repeat size when compared to non-carriers have increased rates of infertility, have a 7-year reduction in mean age at menopause, and an increased prevalence of POF (32%) that initiates at younger ages. Carriers of both smaller and larger premutation repeat sizes also suffer from ovarian insufficiency, but not to as great an extent as women with 80–99 repeats (Allen et al., 2007).

In a post hoc analysis of the data of Allen et al. (2007), it was concluded that more work needed to be done to determine the repeat size definition for alleles that lead to a high risk for FXPOI. For example, it was noted that among women in the low premutation repeat group, defined a priori as 59–79 repeats, 0/17 with 59–70 repeats and 5/22 with 71–79 repeats had POF. Thus, the lower limit of ‘high risk’ alleles may be better defined as ≥70 repeats. The repeat premutation size and its association with the risk of FXPOI is clinically important; however, repeat size does not provide an accurate marker to predict the onset and severity of FXPOI.

Recently, AMH, also referred to as Müllerian inhibitory substance, has been identified both as an important regulator of early follicular growth and as a marker of the size of the primordial follicle pool in women. As reviewed by Dr Themmen and his colleagues (van Rooij et al., 2004; Visser and Themmen, 2005; Visser et al., 2006) and based on the recent work of Visser et al. (2007), the role of AMH in the menstrual cycle involves regulation of the recruitment from the primordial follicle pool and selection for dominance for ovulation. AMH, like activin and inhibin, is a member of the transforming growth factor-β family. Using immunohistochemistry in ovarian sections obtained from healthy regularly cycling women, Weenen et al. (2004) showed that AMH is only expressed in growing follicles and disappears in preovulatory follicles. Thus, serum AMH levels are indicative of the size of the growing follicle pool assuming that the number of follicles is indirectly reflected by the number of growing follicles (Scheffer et al., 1999). Thus, serum AMH levels may provide an excellent prognostic marker for premutation carriers. To test this hypothesis, we compared AMH levels among women with a wide range of FMR1 repeat sizes using frozen serum samples that had been previously collected for FSH studies. Our preliminary data suggest that AMH may be a better marker than FSH to detect early ovarian decline and should be applied in large population studies as a prognostic marker.

Materials and Methods

Study sample
The study sample comprised 219 women who were recruited through the Emory Study of Adult Learning and Reproduction. These participants are a sub-sample of the women reported in Sullivan et al. (2005) and Allen et al. (2007). They were identified through families with fragile X syndrome (n = 80) and from the general population (n = 139), as described in detail in Sullivan et al. (2005). All women were between the ages of 18 and 50 years and English-speaking. Each provided a venous blood sample for DNA to determine carrier status and for serum FSH studies. For women who were still cycling and not on hormonal medication, we obtained fasting blood samples on the third day of their cycle. Women were classified as being on hormone medication if they self-reported taking birth control pills, hormone shots or implants, or estrogen replacement therapy. For women using oral contraceptives, we obtained blood on the third day of their placebos. Medical exclusions for the hormone studies included women who had had their ovaries removed, those who had undergone chemotherapy or radiation therapy, and those who were pregnant or breast feeding at the time of interview. In addition, all women completed a questionnaire regarding her reproductive history. Of the 219 women for whom we had obtained FSH levels, we had stored frozen serum from 74 that could be used to conduct AMH analyses. We had an additional 41 serum samples from women ascertained under the same protocol from which we obtained AMH levels, but not FSH levels. The protocols and consent forms for each enrollment strategy were approved by the Institutional Review Board at Emory University, and informed consent was obtained from all participants.

Data collection

Questionnaires
The questionnaires provided information on women’s menstrual cycle characteristics and fertility. Women also provided information on other factors that may affect ovarian function including age, height and weight, smoking and hormone medication use. The questionnaires were administered in person, on the phone and in a self-administered format.

Hormone assays
Serum was extracted from a fresh 10 ml blood sample and stored at −70˚ until the assay was performed in batch. All samples were collected within the last 6 years.

