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RESEARCH ARTICLE

Non-invasive cell-free DNA-based approach for the diagnosis of clinical miscarriage: A retrospective study

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Abstract

Objective: To evaluate cell-free DNA (cfDNA) testing as a non-invasive approach to detecting aneuploidies in clinical miscarriages.

Design: A retrospective cohort study of women with pregnancy loss.

Setting: Hospitals and genetic analysis laboratories.

Population or sample: Pregnancy losses in the period 2021–2022.

Methods: Results derived from non-invasive cfDNA testing (Veriseq NIPT Solution V2) of maternal blood and invasive analysis of products of conception (POC) (Ion ReproSeq) compared in 120 women who suffered a miscarriage.

Main outcome measures: Concordance rate results, cfDNA testing performance, non-informative rate (NIR) and fetal fraction (FF).

Results: We found no significant differences in the NIR between invasive (iPOC) and non-invasive (niPOC) analysis of POC (10.0% [12/120] versus 16.7% [20/120]). Of 120 samples, 90 provided an informative result in iPOC and niPOC groups (75%). cfDNA analysis correctly identified 74/87 (85.1%) samples (excluding triploidies). Sensitivity and specificity were 79.4% and 100%, respectively; all discordant cases were female. A binomial logistic model suggested fetal sex as the only variable influencing the concordance rate ($P = 0.035$). A Y-chromosome-based FF estimate allowed the optimal reclassification of cfDNA of non-informative male fetuses and a more accurate evaluation of cfDNA testing performance. The difference between the two FF estimates (native algorithm and Y-chromosome-based) suggests that female non-concordant cases may represent non-informative cases.

Conclusions: Cell-free DNA-based testing provides a non-invasive approach to determining the genetic cause of clinical miscarriage.

KEYWORDS
cell-free DNA, chromosomal abnormalities, miscarriage, non-invasive, POC

1 | INTRODUCTION

Spontaneous abortion (or miscarriage) is defined as pregnancy loss before 20–24 gestational weeks, with ~23 million instances occurring yearly worldwide.[1] We currently lack a consensus regarding the number of pregnancy losses required to meet the recurrent miscarriage criteria – values range from two clinical miscarriages (American Society for Reproductive Medicine (ASRM)[2] and European Society for Human Reproduction and Embryology[3]) to three consecutive pregnancy losses (Royal College of Obstetricians and Gynaecologists[4]).

Studies of recurrent pregnancy loss (RPL) have identified causative factors, including parental chromosomal translocations, congenital/acquired uterine abnormalities, endocrine disorders, autoimmune factors, and infectious and thrombophilic causes.[5–15] Chromosomal abnormalities occur in ~60% of miscarriages[16] and <1% of live births

when prenatal diagnosis is not performed.[17] Cytogenetic analyses have traditionally determined genetic causes for miscarriage and the recurrence risk[18,19]; however, they depend on cell culture of products of conception (POC) and a well-standardised methodology in routine laboratory use.[20] While representing the gold standard for studying structural rearrangements, karyotyping suffers from limited resolution in detecting copy number variations (CNVs) below 5–10 Mb,[21,22] a high failure rate (10–40%) due to poor tissue quality, and a significant lag (2–6 weeks) in obtaining results.[18]

The informative rate for POC analysis has increased to ~80% following the advent of DNA-based analytical methods[23–29]; however, molecular/cytogenetic approaches require fresh, uncontaminated and unfixed tissue to identify fetal tissue and perform DNA extraction/cell culture, which carries the potential risk of maternal cell contamination (MCC) and misdiagnosis.[30] POC availability has recently decreased due to increased misoprostol use for miscarriage management,[31,32] making it challenging to use previously noted methods to determine chromosomal causes of early pregnancy loss (EPL) or RPL.[33]

Cell-free DNA (cfDNA) analysis represents an alternative to conventional diagnostic methods. Lo et al.[34] discovered cfDNA in the plasma of pregnant women in 1997, paving the way for cfDNA-based non-invasive prenatal testing (NIPT). This highly utile approach can screen for common aneuploidies and detect rare autosomal aneuploidies/CNVs. As numerical chromosomal abnormalities cause 50–70% of EPL cases (most commonly trisomies and monosomies[35–37]), cfDNA-based analyses have provided proof of concept for investigations into the aetiology of EPL and RPL.[30,32,38]

Despite recent advances, little research has been carried out for non-viable pregnancies; therefore, we sought to explore the potential for chromosomal abnormality detection in sporadic EPL and RPL via cfDNA-based testing.

