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ORIGINAL ARTICLE ***Reproductive genetics***

Maternal plasma genome-wide cell-free DNA can detect fetal aneuploidy in early and recurrent pregnancy loss and can be used to direct further workup

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STUDY QUESTION: Can maternal plasma cell-free DNA (cfDNA) detect chromosomal anomalies in early pregnancy loss (EPL) and recurrent pregnancy loss (RPL)?

SUMMARY ANSWER: Genome-wide cfDNA testing can serve as an alternative to cytogenetic analysis in products of conception (POCs) in RPLs and can guide further management.

WHAT IS KNOWN ALREADY: Random chromosomal anomalies are the single most common cause for EPL and RPL. Cytogenetic analysis in POCs may be used to direct management in RPL because the detection of random chromosomal anomalies can eliminate further unwarranted testing.

STUDY DESIGN, SIZE, DURATION: This was a prospective diagnostic test study from March 2018 to January 2019 of 109 patients experiencing pregnancy loss before 14 weeks gestation at a tertiary-care academic medical center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Blood samples were drawn for genome-wide cfDNA testing prior to chorionic villous sampling for cytogenetic analysis of POCs with both short-term cultures (STCs) and long-term cultures (LTCs). Final analysis included 86 patients with non-mosaic cytogenetic results in POCs and available cfDNA results. Aneuploidy detection rates by cfDNA testing and POC cytogenetic analysis were compared. The first 50 samples served as the *Training Set* to establish pregnancy loss-specific log-likelihood ratio (LLR) thresholds using receiver-operator characteristic (ROC)-like analyses. These were then used for the entire cohort.

MAIN RESULTS AND THE ROLE OF CHANCE: Seventy-eight samples (71.5%) had results available from both STC and LTC; 12 samples (11%) had a result from STC only, and 7 samples (6.4%) had a result from LTC only. A chromosomal anomaly was detected in 55/86 (64%). The rates of chromosomal anomalies were 61, 72, 73 and 44% in patients undergoing their first, second, third and ≥4th pregnancy losses, respectively. The median cfDNA fetal fraction was 5%. With standard LLR thresholds used for noninvasive prenatal screening, the sensitivity of cfDNA in detecting aneuploidy was 55% (30/55) and with a specificity of 100% (31/31). Using pregnancy loss-specific LLR thresholds, the sensitivity of cfDNA in detecting aneuploidy was 82% (45/55), with a specificity of 90% (28/31). The positive and negative likelihood ratios were 8.46 and 0.20, respectively. Fetal sex was correctly assigned in all cases.

LIMITATIONS, REASONS FOR CAUTION: Cases with a false-positive result by cfDNA analysis would not receive the indicated RPL workup. Specificity could be improved by using a fetal fraction (FF) cutoff of 4%, but this would result in exclusion of more than a quarter of cases.

WIDER IMPLICATIONS OF THE FINDINGS: cfDNA-based testing can serve as an alternative to POC cytogenetic analysis and can guide further RPL management: if cfDNA demonstrates aneuploidy, no further action is taken and if no abnormality is detected, the recommended RPL workup is performed.

STUDY FUNDING/COMPETING INTEREST(S): Cell-free DNA testing was funded by Illumina, Inc., San Diego, CA. Y.Y. is a member of Illumina's Clinical Expert Panel and has received travel grants. A.B. has received travel grants from Illumina. All authors have no competing interest to declare.

Key words: cell-free DNA / noninvasive / recurrent pregnancy loss / aneuploidy / chromosome anomalies

Introduction

The etiological investigations of recurrent pregnancy loss (RPL) consists of peripheral karyotype analysis of the parents; screening for lupus anticoagulant, anticardiolipin antibodies and anti beta2-glycoprotein; sonohysterogram, hysterosalpingogram or hysteroscopy to assess the uterine cavity; and screening for thrombophilias, thyroid or prolactin abnormalities (RCOG, 2011; ASRM, 2012; ESHRE, 2018). This workup is costly and identifies an explanation in less than half of the cases (Popescu *et al*., 2018).

