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Trisomic pregnancy and intermediate CGG repeat length at the *FMR1* locus

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BACKGROUND: We hypothesized that trisomy arises as a function of the size of the oocyte pool, with risk increased among women with diminished pools. Diminished pools may cause primary ovarian failure, which has been associated with premutation and intermediate CGG repeat length at the Fragile X mental retardation (*FMR1*) locus. Thus, we hypothesized that the risk of trisomic pregnancy is increased among women with intermediate CGG repeat length on the *FMR1* gene.

METHODS: The analysis drew on data from two hospital-based case–control studies. We compared 207 women with trisomic spontaneous abortions (SAs) to three comparison groups: 82 women with other chromosomally abnormal SAs, 99 women with chromosomally normal SAs and 537 women with live births (LBs), age matched to women with SAs. We defined the length of the CGG repeat in four ways: the biallelic mean, the genotypic mean, the length on allele 2 and the length on allele 1. We analyzed CGG repeat length as a categorical variable. All analyses were adjusted for site, age and ethnicity.

RESULTS: CGG repeat length did not differ significantly between women with trisomic SAs and any of the three comparison groups. For the biallelic mean, the adjusted odds ratio relating trisomy (versus LB controls) to the highest category (35.5–59.5 repeats) versus the modal category (26.5–30.0 repeats) was 1.5 (95% confidence interval (CI): 0.7, 3.1). Comparisons with the two SA control groups also showed increased odds of more repeats among trisomy cases. Results were similar when repeat length was defined by the genotypic mean or by the repeat length on allele 2. For allele 1, the odds of short (9–19) repeat length were lower, but not significantly so, for trisomy cases compared with LB controls. Excluding women with premutations ($n = 2$) from the analysis yielded an adjusted odds ratio of 1.4 (95% CI: 0.7, 2.9) for the biallelic mean.

CONCLUSIONS: Our data are equivocal. The direction of associations is consistent with the hypothesis that repeat length in the intermediate range is associated with trisomy. However, differences between the trisomy cases and the comparison groups are neither large nor statistically significant. Our data rule out odds ratios larger than about 3.

Key words: epidemiology / trisomy / aneuploidy / Fragile X / *FMR1*

Introduction

The processes underlying the association of maternal age with trisomy risk remain unknown. One hypothesis is that this association reflects factors—intra- or extra-ovarian—related to the size of the oocyte pool. Smaller pools might result from formation of fewer oocytes during fetal development or from accelerated atresia. We hypothesized (Kline *et al.*, 2000, 2004, 2010) that trisomy arises as a function of the size of the pool, with risk increased, at any given chronologic age, among women with smaller pools. Thus, risk factors for diminution of the pool may be associated with trisomy risk.

The length of the CGG repeat at the *FMR1* locus is related to premature ovarian failure (POF), which may result from a diminished oocyte pool. *FMR1*, a gene on the X chromosome that underlies the Fragile X mental retardation syndrome, codes for a heterogeneous nuclear ribonucleoprotein, FMRP, that shuttles between the nucleus and the cytoplasm of neuronal cells. FMRP plays a subtle, but critical, role in the translation of mRNA. The 5′ untranslated region of the

FMR1 gene has a polymorphic CGG repeat. In most people, the number of repeats is 11–40. Individuals with repeat lengths of ≈55–200 are considered premutation carriers because repeats of this length have a propensity to expand to the full mutation in the subsequent generation (American College of Medical Genetics, 2006). In premutation carriers, *FMR1* mRNA levels are increased and FMRP levels are slightly decreased (Tassone *et al.*, 2000a,b; Kenneson *et al.*, 2001; Primerano *et al.*, 2002; Loesch *et al.*, 2007). When the repeat length is >200, the *FMR1* locus becomes hypermethylated and transcriptionally silenced, resulting in mental retardation among males and variable phenotypes among females. Repeat lengths between the high end of normal and premutation are considered 'intermediate'. Definitions of the lower bound of intermediate vary from 35 to 45. One study (Loesch *et al.*, 2007) of males suggests that when the number of repeats is between 40 and 60, mRNA levels increase with increasing repeat length.