2 | METHODS

2.1 | Ethical approval

The study was reviewed by the Institutional Review Board (IRB) of the INCLIVA Health Research Institute, and a decision was made on 28 July 2022. Each provider ensured compliance with their own internal ethics and the Ethics Regulation of the clinical diagnosis approved in Spain.

2.2 | Study design and participants

This observational national retrospective study was conducted at the Igenomix facilities in Valencia (Spain) between February 2021 and July 2022 with samples collected in 24 collaborating centres. Eligibility criteria included naturally conceived or assisted reproductive technology-conceived pregnant women over 18 years of age who suffered from spontaneous abortion in the first (≥5 weeks of pregnancy) or second trimester of pregnancy (16 weeks of pregnancy) (for more details on the gestational age, see Table S1). Samples were analysed if they had a next-generation sequencing (NGS) result from the corresponding invasive POC (iPOC) analysis. Exclusion criteria were: (i) missed miscarriage, (ii) vanishing twin pregnancies, (iii) pregnant women with a known immunological disorder, (iv) patients with an altered unbalanced karyotype (i.e., 45, X0 or 47, XXX), and (v) patients with an active neoplastic process.

2.3 | Sample collection

POCs were collected according to the clinician's routine clinical practice. Up to 10 ml of maternal blood was collected in cfDNA BCT® tubes (Streck) for cfDNA analysis and MCC assessment. The POC kit instructions highlight the importance of collecting maternal plasma samples before the expulsion of the fetal remains; however, most collaborating centres did not record this information on the test requisition form. Therefore, this variable could not be included in the study.

2.4 | POC processing and NGS approach (iPOC analysis)

Fetal and maternal tissues were visually identified whenever possible. Samples were cleaned, two sections were taken and DNA was extracted using the Qiagen QIAamp DNA extraction kit. The short tandem repeat (STR) AmpFISTR Identifiler Plus protocol (Life Technologies) was used to detect/rule out MCC and some polyploidies. Genetic testing for POC was performed using the S5 PGS Assay (NGS) for 24 chromosomes to detect numerical chromosomal abnormalities. The Ion ReproSeq PGS kit was used for 24-chromosome aneuploidy screening (Thermo Fisher Scientific). Data analysis was performed using the ION REPORTER software (IRv5.16) (Thermo Fisher Scientific), which aligns reads using the human genome assembly (hg19) (Thermo Fisher Scientific) and a proprietary bioinformatics pipeline (v2.0).

2.5 | cfDNA testing (niPOC analysis)

Cell-free DNA testing for non-invasive POC (niPOC) analysis was performed by genome-wide sequencing using proprietary protocols and data analysis algorithms provided by Illumina (VERISEQ v2 solution). The cfDNA was extracted from 1 ml of plasma using a modified QIAamp DNA blood mini kit (Qiagen) protocol. Sample indexing and library preparation were performed using a TruSeqNano DNA sample preparation kit (Illumina) and then analysed using paired-end technology on a NextSeq 500 system (Illumina). The fetal fraction (FF) was calculated using two estimates: one provided by Illumina's algorithm (FF_i), which considers both the cfDNA fragment size distribution and differences

in genomic coverage between maternal and fetal cfDNA, and another based on normalised chromosome values (NCV) of the Y-chromosome (see Equation 1) for male fetuses only.[39]

$$FF_{ChrY} = (NCVY \bullet 0.0005) \bullet 100 \quad \text{(Eqn 1)}$$

In the main text, FF_r refers to the recalculated FF considering both estimates, i.e. the FF for female fetuses is taken from the native algorithm, whereas the FF for male fetuses is the value calculated with Eqn 1.