It is well established that 50–70% of early pregnancy losses (EPLs) are caused by numeric chromosomal anomalies, mostly trisomies, monosomies and polyploidy (Lathi *et al*., 2011; Goldstein *et al*., 2017; Soler *et al*., 2017). However, even in RPLs, random chromosomal anomalies constitute the single most common etiology (Stephenson *et al*., 2002; Lathi *et al*., 2011; Goldstein *et al*., 2017; Soler *et al*., 2017). Only ~4% are due to *unbalanced* chromosomal rearrangements inherited from a parent carrying a *balanced* chromosomal rearrangement (Stephenson and Kutteh, 2007; Jaslow *et al*., 2010; Tunc *et al*., 2016). Several studies have therefore suggested that product of conception (POC) karyotype analysis should be used to direct further management in RPLs, because the detection of random chromosomal anomalies could be cost-effective by eliminating further unwarranted testing (Bernardi *et al*., 2012; Petracchi *et al*., 2017; Popescu *et al*., 2018).

However, the success rate of POC karyotyping may be as low as 53% due to a 32% culture failure rate (Pauta *et al*., 2018) and a 15% rate of maternal cell contamination (MCC) (Lathi *et al*., 2014). A major problem for successful POC karyotyping, particularly for very early pregnancy losses, is actually identifying suitable material to test. The availability of POC specimens has also declined due to the increasing use of misoprostol for medical management of miscarriage. The use of chromosomal microarray (CMA) is associated with improved results (Pauta *et al*., 2018).

The current study was undertaken in order to assess whether maternal-plasma genome-wide cell-free DNA (cfDNA)-based testing can reliably detect chromosomal anomalies in random early and RPL.

Materials and Methods

Ethical approval

The study was approved by the Internal Review Board (IRB) at Hospital Clinic, Barcelona (IRB number HCB/2017/0726). All patients participating in the study gave their written informed consent.

Study design and participants

This was a prospective study performed at Hospital Clinic, Barcelona. During the study period (March 2018 to January 2019), all consecutive patients experiencing EPL were offered to participate. RPL was defined as ≥2 clinical pregnancy losses (ESHRE, 2018). Study inclusion criteria were as follows: (i) consenting patients, >18 years of age, undergoing EPL or RPL at a *sonographic gestational age* < 14 weeks; (ii) blood samples with sufficient volume for testing; (iii) non-mosaic cytogenetic results available from short-term culture (STC), long-term culture (LTC) or both; and (iv) available cfDNA results. Cases of confirmed mosaicism were removed from the analysis, as is common practice in validation studies (Bianchi *et al*., 2012; Lefkowitz *et al*. 2016), to allow determination of the technical capabilities of the assay. These cases were analyzed separately.

Ultrasound measurements

The standard protocol at the Hospital Clinic Barcelona for all patients experiencing EPL and RPL includes sonographic measurements of the gestational sac and the crown-rump length (CRL) when an embryonic pole is present. The *clinical gestational age* is estimated based on self-reported last menstrual period (LMP). The *sonographic gestational age* is calculated by CRL if a fetal pole was noted. Cases with an empty sac are assigned a *sonographic gestational age* of 5 weeks. The *estimated time-from-demise* is calculated as the *clinical gestational age* minus *sonographic gestational age*. Assessment for the presence of structural abnormalities is also performed after 10 weeks.

Cytogenetic analysis

As a routine practice at the Hospital Clinic Barcelona, POCs are obtained for cytogenetic analysis by chorionic villous sampling (CVS) using a Rodeck fine forceps, prior to medical or surgical evacuation of the uterus, as previously reported (Stergiotou *et al*., 2016; Soler *et al*., 2017). Cytogenetic analysis of POCs is performed at the Cytogenetic Laboratory, Hospital Clinic Barcelona, and includes karyotyping on both STC and LTC.

Blood tests

Patients with pregnancy loss usually undergo tests for complete blood count and clotting factors. For the purpose of this study, quantitative beta-human chorionic gonadotropin (beta-hCG) was also tested. An additional blood sample of 20 mL was drawn into Streck® tubes for genome-wide cfDNA testing, prior to CVS.

