Ultrasound Obstet Gynecol 2024; **63**: 807–814
Published online 30 April 2024 in Wiley Online Library (wileyonlinelibrary.com). **DOI:** 10.1002/uog.26311.

Non-invasive cell-free DNA prenatal screening for trisomy 21 as part of primary screening strategy in twin pregnancy

N. CLAUDEL[1,2,3], M. BARROIS[1,2,3], A. J. VIVANTI[4,5], J. ROSENBLATT[6], L. J. SALOMON[2,7], J.-M. JOUANNIC[8,9], O. PICONE[10,11], L. CARBILLON[12,13], F. VIALARD[14], E. LAUNAY[15], V. TSATSARIS[16], E. CURIS[16,17], L. EL KHATTABI[18,19,20] and APHP non-invasive prenatal screening group[#]

[1]*Maternité Port Royal, AP-HP Hôpital Cochin, FHU Prema, Paris, France;* [2]*Université Paris Cité, Paris, France;* [3]*INSERM UMR_S1139, Paris, France;* [4]*Service de Gynécologie–Obstétrique, DMU Santé des Femmes et des Nouveau-nés, AP-HP Hôpital Antoine Béclère, Clamart, France;* [5]*Université Paris-Saclay, Orsay, France;* [6]*Service de Gynécologie–Obstétrique, AP-HP Hôpital Universitaire Robert-Debré, Paris, France;* [7]*Service de Gynécologie–Obstétrique, AP-HP Hôpital Universitaire Necker–Enfants Malades, Paris, France;* [8]*Département de Médecine Fœtale, Pôle ORIGYNE.6, AP-HP Hôpital Armand Trousseau, Paris, France;* [9]*Université Sorbonne Paris Cité, Paris, France;* [10]*Service de Gynécologie–Obstétrique, AP-HP Hôpital Louis Mourier, Colombes, France;* [11]*Université Paris Diderot, INSERM UMR1137, IAME, Paris, France;* [12]*Service de Gynécologie–Obstétrique, AP-HP Hôpital Jean-Verdier, Bondy, France;* [13]*Université Paris 13, Bobigny, France;* [14]*Service de Cytogénétique, CHI Poissy-Saint Germain en Laye, Poissy, France;* [15]*Service de Cytogénétique et Biologie Cellulaire, CHU Rennes, Rennes, France;* [16]*UR 7537 BioSTM, UFR de Pharmacie, Faculté de Santé, Université Paris Cité, Paris, France;* [17]*Laboratoire d'Hématologie, Hôpital Lariboisière, AP-HP.nord, Paris, France;* [18]*Plateforme de Dépistage Prénatal Non Invasif par Analyse de l'ADN Libre Circulant, AP-HP, Hôpital Cochin and Université Paris Cité, Paris, France;* [19]*Sorbonne Université, Institut du Cerveau - Paris Brain Institute (ICM), Inserm, CNRS, Hôpital Pitié Salpêtrière, Paris, France;* [20]*Unité de Génomique Chromosomique, Département de Génétique Médicale, APHP, Hôpitaux Armand Trousseau et Pitié-Salpêtrière, Paris Brain Institute - ICM, Sorbonne Université, Paris, France*

KEYWORDS: cell-free DNA; chorionicity; failure; NIPS; NIPT VeriSeq; non-invasive prenatal screening; trisomy 21; twin pregnancy

CONTRIBUTION

What are the novel findings of this work?
This retrospective study of the performance of non-invasive cell-free DNA-based prenatal screening for trisomy 21 in a general population of twin pregnancies assessed one of the largest cohorts reported to date. We found high sensitivity 100% (95% CI, 54.1–100%) of screening for trisomy 21 at an extremely low false-positive rate (0.23% (95% CI, 0.06–0.59%)). Additionally, women can be advised that the risk of failure is very low (< 5%) for assays using a dynamic fetal fraction threshold.

What are the clinical implications of this work?
Non-invasive prenatal screening for trisomy 21 can be considered as a primary screening strategy in twin pregnancy.

ABSTRACT

Objectives *The performance of non-invasive prenatal screening using cell-free DNA testing of maternal blood in twin pregnancy is underevaluated, while serum marker-based strategies yield poor results. This study aimed to assess the performance of non-invasive prenatal screening for trisomy 21 in twin pregnancy as a first-tier test. Secondary objectives were to assess its failure rate and factors associated with failure.*

Methods *This retrospective cohort study included twin pregnancies in which non-invasive prenatal screening using cell-free DNA was performed as the primary screening strategy between May 2017 and October 2019. We used the NIPT VeriSeq® test for* in-vitro *diagnosis and set a fetal fraction cut-off of 4% for monochorionic pregnancies and 8% for dichorionic ones. Clinical data and pregnancy outcome were collected from physicians or midwives via a questionnaire or were retrieved directly on-site. We calculated the performance of non-invasive cell-free DNA screening for trisomy 21, analyzed its failure rate and assessed potentially associated factors.*

Results *Among 1885 twin pregnancies with follow-up, there were six (0.32%) confirmed cases of trisomy 21. The sensitivity of non-invasive prenatal screening for*

Correspondence: Dr L. El Khattabi, Non-Invasive Prenatal Screening Unit, Cochin Hospital, APHP.centre, University of Paris, 75014 Paris, France; Unité de Génomique Chromosomique, APHP.Sorbonne, Hôpitaux Armand Trousseau et Pitié-Salpêtrière, 75012 Paris, France (e-mail: laila.el-khattabi@aphp.fr)

#Members of APHP non-invasive prenatal screening group are listed at end of article.

Accepted: 31 May 2023

trisomy 21 was 100% (95% CI, 54.1–100%) and the false-positive rate was 0.23% (95% CI, 0.06–0.59%). The primary failure rate was 4.6%, with 4.0% being due to insufficient fetal fraction. A successful result was obtained for 65.4% of women who underwent a new blood draw, reducing the overall failure rate to 2.8%. Maternal body mass index, gestational age at screening as well as chorionicity were significantly associated with the risk of failure.

