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

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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 pre-term (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 S1a 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 ascertainment 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 S1b 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.

FMRI 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 FMRI 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 FMRI locus based on methylation sensitive restriction digestion and two-
Results

At the New Jersey site, the proportion with long CGG repeat length, whether defined by the biallelic mean ($\geq 35.5$), the genotypic mean ($\geq 35.5$) or the length on allele 2 ($\geq 35$), was higher for trisomy cases than for each of the comparison groups (Table II). The proportion with short length on allele 1 ($\leq 19$) 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),

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type.

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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 \( \approx 1.8 \); 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>
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<th>Losses</th>
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<td>Allele 1: 9–19</td>
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<td>6.2</td>
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apercent may not add to 100% due to rounding.
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., 2005), 196 (7.0%) had lengths >40. Thirty-one (5.8%) of our LB controls had lengths >40. Thus, our data accord 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 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. Of particular relevance are several studies that show moderate to strong associations between intermediate length and POF. Among women aged 40+, POF was reported by 1 of 112 women with length ≤26.5 (compared with 1 of 112 women with length ≥27.5) [odds ratio = 1.6 (95% CI: 0.5, 4.9)]. 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 2.5 (95% CI: 0.9, 6.6). Excluding from the analysis four women with premutations or full mutations does not materially change the odds ratio [odds ratio = 3.4 (95% CI: 1.9, 6.2)] [Sullivan et al., 2005]. 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 2.5 (95% CI: 0.9, 6.6). Excluding from the analysis four women with premutations or full mutations does not materially change the odds ratio [odds ratio = 3.4 (95% CI: 1.9, 6.2)] [Sullivan et al., 2005].

### Table III

<table>
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<th>Adjusted odds ratio (95% CI) for CGG repeat length: trisomy cases versus each comparison group</th>
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<td>Allele 2: 35–89 (versus 30)</td>
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*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).
POF. In Milan (Bodega et al., 2006; 190 POF cases, 200 postmenopausal 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

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<td></td>
<td>30.5–35.0</td>
<td>35.5–59.5</td>
<td></td>
</tr>
<tr>
<td>Adjusted odds ratio (95% CI)^a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35.5–59.5 versus 26.5–30.0</td>
<td>1.5 (0.4, 5.0)</td>
<td>2.2 (0.7, 7.7)</td>
<td>1.6 (0.6, 4.1)</td>
</tr>
<tr>
<td>19.0–22.0 versus 26.5–30.0</td>
<td>1.4 (0.4, 5.5)</td>
<td>NA</td>
<td>0.3 (0.0, 2.0)</td>
</tr>
</tbody>
</table>

^aOdds 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.

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.
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 ≥ 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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