Premutation carriers are at increased risk of POF. POF is usually defined by the occurrence of menopause or two elevated levels of follicle-stimulating hormone (FSH) 1 month apart before age 40 years. Several studies (reviewed in Wittenberger *et al.* (2007) and Sullivan *et al.* (2011); see also Karimov *et al.* (2011) also show associations with other indicators of premature ovarian aging, including early menopause (<45 years), elevated levels of FSH, decreased levels of anti-Müllerian hormone (AMH), decreased levels of inhibin B and occult POF in women treated for infertility. Most studies (Allingham-Hawkins *et al.*, 1999; Hundscheid *et al.*, 2003; Bussani *et al.*, 2004; Sullivan *et al.*, 2005; Bodega *et al.*, 2006; Bennett *et al.*, 2010) support an association with POF; risk ratios range from 5.8 to infinity. POF has been observed among 13–21% of premutation carriers compared with <1% of non-carriers (Allingham-Hawkins *et al.*, 1999; Hundscheid *et al.*, 2003; Sullivan *et al.*, 2005). Among women with normal karotypes, premutation carriers constitute 2–10% of POF cases, although in the general population they constitute <0.5% (Conway *et al.*, 1998; Bussani *et al.*, 2004; Bodega *et al.*, 2006; Bennett *et al.*, 2010). Associations appear stronger among women with family histories of POF. Some studies (Ennis *et al.*, 2006; Allen *et al.*, 2007) suggest that, within the premutation range, associations with POF are strongest for medium length (≈80−100 repeats) premutations. A similar non-linear association is reported for age at menopause (Allen *et al.*, 2007). Because POF is classified as menopause, however, it is unclear whether or not this association is independent of associations with POF.

Some studies further suggest that the association of repeat length with POF (odds ratios: 2.4–5.5) is also present among women with repeat length in the intermediate range: 41–58 (Sullivan *et al.*, 2005), 35–54 (Bretherick *et al.*, 2005), 43–53 (Bodega *et al.*, 2006). A recent study (Bennett *et al.*, 2010) was interpreted to show no association with intermediate repeat length, defined variously as 35–54 and 41–58. We do not, however, concur with this interpretation (see section Discussion).

The most likely causal mechanism for an association between increased CGG repeat length and POF is that *FMR1* mRNA has a toxic gain of function, leading to an increased rate of oocytes atresia and, consequently, a smaller oocyte pool at any given age (see Sullivan *et al.*, 2011). Alternatively, increased repeat length may interfere with oocyte formation, leading to smaller pools.

To date, associations between CGG repeat length and aneuploidy have not been tested with rigor. An early report (Watson *et al.*, 1988) suggests that trisomy 21 births are more common among female obligate carriers of Fragile X. Three studies (Murray *et al.*, 2000; Hundscheid *et al.*, 2003; Allen *et al.*, 2007) tested the hypothesis indirectly, drawing on reported pregnancy history; the rate of unfavorable outcomes (including pregnancy loss) was not increased for premutation carriers. Interpretation is unclear, however, because losses were not karyotyped.

We undertook the present study to test whether the CGG repeat length in the intermediate range is associated with trisomy risk.

Methods

We drew on data from two studies, one in New York and one in New Jersey, to compare the length of the CGG repeat at the *FMR1* locus between women who had trisomic spontaneous abortions (SAs) and women who had other SAs (non-trisomic chromosomally abnormal, chromosomally normal) or who had chromosomally normal live births (LBs). The design and protocols of the studies are similar.

The New York study

The New York study, described in full in Kline *et al.* (2004), was designed to test the hypothesis that the oocyte pool is smaller in women with trisomic pregnancies than in women with pregnancies of other types. Indicators of the size of the pool included antral follicle count, FSH, AMH and inhibin B (see also Kline *et al.*, 2010).

From September 1998 to April 2001, we ascertained a consecutive series of SAs at one hospital. We attempted to karyotype all singleton prefetal (developmental age <9 weeks) SAs to women 18 years or older. If a woman's loss was successfully karyotyped, we asked her to complete a short telephone interview to determine her eligibility for hormone studies. To obtain valid measures, we required no pituitary disorder or hormonal disorder related to ovarian function, no oophorectomy, no hormonal medication, no pregnancy at the time of the study protocol, no breastfeeding or breastfeeding no more than once per day during the menstrual cycle preceding the study assessments. Eligible women who consented to the protocol: (i) completed a more extensive telephone interview regarding demographic characteristics, obstetric and medical histories and common exposures; (ii) recorded the dates of their menstrual periods; (iii) made two visits to the study hospital during the first week of their second or later menstrual cycle, the first on Days 1–4 for a blood draw and the second on Days 5–7 for transvaginal sonography and a brief interview regarding recent exposures and (iv) reported the date of the menses following the sonogram. We saved DNA for later studies.

Women with trisomic SAs constitute the case group. Women with non-trisomic chromosomally abnormal SAs and chromosomally normal SAs constitute two of the comparison groups; women with chromosomally normal LBs constitute the third. For each woman with a trisomic SA (case) who completed the study, we selected an age-matched control with a chromosomally and anatomically normal LB ≥1800 g, no pregnancy loss since the index pregnancy and no known trisomic pregnancy. The LBs were selected from the hospital delivery log of women who delivered during the 7–13 months preceding the date of selection. LB controls were matched to trisomy cases for projected age (±6 months) at the sonography visit. The protocol for LB controls was identical to the protocol for women with SAs. If a selected LB control was ineligible for the study or refused to participate, we replaced her. Fieldwork ended in November 2001.

Supplementary data, Table SIa sets out the number of women identified and their eligibility for this analysis. The analytic sample includes 38 women with trisomic SAs, 17 women with non-trisomic chromosomally abnormal SAs, 11 women with chromosomally normal SAs, and 50 women with LBs.

