

Aneuploidy Screening in Twin Pregnancy: Cell Free DNA Performance and Factors Affecting Fetal Fraction

Rastreio de Aneuploidias na Gravidez Gemelar: Desempenho do DNA Fetal Livre e Factores que Afetam a Fração Fetal

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Abstract

Overview and Aims: The prevalence of multiple pregnancies is increasing. However, data on Noninvasive Prenatal Testing (NIPT) efficacy in twin pregnancies remains limited. Moreover, several studies have indicated a higher incidence of failed results in twin pregnancies due to decreased fetal fraction. This study evaluates the effectiveness of NIPT as a primary aneuploidy screening method in twin pregnancies and identifies predictors of reduced fetal fraction.

Study Design: Observational retrospective study.

Population: Twin pregnancies

Methods: This study analyzed data from twin pregnancies screened with NIPT as the primary method for detecting trisomies 13, 18, and 21 at a tertiary hospital between 2014 and 2019. Women with a high risk of aneuploidies (>1%) were advised to undergo invasive testing. The definitive diagnosis was obtained through prenatal karyotyping or the phenotypic assessment of newborns. A logistic regression model was calculated to identify predictors of fetal fraction <6% (failure) or ≥6% (success), with significance set at $p < 0.05$.

Results: A total of 76 cases were analyzed, with conclusive results obtained from the initial sample in 95% of cases. Two cases of trisomy 21 were detected and confirmed by amniocentesis, with no false positives or negatives. Body Mass Index (BMI) and parity were significant predictors of success: each unit increase in BMI reduced the odds of success by 21% (OR 0.79, 95% C.I: 0.69-0.92, $p = 0.002$), and multiparity reduced the odds of success by 87% compared to nulliparity (OR 0.13, 95% C.I: 0.03-0.67, $p = 0.014$).

Conclusions: NIPT demonstrated high accuracy in detecting trisomy 21 in twin pregnancies, though its efficacy for trisomies 13 and 18 could not be assessed. Higher maternal BMI and multiparity were associated with reduced fetal fractions and a higher likelihood of test failure.

Keywords: Aneuploidy; Non-invasive prenatal testing; Multiple pregnancy; Prenatal diagnosis; Trisomy.

Resumo

Introdução e Objetivos: A prevalência de gravidezes múltiplas está a aumentar. No entanto, a evidência sobre a eficácia do Teste Pré-Natal Não Invasivo (NIPT) em gravidezes gemelares permanece limitada. Além disso, estudos sugerem maior incidência de resultados inconclusivos nestas gravidezes devido à redução da fração fetal. Este estudo tem como objetivo avaliar a eficácia do NIPT como método primário de rastreio de aneuploidias em gravidezes gemelares e identificar fatores preditores de redução da fração fetal.

Desenho de Estudo: Estudo observacional retrospectivo.

População: Gravidezes gemelares.

Métodos: Este estudo analisou os resultados do NIPT, usado como rastreio primário das trissomias 13, 18 e 21 num hospital terciário, entre 2014 e 2019. Mulheres com risco elevado de aneuploidias (>1%) foram aconselhadas a realizar teste invasivo. O diagnóstico definitivo foi obtido por cariótipo pré-natal ou fenótipo dos recém-nascidos. Calculou-se um modelo de regressão logística para identificar preditores de fração fetal <6% (falha) ou ≥6% (sucesso), considerando um nível de significância de $p<0.05$.

Resultados: Foram analisados 76 casos, com resultados conclusivos após uma amostra inicial em 95%. Dois casos de trissomia 21 foram identificados e confirmados por amniocentese, sem falsos positivos ou negativos. O Índice de Massa Corporal (IMC) e a paridade foram preditores significativos de sucesso: cada unidade adicional no IMC reduziu as probabilidades de sucesso em 21% (OR 0.79, 95% C.I: 0.69-0.92, $p=0.002$), e a multiparidade reduziu as probabilidades de sucesso em 87% em comparação com a nuliparidade (OR 0.13, 95% C.I: 0.03-0.67, $p=0.014$).