Conclusion *This study provides further evidence of the high performance, at an extremely low false-positive rate, of non-invasive prenatal screening in twins as part of a primary screening strategy for trisomy 21.* © *2023 The Authors. Ultrasound in Obstetrics & Gynecology published by John Wiley & Sons Ltd on behalf of International Society of Ultrasound in Obstetrics and Gynecology.*

INTRODUCTION

Compared with that in singleton pregnancy, conventional prenatal screening for trisomy 21 in twin pregnancy, based on measurement of nuchal translucency thickness and first-trimester biochemical serum markers (free β-subunit of human chorionic gonadotrophin and pregnancy-associated plasma protein-A), is less effective. In a meta-analysis, the pooled sensitivity of such a combined screening strategy was 89.3% (95% CI, 79.7–94.7%) and the false-positive rate was 5.4% (95% CI, 4.3–6.7%)[1], compared with 92–94% and 3–5%, respectively, in singleton pregnancies[2]. This leads to the performance of unnecessary invasive procedures, although recent studies suggest that the risk of induced fetal loss in twin pregnancy, when weighted by the background risk, is less than that originally reported[3–6].

The discovery of cell-free fetal DNA (cfDNA) in maternal blood[7] allowed the development of non-invasive prenatal screening (NIPS) tests[8,9]. Because of their high-performance metrics in singleton pregnancy[10,11], NIPS assays are now part of the prenatal screening scheme for this population, as either a first- or second-line test in many countries[12–14], leading to a significant decrease in the rates of invasive procedures[15–18]. In France, since the introduction of this test, invasive procedures have been more than halved (from 31 422 in 2015 to 15 249 in 2020)[19].

Many studies have shown that cfDNA-based NIPS is efficient as a replacement for screening using biochemical markers in singletons, and even outperforms it, but far smaller cohorts have been evaluated for twin pregnancy[20–24], particularly for the commercial assays which are now being used widely[25]. These studies report variable performance results[20,22]. Consequently, international guidelines recommend unequivocally cfDNA NIPS and approve its clinical utility in singleton pregnancies, while their statement for twins is less consensual: some remain silent on this point (reviewed by Palomaki *et al.*[26]), while other statement positions have been reviewed and now endorse cfDNA screening in twin pregnancies, for example the International Society of Prenatal Diagnosis and the American College of Obstetricians and Gynecologists. Additionally, performing NIPS in multiple pregnancies raises specific questions, such as regarding the impact of chorionicity, pregnancy origin or fetal fraction (FF) on performance or failure rate[27,28].

The primary objective of our study was to assess the performance of a paired-end polymerase chain reaction (PCR)-free NIPS assay for trisomy 21 as part of the primary screening strategy, in a large cohort of twin pregnancies. Secondary objectives were to assess the failure rate and investigate potential associated factors.

METHODS

Study design and population

This retrospective cohort study included pregnant women referred to one of the 13 maternity units of the AP-HP (Assistance Publique des Hôpitaux de Paris; Parisian Hospitals) and for whom NIPS was performed in the context of twin pregnancy, between May 2017 and October 2019. Pregnancies with nuchal translucency thickness ≥ 3.5 mm or any other ultrasound abnormality and higher-order multiple pregnancies were not included. We excluded pregnancies with a vanishing twin.

Clinical data were collected from either physicians or midwives using a questionnaire, or were retrieved directly on-site. These included the patient's weight and height, mode of conception (spontaneous or following assisted reproduction), chorionicity (as determined by first-trimester sonography[29]), fetal karyotype, when available, and pregnancy outcome until birth. The study was approved by the local ethics committee under the following number: AAA-2022-08005.

In addition, NIPS performance as well as failure rate and reasons for failure were compared to those of a population of high- to moderate-risk singleton pregnancies in which NIPS was performed during the same study period. This consecutive population was composed of singletons referred because of a risk ≥ 1/1000 following biochemical marker screening, history of a previous pregnancy with a common trisomy or a paternal Robertsonian translocation involving chromosome 21 or 13.

Non-invasive prenatal screening

cfDNA-based screening for trisomy 21 was performed at the AP-HP NIPS laboratory. This is a public laboratory located in one of the AP-HP hospitals and it performs NIPS analyses for patients from all AP-HP maternity units. Our laboratory has implemented NIPS since 2015, initially using an inhouse protocol[8] and, since May 2017, using the NIPT VeriSeq workflow. We used the NIPT VeriSeq® assay (Illumina, San Diego, CA, USA), which has been approved for *in-vitro* diagnostic use in Europe, in line with the requirements of French policy. Following blood collection in a cfDNA

blood collection tube (Streck©, Omaha, NE, USA), sample transportation and processing until a result was obtained were performed following the manufacturer's recommendations and protocol. DNA extraction and PCR-free library preparation were performed using the Microlab STAR system (Hamilton©, Reno, NV, USA). Paired-end sequencing was performed using the NextSeq500 system (Illumina) and sequencing data were analyzed with the NIPT VeriSeq® analysis software. A log likelihood ratio was calculated by integrating a normalized chromosomal score and the estimated FF.

The NIPS result was either negative for the three common trisomies (T21, T18, T13), positive for one of them, or failed for one of the following reasons: insufficient FF regarding sequencing coverage; abnormal representation of at least one chromosome other than chromosome 21, 18 or 13, suggesting complete or partial aneuploidy of that chromosome; or abnormal distribution of read size, which may reflect contamination with maternal genomic DNA from cell lysis. Since sex chromosomal analysis during NIPS is not recommended in France, this was not performed.

Fetal fraction estimation and threshold

FF was estimated by the NIPT VeriSeq® analysis software based on read size distribution, as described previously[30]. The threshold is dynamic depending on coverage for each sample based on previous work showing the link between FF, coverage and trisomy risk[31,32]. At the time when this study started, the NIPT VeriSeq® test was newly marketed and no data existed on the reliability of a dynamic FF threshold for NIPS for trisomy 21 in twin pregnancy. Most studies applied a fixed FF threshold of 4% in singletons although some argued for a lower threshold[33]. We therefore decided to play it safe by not reporting NIPS results for which the FF was $< 4\%$ for monochorionic pregnancies and $< 8\%$ for dichorionic ones, assuming that, in monochorionic pregnancy, cfDNA is released by a single placenta, as it is in singletons, and that these pregnancies are mainly monozygotic, while dichorionic pregnancies are always dizygotic. Besides, studies comparing FF in dichorionic/dizygotic (mainly dichorionic) and monochorionic/monozygotic pregnancies showed no significant difference between these groups[34,35], consistent with our results (monochorionic *vs* dichorionic: 13.7% *vs* 14.1% (SD = 4.5% in both), $P = 0.1148$). This supports the fact that, in twin pregnancy, each placenta releases equivalent total amounts of cfDNA. Finally, our choice of FF cut-off is in line with general practice, according to which the FF cut-off is set twice as high in twins than in singletons for non-single nucleotide polymorphism (SNP)-based NIPS tests and twice as high in dizygotic than monozygotic twins when a SNP-based NIPS test is performed[23,36,37]. For the few cases in which chorionicity was unknown, the threshold was set to the maximum, i.e. 8%. For singletons, however, the threshold was dynamic and determined by the NIPT VeriSeq® analysis software according to coverage.