Cell-free DNA testing

Samples were submitted for the Verifi® Plus prenatal aneuploidy screening test at the College of American Pathologists-accredited and Clinical Laboratory Improvement Act-certified Illumina Laboratory (Verinata Health, Inc., a wholly owned subsidiary of Illumina, Inc.,

Redwood City, CA). Verifi® Plus involves isolation of plasma from maternal whole blood samples and preparation of sequencing libraries using TruSeq DNA Nano LP kit (Illumina, Inc., San Diego, CA). Sequencing was carried out using TruSeq SBS Kit v3-HS (50 cycles) and HiSeq 2000 instrument (96 samples-plex), with single-end reads of 36 base pairs obtained and an average of 22 million reads per sample. The sequence reads are mapped to the human reference genome (hg19) using the Bowtie software program (Langmead *et al*., 2009). Data are filtered to remove nonunique alignments and high-variation genomic regions. A log-likelihood ratio (LLR) score is then calculated by evaluating the likelihood of the observed sequence data under two competing hypotheses of 'no aneuploidy present' and 'aneuploidy present' for each chromosome; LLR scores consider the observed coverage and the estimated fetal fraction (FF) for the sample in question. Aneuploidy classification status for all chromosomes is then determined by comparing the LLR against a classification threshold; a score above the LLR threshold indicates the presence of aneuploidy. This test screens for the presence of aneuploidy on all 22 autosomes as well as for sex chromosome aneuploidy and reports sample FF. FF is determined using the SeqFF method developed by Kim *et al*. (2015). This method estimates FF by counting the number of reads aligned within specific autosomal regions and applying a weighting scheme derived from a multivariate model. FF is estimated by inferring discrete regions in the genome that are overrepresented in fetal cfDNA. Verifi® Plus uses a sample-specific quality control (QC) metric known as the *individualized Fetal Aneuploidy Confidence Test* (iFACT) that considers the estimated FF to determine if the system has generated sufficient sequencing coverage for each sample; samples that fail to meet this threshold do not report out a result.

Determining pregnancy loss-specific LLR thresholds

In ongoing pregnancies, even those at high risk, the likelihood of a fetal chromosome anomaly is much lower than among patients experiencing pregnancy loss. Thus, for noninvasive prenatal screening (NIPS), LLR thresholds are set relatively high to eliminate false-positive results. In contrast, in early pregnancy loss more than half of cases are expected to be aneuploid. Therefore, pregnancy loss-specific exploratory LLR thresholds were established to increase sensitivity while still maintaining a low false positive rate. These were determined using a receiver-operator characteristic (ROC)-like analyses (Supplementary Fig. S1). The first 50 samples served as the *Training Set* to establish a single LLR threshold for all trisomy events, a second threshold for all monosomy events and third for 45,X and 47,XXX. After applying these thresholds to the entire cohort, we slightly modified the LLR threshold for trisomy 16 to improve sensitivity without compromising specificity.

Statistical analysis

Maternal and pregnancy characteristics and data obtained from the first-trimester ultrasound examination were entered in the Statistical Package for the Social Science (SPSS) database (SPSS, Chicago, IL, USA), which was then used for statistical analyses. When missing at random, data were imputed using multiple imputations by marginal long style creating 40 subsets including all predictors, outcomes and passive variables in the analysis. The Kolmogorov–Smirnov test was used to demonstrate whether variables were normally distributed. Normally distributed variables were compared using *T* test and expressed as mean and standard deviation, while not normally distributed were compared using the Mann–Whitney test and expressed as median and interquartile range (IQR) or range. To determine significant differences between groups, chi-square statistics or the Fisher exact test was used to examine differences between proportions. Test for trend across ordered group was calculated by Wilcoxon-type test for trends using a nonparametric approach. Test performance was expressed as sensitivity, specificity and AUC and was compared using the DeLong test. For this proof-of-concept study, a sample size of 100 patients was chosen assuming at least 50% would have a chromosomal anomaly. A P value <0.05 was considered statistically significant. Error values shown in the text are SD.