Conclusão: O NIPT foi eficaz na deteção de trissomia 21 em gravidezes gemelares. Não foi possível tirar conclusões sobre a deteção de trissomias 18 e 13. O aumento do IMC materno e a multiparidade foram fatores associados a frações fetais mais baixas e maior probabilidade de falha no teste.

Palavras-chave: Aneuploidia; Teste pré-natal não invasivo; Gravidez gemelar; Diagnóstico pré-natal; Trissomia.

INTRODUCTION

The incidence of multiple pregnancies is rising, primarily because of the increased use of medically-assisted procreation techniques and the higher average maternal age¹.

In aneuploidy screening, the traditional combined test has been less effective for twin pregnancies. Studies have shown a lower detection rate and a higher false positive rate for detecting trisomy 21 compared to singleton pregnancies^{2,3}. This results in the performance of unnecessary invasive procedures, although recent studies suggest that the risk of induced fetal loss in twin pregnancies, when adjusted for background risk, is lower than previously reported^{4,7}.

Noninvasive Prenatal Testing (NIPT) using the analysis of cell-free DNA in maternal blood performs well for screening trisomies 13, 18 and 21 in singleton pregnancies^{8,9}. NIPT assays are now integrated into prenatal screening protocols in many countries, either as a first- or second-line test^{10,11}, resulting in a significant reduction in the rates of invasive procedures¹²⁻¹⁵.

Regarding twin pregnancies, data on the effectiveness of NIPT is more limited. Most of published re-

sults on twins have focused on high risk pregnancies, showing a detection rate of 94%-100% and a test failure rate of 5-11% for trisomy 21¹⁶⁻¹⁹. Although earlier guidance reflected uncertainty, more recent ISUOG recommendations recognise that NIPT is currently the most effective screening test for trisomy 21 in twin pregnancies.²⁰ Similarly, other societies, such as the International Society of Prenatal Diagnosis and the American College of Obstetricians and Gynecologists (ACOG), have reviewed their positions and now endorse cell free DNA screening in twin pregnancies^{11,21}.

In noninvasive prenatal screening, test performance is directly related to the relative proportion of maternal/fetal DNA (fetal fraction)²². A minimum fetal fraction of 4% was defined as necessary for an adequate analysis in singleton pregnancies^{23,24}. However, one study suggested that the minimum fetal fraction considered for trisomy discordant twin pregnancies should be 6%²⁵.

In twin pregnancies, the application of NIPT is more complex, since in dizygotic twins only one fetus may have the trisomy and the contribution of fetal DNA from each fetus to the maternal circulation can vary approximately twice²⁶. Consequently, if the fetal fraction of the affected twin is less than the 4% threshold required for analysis, and if there is a high contribution from the unaffected fetus (total fetal fraction is

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satisfactory), a wrong conclusion that the fetuses are euploid may be drawn. To avoid this, it has been proposed that in dichorionic twin pregnancies, the lower fetal fraction of the two fetuses should be considered instead of the total fraction. Thus, for an adequate analysis, a minimum fetal fraction of 4% must be achieved by both fetuses²⁷. An inevitable consequence is that test failure rate will be higher in dichorionic twin pregnancies^{27,28}.

The failure to provide a result is one of the problems of NIPT. It may be caused, essentially, by three reasons: 1) Inadequate collection and transport to the laboratory, including low blood volume, hemolysis, incorrect labeling of tubes and delay in arrival at the laboratory; 2) Low fetal fraction, usually less than 4%; 3) Processing failure for a variety of causes, including failure in extraction, amplification, and DNA sequencing²⁹.

The most common cause of test failure is a low fetal fraction^{28,30}. Studies have indicated that twin pregnancies tend to have a lower fetal fraction^{17,28}. Additionally, several other factors are associated with lower fetal fractions, including earlier gestational age^{28,31}, increased maternal weight^{17,32}, impaired placentation, and pregnancies resulting from In Vitro Fertilization (IVF)^{17,28,31}.