Invasive procedure and pregnancy outcome

Invasive sampling following positive or failed NIPS results consisted of amniotic fluid withdrawal from each amniotic sac. For positive NIPS results, fluorescence *in-situ* hybridization on amniotic cell nuclei and fetal karyotyping following cell culture were performed according to standard protocols, while, in case of NIPS failure, DNA microarray analysis was performed in addition to conventional cytogenetics.

For all pregnancies for which fetal karyotyping was not available (negative NIPS, or failed or positive NIPS following which the woman rejected invasive sampling), we collected information on the pregnancy outcome (miscarriage, (selective) termination of pregnancy, *in-utero* fetal death, stillbirth, neonatal death, healthy or affected newborn), as well as the clinical examination at birth or postnatal karyotyping. Reason for *in-utero* death or termination of pregnancy was noted when available. When a positive NIPS was not confirmed by fetal karyotyping from amniotic cells, we requested placenta samples at birth, but were not always able to obtain them. It is difficult for the clinical teams to organize such sampling systematically for these situations.

Data analysis and statistics

Descriptive analysis included calculation of mean (± SD), median and range for quantitative parameters and percentages for qualitative ones. It is worth noting that we use the term 'positivity rate' to refer to the percentage of positive NIPS (both true and false) among all NIPS performed, while the 'detection rate' corresponds to the rate of positives correctly detected among the true-positive ones. We calculated performance metrics of NIPS, including sensitivity, specificity, positive and negative predictive values and false-positive rate, based only on pregnancies for which strong evidence was available. Hence, we excluded pregnancies which underwent miscarriage, *in-utero* death, stillbirth or neonatal death, without undergoing prenatal invasive cytogenetic investigation to define chromosomal status, leaving for analysis 1747 twin pregnancies and 3877 singletons in the control population. The corresponding 95% CIs were calculated using the 'exact' binomial confidence interval (Clopper–Pearson method). In addition, the failure rate and reason for failure were compared between twin and singleton pregnancies, and between monochorionic and dichorionic pregnancies, using Fisher's exact test for 2×2 contingency tables. Given the low incidence of trisomy 13 and trisomy 18, we did not calculate their performance metrics. Comparison of population characteristics between twin and singleton pregnancies, and between mono- and dichorionic twin pregnancies, was carried out using either Wilcoxon–Mann–Whitney tests (for quantitative variables) or Fisher's exact test (for qualitative variables). Analysis of NIPS failure was performed using logistic regression, on cases with complete data; *P*-values were obtained using the asymptotic chi-square law of the

likelihood-ratio test for nested models. Univariate and multivariate analyses were carried out using all variables reported in the literature to be associated with NIPS failure (chorionicity, maternal age, body mass index (BMI), mode of conception, gestational age) except for FF, since this is a major cause of failure and its integration would lead to instable regressions and quasiseparation.

All analyses were conducted using *R* Statistical Software (v4.1.2; R Core Team 2021, R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/). All tests were performed at a Type-I error rate of 0.05 ($P < 0.05$); all confidence intervals are at 95%; *P*-values and confidence intervals are given without multiplicity corrections.

RESULTS

Study population

During the study period, 2577 women with multiple pregnancy had a NIPS test. After exclusions, 2223 patients were included in the study (Figure 1). Among these, pregnancy outcome was successfully collected for 1885 (85%) women (with 3770 fetuses), allowing analysis of NIPS results in this population. During the same period, 8444 singleton pregnancies were referred for NIPS and included in this study. Because this number of singletons was so large, with the difficulty in collecting pregnancy outcome information retrospectively, we were able to obtain follow-up for only 3877 (46%) singletons.

The descriptive characteristics of both twin and singleton pregnancies and their comparison are provided in Table 1. Maternal age was significantly higher in singletons. As expected, conception following the use of assisted reproductive technology was more frequent in the twin group. There was a statistically significant, but not clinically relevant, difference in gestational age at NIPS (median (range), 15 (11–34) weeks and 15 (10–37) weeks in twins and singletons, respectively). It is of note that the gestational-age range at screening was large for both populations; this corresponds to reality.