The New Jersey study

The New Jersey study, described in full in Warburton (Warburton *et al.*, 2009), was designed to examine the relation of highly skewed X chromosome inactivation to trisomy. We also collected sera in anticipation of analyses to examine the relation of hormonal indicators of the size of the oocyte pool to trisomy (Kline *et al.*, 2010).

From February 2003 to November 2005, we ascertained a consecutive series of SAs at one hospital. The New Jersey study was similar in design to the New York study. It differed in the following ways: (i) it included women with singleton SAs <18 weeks (rather than <9 weeks) developmental age; (ii) it included women ineligible for hormone measures (hormone levels were irrelevant to the primary aim of the study); (iii) age-matched women with LBs were selected for all women with SAs (rather than only for women with trisomic SAs); (iv) we drew blood on Days 2–4 (rather than Days 1–4); (v) in the event that a woman with an SA was eligible for hormone studies but her first LB control was not, we enrolled a second LB control who was eligible for hormone studies; (vi) LB controls delivered 6–12 months (rather than 7–13 months) preceding the date of their selection.

Supplementary data, Table SIb sets out the number of women identified and their eligibility status for this analysis. The analytic sample includes 169 women with trisomic SAs, 65 women with non-trisomic chromosomally abnormal SAs, 88 women with chromosomally normal SAs and 487 women with LBs.

Both studies

Each study was approved by the Institutional Review Boards of the study hospital and of our institution.

Table I shows selected characteristics of the trisomy cases and the three comparison groups for the two studies. Mean maternal age is younger for the New York sample. For the New Jersey sample, trisomy cases are older than LB controls, as expected given that LB controls were age matched to all SAs, rather than to trisomy cases only. At both settings, the majority of women were white, non-Hispanic; trisomy cases do not differ from the three comparison groups in the proportion white, non-Hispanic.

FMR1 CGG repeat size

CGG repeat length was determined using PCR and capillary electrophoresis (CE) procedures as previously described (Filipovic-Sadic *et al.*, 2010) using prototype *FMR1* PCR reagents obtained from Asuragen, Inc. Samples were prepared for CE analysis by mixing 2 μl of unpurified PCR products with 11 μl of Hi-Di formamide (Applied Biosystems) and 2 μl of ROX-1000 Size Ladder (Asuragen, Inc.). All samples were heat denatured at 95°C for 2 min, followed by cooling at 4°C for at least 2 min. Injections were at 1.2 kV for 15 s, with a run time of 45 min at 15 kV. PCR products were resolved by CE with a 3100-Avant Capillary Array (Applied Biosystems, Foster City, CA) running POP-4 polymer (Applied Biosystems) in a 36-cm array. Quantification of the repeat number was achieved using PeakScanner software after a comparison of PCR product lengths to a ladder of ROX-labeled size standards. All assay runs included a pooled mixture of five alleles ranging from 20 to 120 CGG repeats for which repeat length had been previously verified by sequencing. These process controls were used to estimate repeat length in the sample.

Randomly ordered samples were run in 30 batches. The number of CGGs was estimated based on batch-specific linear regression equations relating mobility of the peaks from the CE analysis to the true repeat lengths of the process controls. Because the computation yields a non-integer estimate of CGG repeat length, we rounded these values to the nearest integer for analysis.

To test assay validity, we performed two blind analyses of a set of 25 sequence-verified control samples. In both analyses, the agreement between estimated repeat length and true repeat length was perfect for 96% and within ±1 repeat unit for all. To test assay reliability, we re-assayed 25 randomly chosen samples. The assay performed consistently over the 11 months of analysis, with perfect agreement between estimated repeat length from the original and repeat runs.

X chromosome inactivation

We determined the X chromosome inactivation (XCI) percent at the *FMR1* locus based on methylation sensitive restriction digestion and two-

Table I Selected characteristics of women who completed the protocol classified by the outcome of the index pregnancy.

	Losses			Births
	Trisomy	Non-trisomy abnormal	Chromosomally normal	
(1) New York sample				
Number of women	38	17	11	50
Age (years) at the blood draw[a] [mean (SD)]	34.4 (5.9)	32.2 (4.2)	30.8 (5.5)	35.0 (6.2)
Ethnicity: White, non-Hispanic[b,c] (%)	97.4	94.1	90.9	94.0
(2) New Jersey sample				
Number of women	169	65	88	487
Age (years) at the blood draw[d] [mean (SD)]	37.1 (4.6)	33.6 (4.2)	33.3 (4.9)	35.4 (4.8)
Ethnicity: White, non-Hispanic[b,c] (%)	85.2	86.2	81.8	86.9