Furthermore, recent studies suggested that NIPT failure is associated with adverse pregnancy outcomes, including trisomies 18 and 13³³, pregnancy-induced hypertension, pre-eclampsia after 34 weeks, gestational diabetes and congenital structural anomalies³⁴.

The primary objectives of this study are to determine the success rate and accuracy of NIPT as a primary screening method for aneuploidies in twin pregnancies, and to identify maternal and pregnancy factors that predict free fetal DNA fractions below 6%.

METHODS

This retrospective study involves pregnant women with twin pregnancies who attended the Obstetrics Service at a tertiary hospital for routine care between October 2014 and December 2019. Since October 2014, our hospital has used the NIPT test as the initial screening tool for trisomies 13, 18 and 21 in twin pregnancies.

During a routine visit at 11-13 weeks' gestation, we recorded maternal demographic characteristics and

medical history. An ultrasound scan was performed to determine gestational age by measuring the fetal crown-rump length of the larger fetus and to assess chorionicity by examining the junction of the intertwin membrane with the placenta. The scan also aimed to diagnose any major fetal abnormalities, exclude the possibility of "vanishing twin" (abortion of one of the twins), and measure fetal nuchal translucency thickness.

NIPT was routinely offered as the initial screening tool for trisomies 13, 18 and 21, provided the exclusion criteria were not met. The exclusion criteria included the presence of a "vanishing twin", known maternal chromosomal disorders, metastatic cancer, bone marrow or organ transplants, and recent blood transfusions (within the last six months).

A written informed consent was provided and signed by the mother. The blood sample was taken at our hospital and sent via courier to Ariosa Diagnostics, Inc. (San Jose, CA, USA) for analysis. Harmony™ Prenatal Test uses Digital ANALysis of Selected Regions (DANSR) assays targeting sequences on chromosomes 13, 18 and 21 for chromosome quantitation and single-nucleotide polymorphisms on chromosomes 1 to 12 for fetal-fraction measurement. Products of the DANSR assays can be quantified using either next-generation sequencing or a custom microarray; both were used during the course of this study. The data were analyzed using the fetal fraction-optimized risk of trisomy evaluation (FORTE) algorithm, which calculates probability scores for fetal trisomy, with >1% considered to be high probability.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by our Hospital's Ethics Committee (Reference: S-HDES/2022/215).

For each case, the following variables were collected: date of blood sample collection, date of arrival at the laboratory, date of issuance of the report, obtaining a result (yes/no), need to repeat the collection (yes/no), fetal fraction, NIPT test result, final result (obtained by karyotype or phenotype assessment after birth), maternal age (or age of the oocyte donor in cases of oocytes donation), parity (nulliparous/parous), gestational age, race, smoking habits (yes/no), maternal weight, height, Body Mass Index (BMI), method of conception,

oocyte donation (yes/no) and chorionicity.

The fetal fraction reported for twin pregnancies specifically refers to the fetus with the lowest contribution of fetal DNA. It was not a combined measure of both fetuses. Thus, a minimum fetal fraction of 4% had to be achieved by both fetuses for the analysis to be considered adequate.

If the NIPT did not yield results after the first sampling, the parents were offered the options of repeating the NIPT, undergoing invasive testing, or pursuing no further investigation. In cases where the NIPT failed a second time, the choices were limited to invasive testing or no further evaluation. In the scenario of a high probability test, invasive fetal karyotyping was recommended to the parents.

The final results were classified in: 1) Trisomy 13, 18 or 21 after karyotype; 2) No trisomies after karyotype or phenotypically normal newborn; 3) No known karyotype because of abortion or stillbirth and no study performed; 4) Outcome unknown due to loss to follow-up.

For continuous variables, the median and interquartile value were calculated. For categorical variables, absolute and relative frequency were used. Logistic regression was employed to identify maternal and pregnancy factors that significantly predict lower fetal fractions (less than 6%).