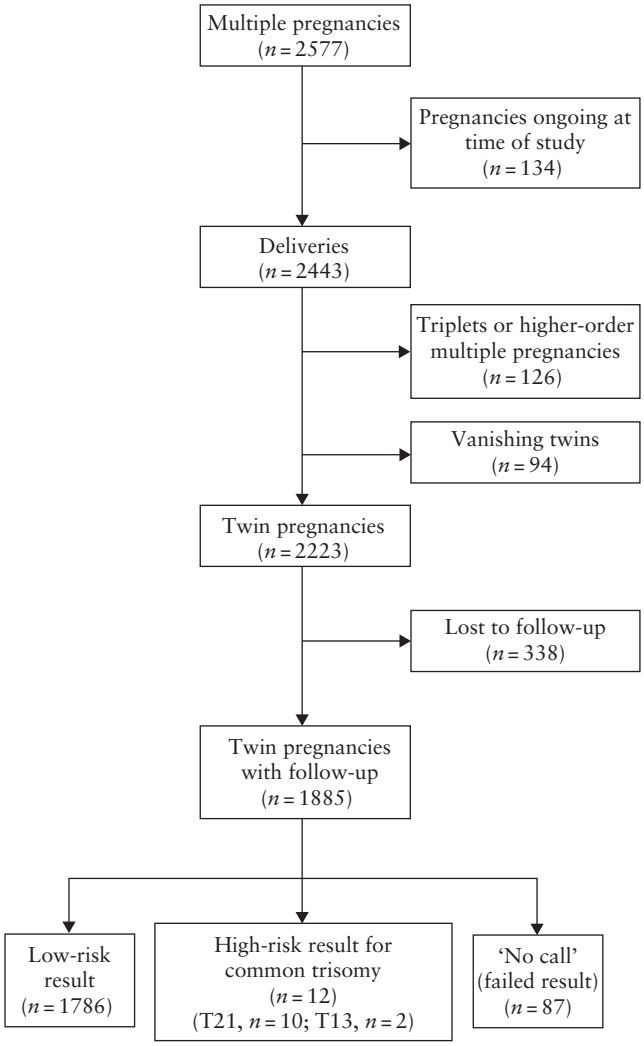

Figure 1 Flowchart summarizing study population of twin pregnancies. T13, trisomy 13; T21, trisomy 21.

Table 1 Description of twin and singleton pregnancy populations which underwent non-invasive prenatal screening (NIPS)

	Twin pregnancy (n = *1885*)		*Singleton pregnancy* (n = *8444*)		
Parameter	n	*Value*	n	*Value*	P
MA (years)	1873		8403		< 0.0001*
Mean ± SD		33.0 ± 5.4		35.6 ± 4.9	
Median (range)		33 (17–56)		36 (15–50)	
Maternal BMI (kg/m^2)	1827		8209		0.2923*
Mean ± SD		24.4 ± 4.8		24.6 ± 5.1	
Median (range)		23.4 (15.1–44.5)		23.5 (15.0–58.3)	
Conception following ART (*n* (%))	1572	464 (29.5)	7619	622 (8.2)	< 0.0001†
GA at NIPS (weeks)	1848		8357		< 0.0001*
Mean ± SD		16.2 ± 4.2		16.3 ± 4.0	
Median (range)		15 (11–34)		15 (10–37)	
Chorionicity (*n* (%))	1868		—	—	—
Monochorionic		426 (22.8)			
Dichorionic		1442 (77.2)			

*Wilcoxon–Mann–Whitney test for independent samples. †Fisher's exact test for 2 × 2 contingency tables. ART, assisted reproductive technology; BMI, body mass index; GA, gestational age; MA, maternal age at presentation.

NIPS results and performance metrics

The overall rate of positive NIPS for the common trisomies in twin pregnancies was 0.64% (trisomy 21, $n = 10$; trisomy 13, $n = 2$; trisomy 18, $n = 0$). Four (0.2%) false-positive calls were reported for trisomy 21 (three dichorionic and one monochorionic) and two (0.1%) were reported for trisomy 13 (both dichorionic). Among the six true-positive results for trisomy 21, only one of the twins was affected in five (83.3%) and all of these pregnancies were dichorionic. Both twins were affected in the one pregnancy that was monochorionic. Five of these women opted for selective termination of pregnancy (dichorionic pregnancy) or termination of pregnancy (monochorionic pregnancy), while one wished to pursue the pregnancy (dichorionic pregnancy with one affected twin). The calculated performance metrics for trisomy 21, in both twin and singleton pregnancies, are provided in Table 2.

Although we were unable to calculate the performance metrics of screening for trisomies 13 and 18 because of their low prevalence in this population, it is important to highlight that, among the 3770 fetuses that were analyzed, we did not observe any false-negative result for either of these trisomies as based on neonatal clinical examination and/or karyotyping.

The overall rate of positive NIPS for the common trisomies in dichorionic pregnancies was 0.69% (10/1442) compared with 0.47% (2/426) in monochorionic ones ($P = 0.06$) (Table 3). The false-positive rates, for trisomies overall, were 0.35% (5/1442) and 0.23% (1/426), respectively ($P = 0.79$). It is worth noting that this study was not powered for such analysis. The gestational age at testing for the false-positive cases was 33 weeks and above.

Analysis of NIPS failures

The primary failure rate after a first NIPS test was 4.6% (87/1885) in twin pregnancies *vs* 1.4% (115/8444) in singletons ($P < 0.0001$). Among the 87 women for whom NIPS failed, 52 (59.8%) chose to undergo secondary NIPS on a new sample, two (2.3%) decided to undergo chorionic villus sampling and the remaining 33 (37.9%) women opted for regular pregnancy follow-up. We obtained a successful result for 34 of the 52 (65.4%) women who underwent a second NIPS, reducing the overall failure rate to 2.8% (53/1885).

The rate of NIPS failure due to insufficient FF was significantly higher in twin pregnancies compared with singletons (4.0% (76/1885) *vs* 0.7% (55/8444); $P < 0.0001$). NIPS failure due to insufficient FF was also significantly higher in dichorionic compared with monochorionic pregnancies (68/1442 (4.7%) *vs* 8/426 (1.9%), respectively; $P = 0.00761$). However, conclusions

Table 2 Performance of non-invasive prenatal screening for trisomy 21 in twin compared with singleton pregnancy

Parameter	*Twin pregnancy* (n = *1747*)	*Singleton pregnancy* (n = *3877*)
Sensitivity (%)	100 (54.1–100)	98.2 (90.3–99.6)
Specificity (%)	99.8 (99.4–99.9)	99.8 (99.6–99.9)
Positive predictive value (%)	60.0 (26.2–87.8)	88.5 (77.8–95.3)
Negative predictive value (%)	100 (99.8–100)	100 (99.9–100)
False-positive rate (%)	0.23 (0.06–0.59)	0.18 (0.07–0.38)

Values in parentheses are 95% CI.