Results

During the study period, 118 consecutive patients experiencing EPL or RPL were offered to participate in the study; 9 patients declined participation. Among the 109 consenting patients, the success rates of the cytogenetic analysis were as follows: 78 samples (71.5%) had results available from both STC and LTC; 12 samples (11%) had a result from STC only; and 7 samples (6.4%) had a result from LTC only. Of the 97 cases with cytogenetic results, there were 9 cases with mosaicism, which were not included in the final analysis. Of the remaining 88 cases, 2 did not receive cfDNA results: in one case, the tube was broken during shipping and another sample failed the presequencing DNA quantification QC threshold. The final analysis included 86 cases with complete non-mosaic cytogenetic results and available cfDNA results. The median maternal age was 37 years (range: 21–46 years). For 41 patients (48%), this was the first pregnancy loss; 25 (29%) were undergoing their second loss, 11 (13%) were having their third loss and 9 (10%) experienced four or more losses. The mean *clinical gestational age* was 9.6 ± 1.9 weeks (range: 5.1–13.6 weeks) and the mean *sonographic gestational age* was 6.4 ± 1.6 weeks (range: 5.0–11.3 weeks). The mean *estimated time from embryo demise* was 3.3 ± 1.9 weeks.

A chromosomal anomaly was detected in 64% (55/86). Patient characteristics among those with and without a chromosomal anomaly are presented in Table I. As expected, the rate of chromosomal anomalies increased with maternal age, from 25% (1/4) in patients 25 years of age or younger to 90% (9/10) in patients over 40 years of age (Table II). The same trend was observed when restricted to RPL patients. The rate of chromosomal anomalies was not significantly different between RPL patients (30/45, 67%) and those experiencing their first pregnancy loss (25/41, 61%; chi-square statistic 0.3014, $P = 0.583$) (Supplementary Table SI).

With standard LLR thresholds commonly used for NIPS, the sensitivity of cfDNA in detecting aneuploidy in pregnancy loss was 55% (30/55) with a specificity of 100% (31/31) and an accuracy of 71% (61/86) (Table III). The first 50 cases were used as a *Training Set* to establish pregnancy loss-specific LLR thresholds. A uniform threshold was set at 1.5 for all autosomal trisomies, a threshold of 3.5 for all autosomal monosomies and a threshold of 0.88 for 45,X and 47,XXX. After applying these LLR thresholds to the entire cohort, the LLR threshold for trisomy 16 was reset at 1.0, to improve sensitivity without compromising specificity. Using these LLR thresholds,

Table I Patient and pregnancy characteristics.

	Total ($n = 86$)	Chromosomally abnormal ($n = 55$)	Chromosomally normal ($n = 31$)	*P* value
Maternal age* (years)	37 (IQR: 5)	38 (IQR: 5)	35 (IQR: 8)	0.015
BMI†	24.1 ± 4.1	24.7 ± 4.3	23.1 ± 3.4	0.090
Recurrent loss (%)	45 (52.3%)	30 (54.5%)	15 (48.4)	0.583
Clinical gestational age† (weeks)	9.6 ± 1.9	9.7 ± 1.7	9.3 ± 2.1	0.355
Sonographic gestational age† (weeks)	6.4 ± 1.6	6.5 ± 1.5	6.3 ± 1.6	0.460
Time from embryo demise† (weeks)	3.3 ± 1.9	3.3 ± 1.9	3.2 ± 1.9	0.899
Bleeding	36 (41.9%)	26 (42.3%)	10 (32.3)	0.175
Sac volume* (cm)	11.7 (IQR: 26)	10.9 (IQR: 26)	12.4 (24.5)	0.956
hCG* (mIU/mL)	20 184 (IQR: 28752)	18 750 (IQR: 29114)	23 552 (IQR: 24729)	0.590
Medical management (%)	46 (53.5%)	31 (56.4%)	15 (48.4%)	0.401
Fetal fraction* (%)	5% (IQR: 4%)	5% (IQR: 3%)	5% (IQR: 4%)	0.446

*Values are median and interquartile range (IQR)
†Values are means ± SD

Table II Rate of chromosomal anomalies in all patients and in patients with RPL according to age groups (at 5-year intervals).