[a]Mean age does not vary significantly with the outcome of the index pregnancy.
[b]Adjusted for age, ethnicity does not vary significantly with the outcome of the index pregnancy.
[c]In the total sample, the 117 women who were not white, non-Hispanic include 59 Hispanics, 37 Asians, 13 blacks and 8 women of other or unknown ethnicity.
[d]Mean age varies significantly ($P < 0.0001$) with the outcome of the index pregnancy. As expected, women with trisomy losses are significantly older than women with non-trisomy losses. Because live birth controls were age matched to women with losses, women with births are significantly younger than women with trisomy losses and significantly older than women with non-trisomy losses.

color PCR as described (Hatakeyama *et al.*, 2004) for the *HUMARA* locus. In brief, aliquots of 0.5 μg of each DNA sample were split and either digested with HpaII or mock digested for 12–16 h at 37°C. After desalting, PCR of the FRAXA CGG was performed on both HpaII-digested and mock-digested samples, using different fluorochromes to distinguish between the two amplification products.

The XCI skewing ratio was determined by comparing the ratio of allele peak heights in the HpaII-digested sample (d_1 and d_2, for smaller and larger PCR product sizes, respectively) with the ratio in the sample digested by HpaII alone (u_1 and u_2). The method corrects for differences in amplification efficiency of the two alleles. The XCI skewing ratio equals $(d_1/u_1)/(d_2/u_2)$. We converted the ratio to an XCI skewing percent by computing $P = [(d_1/u_1)/\{(d_1/u_1) + (d_2/u_2)\}] \times 100$, a value which ranges from 0 to 100%. We refer to P as the XCI skewing percent for allele 1. To compute the genotypic mean of the CGG repeat lengths, we took a weighted average of the two lengths, where the length of the repeat on allele 1 is weighted by P and the length of the repeat on allele 2 by $1 - P$.

Among 925 samples analyzed, we determined the XCI skewing percent in 746 (81%) heterozygous samples at the *FMR1* locus. Repeat measures of the XCI skewing percent showed excellent agreement; the intra-class correlation coefficient was 0.99 in the reliability analysis described above. The analyses exclude four homozygous samples because the XCI skewing percent cannot be determined for homozygotes. Agreement on homozygosity between repeat runs was perfect.

Definition of CGG repeat length

We defined CGG repeat length in four ways: the biallelic mean, the genotypic mean, the length on allele 2, the length on allele 1. We consider the biallelic mean the primary measure because both X chromosomes are active in the oocyte. Supplementary data, Fig. S1 shows the distribution of the biallelic means among LB controls. We analyzed each of the four measures as categorical variables defined, approximately, by percentiles—lower 5th, 5 to <25th, 25 to <75th, 75 to <95th, upper 5th—of length in the LB controls. We defined the middle category (25th to <75th percentile) as the reference group. The lower bound of the upper 5th percentile was 35.5 for both the biallelic mean and the genotypic mean, and 35 for allele 2. Because one study (Chen *et al.*, 2003) suggests that FMRP expression levels are low for short alleles, we also examined associations with short repeat length on allele 1; the upper bound of the lowest 5th percentile was 19.

Statistical analyses

We used conditional logistic regression (Breslow and Day, 1980; Levin, 1988) to test the null hypothesis that, at any maternal age, there is no difference in CGG repeat length between trisomy cases and the three comparison groups. For the biallelic mean, the genotypic mean and allele 2 length, we hypothesized that the odds of the longest category is increased among trisomy cases; for allele 1 length, we hypothesized that the odds of the shortest category is increased among trisomy cases. The analysis adjusted by stratification for site and age (single years) at blood draw and by indicator variable for ethnicity (non-Hispanic white, other). We repeated the analysis among non-Hispanic white women. Adjusting for site and pregnancy outcome, ethnicity was not significantly related to the biallelic mean, the genotypic mean or the length on allele 2, each defined categorically. Ethnicity was significantly related to the length on allele 1: compared with non-Hispanic white women, the allele 1 length of women of other races were more concentrated in the reference category of 23–29 repeats than in the category of 30 repeats.

We also carried out two secondary analyses of associations with repeat length defined by the biallelic mean: (i) limiting LB controls to women without prior SAs, since women with prior SAs include women with undiagnosed trisomic pregnancies and (ii) dividing trisomic SAs into three groups: trisomy 16, other non-acrocentric trisomies and acrocentric trisomies.

Results

At the New Jersey site, the proportion with long CGG repeat length, whether defined by the biallelic mean (≥35.5), the genotypic mean (≥35.5) or the length on allele 2 (≥35), was higher for trisomy cases than for each of the comparison groups (Table II). The proportion with short length on allele 1 (≤19) was lower for trisomy cases than for each comparison group. For the smaller New York sample, the proportion with long repeat length was higher for trisomy cases than for non-trisomy abnormal SAs and LBs; the proportion with short allele 1 length was lower for trisomy cases than for non-trisomy abnormal SAs and LBs.