Table 3 Comparison between dichorionic and monochorionic twin pregnancies which underwent non-invasive prenatal screening (NIPS)

	Dichorionic (n = *1442*)		*Monochorionic* (n = *426*)		
Parameter	n	*Value*	n	*Value*	P
MA (years)	1430		426		< 0.0001*
Mean ± SD		33.3 ± 5.3		32.0 ± 5.3	
Median (range)		33 (18–56)		32 (17–48)	
Maternal BMI (kg/m^2)	1398		415		0.0659*
Mean ± SD		24.4 ± 4.8		24.0 ± 4.8	
Median (range)		23.5 (15.6–44.5)		23.2 (15.1–42.2)	
Conception following ART (*n* (%))	1204	408 (33.9)	361	56 (15.5)	< 0.0001†
GA at NIPS (weeks)	1412		419		0.0043*
Mean ± SD		16.3 ± 4.2		15.7 ± 4.1	
Median (range)		15 (11–34)		14 (11–33)	
Fetal fraction (%)	1437		425		0.1148*
Mean ± SD		14.1 ± 4.5		13.7 ± 4.5	
Median (range)		13.7 (2.3–37.0)		13.1 (1.9–35.0)	
Positivity rate (% (*n*))	1442	0.69 (10)	426	0.47 (2)	0.0592†
False-positive rate (% (*n*))	1442	0.35 (5)	426	0.23 (1)	0.7907†
Primary failure rate (% (*n*))	1442	5.27 (76)	426	2.58 (11)	0.0207†

*Wilcoxon–Mann–Whitney test for independent samples. †Fisher's exact test for 2 × 2 contingency tables. ART, assisted reproductive technology; BMI, body mass index; GA, gestational age; MA, maternal age at presentation.

Table 4 Analysis of predictors of failure of non-invasive prenatal screening (NIPS) in twin pregnancies with known chorionicity

Parameter	*Failed NIPS* (n = *87*)	*Successful NIPS* (n = *1781*)	*Univariate analysis* n	*OR (95% CI)*	P	*Multivariate analysis* (n = *1497*) *OR (95% CI)*	P
MA (years)			1856		0.0520		0.9533
Mean ± SD	34.1 ± 6.1	33.0 ± 5.3		1.040		0.999	
Median (range)	34 (24–56)	33 (17–54)		(1.000–1.082)		(0.951–1.047)	
Maternal BMI (kg/m^2)			1831		< 0.0001		< 0.0001
Mean ± SD	27.7 ± 5.6	24.2 ± 4.7		1.127		1.125	
Median (range)	26.7 (19–43.7)	23.4 (15.1–44.5)		(1.084–1.171)		(1.077–1.174)	
Conception following ART (*n*)*	28	435	1565	1.478 (0.902–2.380)	0.1190	1.246 (0.715–2.136)	0.4327
GA at NIPS (weeks)			1831		0.0880		0.0069
Mean ± SD	15.4 ± 2.8	16.2 ± 4.3		0.952		0.911	
Median (range)	15 (11–26)	15 (11–34)		(0.896–1.007)		(0.842–0.976)	
Chorionicity (*n*)*			1868		0.0138		0.0019
Monochorionic	11	415		2.099		3.293	
Dichorionic	76	1366		(1.153–4.214)		(1.499–8.701)	
Fetal fraction (%)			1879		< 0.0001	—†	—†
Mean ± SD	9.4 ± 4.6	14.3 ± 4.4		8.82×10^{-16}			
Median (range)	8.7 (2.8–23.9)	13.8 (1.9–37.0)		(4.00×10^{-19} to 1.20×10^{-12})			

*References for statistical analyses were spontaneous pregnancy and monochorionic twins, respectively. †Excluded from multivariate analysis. ART, assisted reproductive technology; BMI, body mass index; GA, gestational age; MA, maternal age at presentation; OR, odds ratio.

should be drawn with caution because of the small number of failures in the monochorionic pregnancies.

If we considered a dynamic FF threshold according to coverage, as calculated by the VeriSeq® software, all 76 tests that failed because of insufficient FF had a FF above the dynamic threshold, which would have resulted in a primary failure rate of 0.6% (11/1885). The corresponding calls of the 76 original failures due to insufficient FF were negative for the common trisomies, and pregnancy outcome and birth were uneventful except in two cases for which *in-utero* fetal death was reported without any evidence of a chromosomal anomaly.

All univariate and multivariate analyses were limited to pregnancies with known chorionicity ($n = 1868$, including 87 NIPS failures) and complete data. The final multivariate analysis was performed on 1497 twin pregnancies (including 69 NIPS failures). According to univariate analysis, factors predicting failure were BMI, chorionicity and FF (Table 4). However, after multivariate analysis, from which FF was excluded because of its high dependence on the test itself, earlier gestational age at testing, higher BMI and dichorionicity were shown to be factors predictive of failure (Table 4).

DISCUSSION

In this academic study, we assessed the performance of a PCR-free NIPS test, which is approved for *in-vitro* diagnosis, as a primary screening strategy in twin pregnancies with no ultrasound anomaly and a normal first-trimester nuchal translucency measurement. Our findings support high performance of NIPS in this population of twin pregnancies, with a sensitivity for trisomy 21 of 100% (95% CI, 54.1–100%), a specificity of 99.8% (95% CI, 99.4–99.9%), a positive predictive value of 60.0% (95% CI, 26.2–87.8%) and a false-positive rate of 0.23% (95% CI, 0.06–0.59%). Our population was at lower risk than those in most previous reports, which were conducted in high-risk populations[28,38–41] or in mixed populations and thus had an inherent bias in the proportions of high-risk *vs* low- or moderate-risk pregnancies[20,22,25,27,35,42–48]. This may explain the lower proportion of trisomy cases and higher false-positive rate in our study compared with the findings of a recent meta-analysis[22], particularly as placenta-confined mosaicism, which is a major source of false positives, may be more frequent in low-risk populations. Our calculated performance was also close to that reported by a recent meta-analysis of NIPS results in early-gestation twins[20], which found a detection rate of 99.0% (95% CI, 92.0–99.9%) and a false-positive rate of 0.02% (95% CI, 0.001–0.43%). Additional large studies in the general twin population are needed to improve the performance estimation.

In their meta-analysis evaluating the performance of first-trimester screening markers in twin pregnancies, Prats *et al.*[1] found a sensitivity of 89.3% (95% CI, 79.7–94.7%), specificity of 94.6% (95% CI, 93.3–95.7%) and 5.5% false-positive rate. For second-trimester screening markers, the results were even worse, with a sensitivity of 63.0% (95% CI, 44.8–81.2%) and a 10.8% false-positive rate[49]. Our findings, along with previously published results, suggest that, in twin pregnancy, NIPS outperforms the conventional screening strategy based on first- or second-trimester maternal serum markers.