Data analysis was performed using Illumina software to determine POC classification. To make chromosomal representation calls, the VERISEQ NIPT ASSAY SOFTWARE v2 uses the individualised Fetal Aneuploidy Confidence Test (iFACT), a dynamic threshold metric that indicates whether the system has generated sufficient sequencing coverage, given the FF estimate for each sample. If a sample does not meet this threshold, the quality control (QC) assessment displays 'FAILED iFACT' and is considered a non-informative result. Similarly, all cases with an FF_{ChrY} of less than 2% were classified as non-informative. In addition to iFACT, the VERISEQ NIPT ASSAY SOFTWARE v2 assesses several additional QC metrics during analysis. The additional metrics include assessments of coverage uniformity on reference genome regions and the distribution of cfDNA fragment lengths. The QC assessment displays either a QC flag or failure for any metrics outside the acceptable range. In the case of QC failure, the system does not generate a sample result. Based on the NCVs and T-score values, samples were classified as screen-negative (no aneuploidy detected) or screen-positive (aneuploidy detected).

2.6 | Statistical analysis

Variables were expressed as the median and interquartile range (IQR). Binomial logistic regression was used to determine the effect of qualitative variables (presence of anomaly and fetal sex) and quantitative variables (gestational age [GA], maternal age [MA] and FF) on the concordance rate between the niPOC and iPOC approaches. Test performance was assessed by estimating the values of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Statistical analyses were performed using XLSTAT software (Addinsoft).

3 | RESULTS

We simultaneously analysed 120 POCs by low-pass NGS using both direct tissue DNA (iPOC) and maternal blood cfDNA (niPOC). Table 1 summarises baseline patient characteristics stratified by the informativity results. There were no differences in non-informativity rates between iPOC and niPOC (10.0% [12/120] versus 16.7% [20/120]). We found a lower FF value estimated by the native algorithm (FF_i) or Y-based chromosome (FF_r) in non-informative niPOC cases (FF_i: 6.0% versus 2.7%; FF_r: 5.9% versus 1.4%). Of the 20 non-informative niPOC cases, 17 were male (85.0% [17/19]) and three female (15.0% [3/20]). FF disaggregation by GA suggested that the time of fetal arrest may not determine informativeness (Figure 1). We observed no noticeable differences between the MA and GA values of the involved patients.

We evaluated concordance rates between iPOC and niPOC after removing the non-informative cases (Table 2) and found no differences for FF, GA (Figure 2) or MA values (Table 2). The iPOC detected specific abnormalities: trisomy 13, 18 or 21 ($n = 13$); monosomy X ($n = 12$); other trisomies ($n = 34$); CNVs ($n = 1$); multi-aneuploidy ($n = 3$); and triploidy ($n = 3$) (Table S1). niPOC correctly identified 74/90 (82.2%) samples, including 50 abnormal and 24 normal cases (Table S1) but did not correctly classify 16 abnormalities: monosomy X ($n = 3$), trisomy 13 ($n = 1$), trisomy 4 ($n = 1$), trisomy 11 ($n = 1$), trisomy 16 ($n = 1$), trisomy 20 ($n = 1$), trisomy 22 ($n = 5$) and triploidy ($n = 3$) (Table S1). Fetal sex was correctly assigned in all cases. All discordant cases (except triploids) were female, with a median FF_i of 4.6%. A univariate model suggested that fetal sex influenced discordance

TABLE 1 Comparison of demographics between informative and non-informative cases within the niPOC and iPOC cases.

	iPOC approach		niPOC approach	
Variable	**Informative ($n = 108$)**	**Non-informative ($n = 12$)**	**Informative ($n = 100$)**	**Non-informative ($n = 20$)**
MA	36.0 (33.8–39.0)	38.5 (37.0–39.3)	37.0 (34.0–39.0)	36.0 (32.5–39.0)
GA	8.0 (7.0–9.4)	8.0 (7.6–8.5)	8.0 (7.0–9.3)	8.0 (7.5–9.0)
FF_i	5.6 (3.9–8.9)	3.6 (3.0–5.3)	6.0 (4.1–9.0)	2.7 (2.3–4.3)
FF_r	5.0 (3.0–8.2)	3.5 (2.5–5.3)	5.9 (4.0–8.8)	1.4 (0.6–1.7)
Fetal sex				
Male, *n* (%)	51 (47.0)		38 (38.0)	17 (85.0)
Female, *n* (%)	57 (53.0)	12 (100)	62 (62.0)	3 (15.0)

Note: Data presented as median (interquartile range) or *n* (%) unless otherwise specified.