For clinical reporting in our cohort, a fetal fraction cut-off of 4% was applied. To explore predictors of lower fetal fractions in the statistical analysis, a cut-off of <6% was used as the dependent variable in the logistic regression model. This threshold was selected based on previous evidence suggesting that the minimum fetal fraction considered for trisomy discordant twin pregnancies should be 6%²⁵. Logistic regression was employed to identify maternal and pregnancy factors that significantly predicted lower fetal fractions (<6%). The variables included in the analysis were maternal age, parity, gestational age, maternal BMI, smoking status, conception via IVF, and the origin of the oocyte (maternal or donor).

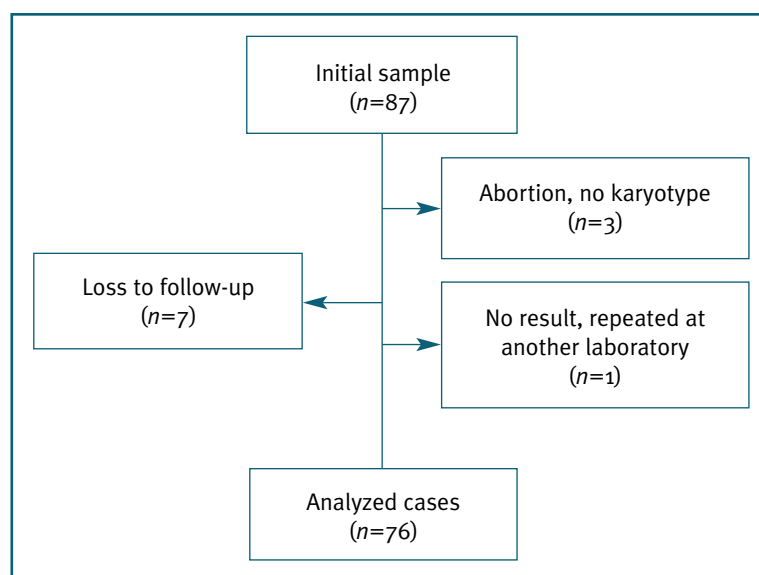


FIGURE 1. Selection of cases for inclusion in the study.

RESULTS

During the study period, we performed NIPT on 87 twin pregnancies. Of these, 11 cases (12,6%) were excluded (Figure 1) for the following reasons: 7 cases were lost to follow-up, resulting in unknown pregnancy outcomes; 3 cases involved the miscarriage of one or both fetuses without obtaining their karyotype; and 1 case involved a second sample collection using a different laboratory after the first attempt failed to yield results. Among these excluded cases, fetal DNA results were as follows: 9 showed low-risk results for trisomies 21, 18, or 13, and two did not yield a result after the first collection (one was repeated in another laboratory – reason for exclusion – and the other had insufficient sample and was classified as lost to follow-up). Therefore, no positive results were observed among the excluded cases.

A total of 76 cases were analyzed. Of these, as illustrated in Figure 2, no result was obtained after the first collection in 4 cases: 3 related to problems with transport to the laboratory, and one case of unknown cause. Of these 4 cases, a result was subsequently obtained after a second collection in 3 cases. In the remaining case, no result was obtained even after the second collection; the exact management of this case is unknown, although the phenotype of the newborn was reported