For the biallelic mean, adjusted odds ratios for the longest repeat length category (35.5–59.5) versus the modal category (26.5–30.0) ranged from 1.5 to 3.8, with all 95% confidence intervals (CIs) including 1.0 (Table III). Among non-Hispanic white women, adjusted odds ratios ranged from 1.9 to 3.7. In comparison with LB controls, the largest comparison group, the adjusted odds ratio was 1.5 for the total sample and 1.9 for the sample of non-Hispanic white women. The trisomy–LB odds ratio did not differ significantly with ethnicity. Analyses using the genotypic mean or the length on allele 2 yielded results similar to analyses using the biallelic mean. The trisomy–chromosomally normal odds ratio is larger for the longest category of the biallelic mean than for the genotypic mean.

Two women (one with a trisomic loss, one with a chromosomally normal loss) had CGG repeat lengths in the premutation range. When we excluded these two women from the analysis, the adjusted odds ratio for the longest category of the biallelic mean, comparing trisomy cases with LB controls, was 1.4 (95% CI: 0.7, 2.9).

For the shortest allele 1 length category of 9–19 (versus the modal category of 23–29), the adjusted odds ratios comparing trisomy cases with each of the three comparison groups ranged from 0.3 to 0.6, with all 95% CIs including 1.0. In comparison with LB controls, the adjusted odds ratio was 0.5. Among non-Hispanic white women, adjusted odds ratios ranged from 0.4 to 2.2; in comparison with LB controls, the adjusted odds ratio was 0.6.

We repeated the primary analysis for the biallelic mean excluding women with prior SAs from the LB control group (data not shown). The adjusted odds ratio for the longest category of the biallelic mean comparing trisomy cases with LB controls was 1.4 (95% CI: 0.7, 3.0).

The adjusted odds ratios relating the longest biallelic mean category to trisomy type (versus LB controls) were 1.5 for trisomy 16, 2.2 for other non-acrocentric trisomies and 1.6 for acrocentric trisomies; all 95% CIs included 1.0 (Table IV). The adjusted odds ratios for the shortest biallelic mean category were 1.4 for trisomy 16 and 0.3 for acrocentric trisomies.

Discussion

Our data show a modest, statistically non-significant association of intermediate CGG repeat length with trisomy. For biallelic means of 35.5–59.5, adjusted odds ratios ranged from 1.5 to 3.8, depending on the comparison group. The largest odds ratio of 3.8 (95% CI: 0.96, 15.4),

Table II Length of the CGG repeat at the *FMR1* locus by karyotype: Percent distribution of five categories of the biallelic mean; the proportion with long repeats (genotypic mean, allele 2) and short repeats (allele 1) among women with trisomic losses (cases) compared with women with non-trisomy chromosomally abnormal losses, with chromosomally normal losses, and with live births for (1) the New York sample and (2) the New Jersey sample

	Losses			Births
	Trisomy	Non-trisomy abnormal	Chromosomally normal	
(1) The New York sample				
Number of women	38	17	11	50
Biallelic mean (%)[a]				
19.0–22.0	2.6	5.9	0.0	6.0
22.5–26.0	13.2	11.8	18.2	18.0
26.5–30.0	52.6	58.8	27.3	50.0
30.5–35.0	21.0	17.6	45.4	20.0
35.5–59.5	10.5	5.9	9.1	6.0
Genotypic mean: 35.5–58.0	10.5	5.9	18.2	4.0
Allele 2: 35–89	18.4	5.9	27.3	14.0
Allele 1: 9–19	2.6	5.9	0.0	4.0
(2) The New Jersey sample[a]				
Number of women	169	65	88	487
Biallelic mean (%)[a]				
19.0–22.0	1.8	7.7	3.4	4.7
22.5–26.0	16.6	15.4	12.5	19.3
26.5–30.0	51.5	30.8	53.4	46.2
30.5–35.0	23.1	41.5	28.4	24.6
35.5–59.5	7.1	4.6	2.3	5.1
Genotypic mean: 35.5–58.0	7.1	4.6	3.4	5.1
Allele 2: 35–89	16.6	15.4	15.9	14.2
Allele 1: 9–19	1.8	6.2	3.4	3.7

[a]Percents may not add to 100% due to rounding.

from the comparison of trisomy cases with chromosomally normal SAs, is compatible with no association. In comparison with LB controls, the largest control group, the adjusted odds ratio was 1.5 (95% CI: 0.7, 3.1). This result was essentially unchanged when we (i) excluded two women with premutations (because our goal was to examine the associations with intermediate length), (ii) limited LB controls to women with no prior SAs (to exclude women with undetected trisomic loss) and (iii) limited the sample to non-Hispanic white women. Our results were also essentially the same when we defined length by the genotypic mean or by the length on allele 2. The sizes of associations with trisomy 16, other non-acrocentric trisomies and acrocentric trisomies were similar to the size of the association for all trisomies combined. Our data, which are consistent with no association between intermediate CGG repeat length and trisomy, rule out odds ratios greater than about 3. The detectable effect size (80% power, $\alpha = 0.05$, two-tailed) is 2.8.