FF_i, fetal fraction estimated by the Illumina algorithm; FF_r, FF recalculated (FF for female fetuses taken from the native algorithm and FF for male fetuses based on the Y-chromosome); GA, gestational age; iPOC, invasive products of conception (low-pass NGS); MA, maternal age; niPOC, non-invasive products of conception (cfDNA testing).

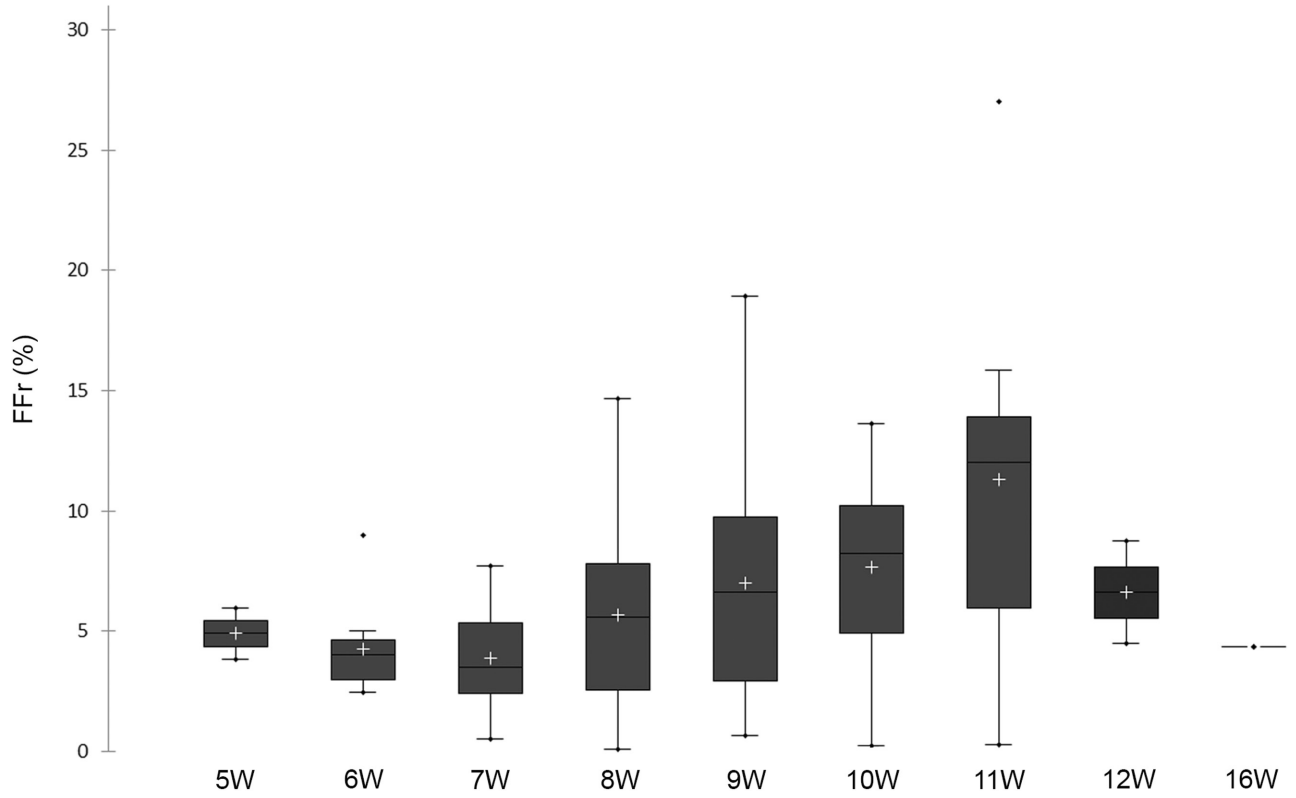

FIGURE 1 Fetal fraction variation according to gestational age. No significant differences were found in FF among the different GAs studied, suggesting that the time of fetal arrest does not influence the informativeness of the results. FF_r: FF recalculated (FF for female fetuses taken from the native algorithm, and FF for male fetuses based on the Y-chromosome); W, weeks.