An immunoradiometric procedure (Diagnostic Products; Los Angeles, CA, USA) was used to determine FSH levels for 178 samples that were reported in Sullivan et al. (2005). The assay sensitivity was <0.2 mIU/ml and within assay coefficient of variation (CV) was 2−5%.

AMH levels were also obtained using a commercially prepared kit produced by Diagnostic Systems Laboratories (Webster, TX, USA). The normal assay range was 0.05–14.0 ng/ml given a 20 μl assay volume. The inter-assay quality control was between 6 and 11% depending on the concentration. Measures were repeated for samples whose CV between the replicates exceeded 20%. AMH values less than 0.05 ng/ml were recorded as zero for our statistical analyses. We also conducted those same analyses with the value recorded as 0.05 ng/ml instead of 0 ng/ml and found essentially the same results (data not shown).

FMR1 CGG repeat size
DNA was extracted from buccal samples or blood using Qiagen QiAmp DNA Blood Mini Kit. FMR1 CGG repeat sizes were determined by a fluorescent-sequencer method, as described elsewhere (Meadows et al., 1996), using the ABI Prism 377 DNA Sequencer. For females with only one allele, a second PCR-based, hybridization technique was used to identify a possible long repeat size band (Brown et al., 1993). The PCR reaction consisted of 1X PCR Buffer, 10% dimethylsulphoxide, 370 μM deazaG, 500 μM d(TAC), 0.3 μM each primer, 15 ng T4 gene 32 and 1.05 U Roche Expand Long Tag. Primers for the FMR1 repeat region were 5’Cy5GCTCAGC
TCCGGTTCTTTCACTTCCCCACACGCTCCTCCA3’ and 5’AGCCCCGCACCTCCC
used hormones and those who did not. No significant
group showed a significant difference by repeat size alleles
women: only women in the 31–40 year age
FSH levels, the patterns were the same with and without
USA, or using SAS V9.
lyses were computed using the Statistical Package for the Social
alternative hypotheses were directional. Statistical ana-
in the model. The adjusted OR and 95% CI for longer repeat
alleles on low ovarian reserve. Owing to the limited sample
size and the non-normal distributions of FSH and AMH, a simple stat-
ical approach was taken. Women were defined as having low
ovarian reserve if they had AMH levels = 0 ng/ml or if they had
FSH levels ≥10 mIU/ml. Logistic regression analyses were con-
ducted to determine the strength of association between repeat size
group and low ovarian reserve. We examined the following covariates
as possible confounders or effect modifiers: age at blood draw, current
hormone use, body mass index and current smoking. If the 95% con-
fidence interval (CI) of the point estimate of the odds ratio (OR) for the
covariate in the model did not include one, that variable was included
in the model.

For all statistical tests, P < 0.05 was considered significant,
although all alternative hypotheses were directional. Statistical ana-
lyses were computed using the Statistical Package for the Social
Sciences (SPSS) 15.0, released in 2006 by SPSS Inc., Chicago, IL,
USA, or using SAS V9.

Results
On the basis of our previous data related to the identification of
high-risk alleles for FXPOI (Allen et al., 2007), we defined
high-risk allele carriers as women with ≥70 repeats. We will
refer to these allele groups as ‘shorter’ and ‘longer’ repeat
size groups to be clear that these repeat-size definitions differ
from those used to define premutation carriers. We first com-
pared FSH levels (Table I, Fig. 1a) and AMH levels
(Table II, Fig. 1b) among repeat size groups stratified by age
at blood draw among all women, irrespective of hormone
use. Among all women, AMH levels were significantly lower
in women carrying longer repeat alleles (P = 0.002, 0.006
and 0.020 for women ages 18–30, 31–40 and 41–50 years,
respectively). FSH values were significantly higher in women
carrying longer repeat alleles, only for those women in the
31–40 year group (P = 0.001) as reported in Sullivan et al.
(2005).