We also examined whether short CGG repeat length on allele 1 is associated with trisomy. We undertook this analysis because an *in vitro* study (Chen *et al.*, 2003) showed that for alleles with $\leq$30 repeats, short length was associated with less efficient expression of a reporter gene. In addition, data from an assisted reproduction sample (Gleicher *et al.*, 2009) were interpreted to suggest that short length is associated with decreased levels of AMH. In our data, adjusted odds ratios relating trisomy to short length range from 0.3 to 0.6 for allele 1 and 0.3–0.8 for the biallelic mean. Results were similar when we limited the sample to non-Hispanic white women. All 95% CIs are compatible with no association. For short length on allele 1, we can rule out associations in excess of $\sim$1.8; for the biallelic mean, we can rule out associations $>$1.2.

Strengths of our study include excellent validity and reliability of our assay; laboratory analyses blind to birth outcome and karyotype; random ordering of samples to guard against potential confounding by assay batch; a sample unselected for family history of Fragile X syndrome; face validity (i.e. the distribution of repeat length in our data from LBs is similar to the distribution in other samples).

The unselected sample allows us to generalize our findings to the majority of women rather than only to women from families in which the premutation has demonstrated the capacity to expand. This aspect is important in light of a recent paper (Nolin *et al.*, 2011) indicating that the risk of expansion to the full mutation in a single generation for lengths of 55–59 is lower for women unselected for a family history of Fragile X syndrome than for women from Fragile X families. On the other hand, because our sample is unselected, the

Table III Adjusted odds ratios (New York and New Jersey samples combined) for CGG repeat length at the *FMR1* locus for cases (trisomy loss) versus controls (non-trisomy chromosomally abnormal loss, chromosomally normal loss, live birth): (1) for the total sample; (2) for white non-Hispanic women.

	Adjusted odds ratio (95% CI) for CGG repeat length: trisomy cases versus each comparison group		
	Non-trisomy abnormal loss	**Chromosomally normal loss**	**Birth**
(1) Total sample[a]			
Biallelic mean			
19.0–22.0	0.3 (0.1, 1.0)	0.8 (0.2, 3.7)	0.4 (0.1, 1.2)
22.5–26.0	0.7 (0.3, 1.5)	1.2 (0.6, 2.5)	0.7 (0.5, 1.2)
26.5–30.0	1.0 (reference)	1.0 (reference)	1.0 (reference)
30.5–35.0	0.4 (0.2, 0.8)	0.8 (0.4, 1.4)	0.9 (0.6, 1.3)
35.5–59.5	1.6 (0.5, 5.3)	3.8 (0.96, 15.4)	1.5 (0.7, 3.1)
Genotypic mean: 35.5 to <58.0 (versus 26.5 to <30.5)	1.5 (0.4, 5.2)	1.8 (0.6, 5.7)	1.5 (0.8, 3.1)
Allele 2: 35–89 (versus 30)	1.4 (0.6, 3.2)	1.3 (0.6, 2.8)	1.3 (0.8, 2.1)
Allele 1: 9–19 (versus 23–29)	0.3 (0.1, 1.3)	0.6 (0.1, 2.9)	0.5 (0.2, 1.5)
(2) White, non-Hispanic women[b]			
Biallelic mean			
19.0–22.0	0.3 (0.1, 1.3)	1.2 (0.2, 6.9)	0.5 (0.2, 1.6)
22.5–26.0	0.7 (0.3, 1.7)	1.2 (0.5, 2.6)	0.7 (0.4, 1.2)
26.5–30.0	1.0 (reference)	1.0 (reference)	1.0 (reference)
30.5–35.0	0.5 (0.3, 1.04)	0.7 (0.4, 1.4)	0.9 (0.6, 1.5)
35.5–59.5	2.1 (0.5, 8.2)	3.7 (0.9, 15.7)	1.9 (0.9, 3.9)
Genotypic mean: 35.5 to <58.0 (versus 26.5 to <30.5)	2.0 (0.5, 7.5)	1.7 (0.5, 5.4)	2.0 (0.96, 4.2)
Allele 2: 35–89 (versus 30)	2.1 (0.8, 5.4)	1.4 (0.6, 3.0)	1.5 (0.9, 2.5)
Allele 1: 9–19 (versus 23–29)	0.4 (0.1, 1.7)	2.2 (0.2, 19.4)	0.6 (0.2, 1.8)

[a]Odds ratios from conditional maximum likelihood logistic regression. Analyses adjust by stratification for site (New York, New Jersey) and age in single years and by indicator variable for race (White non-Hispanic versus Other).
[b]Odds ratios from conditional maximum likelihood logistic regression. Analyses adjust by stratification for site (New York, New Jersey) and age in single years. Analyses of White, non-Hispanic women are based on 181 women with trisomy losses, 72 women with non-trisomy chromosomally abnormal losses, 82 women with chromosomally normal losses and 470 women with births.

number of women with premutations was too few for analysis. Of the two women with premutations, one had a trisomy 22 loss (repeat lengths 23/73) and the other a chromosomally normal loss (repeat lengths 30/89).