None of the traditional serum markers has proven efficient in screening for trisomy 13 or trisomy 18 in twin pregnancy. Assessing the performance of NIPS in detecting these less common trisomies is difficult, because

of their low prevalence in the general population. In two recent meta-analyses, Gil *et al.*[22] and Judah *et al.*[20] were unable to assess the performance of NIPS for these trisomies because the number of cases was too small. In our study population, the prevalence was even lower, since NIPS was performed in the general twin population at 15 weeks, on average, excluding cases with elevated first-trimester nuchal translucency or any other ultrasound abnormality, while trisomy 13 and trisomy 18 are usually associated with such features. This likely explains our lower positivity rates for the common trisomies compared with some previous reports (0.64% (12/1885) in our cohort compared with 2.58% in the combined cohorts in the meta-analysis of Gil *et al.*[22]). Although we were unable to calculate performance for trisomies 13 and 18, indirect evidence suggests that NIPS could perform well for these trisomies. In fact, its performance for such trisomies in singleton pregnancy is well established and considered reasonably good, in particular for trisomy 18[10]. It is reasonable to expect similar to slightly lower performance metrics for trisomies 13 and 18 in twin compared with singleton pregnancies.

The primary NIPS failure rate in our cohort was 4.6%, the majority of cases being failures due to insufficient FF (4.0%). This failure rate is three times higher than that in our singleton population (1.4%), in which the failure rate due to low FF was 0.7% of pregnancies ($P < 0.0001$). Previous studies have also reported a higher failure rate in twins compared with singletons[28,42] and FF is usually reported as a major contributing factor[26]. In our study, the difference was magnified because of the higher FF cut-offs that we adopted for twins (4% per chorion) compared with singletons (dynamic threshold according to coverage), choosing to err on the side of caution. It is worth noting that all cases with FF below 4% per chorion passed the NIPT VeriSeq FF dynamic threshold and were negative for the three main trisomies, in line with pregnancy follow-up and birth clinical data. Applying a dynamic FF threshold brought the failure rate down to levels similar to those in singletons. However, the implications of this in terms of detection rate need to be evaluated. In addition, one should keep in mind that FF measurements and test failure rates vary depending on the technology, so these results may not apply to all providers.

In terms of factors predicting NIPS failure, our multivariate analysis validates maternal BMI, gestational age and chorionicity as major factors associated with failure, in accordance with some previously published results[27,28,42,46], and discards maternal age and pregnancy origin, in contrast with previously published results[27,28,42,46]. However, the study of the impact of pregnancy origin in our cohort may have been limited by the high proportion (16%) of data missing for this variable. BMI and gestational age have been shown to be correlated with FF (reviewed by Deng and Liu[50]), which was the main reason for failure in our cohort. This may explain why these two parameters were significant predictors of NIPS failure.

The strengths of our study are its large size, the high rate of pregnancy follow-up in the twin cohort, the high proportion of clinical data available, including chorionicity, and the comprehensive data on FF and failure (primary and secondary rates as well as cause of failure). This valuable source of data can be integrated into future meta-analyses or systematic studies evaluating NIPS in twin pregnancy. However, there were some limitations, including the small number of positive cases, the difference in risk level between twins and singletons and the small number of failures in monochorionic pregnancies. Analysis of additional, similarly large cohorts is needed to improve the estimation of NIPS performance.

To conclude, our study adds further evidence that implementing NIPS as the primary prenatal screening strategy in twin pregnancies enables highly efficient screening for trisomy 21, and that BMI, gestational age and chorionicity are predictors for NIPS failure in twin pregnancy.

APHP non-invasive prenatal screening group

Audrey Rosefort, *Service de Gynécologie-Obstétrique et de Médecine de la Reproduction, AP-HP Hôpital Universitaire Tenon, Paris, France*
Marc Dommergues, *Service de Gynécologie-Obstétrique, AP-HP Hôpital Universitaire Pitié-Salpêtrière-Charles Foix; Université Sorbonne Paris-Cité, Paris, France*
Morgane Valentin, *Service de Gynécologie-Obstétrique, AP-HP Hôpital Bichat, Université Denis-Diderot-Paris VII, Paris, France*
Hanane Bouchghoul, *Service de Gynécologie-Obstétrique, AP-HP Hôpital Bicêtre, Université Paris Saclay, Le Kremlin-Bicêtre, France*
Pierre François Ceccaldi, *Service de Gynécologie-Obstétrique, AP-HP Hôpital Beaujon, Hôpitaux Universitaires Paris-Nord Val de Seine, Clichy; Université Paris Cité, Paris, France*
Jeremy Sroussi, *Service de Gynécologie-Obstétrique, AP-HP Hôpital Universitaire Lariboisière, Paris, France*
Jocelyn Brayet, *Plateforme Bioinformatique MOABI, AP-HP, Paris, France*
Nathalie Le Dû, *Plateforme de Dépistage Prénatal Non Invasif par Analyse de l'ADN Libre Circulant, APHP, Centre Hôpital Cochin; Université Paris Cité, Paris, France*

ACKNOWLEDGMENTS

We thank our colleagues, doctors, midwives, nurses and all those who were involved in the process of NIPS at the 13 maternity units of the AP-HP hospitals. We also thank our colleagues in the different cytogenetics laboratories who performed cytogenetic analyses in prenatal or postnatal situations.

Disclosure

A.V. declares an advisory role for Norgine Pharma (headquarters in The Netherlands).