Age range	All patients*	Patients with RPLs†
≤25	1/4 (25%)	0/0
26–30	3/6 (50%)	2/4 (50%)
31–35	11/21 (52%)	8/14 (57%)
36–40	31/45 (69%)	14/20 (70%)
>40	9/10 (90%)	6/7 (86%)
Total	**55/86 (64%)**	**30/45 (67%)**

RPL, recurrent pregnancy loss
*$Chi^2 = 7.777$; *P* value = 0.100.
Test for trend across groups rank-sum test: $z = 2.66$; $P = 0.008$.
†$Chi^2 = 2.3143$. *P* value = 0.510.
Test for trend across groups rank-sum test: $z = 1.49$; $P = 0.136$.

the sensitivity of cfDNA in detecting aneuploidy was 82% (45/55), with a specificity of 90% (28/31). The area under the curve (AUC) was 86% (79–94%), which correctly classified 85% (73/86) of the observations (Table II). The positive and negative likelihood ratios were 8.46 and 0.20 respectively. Of the 31 normal cases, 28 were correctly assigned, including 14/16 of the 46,XX cases and 14/15 of the 46,XY cases. Among the three false positive cases, two had a trisomy 4 result and one case had a result of triple trisomy for chromosomes 15, 17 and 20 (Supplementary Table SII). Fetal sex was correctly assigned in all concordant cases as well as among the 13 discordant cases.

While most of the affected cases had a single anomaly, two cases had a double trisomy: one case of 48,XX,+15,+21 was correctly detected and another case of 48,XX,+7,+22 was a false negative. An additional case had both triploidy and trisomy 16 (70,XXY,+16) wherein only trisomy 16 was detected by cfDNA. In the entire cohort, there was only one case of an unbalanced chromosomal rearrangement: an unbalanced Robertsonian translocation resulting in trisomy 15. Parental karyotyping performed in this case was negative. Of the nine mosaic cases, only one was correctly ascertained (Supplementary Table SIII).

The median FF was 5% (range <1 to 28%), the highest level detected in a case of triploidy. Among the 73 correctly classified cases, the median FF was 5% (IQR: 3%), compared to 4% (IQR: 2%) among the 13 incorrectly classified ones (Supplementary Table SII, Mann–Whitney *U* test, $P = 0.169$). Performance was evaluated with different possible FF cutoffs (<1, <2, <3 and <4%), below which cases would be excluded (Supplementary Table SIV). No improvement in sensitivity was noted for any of these thresholds compared to using no threshold at all. An improvement was noted in specificity only when a threshold of <4% FF was employed. This however resulted in exclusion of over one-quarter of samples. Similarly, we found no clinically useful cutoff value for beta-hCG levels, patient BMI or sac size. The sensitivity, specificity and AUC were similar in patients with and without bleeding at the time of testing (sensitivity 81 vs. 83%; specificity 91 vs. 90%; AUC: 85 vs. 87%, respectively; DeLong test: $P = 0.877$).

Discussion

In this study, 109 patients experiencing early pregnancy loss were evaluated for chromosomal anomalies by cytogenetics of POCs and maternal plasma cfDNA analysis. Of these, 86 patients met the inclusion criteria. The rate and type of chromosomal anomalies in this cohort concurs with other similar studies, as did the sex distribution, indicating that it is appropriately representative (Jaslow *et al.*, 2010, Lathi *et al.*, 2011; Bernardi *et al.*, 2012; Tunc *et al.*, 2016; Goldstein *et al.*, 2017; Soler *et al.*, 2017; Popescu *et al.*, 2018). Cell-free DNA testing achieved a sensitivity of 82% and a specificity of 90%, an overall accuracy of 85%. This rate compares favorably with that of routine cytogenetic analysis of POCs (Pauta *et al.*, 2018). It is of note that pregnancy loss-specific LLR thresholds had to be established to achieve this rate of detection.

To the best of our knowledge, this is the first systematic study of its kind. A few small studies evaluated the levels of fetal cfDNA in EPLs.

Table III Detection rates for EPLs using maternal plasma cfDNA: standard log likelihood ration (LLR) thresholds vs. pregnancy loss-specific LLR thresholds.