When women on hormones were excluded from the ana-
lyses, AMH levels were significantly lower for longer repeat
allele carriers among women ages 31–40 (P = 0.015), and
not significant among women in the young or older age
groups (P = 0.243 and 0.089, respectively, Table II). For
FSH levels, the patterns were the same with and without
women on hormones: only women in the 31–40 year age
group showed a significant difference by repeat size alleles
(P = 0.001, Table I).

We further examined FSH and AMH values within age and
repeat size groups to determine if levels differed among women
who used hormones and those who did not. No significant
differences were found in any of the groups, although sample
sizes were small and limited the ability to detect small differ-
ences (data not shown).

Lastly, we evaluated the OR associated with carrying the
longer repeat alleles on low ovarian reserve. AMH and FSH
levels were dichotomized to indicate low ovarian reserve and
logistic regression analyses applied as described in Materials
and Methods. For AMH levels = 0 ng/ml as the indicator vari-
able for low ovarian reserve, age at blood draw and current
hormone use were significant covariates and were included
in the model. The adjusted OR and 95% CI for longer repeat
group was 22.8 (4.2–122.5). For FSH levels ≥10 mIU/ml as
the outcome indicator variable for low ovarian reserve, only
age at blood draw was a significant covariate. The age-adjusted
OR for the longer repeat group was 1.6 (0.7–3.4).

Discussion
Taken together, these preliminary data are intriguing. We
tested the hypothesis that AMH levels may be a better
mark of early ovarian decline compared with FSH. The
data from this study suggest that this is true. This is the first
report to show that AMH levels are significantly lower
among women with longer FMR1 repeat alleles compared to
those with shorter repeats, especially at the younger ages
(Table II and Fig. 1b). Of importance, we report that FSH
levels appear to be a later marker of ovarian decline (Table II
and Fig. 1a). The greatest difference in FSH levels between
women with longer and shorter repeat alleles occurred in the
group aged 31–40 years. AMH levels in the oldest women
showed only small differences among repeat size groups, as
most women >35 years had AMH near or at zero, irrespective
of repeat size. Thus, AMH levels may be a more sensitive

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>&lt;70 Repeats</th>
<th>≥70 Repeats</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>All women</td>
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<tr>
<td>18–30</td>
<td>5.20</td>
<td>6.65</td>
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</tr>
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<td>20–30</td>
<td>0.2–39.8</td>
<td>0.6–10.4</td>
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<td>31–40</td>
<td>6.45</td>
<td>10.35</td>
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<td>5.2–38.1</td>
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<td>10.60</td>
<td>9.00</td>
<td>0.261</td>
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<td>18</td>
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<td>Excluding women on hormone treatment</td>
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<td>18–30</td>
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<td>11.26</td>
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<td>35–40</td>
<td>10.0</td>
<td>8.60</td>
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</tr>
<tr>
<td>41–50</td>
<td>3.6–38.2</td>
<td>3.4–22.2</td>
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<td>n = 25</td>
<td>n = 11</td>
<td>n = 11</td>
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P-values are provided for a one-sided test (Wilcoxon Rank Sum Test,
Normal or Exact Approximations where appropriate) comparing women with
<70 repeats to those with ≥70 repeats.

Table I. Median FSH values (mIU/ml), range and sample size by age group
and fragile X mental retardation (FMR1) gene CGG repeat size group.
marker for predicting the earliest signs of FXPOI rather than onset of menopause among women with the premutation.

Importantly, differences in AMH levels in women under 30 years of age suggest that ovarian reserve is reduced in premutation carriers from a young age. In our previous study using FSH as a marker of ovarian reserve, we concluded that women under age 30 may not be at risk of reduced ovarian reserve (Sullivan et al., 2005). Unfortunately, this conclusion does not hold when we use AMH, which is a more sensitive marker for this population and did show a reduction of ovarian reserve in the younger age group.