REFERENCES

1. Prats P, Rodríguez I, Comas C, Puerto B. Systematic review of screening for trisomy 21 in twin pregnancies in first trimester combining nuchal translucency and biochemical markers: a meta-analysis. *Prenat Diagn*. 2014;34(11):1077-1083. doi:10.1002/pd.4431
2. Kagan KO, Wright D, Baker A, Sahota D, Nicolaides KH. Screening for trisomy 21 by maternal age, fetal nuchal translucency thickness, free beta-human chorionic gonadotropin and pregnancy-associated plasma protein-A. *Ultrasound Obstet Gynecol*. 2008;31(6):618-624. doi:10.1002/uog.5331
3. Vink J, Fuchs K, D'Alton ME. Amniocentesis in twin pregnancies: a systematic review of the literature. *Prenat Diagn*. 2012;32(5):409-416. doi:10.1002/pd.2897
4. Akolekar R, Beta J, Picciarelli G, Ogilvie C, D'Antonio F. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. *Ultrasound Obstet Gynecol*. 2015;45(1):16-26. doi:10.1002/uog.14636
5. Di Mascio D, Khalil A, Rizzo G, et al. Risk of fetal loss following amniocentesis or chorionic villus sampling in twin pregnancy: systematic review and meta-analysis. *Ultrasound Obstet Gynecol*. 2020;56(5):647-655. doi:10.1002/uog.22143
6. Elger T, Akolekar R, Syngelaki A, et al. Fetal loss after chorionic villus sampling in twin pregnancy. *Ultrasound Obstet Gynecol*. 2021;58(1):48-55. doi:10.1002/uog.23694
7. Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet*. 1997;350(9076):485-487. doi:10.1016/S0140-6736(97)02174-0
8. El Khattabi LA, Brun S, Gueguen P, et al. Performance of semiconductor sequencing platform for non-invasive prenatal genetic screening for fetal aneuploidy: results from a multicenter prospective cohort study in a clinical setting. *Ultrasound Obstet Gynecol*. 2019;54(2):246-254. doi:10.1002/uog.20112
9. El Khattabi LA, Rouillac-Le Sciellour C, Le Tessier D, et al. Could digital PCR be an alternative as a non-invasive prenatal test for trisomy 21: a proof of concept study. *PLoS One*. 2016;11(5):e0155009. doi:10.1371/journal.pone.0155009
10. Gil MM, Accurti V, Santacruz B, Plana MN, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for aneuploidies: updated meta-analysis. *Ultrasound Obstet Gynecol*. 2017;50(3):302-314. doi:10.1002/uog.17484
11. Mackie F, Hemming K, Allen S, Morris RK, Kilby MD. The accuracy of cell-free fetal DNA-based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis. *BJOG*. 2017;124(1):32-46. doi:10.1111/1471-0528.14050
12. American College of Obstetricians and Gynecologists. ACOG Practice Bulletin No. 163: screening for fetal aneuploidy. *Obstet Gynecol*. 2016;127:e123-e137.
13. BeSHG. NIPT good clinical practice guidelines. 2017 consulté le mai 24, 2021. www.college-genetics.be/assets/recommendations/fr/guidelines/BeSHG%20prenatal%20consortium_guidelines%20for%20NIPT%20good%20clinical%20practice.pdf
14. Audibert F, De Bie I, Johnson JA, et al. No. 348-Joint SOGC-CCMG guideline: update on prenatal screening for fetal aneuploidy, fetal anomalies, and adverse pregnancy outcomes. *J Obstet Gynaecol Can*. 2017;39(9):805-817. doi:10.1016/j.jogc.2017.01.032
15. Song K, Musci TJ, Caughey AB. Clinical utility and cost of non-invasive prenatal testing with cfDNA analysis in high-risk women based on a US population. *J Matern Fetal Neonatal Med*. 2013;26(12):1180-1185. doi:10.3109/14767058.2013.770464
16. Fairbrother G, Burigo J, Sharon T, Song K. Prenatal screening for fetal aneuploidies with cell-free DNA in the general pregnancy population: a cost-effectiveness analysis. *J Matern Fetal Neonatal Med*. 2016;29(7):1160-1164. doi:10.3109/14767058.2015.1038703
17. Nshimyumukiza L, Beaumont JA, Duplantie J, et al. Cell-free DNA-based non-invasive prenatal screening for common aneuploidies in a Canadian Province: a cost-effectiveness analysis. *J Obstet Gynaecol Can*. 2018;40(1):48-60. doi:10.1016/j.jogc.2017.05.015
18. Benn P, Curnow KJ, Chapman S, Michalopoulos SN, Hornberger J, Rabinowitz M. An economic analysis of cell-free DNA non-invasive prenatal testing in the US general pregnancy population. *PLoS One*. 2015;10(7):e0132313. doi:10.1371/journal.pone.0132313
19. https://rams-archives2020.agence-biomedecine.fr/sites/default/files/2021-08/ABM_PEGH_DPN2020_1.pdf.
20. Judah H, Gil MM, Syngelaki A, et al. Cell-free DNA testing of maternal blood in screening for trisomies in twin pregnancy: updated cohort study at 10-14 weeks and meta-analysis. *Ultrasound Obstet Gynecol*. 2021;58(2):178-189. doi:10.1002/uog.23648
21. van Riel M, Brison N, Baetens M, et al. Performance and diagnostic value of genome-wide noninvasive prenatal testing in multiple gestations. *Obstet Gynecol*. 2021;137(6):1102-1108. doi:10.1097/AOG.0000000000004385
22. Gil MM, Galeva S, Jani J, et al. Screening for trisomies by cfDNA testing of maternal blood in twin pregnancy: update of The Fetal Medicine Foundation results and meta-analysis. *Ultrasound Obstet Gynecol*. 2019;53(6):734-742. doi:10.1002/uog.20284
23. Dyr B, Boomer T, Almasri EA, et al. A new era in aneuploidy screening: cfDNA testing in > 30,000 multifetal gestations: experience at one clinical laboratory. *PLoS One*. 2019;14(8):e0220979. doi:10.1371/journal.pone.0220979
24. Kantor V, Mo L, DiNonno W, et al. Positive predictive value of a single nucleotide polymorphism (SNP)-based NIPT for aneuploidy in twins: experience from clinical practice. *Prenat Diagn*. 2022;42(13):1587-1593. doi:10.1002/pd.6262
25. Gottardi E, Costa JM, Kleinfinger P, et al. First-trimester accuracy of cell-free DNA test for trisomy 21 screening in twin pregnancies. *Ultrasound Obstet Gynecol*. 2020;58:327-328. doi:10.1002/uog.23102