TABLE 2 Comparison of demographics between concordant and non-concordant cases.

Variable	Concordant ($n=74$)	Non-concordant ($n=16$)
MA	37.0 (34.0–39.5)	35.0 (32.0–38.5)
GA	8.1 (7.0–9.4)	8.1 (7.8–9.1)
FF_i	7.0 (4.8–9.1)	5.5 (4.0–6.9)
FF_r	6.9 (4.1–9.0)	5.5 (4.0–6.9)
Fetal sex		
Male, *n* (%)	33 (44.6)	3 (18.7)
Female, *n* (%)	41 (55.4)	13 (81.3)

Note: Data presented as median (interquartile range) or *n* (%) unless otherwise specified.

FF_i, fetal fraction estimated by the Illumina algorithm; FF_r, FF recalculated (FF for female fetuses taken from the native algorithm and FF for male fetuses based on the Y-chromosome); GA, gestational age; MA, maternal age.

rates (Table 2). We used a binomial logit model to predict the effect of study variables (i.e. MA, GA, FF and fetal sex) on concordance rates. Based on the Type II sum of squares (Table 3), only fetal sex affects concordance rates (adjusted odds ratio [aOR] 0.26, 95% CI 0.05–0.92).

We found a sensitivity and specificity of the test (considering only informative cases) of 0.76 (95% CI 0.64–0.85) and 1.00 (95% CI 0.86–1.00), respectively; a PPV of 1.00 (95% CI 0.93–1.00); and an NPV of 0.65 (95% CI 0.47–0.8). However, test performance sensitivity increased to 0.79 (95% CI 0.67–0.89) with a concordance rate of 85.1% after excluding triploids; a PPV of 1.00 (95% CI 0.93–1.00); and an NPV of 0.65 (95% CI 0.47–0.8). Finally, sensitivity increased to 100% (33/33) when only assessing concordance for male fetuses.

4 | DISCUSSION

4.1 | Main findings

Our data support cfDNA testing as a non-invasive means to assess aneuploidy in clinical miscarriages. Non-informative rates for niPOC displayed insignificant differences from iPOC and were lower than for karyotyping.[5]

4.2 | Strengths and limitations

Non-invasive POC analysis will benefit pregnant women undergoing medical management with misoprostol or requiring surgical extraction to retrieve POCs.[31] This alternative approach positively impacts patient well-being, as knowing that loss derives from chromosomal and not maternal problems can provide comfort/relief.[40]

We used a Y-chromosome-based FF estimator and the native algorithm default. Y-chromosome-based methods provide accurate FF estimates in male fetus pregnancies,[41,42]