Karyotype	Detected by standard LLR thresholds	Detected by pregnancy loss-specific LLR thresholds
Trisomy 2	0/1	1/1
Trisomy 4	0/1	1/1
Trisomy 7	1/1	1/1
Trisomy 9	2/2	2/2
Trisomy 10	3/4	3/4
Trisomy 11	1/1	1/1
Trisomy 12	2/2	2/2
Trisomy 13	1/2	2/2
Trisomy 14	1/1	1/1
Trisomy 15	3/5	5/5
Trisomy 16*†	4/10	8/10
Trisomy 17	0/1	1/1
Trisomy 18	2/2	2/2
Trisomy 20	2/4	3/4
Trisomy 21	2/2	2/2
Trisomy 22	1/4	3/4
Monosomy X	4/7	6/7
Monosomy 21	0/1	0/1
Double trisomy	1/2	1/2
Triploidy*	0/2	0/2
Total	30/55 (55%)	**45/55 (81.8%)**

EPL, early pregnancy loss
cfDNA, cell-free DNA
*In a case of 70,XXY,+16 the trisomy 16 was detected but the triploidy was not
†One case of trisomy 16 was called 47,XXX

One study found that levels of fetal cfDNA and total cfDNA were significantly higher in patients with pregnancy loss (both euploid and aneuploid) in comparison with the normal controls (Lim *et al*., 2013). Another prospective study examined whether cfDNA can be used for diagnosis in nonviable pregnancies at *all* gestational ages and found that 76% had FFs within the detectable range (>3.7%) (Clark-Ganheart *et al*., 2015). They recommended that cfDNA be used only after 8 weeks of gestation when FF is expected to be above their suggested cutoff in most cases. In that study, however, prior cytogenetic analysis or analysis of POCs was only available in 38% of cases. In contrast, we found no clinically useful cutoff for exclusion of low FF cases and cfDNA testing yielded results even before 8 weeks and at low FFs. This is in accordance with previous publications demonstrating that genome-wide sequencing for cfDNA analysis has >80% sensitivity for trisomies even in low FF samples (Artieri *et al*., 2017).

The high rate of successful genetic POC analysis (97/109, 89%) achieved here was through a combination of meticulous CVS prior to uterine evacuation, followed by cytogenetic analysis of both STC and LTC. This degree of lab effort is not characteristic of most routine centers, and the realistic success rate of karyotype analysis of POCs may be as low as 53% (Pauta *et al*., 2018). Other limitations of cytogenetic analysis of POCs include failed culture due to contamination or the extraction of nonviable tissue**.** This may be overcome, to some extent, by the use of SNP-based chromosomal microarray analysis (CMA) that obviates the need for culture while concurrently detecting MCC (Liu *et al*., 2015; Pauta *et al*., 2018). Consistent with this, the aforementioned meta-analysis showed that CMA improved POC analysis success rates by 27% (from 68 to 95%). However, the incremental yield of CMA in detecting pathogenic submicroscopic variants undetectable by karyotyping was only 2% (106/5507) (Pauta *et al*., 2018).

Regardless of the technology used for POC analysis, obtaining a POC sample is challenging: some patients miscarry before a proper sample is obtained. Moreover, many patients with an early missed abortion now undergo medical management using misoprostol, rather than surgical extraction (Wu *et al*., 2017). Indeed, in our series, 55% of the patients were managed medically. For such cases, a tissue-collection kit has been developed but its success rate in obtaining a proper sample is lower than that of surgical extraction (84 vs 100%, respectively) (Kucherov *et al*., 2018).