26. Palomaki GE, Chiu RWK, Pertile MD, et al. International Society for Prenatal Diagnosis Position Statement: cell free (cf)DNA screening for Down syndrome in multiple pregnancies. *Prenat Diagn*. 2021;41(10):1222-1232. doi:10.1002/pd.5832
27. Sarno L, Revello R, Hanson E, Akolekar R, Nicolaides KH. Prospective first-trimester screening for trisomies by cell-free DNA testing of maternal blood in twin pregnancy. *Ultrasound Obstet Gynecol*. 2016;47(6):705-711. doi:10.1002/uog.15913
28. Galeva S, Gil MM, Konstantinidou L, Akolekar R, Nicolaides KH. First-trimester screening for trisomies by cfDNA testing of maternal blood in singleton and twin pregnancies: factors affecting test failure. *Ultrasound Obstet Gynecol*. 2019;53(6):804-809. doi:10.1002/uog.20290
29. Maruotti GM, Saccone G, Morlando M, Martinelli P. First-trimester ultrasound determination of chorionicity in twin gestations using the lambda sign: a systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol*. 2016;202:66-70. doi:10.1016/j.ejogrb.2016.04.023
30. Yu SCY, Chan KCA, Zheng YWL, et al. Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proc Natl Acad Sci U S A*. 2014;111(23):8583-8588. doi:10.1073/pnas.1406103111
31. Benn P, Cuckle H. Theoretical performance of non-invasive prenatal testing for chromosome imbalances using counting of cell-free DNA fragments in maternal plasma. *Prenat Diagn*. 2014;34(8):778-783. doi:10.1002/pd.4366
32. McKanna T, Ryan A, Krinshpun S, et al. Fetal fraction-based risk algorithm for non-invasive prenatal testing: screening for trisomies 13 and 18 and triploidy in women with low cell-free fetal DNA. *Ultrasound Obstet Gynecol*. 2019;53(1):73-79. doi:10.1002/uog.19176
33. Fiorentino F, Bono S, Pizzuti F, et al. The importance of determining the limit of detection of non-invasive prenatal testing methods. *Prenat Diagn*. 2016;36(4):304-311. doi:10.1002/pd.4780
34. Norwitz ER, McNeill G, Kalyan A, et al. Validation of a single-nucleotide polymorphism-based non-invasive prenatal test in twin gestations: determination of zygosity, individual fetal sex, and fetal aneuploidy. *J Clin Med*. 2019;8(7):937. doi:10.3390/jcm8070937
35. Leung TY, Qu JZZ, Liao GJW, et al. Noninvasive twin zygosity assessment and aneuploidy detection by maternal plasma DNA sequencing. *Prenat Diagn*. 2013;33(7):675-681. doi:10.1002/pd.4132
36. Chen M, Jiang F, Guo Y, et al. Validation of fetal DNA fraction estimation and its application in noninvasive prenatal testing for aneuploidy detection in multiple pregnancies. *Prenat Diagn*. 2019;39(13):1273-1282. doi:10.1002/pd.5597
37. Hedriana H, Martin K, Saltzman D, Billings P, Demko Z, Benn P. Cell-free DNA fetal fraction in twin gestations in single-nucleotide polymorphism-based noninvasive prenatal screening. *Prenat Diagn*. 2020;40(2):179-184. doi:10.1002/pd.5609
38. Canick JA, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to identify Down syndrome and other trisomies in multiple gestations. *Prenat Diagn*. 2012;32(8):730-734. doi:10.1002/pd.3892
39. Lau TK, Jiang FM, Stevenson RJ, et al. Secondary findings from non-invasive prenatal testing for common fetal aneuploidies by whole genome sequencing as a clinical service. *Prenat Diagn*. 2013;33(6):602-608. doi:10.1002/pd.4076
40. Huang X, Zheng J, Chen M, et al. Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies. *Prenat Diagn*. 2014;34(4):335-340. doi:10.1002/pd.4303
41. Du E, Feng C, Cao Y, Yao Y, Lu J, Zhang Y. Massively Parallel Sequencing (MPS) of cell-free fetal DNA (cffDNA) for trisomies 21, 18, and 13 in twin pregnancies. *Twin Res Hum Genet*. 2017;20(3):242-249. doi:10.1017/thg.2017.23
42. Bevilacqua E, Gil MM, Nicolaides KH, et al. Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies. *Ultrasound Obstet Gynecol*. 2015;45(1):61-66. doi:10.1002/uog.14690
43. Zhang H, Gao Y, Jiang F, et al. Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146 958 pregnancies. *Ultrasound Obstet Gynecol*. 2015;45(5):530-538. doi:10.1002/uog.14792
44. Tan Y, Gao Y, Lin G, et al. Noninvasive prenatal testing (NIPT) in twin pregnancies with treatment of assisted reproductive techniques (ART) in a single center. *Prenat Diagn*. 2016;36(7):672-679. doi:10.1002/pd.4837
45. Fosler L, Winters P, Jones KW, et al. Aneuploidy screening by non-invasive prenatal testing in twin pregnancy. *Ultrasound Obstet Gynecol*. 2017;49(4):470-477. doi:10.1002/uog.15964
46. Le Conte G, Letourneau A, Jani J, et al. Cell-free fetal DNA analysis in maternal plasma as screening test for trisomies 21, 18 and 13 in twin pregnancy. *Ultrasound Obstet Gynecol*. 2018;52(3):318-324. doi:10.1002/uog.18838
47. Hartwig TS, Ambye L, Werge L, et al. Non-Invasive Prenatal Testing (NIPT) in pregnancies with trisomy 21, 18 and 13 performed in a public setting–factors of importance for correct interpretation of results. *Eur J Obstet Gynecol Reprod Biol*. 2018;226:35-39. doi:10.1016/j.ejogrb.2018.04.042
48. Khalil A, Archer R, Hutchinson V, et al. Noninvasive prenatal screening in twin pregnancies with cell-free DNA using the IONA test: a prospective multicenter study. *Am J Obstet Gynecol*. 2021;225:79.e1-79.e13. doi:10.1016/j.ajog.2021.01.005
49. Garchet-Beaudron A, Dreux S, Leporrier N, et al. Second-trimester Down syndrome maternal serum marker screening: a prospective study of 11 040 twin pregnancies. *Prenat Diagn*. 2008;28(12):1105-1109. doi:10.1002/pd.2145
50. Deng C, Liu S. Factors affecting the fetal fraction in noninvasive prenatal screening: a review. *Front Pediatr*. 2022;10:812781. doi:10.3389/fped.2022.812781