With respect to face validity, among 537 LB controls, none had a premutation. In samples of females unselected for a family history of Fragile X syndrome or developmental problems in their relatives, the expected rate of premutation (61–200 repeats) is about 2.8 per 1000 (computed from a review by Crawford *et al.*, 2001). In our total sample of 925 women, two (2.2 per 1000) have premutations, consistent with previous observations. Among our LB controls, 76 (14.2%) women had at least one allele of intermediate (35–59) length. In a survey of 2781 unselected Atlanta women (Sullivan *et al.*, 2005), 196 (7.0%) had lengths >40. Thirty-one (5.8%) of our LB controls had lengths >40. Thus, our data accord with previous studies and add information on the expected frequency of women with alleles of length 35–39.

We hypothesized that the maternal age association with trisomy is the result of a causal relation between intra- or extra-ovarian factors related to the size of the oocyte pool. The extent to which the results of the current analysis refute this hypothesis depends on the strength of the evidence relating intermediate CGG repeat length to POF. Of particular relevance are several studies that show moderate to strong associations between intermediate length and POF. Data from a cohort study (Sullivan *et al.*, 2005) comprising women from Fragile X families and women from the general population contribute only limited information because POF is rare: among women aged 40+, POF was reported by 1 of 112 women with length ≤40 versus 1 of 45 women with length of 41–58 [odds ratio = 2.5 (95% CI: 0.2, 41.2]. In Vancouver (Bretherick *et al.*, 2005; 53 POF cases, 161 in the primary control group), the authors analyzed their data using, variously, chromosomes and women as the unit of analysis. The first approach, which doubles the sample size, does not allow computation of appropriate CIs or significance tests; the reported odds ratio was 2.4 for length 35–54 (i.e. excluding premutations and full mutations). For biallelic means 35+, where the unit of analysis was women, the reported odds ratio is 3.4. Excluding from the analysis four women with premutations or full mutations does not materially change the odds ratio [odds ratio = 3.6, 95% CI: 1.2, 10.9 (our computation)], supporting a strong association of intermediate length with

Table IV Length of the CGG repeat at the *FMR1* locus by trisomy type: percent distribution of five categories of the biallelic mean among women with trisomic losses (cases) classified by trisomy type and women with live births for (1) the New York sample and (2) the New Jersey sample; adjusted odds ratios [95% confidence intervals (CI)] relating length in the longest and shortest categories to trisomy type.

	Losses			Births
	Trisomy 16	Other non-acrocentric trisomy	Acrocentric trisomy	
(1) The New York sample				
Number of women	14	7	15	50
Biallelic mean (%)				
19.0–22.0	7.1	0.0	0.0	6.0
22.5–26.0	7.1	14.3	13.3	18.0
26.5–30.0	35.7	57.1	66.7	50.0
30.5–35.0	28.6	14.3	20.0	20.0
35.5–59.5	21.4	14.3	0.0	6.0
(2) The New Jersey sample				
Number of women	41	40	78	487
Biallelic mean (%)				
19.0–22.0	4.9	0.0	1.3	4.7
22.5–26.0	19.5	15.0	17.9	19.3
26.5–30.0	46.3	52.5	48.7	46.2
30.5–35.0	24.4	25.0	23.1	24.6
35.5–59.5	4.9	7.5	9.0	5.1
Adjusted odds ratio (95% CI)[a]				
35.5–59.5 versus 26.5–30.0	1.5 (0.4, 5.0)	2.2 (0.7, 7.7)	1.6 (0.6, 4.1)	
19.0–22.0 versus 26.5–30.0	1.4 (0.4, 5.5)	NA	0.3 (0.0, 2.0)	

NA, not estimated because there were no other non-acrocentric trisomies with biallelic mean 19.0–22.0.
[a]Odds ratios from conditional maximum likelihood logistic regression. Analyses adjust by stratification for site (New York, New Jersey) and age in single years and by indicator variable for race (White non-Hispanic versus Other). Analyses exclude 12 women (2 New York, 10 New Jersey) with double trisomies.