The etiological investigations of RPLs have traditionally not centered on the abortus. Except for karyotyping *both* parents to screen for chromosomal rearrangements, most have focused on defects in the *female partner*, namely congenital or acquired uterine abnormalities, autoimmune factors, endocrine imbalances and thrombophilia (Jaslow *et al*., 2010, Stephenson and Kutteh, 2007). More recently, several groups challenged this approach and promoted the concept that RPL workup should be guided by genetic analysis of POCs (Bernardi *et al*., 2012; Kutteh 2015; Petracchi *et al*., 2017; Popescu *et al*., 2018)**.** Their suggested model stipulates that genetic analysis of POCs be performed in the second and subsequent pregnancy loss: if aneuploidy is demonstrated, no further evaluation is needed; if an unbalanced chromosomal rearrangement (such as a translocation or inversion) is detected, parental karyotyping is performed; and if no chromosomal aberration is detected and MCC has been ruled out, then the American Society for Reproductive Medicine (ASRM) RPL workup should be performed. With this approach, a definitive or probable cause for RPL could be identified in 95% of cases, compared with 45% using the classical approach (Popescu *et al*., 2018). This approach has been shown to produce cost savings of around $1100 per case by eliminating unnecessary investigations (Wolf and Horger, 1995; Bernardi *et al*., 2012; Petracchi *et al*., 2017). Finally, Popescu *et al*. (2018) calculated that if POC genetic analysis was used to guide workup, the total cost to make a diagnosis would be $1879.16 per patient whereas if the ASRM RPL evaluation were performed for all RPL patients, the total cost to make a diagnosis would be $3866.84 per patient.

Based on our results and these cost-effectiveness analyses, we suggest an alternative, modified algorithm for RPL evaluation, guided by cfDNA results rather than karyotyping or CMA analysis of POCs (Fig. 1): if cfDNA in the second and subsequent RPL demonstrates aneuploidy, no further action is taken; and if no abnormality is detected, then the recommended RPL workup is performed. While speculative as regards this cohort, cfDNA testing can also detect unbalanced rearrangements such as unbalanced reciprocal translocations, provided they are of a substantial size (Lefkowitz *et al*., 2016; Van Opstal *et al*., 2017; Wapner *et al*., 2015; Hu *et al*., 2019)**.** When detected, this would be the only direct prompt for parental karyotyping. If an unbalanced rearrangement is not detected by cfDNA testing, parental karyotyping would be performed, at any rate, as a part of the recommended RPL

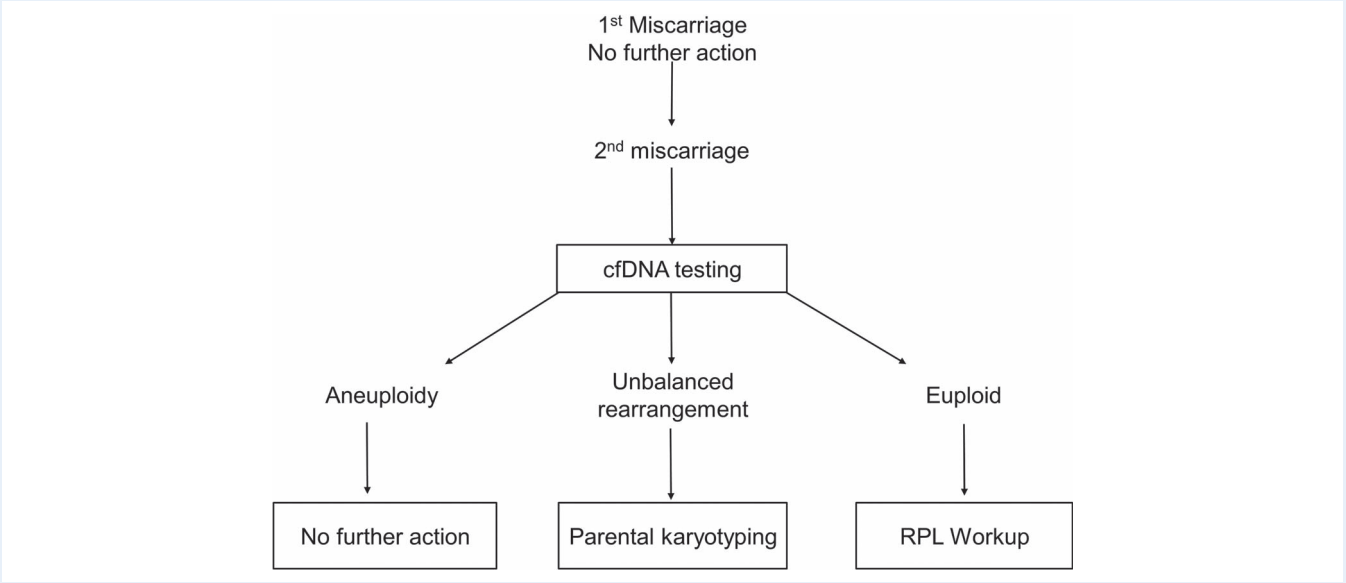

Figure 1 Proposed algorithm for recurrent pregnancy loss workup based on cfDNA results. cfDNA, cell-free DNA; RPL, recurrent pregnancy loss.