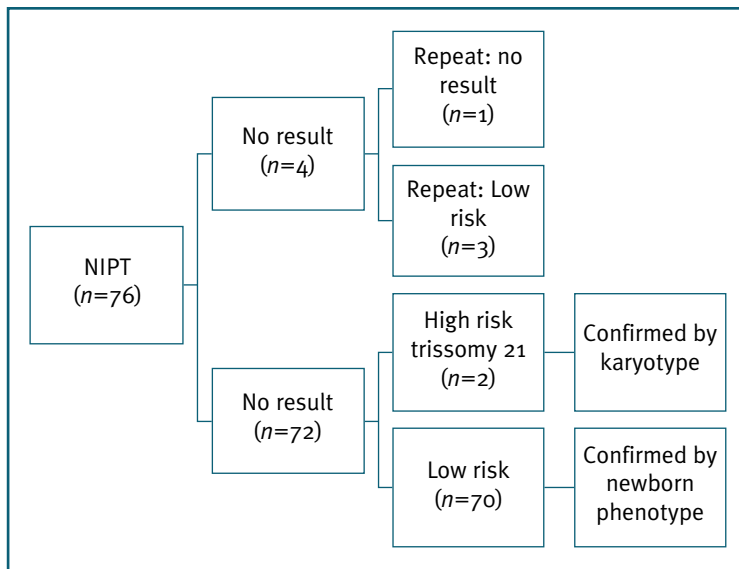


FIGURE 2. Results of cell-free DNA screening for trisomies 13, 18 and 21 in twin gestations.

as normal. Thus, a success rate of 95% was achieved, after the first collection.

The maternal and pregnancy characteristics of the study population are summarized in Table I. The median maternal age was 32 years, and the median maternal BMI was 25.3 kg/m². The majority of pregnant women who underwent NIPT were nulliparous. In the cohort, 82.9% of the twins were dichorionic, and 27.7% of the twin pregnancies were conceived through medically-assisted procreation techniques. NIPT was conducted on twin pregnancies at a median gestational age of 13.4 weeks, ranging from 11 to 20 weeks.

Of the 75 cases in which a result was obtained after the first or second collection, the average time between collection and arrival at the laboratory was 3.3 days (ranging from 3 to 7 days), and the average time between collection and issuance of the report was 8.4 days (ranging from 6 to 16 days), with 72% of results provided before 14 days.

The median fetal fraction was 7.5%. Two high risk results for trisomy 21 were obtained with NIPT. In the first case, NIPT was performed at 13.3 weeks with a fetal fraction of 11.6%, resulting in a 99% risk for trisomy 21. A subsequent amniocentesis at 15 weeks confirmed a discordant result for trisomy 21. The couple chose to continue the pregnancy with both fetuses; ho-

wever, they experienced a spontaneous late miscarriage at 22.9 weeks.

In the second case, NIPT was performed at 14.4 weeks, with a fetal fraction of 4.5%. The result indicated a 99% risk for trisomy 21 and was communicated to the patient at 16 weeks. Amniocentesis was initially suggested; however, the patient opted for a wait-and-see approach and declined the procedure at that time. At 19 weeks, she was referred to a prenatal diagnostic reference center, where amniocentesis was performed at 22 weeks, confirming the discordant result for trisomy 21. Selective feticide was performed at 32 weeks in accordance with the hospital protocol. Delivery occurred at 37 weeks, resulting in the birth of a deceased fetus with trisomy 21 and a male newborn with normal phenotype.

In 73 cases a low risk for trisomies 13, 18 and 21 was determined, which was subsequently confirmed by normal newborn phenotypes. Therefore, no false positive or false negative results were obtained.

Logistic regression was employed to construct a predictive model of fetal fraction less than 6% (failure) or equal or greater than 6% (success). The analysis revealed that parity and BMI variables were statistically significant predictors, whereas maternal age, gestational age, smoking habits, IVF conception and oocyte donation did not demonstrate statistical significance in predicting fetal fraction.

When controlling for parity, for each one-unit increase in BMI, the odds of success decreased by a factor of 0.79, indicating a 21% decrease (OR 0.79, 95% C.I: 0.69-0.92, $p=0.002$).

Similarly, when controlling for BMI, the odds of obtaining a successful test for parous women were 87% lower compared to nulliparous women (OR 0.13, 95% C.I: 0.03-0.67, $p=0.014$).

The following model was thus obtained (Equation 1):

$\text{logit}(y=1) = 8,788 + (-2,019)M + (-0,232)BMI$, where $Y=1$ means success, M means Multipara and BMI means Body Mass Index.