The results of this study are dependent on inclusion or exclusion of women on hormone medication. First, the sample size is reduced; thus, we cannot draw any strong conclusions. Second, we need to consider if excluding women who were prescribed hormone medication may be excluding those women who are already experiencing cycle irregularity and/or other symptoms of FXPOI. Thus, results would be biased. Further studies need to be conducted to both increase sample size and examine the effect of hormone use on AMH and FSH levels.

There are at least two viable mechanisms to explain FXPOI based on our knowledge of the function of FMRP, the protein produced by FMR1, and the molecular consequence of long repeat alleles in the 5' untranslated region of FMR1. First, the premutation allele produces a messenger RNA (mRNA) that includes a large repeat track. This premutation mRNA may cause a time-related cumulative toxic effect that eventually leads to an increased rate of follicular atresia during a woman’s reproductive life. Fragile X-related tremor/ataxia syndrome, another FMR1 premutation-associated disorder, has now been shown to be due to this type of mechanism (for review, see Hagerman and Hagerman, 2007). Alternatively, the protein may be involved. FMRP regulates translation of a subset of mRNAs using a suppression mechanism (Jin et al., 2004). Perhaps increased levels of FMRP at specific times during development could lead to haploinsufficiency of the proteins needed in oocyte or follicle development. Our preliminary data suggest that whatever the effect, this premutation plays a role early in a woman’s reproductive life, evidenced not by FSH fluctuations but by low AMH levels. One limitation of the current study is that we have few women in the younger age groups and we have not tested women less than 18 years of age. Additional studies of younger women are important to help identify when premutation carriers begin to differ from non-carriers with respect to their ovarian reserve. Such studies will also provide insight into the toxic effect of the premutation allele on ovarian function.

Not only is AMH a useful marker of ovarian aging since changes are evident earlier in the reproductive lifespan when compared with other ovarian hormone markers (e.g. Hale et al., 2007), but additionally it is not regulated by other hormones (e.g. van Rooji et al., 2002), it does not fluctuate

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**Figure 1:** (A) FSH (mlU/ml) and (B) anti-Müllerian hormone (AMH) (ng/ml) levels by age (years) at blood draw for women who carry <70 CGG repeats (white diamond) and ≥70 repeats (gray square) in the 5' untranslated region of the fragile X mental retardation (FMR1) gene.
during the menstrual cycle (e.g. Hekenkamp et al., 2006; La Marca et al., 2006; Tsipelidis et al., 2007), and it is not changed by pregnancy (La Marca et al., 2005). In conclusion, AMH appears to be an excellent marker for clinical studies of FXPOI. AMH may prove to be a non-invasive prognostic marker for premutation carriers to help provide them with additional information on their given risk of ovarian insufficiency beyond that offered by repeat size alone.

Our data and others indicate that women with the premutation, especially those with mid-size repeats, experience an earlier onset of FXPOI. Thus, all premutation carriers are at an increased risk for infertility and having earlier exposure to estrogen deficiency, the latter being a known risk factor for osteoporosis (Richelson et al., 1984; Kritz-Silverstein and Baron, 1991; Jacobsen et al., 2002), colorectal cancer (van der Schouw et al., 2005). In conclusion, estrogen exposure and cardiovascular mortality risk in postmenopausal women. Am J Epidemiol 2002;155:339–345. Ennis S, Ward D, Murray A. Nonlinear association between CGG repeat number and age of menopause in FMR1 premutation carriers. Eur J Hum Genet 2006;14:253–255.

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<th>≥70 repeats</th>
<th>P-value</th>
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<td>31–40</td>
<td>1.60</td>
<td>0.52</td>
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<td>n = 15</td>
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<td>0.23</td>
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<td>31–40</td>
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<td>31–40</td>
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<td>n = 12</td>
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P-values are provided for a one-sided test (Wilcoxon Rank Sum Test, Normal or Exact Approximations where appropriate) comparing women with <70 repeats to those with ≥70 repeats.

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### References

### Acknowledgements
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