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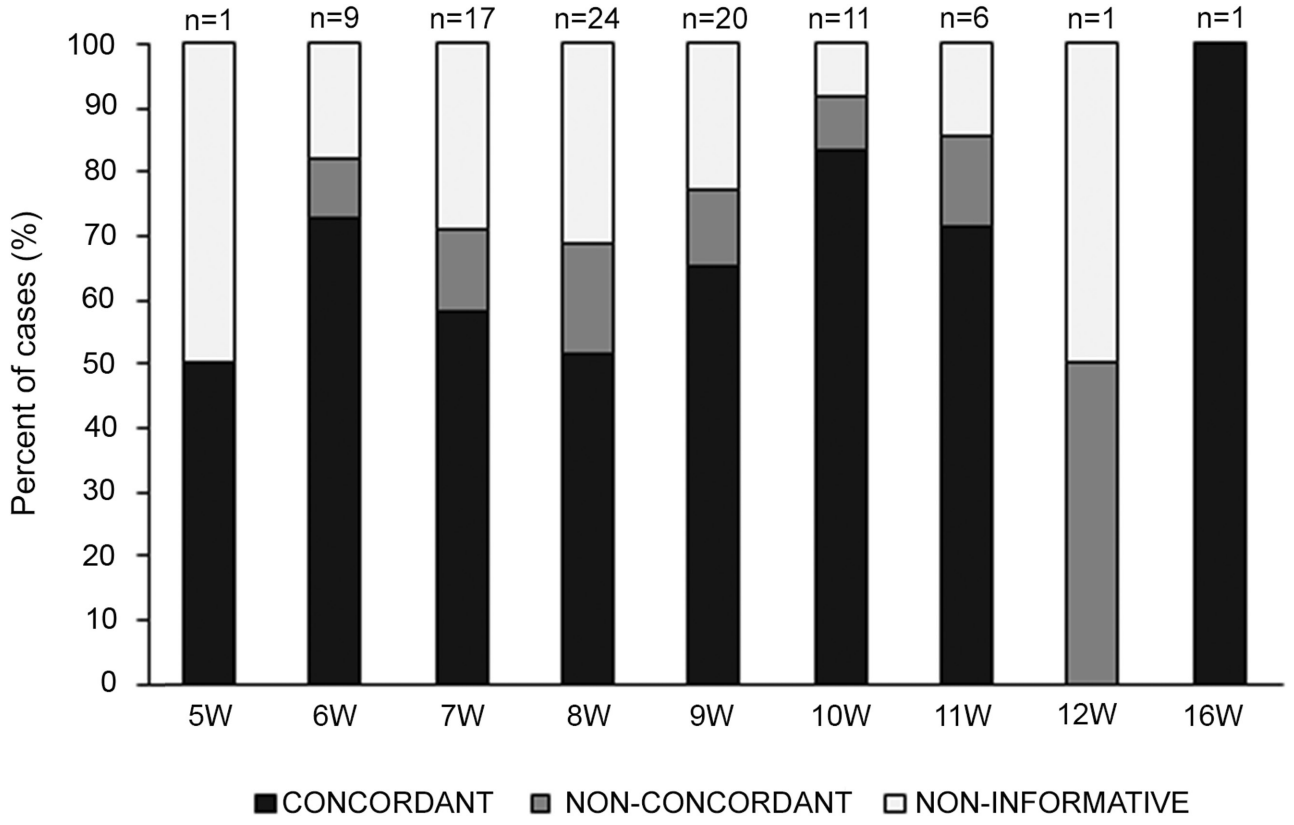

FIGURE 2 Stratification of the concordance variable by gestational age. The graphs show no higher rate of discordant results with decreasing GA; therefore, GA does not affect concordance rates. The number ofinformative cases at each gestational age (concordant and non-concordant) is shown at the top of each bar. NI, non-informative.

TABLE 3 Model parameters influencing the concordance rate between niPOC and iPOC cases.

Variable	aOR	95% CI	*P*-value
MA	1.16	0.98–1.41	0.08
FF_r	1.08	0.93–1.30	0.31
GA	1.05	0.73–1.57	0.78
Fetal sex			
Male	Reference	Reference	Reference
Female	0.26	0.05–0.92	**0.03**

Note: Bold numbers indicate statistical significance.

aOR, adjusted odds ratio; CI, confidence interval; FF_r, FF recalculated (FF for female fetuses taken from the native algorithm and FF for male fetuses based on the Y-chromosome); GA, gestational age; MA, maternal age.

allowing non-informative case reclassification and more accurate/objective assessments of concordance rates and cfDNA testing performance.

A lack of information regarding the timing of maternal blood sample collection represents a limitation; most collaborating centres did not provide this data, so we cannot know whether samples were taken before or after POC evacuation. This represents a challenge, given the rapid clearance of fetal DNA from maternal plasma after delivery (mean half-life of 16.3 minutes) in cases without medical conditions/antenatal complications.[43] Schlaikjær Hartwig et al.[44] described a three-fold increase in cfDNA non-informative rates 12–24 hours after evacuating POCs; therefore, we can assume that some non-concordant samples represent non-informative cases (note: all cases were females [except the triploids] with low median FF_i). For this subset, we lacked tools such as the Y-chromosome-based FF to identify female fetuses with an overestimated FF and, therefore, concordance rates may be underestimated and test performances could be significantly improved. The 100% concordance rate for male fetuses after excluding triploids supports this hypothesis. FF estimation and/or short DNA fragment enrichment improvements may significantly impact cfDNA performance in POC analysis.