TABLE I. DEMOGRAPHIC CHARACTERISTICS OF THE STUDY POPULATION

Characteristic	Twin pregnancies (n = 76)
Maternal age (years)	32,0 (28,3 - 35,0)
BMI (kg/m ²)	25,3 (23,2 - 29,7)
Obesity (IMC ≥ 30 kg/m ²)	
Yes	15 (19,7)
No	49 (64,5)
Unknown	12 (15,8)
Race	
Caucasian	58 (76,3)
Unknown	18 (23,7)
Nuliparous	
Yes	40 (52,6)
No	36 (47,4)
Smoking habits	
Yes	15 (19,7)
No	46 (60,5)
Unknown	15 (19,8)
Conception method	
Spontaneous	55 (72,4)
IVF	17 (22,4)
ICSI	4 (5,2)
Oocyte origin	
Self	71 (93,4)
Not self	5 (6,6)
Chorionicity	
Bichorionic	63 (82,9)
Monochorionic/Biamniotic	12 (15,8)
Unknown	1 (1,3)
Gestacional Age (weeks)	13,4 (12,6 - 15,1)

The probability of success is determined by the formula (Equation 2):

$$p(y=1) = \frac{\text{odds}}{1+\text{odds}} = \frac{e^{\text{logit}}}{1+e^{\text{logit}}}$$

For example, for a nulliparous woman with a BMI of 29 kg/m², the probability of success (i.e., obtaining a fetal DNA fraction above 6%), would be 89% as shown below:

$$\text{Logit}(y=1) = 8,788 + (-2,019)(0) + (-0,232)(29) = 2,06$$

$$p(y=1) = \frac{e^{2,06}}{1+e^{2,06}} = 0,89 \times 100 = 89\%$$

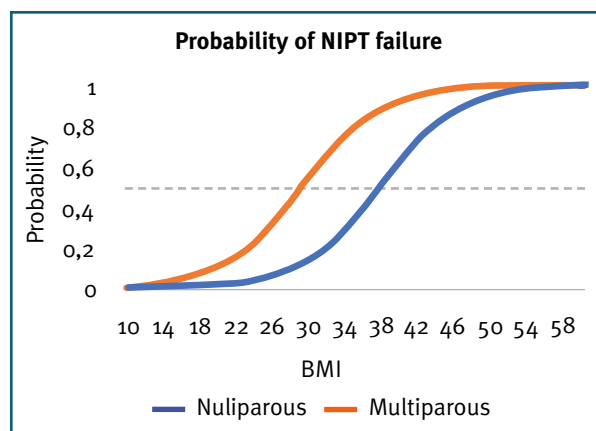


FIGURE 3. Probability of failure, according to the logistic regression model, as a function of Body Mass Index (BMI) and parity. NIPT, Noninvasive Prenatal Testing; BMI, Body Mass Index.

Applying the same method for a nulliparous with a BMI of 35 kg/m² and BMI of 40 kg/m², we would obtain a probability of success of 66% and 38%, respectively.

The model is depicted in Figure 3. It demonstrates that the probability of failure, indicated by obtaining a fetal DNA fraction < 6%, escalates with increasing BMI. Specifically, for nulliparous woman, the probability of failure surpasses 50% when BMI reaches 37.9 kg/m² or higher. In contrast, for multiparous woman, this critical BMI threshold decreases to 29.2 kg/m².

With this model, we obtained an accuracy of 81%, a positive predictive value of 83%, a negative predictive value of 73%, a sensitivity of 94% and a specificity of 47%.

DISCUSSION

Summary of Main Results

In this study, the NIPT test showed good accuracy in detecting trisomy 21 in twin pregnancies. Factors such as increased maternal BMI and multiparity were associated with lower fetal fractions, consequently increasing the probability of failure to obtain a result.

Results in the Context of Published Literature

The median gestational age for NIPT was 13.4 weeks, slightly higher than that reported in other studies,

which ranged between 10.6 and 12.1 weeks^{19,28,35,36}. The average time interval between blood collection and arrival at the laboratory was 3.3 days, while the interval between blood collection and result issuance was 8.4 days. These times are consistent with those observed in previous studies¹⁹.