workup, and thus a carrier parent would not be missed. For trisomies involving the acrocentric chromosomes (13, 14, 15, 21 and 22), one could still consider the rare occurrence of an unbalanced Robertsonian translocation, which may be detected on parental karyotyping, but these would account for ~0.5% of cases (Soler *et al*., 2017)**.** A result compatible with other random trisomies (such as the common trisomy 16) would clearly obviate parental karyotyping.

In our series, a positive result for a chromosomal aberration was given in 48 cases (56%), 45 of which were true positives, for a positive predictive value (PPV) of 94%. This implies that in more than half of the cases, the RPL workup would be averted, because a definite cause for miscarriage had been revealed. Conversely, in those with a negative result, the yield of standard RPL workup would expectedly be higher.

Nonetheless, the approach suggested in this proof-of-concept study has several limitations: an important implication is that cases with a false-positive result by cfDNA analysis would not receive the indicated RPL workup, and a biological explanation for the loss may not be identified. A larger cohort may promote additional refinement of chromosome-specific LLR thresholds to further improve cfDNA test performance. Specificity could be improved by using an FF cutoff of 4%, but in this case more than a quarter of the cases would be excluded.

This approach is also unable to discriminate true fetal monosomy X from low-grade, maternal monosomy X mosaicism, which increases with maternal age (Machiela *et al*., 2016). This is a potential source of false positive results in as much as 8.6% of sex chromosome anomalies reported by NIPS (Wang *et al*., 2014). This issue may be overcome by employing paired-end sequencing, which enables determination of cfDNA fragment length, as fetal cfDNA fragments are generally shorter than the corresponding maternal ones (Yu *et al*., 2014). This methodology allows deduction of FF as well as improvement in the sensitivity and specificity of autosomal aneuploidy as well as of monosomy X. Another limitation of genome-wide cfDNA sequencing is that it does not detect triploidy, the incidence of which was 3.4% in our series but may account for 8% of cases (Soler *et al*., 2017). Such missed cases of triploidy however would undergo the standard RPL workup with no contribution to cost-savings. Despite these limitations, cfDNA testing could prove to be of value particularly in cases where medical management is used and in the absence of POC sample for cytogenetic analysis.

Finally, cfDNA testing may also be used in sporadic EPLs. While not significantly impacting further clinical management, it is likely to have a positive influence on patient well-being. The psychological impact of EPL is often neglected. Farren *et al*. have recently found evidence of significant depression and anxiety in the first month following EPL in women (Farren *et al*., 2018). For some patients, knowing that the cause for loss is chromosomal rather than maternal may provide comfort. In summary, genome-wide cfDNA-based screening provides a noninvasive approach for determining whether fetal aneuploidy could explain the loss in patients experiencing early or RPL.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Y.Y.: substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the

version to be published. **M.P.**: substantial contributions to conception and design, acquisition of data and analysis and interpretation of data; revising it critically for important intellectual content; and final approval of the version to be published. **C.B.**: substantial contributions to acquisition of data revising it critically for important intellectual content; and final approval of the version to be published. **A.S.**: substantial contributions to acquisition of data; revising it critically for important intellectual content; and final approval of the version to be published. **V.B.**: substantial contributions to acquisition of data; revising it critically for important intellectual content; and final approval of the version to be published.**C.I.**: substantial contributions to acquisition of data; revising it critically for important intellectual content; and final approval of the version to be published. **F.P.y.M.**: substantial contributions to acquisition of data; revising it critically for important intellectual content; and final approval of the version to be published. **R.M.-P.**: substantial contributions to acquisition of data, and analysis and interpretation of data; revising it critically for important intellectual content; and final approval of the version to be published. **A.B.**: substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published.

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Conflict of interest

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