The non-detection of polyploidy represents another limitation. A triploid detection rate of 5% lies within the expected range (5–10%) for triploids in the first trimester as natural pregnancy losses.[45–47] Therefore, in cases of molar pregnancy/suspected altered ploidy, cfDNA analysis for genetic POC evaluation should be discouraged.

Genetic abnormalities may be missed with mosaicism and low FF. In fact, according to the Illumina VERISEQ NIPT SOLUTION v2 package insert, the minimum FF required to detect 95% of trisomies 22 and 16 (the latter accounting for 37.25% of trisomies causing miscarriages) is 4.87% and 3.10%, respectively. These limits of detection (LOD) have been postulated as a major handicap when using cfDNA to detect abnormalities in early miscarriage.

However, our study detected 50% and 80% of trisomies 16 and 22. Although this is a rate with room for improvement, the performance is quite good, especially when compared with iPOC and traditional cytogenetic studies, where poor tissue quality may affect the final diagnosis and where the limits of mosaicism are around 30%.[48,49]

Neither cfDNA analysis nor other available cytogenetic studies cover all genetic alterations causing pregnancy loss. The resolution limit of karyotypes or low-pass NGS does not support the detection of subchromosomal alterations of <7 Mb,[21,22] and microarrays do not identify specific structural rearrangements.[50,51] None of these approaches detects potentially lethal monogenic mutations; therefore, cfDNA provides helpful complementary information, especially in cases where POC tissue remains unavailable or in IVF, where triploid incidence remains lower than natural conceptions.[52]

4.3 | Interpretation

We screened 120 patients for chromosomal abnormalities via iPOC using low-pass NGS and niPOC via cfDNA testing of maternal blood and assessed non-informative rates through MCC and FF values. Approximately 20% of POCs processed by low-pass NGS remain undiagnosed due to unwanted contamination.[53] We observed a low percentage of non-informative results (10.0%), a value a priori not different from cfDNA testing (16.7%). Precise tissue processing (not commonly available in most centres) supported a high rate of successful iPOC analysis; however, success rates of common POC techniques (e.g. karyotyping or microarray analysis) can also be as low (e.g. 53%[23] and 70%[54]).

Our niPOC informative rate was lower than that reported in a single-centre study by Yaron et al.[32] (99%) that controlled all process variables. Our research involved samples from multiple centres, with POC instructions specifying that maternal blood should be isolated before expulsion or chorionic villous sampling (CVS). While our model resembles a realistic clinical diagnosis scenario (not all centres use the same clinical practice), technique performance rates remained similar to reported data.

Additionally, cfDNA-based techniques should not be ruled out for aneuploidy assessment at GA of <10 weeks based on insufficient cfDNA. Although studies suggest a high proportion of low FF cases at <8 weeks' gestation,[38,44] we found no differences in FF between GA values, suggesting that the earliest miscarriages may not necessarily increase the likelihood of non-informative results. As in other prospective/more recent studies,[30,32] cfDNA testing provides informative results before 8 gestational weeks, suggesting no clinically valuable cut-off excluding low FF cases. This agrees with genome-wide sequencing studies for cfDNA analysis with >80% sensitivity for trisomies, even in low FF samples.[55]

We classified 13 non-concordant cases as female non-viable pregnancies with a low median FF, suggesting female POCs as the most likely to have discordant outcomes when using a Y-chromosome-based FF cut-off with a standard FF estimator. A multivariable model demonstrated fetal sex as the only variable influencing cfDNA testing misclassification. The abnormality discordance may arise from an overrepresentation of informative cases associated with an overestimated FF in female pregnancies. In support, we found a different non-informative case distribution in male fetuses when basing classification on the native algorithm FF rather than the Y-chromosome-based FF.