We achieved a 95% success rate in obtaining a result after the first collection, a figure comparable to that reported in a cohort study by H. Judah et al. and in a large retrospective cohort study of Claudel et al.^{36,37}. The median fetal fraction obtained was 7.5%, a value that closely resembles that observed by Mar Gil M et al. (7.4%)¹⁹.

In this study, NIPT demonstrated excellent performance by detecting all cases of trisomy 21. Additionally, a recent meta-analysis by Judah et al. showed promising results for NIPT screening in twin pregnancies, with a pooled weighted detection rate of 99.0% and a false positive rate of 0.02% for trisomy 21³⁶. A systematic review and meta-analysis by Valle et al. also found a sensitivity of 98.8% and a specificity of 100%³⁸. Moreover, a large retrospective cohort study by Claudel et al. reported a sensitivity of 100% and a false positive rate of 0.23% for NIPT in detecting trisomy 21³⁷.

The inverse association between fetal fraction and maternal weight/BMI in single and twin pregnancies has been reported in numerous studies^{17,28,31,37}. Several explanations have emerged, including the dilution effect³⁰. Another theory suggests that in obese women, there's an accelerated turnover of adipocytes, leading to a higher release of maternal free DNA into circulation, consequently reducing the proportion of fetal free DNA³⁹.

Unlike the present study, several studies have shown an association between earlier gestational age and lower fetal DNA fractions³⁷. This is due to the reduction in placental mass at lower gestational ages, because the likely origin of fetal DNA in maternal plasma is dead placental cells³⁰.

In contrast to previous studies^{17,28,31}, no relationship was found between IVF conception and lower fetal fractions in this study. Additionally, similar findings were reported in studies by Le Conte et al., and Claudel et al., where no differences in the test failure rate were observed based on the method of conception^{32,37}.

In this study, it was demonstrated that multiparity is associated with lower free fetal DNA fractions, leading

to a higher probability of test failure. Interestingly, similar studies have indicated a higher risk of test failure in nulliparous women³⁴, although explanations for this discrepancy were not provided. Additionally, the wide confidence interval for parity in our model underscores the need for larger sample sizes to gain a clearer understanding of this relationship.

Strengths and Weaknesses

One notable strength of our study is our experience with NIPT as the primary screening method for aneuploidies in twin pregnancies over an extended period. Additionally, the study boasts a high rate of pregnancy follow-up, substantial availability of clinical data, including chorionicity information, and comprehensive data on fetal fraction and failure (covering primary and secondary failure rates).

Regarding limitations of our study, despite the extended duration of the study, our sample size was relatively small, encompassing only 87 twin pregnancies and just two cases of trisomy 21. No cases of trisomy 13 or 18 were observed, precluding any conclusions about the test's performance in these scenarios. Additionally, the defined 4% cut-off for test failure may be subject to discussion, particularly in light of recent studies suggesting a dynamic fetal fraction threshold^{40,41}. Furthermore, our study focused on generalizing the results of the Harmony for NIPT, whereas other methods of performing NIPT are also available.

Implications for Practice and Future Research

Regarding the practical implications of our findings, this study adds to the growing body of evidence supporting the high performance and exceptionally low false-positive rate of NIPT in twin pregnancies, particularly as a primary screening tool for trisomy 21. The challenge posed by reduced fetal fraction in overweight and obese women may present difficulties in current NIPT techniques. Consequently, further research is warranted to explore the optimal screening approach for aneuploidies in this population and to delineate the role of NIPT in obese women.

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Mariana Teves – Conception and design; data collection, analysis and interpretation of data; drafting of the manuscript. Sara Dias Leite – Critical review of the intellectual content. Catarina Frias – Contributions to conception and design; data collection, analysis and interpretation of data. Maria José Rego de Sousa – Critical review of the intellectual content and final approval of the version to be published. Joana Sampaio – Critical review of the intellectual content and final approval of the version to be published. Nuno Maciel – Critical review of the intellectual content and final approval of the version to be published. André Sampaio – Critical review of the intellectual content and final approval of the version to be published. Carlos Ponte – Critical review of the intellectual content and final approval of the version to be published.

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