POF. In Milan (Bodega *et al.*, 2006; 190 POF cases, 200 post-menopausal non-carrier controls aged >50), the odds ratio was 5.5 [95% CI: 1.2, 25.8 (our computation)] for lengths of 43–52. In a London and Salisbury sample (Bennett *et al.*, 2010), repeat length on both X chromosomes of 366 POF cases was compared with length on the single, untransmitted X chromosome of 2779 mothers screened because their sons had learning problems. In this study, to replicate previous analyses, intermediate length was defined both as 35–54 and 41–58. The authors, who report non-significant odds ratios of 0.9 and 1.3, respectively, for the two definitions, conclude that their data do not support an association of POF with intermediate length. However, we consider this analysis erroneous because the case and control groups were not analyzed in the same way. Each case contributed two chromosomes to the analysis, whereas each control contributed only one. Moreover, to estimate confidence intervals for the odds ratio, the correct unit of analysis is the woman, not the chromosome. The published data are not sufficiently detailed to limit the analysis to one chromosome per case to compare with one chromosome per control. However, assuming that individual cases contributed each of the intermediate length alleles, we estimate odds ratios of 1.8 and 2.6 for lengths of 35–54 and 41–58, respectively. Since some women may have two alleles in the intermediate range, these computations may slightly overestimate associations. Thus, this study supports a moderate association of POF with intermediate length.

Few studies have examined connections between intermediate CGG repeat length and other indicators of premature ovarian aging. In data from both The Netherlands and Atlanta, AMH levels were lower, suggesting smaller oocyte pools, in premutation carriers than in non-carriers. In their discussion, the authors note that AMH levels did not differ between women with intermediate lengths (35–45, 46–55) and women with lengths <45, although this result is based on an analysis with less robust adjustment for age and a smaller sample than the primary analysis (Spath *et al.*, 2011). This result contrasts with results from two studies of patients seeking infertility treatment. In a large Boston sample (Karimov *et al.*, 2011), women with occult POF (elevated FSH, elevated early follicular phase estradiol or poor response to gonadotrophin stimulation) were compared with controls (infertility patients not meeting the criteria for occult POF and oocyte donors). Intermediate length (45–54) was more common among cases [odds ratio 2.4, 95% CI: 1.0, 5.9 (our computation)]. This study limited the potential for selection bias by excluding women with family histories indicative of Fragile X syndrome from both case and control groups. Similar results obtained in a

smaller sample of Swiss patients (Streuli *et al.*, 2009), although the authors acknowledge potential selection bias because FMR1 testing was part of routine clinical care for women with occult POF.

In sum, several observations support an association of POF with intermediate CGG repeat length. Observations on other indicators of premature ovarian aging are few and inconsistent.

For premutations, the mechanism underlying associations with POF is not known. However, the association probably reflects the toxicity of mRNA on either the oocyte pool or follicle survival. This mechanism may be relevant to associations of intermediate CGG repeat length with POF. A study (Loesch *et al.*, 2007) shows increased mRNA transcriptional activity in males with intermediate (41–60) length.

The absence of a statistically significant association between trisomy and intermediate CGG repeat length may be interpreted in two ways. First, given the number of women with long repeats (e.g. 51 women with biallelic mean $\geq$35.5), we cannot rule out odds ratios $<$3. Risk ratios for associations between intermediate length and POF range from about 1.8 to 5.5. We expect associations with trisomy, if any, to be weaker than associations with POF because POF is a direct indicator of the size of the oocyte pool. Thus, if the true association of intermediate length with POF is on the order of 2–3 (obtained from our analysis of the data from the UK; Bennett *et al.*, 2010), then our study does not provide strong evidence against an association of intermediate length with trisomy. On the other hand, if the true association with POF is $>$3, as suggested by data from Vancouver and Milan, our data are inconsistent with associations of this magnitude for trisomy. Second, if our data show no association of trisomy with intermediate length then, contrary to our hypothesis, trisomy may not arise as a function of the size of the oocyte pool. This inference is compatible with our observation that elevated FSH, but not lowered AMH, is associated with trisomic spontaneous abortion. Since AMH is probably a better indicator of the size of the underlying oocyte pool than FSH, we think that our data do not support the limited oocyte pool hypothesis. Rather, they suggest that elevated FSH might alter the fidelity of meiosis (Kline *et al.*, 2010). [An alternative interpretation, that the quality of the entire antral follicle cohort or the quality of the dominant follicle affects trisomy risk, is not compatible with the observations that trisomy is unrelated to antral follicle count (Kline *et al.*, 2004) or to inhibin B (van Montfrans *et al.*, 2001; Kline *et al.*, 2004; Kline *et al.*, 2010)]. From a practical point of view, the small, non-significant association between CGG repeat length and trisomy indicates that there is no reason to add repeat length to the battery of screening tests for trisomic pregnancy.

Supplementary data

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

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Authors' roles

J.K. designed the study and analysis and wrote the manuscript. A.K. collaborated in the design of the study and analysis, carried out the statistical programming and helped write the manuscript. B.L. collaborated in the design of the study and the analysis and helped write the manuscript. S.B. collaborated in the design of the laboratory analyses and assessment of validity and reliability, oversaw the laboratory analyses for CGG repeat length and helped write the manuscript. K.O. collaborated in the design of the laboratory analyses, carried out the assays and collaborated in their interpretation. D.W. collaborated in the design of the study, oversaw the laboratory that karyotyped spontaneous abortion specimens, collaborated in the interpretation of results and writing of the manuscript.

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Conflict of interest

None declared.

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