Despite this setback, we observed promising niPOC performance, with sensitivity and specificity values of 0.79 (95% CI 0.67–0.89) and 1.00 (95% CI 0.86–1.00) and a concordance rate of 85.1%; this compares favourably with cytogenetic analysis of POCs and argues against studies questioning the utility of cfDNA testing for POC studies.[30,32,38,44]

Although cfDNA testing cannot currently replace cytogenetic testing, this approach could improve the diagnostic yields of current approaches. Studies have reported the cost-effectiveness of cfDNA testing to guide RPL work-up.[56] In this regard, cfDNA testing represented the second-highest diagnostic yield pathway for identifying POC aetiology in RPL (ahead of ASRM work-up and POC karyotyping) and the most cost-effective system (avoiding costs associated with invasive procedures required for POC collection).

Non-invasive POC can be used for both EPL and RPL. The niPOC could determine the aetiology of fetal remains in first miscarriages. A screen-positive result provides a high probability that the detected abnormality caused the miscarriage, with appropriate genetic counselling offered. Finding fetal aneuploidy could be important, given its association

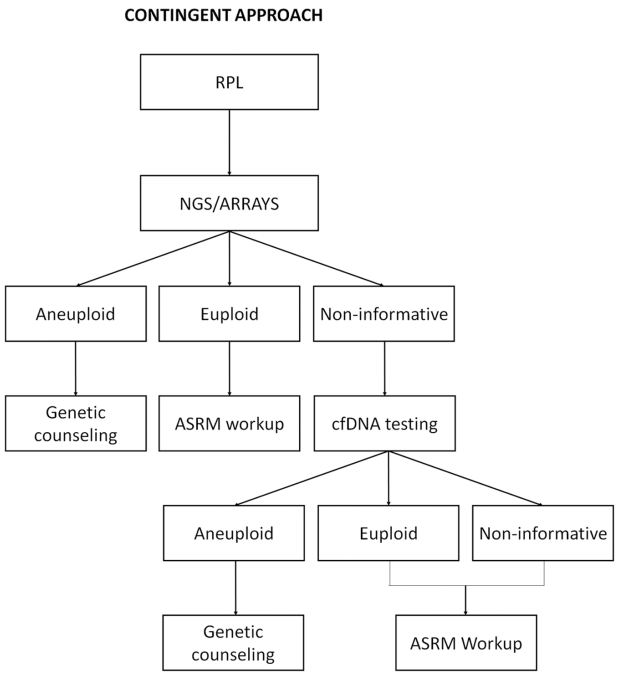

FIGURE 3 Flowchart proposing the path of choice for genetic analysis of POCs based on the number of abortions. Application of the contingent approach through cfDNA testing.

with good prognoses for future pregnancies compared with euploid pregnancy loss.[44]

InvasivePOC analysis may represent the method of choice in RPL, given its higher diagnostic yield in informative cases (Figure 3). In this scenario, given a euploid result, patients should be referred for an ASRM work-up, but given an aneuploid result, the patient should receive appropriate genetic counselling. In non-informative cases caused by MCC, the patient could opt to use cfDNA testing to increase the diagnostic yield. In these cases, if we obtain a screen-positive result, the patient would receive appropriate genetic counselling and avoid referral to the ASRM-work-up,[2] which would not have happened if only the IPOC pathway had been used. Based on our results, this contingent approach increases iPOC informative rates from 80–90% to 98%.

5 | CONCLUSION

Genome-wide cfDNA-based screening provides a straightforward, non-invasive approach to elucidate whether fetal aneuploidy explains loss in EPL or RPL patients. Clinicians must consider maternal blood sampling before POC collection to ensure niPOC effectiveness. Y-chromosome-based methods better classify non-informative cases in male fetus pregnancies and, thus, provide a more accurate assessment of cfDNA testing performance. Used contingently, cfDNA testing can significantly improve diagnostic yields of molecular approaches in MCC.

AUTHOR CONTRIBUTIONS

NB: experimental conception and design, data acquisition/analysis/interpretation, writing/review/final approval of the paper. LR: experimental conception and design, writing/review/final approval of the paper. EM-B: experimental conception and design, data acquisition/analysis/interpretation, review and final approval of the paper. IC-G: data acquisition, review and final approval of the paper. JAC: statistical analysis, writing/review/final approval of paper. NA-A: review and final approval of the paper. CR: review/final approval of paper. MM: experimental conception and design, data acquisition/analysis/interpretation, writing/review/final approval of the paper. All authors have agreed to be accountable for all aspects of the work and in ensuring that questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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