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Test performance and clinical utility of expanded non-invasive prenatal test: Experience on 71,883 unselected routine cases from one single center

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Abstract

Objective: The balance between benefits and risks of discordant outcomes makes the Genome-Wide Non-Invasive Prenatal Test (GW-NIPT) controversial. This study aims to evaluate performance and clinical utility in a wide cohort of unselected clinical cases from a single center when a standardized protocol is applied and integrated with a secondary algorithm for data interpretation.

Method: In 2 years, over 70,000 pregnant patients underwent GW-NIPT for fetal common trisomies, sex chromosome aneuploidies, rare autosomal aneuploidies, segmental abnormalities (CNVs $\geq$ 7 Mb) and microdeletions (CNVs $<$ 7 Mb). All samples were uniformly processed with *Veriseq NIPT Solution v2* and analyzed using all data metrics along with a home-made algorithm for sequencing data analysis. Results were retrospectively reviewed for clinical outcomes.

Results: Among 71,883 eligible cases including twin pregnancies, 1011 (1.4%) received a positive result and 781 were confirmed by invasive prenatal diagnosis. Clinical sensitivity ranged from 99.65% for common trisomy (T21, T18, T13) to 83.33% for microdeletions, while specificity remained high (99.98%) for each class of fetal abnormalities detected.

Conclusions: Integrating a standardized protocol with an internal algorithm allowed discordant results to be reduced, yielding high accuracy. Observed reliability in detecting genome-wide chromosomal conditions reinforced the expanded NIPT utility in clinical practice.

Key points

What is already known about this topic?

- Both accuracy and scope of GW-NIPT are detailed in several publications, often based on multisite assessment, selected study cohorts, or data clustered from different protocols, introducing bias in estimating test performance, and making expanded Non-Invasive Prenatal Testing (NIPT) still a matter of debate.

What does this study add?

- Clinical experience on a wide cohort of unselected cases from a single center proved that accurate data metrics evaluation, combined with a dedicated home-made algorithm for secondary data analysis, could reduce discordant results and allow to reconsider the clinical utility of genome-wide NIPT, even when widened to microdeletions.

1 | INTRODUCTION

In over 10 years since its introduction, NIPT has led to a dramatic shift in prenatal care, allowing early and safer detection of fetal genetic conditions through the analysis of circulating cell-free DNA (cfDNA) derived from the placenta and found in maternal plasma.[1] An increasing number of clinical studies have shown high Positive Predictive Values (PPVs) for Trisomy 21, 18, 13, and for sex chromosome aneuploidies (SCAs).[2–6]

Over time, NIPT has widened from well-established common fetal trisomies (T21, T18, T13) and SCA testing[7] to rare autosomal aneuploidies (RAAs), partial deletions/duplications (CNVs), and selected microdeletions/duplications <7 Mb with clinical relevance, including but not limited to DiGeorge (22q11.2), Prader–Willi/Angelman (15q11.2), Cri du Chat (5p-), and 1p36 deletion syndromes.[8–10] About 40% of these abnormalities are often restricted to the placenta only, while the fetus has a normal karyotype, a condition known as Confined Placental Mosaicism (CPM). CPM, affecting about 1%–2% of pregnancies, is associated with adverse outcomes, including early miscarriage, intrauterine fetal death (IUFD), fetal growth restriction (IUGR), fetal structural abnormalities, and preterm delivery.[11,12]

Despite the ceaseless improvement in both accuracy and scope of genome-wide NIPT (GW-NIPT), the origin of the cfDNA (maternal and placental, rather than fetal) poses an intrinsic limitation to the test. A chromosomal abnormality detected by GW-NIPT may not always be fetal and therefore requires confirmation.[13] In some cases, false-positive results have been attributed to CPM or other biological confounders, such as aberrations of maternal origin, including fibroids and malignancies, or vanishing twins (caused by silent twin miscarriage). Dealing with one of these conditions while seeking chromosomal aberrations beyond common trisomies adds a further level of complexity and makes expanded NIPT more controversial.[14] The American College of Medical Genetics and Genomics (ACMG)[15] and the International Society for Prenatal Diagnosis (ISPD)[5] have argued against routine screening for autosomal aneuploidies other than commons and CNVs, despite their potential, due to the risk of discordant results, parental anxiety, and the possible increase in invasive diagnostic procedures.

Moreover, most studies on the exponential use of GW-NIPT in routine clinical settings come from multicenter studies and various laboratories.[16–18] Multisite assessments introduce inevitable bias resulting from the uneven origin of data and differences in test performance. That makes it difficult to accurately define test reliability and GW utility, both crucial when implemented in clinical practice.

Recently, large-scale national studies supporting the clinical relevance of GW-NIPT for pregnancy management in both high-risk and general obstetric populations (TRIDENT 1-2),[12,19,20] have rekindled the debate on the implementation of expanded NIPT in routine clinical practice.

Here we report the clinical experience of 71,883 unselected routine cases from a single center, all of which underwent GW-NIPT by a standardized paired-end sequencing-based assay, together with an internally developed algorithm for data analysis. The aim of the study was to evaluate NIPT test performance in detecting common trisomies, RAAs, SCAs, CNVs ≥ 7 Mb, and microdeletions (<7 Mb), and to reconsider the utility of expanded NIPT in prenatal obstetric care.

2 | METHODS

2.1 | Study population and laboratory workflow

The study cohort encompassed 71,883 consecutive unselected cases from pregnant patients undergoing NIPT from November 2019 to December 2021, including singleton and twin pregnancies, retrospectively reviewed for demographics and clinical outcomes.

Eligible samples were analyzed by the CE-IVD certified *Veriseq NIPT Solution v2* (Illumina, Inc. San Diego, CA, USA), a paired-end whole-genome sequencing assay to detect fetal anomalies, as described in Supplementary appendix 1 in Supporting Information S1. The entire laboratory workflow from extraction to data analysis was uniformly carried out at Eurofins Genoma Laboratory.

Veriseq NIPT v2 calculates fetal fraction (FF) and its combination with sequencing depth, judging whether sequencing coverage and data quality generated are reliable for the aneuploidy call (*IFACT*, *Individualized Fetal Aneuploidy Confidence Test*) or rather causing test failure. Therefore, no fixed but dynamic FF threshold is applied. Given the estimated FF and sequencing coverage, the probability of a specimen being affected is evaluated and expressed as *log likelihood ratio* (LLR). Along with FF and LLR, the Mosaic Ratio (MR) is also estimated by dividing the FF affected by the chromosomal aberration by the overall FF of the sample. As reported, whenever the MR is roughly 1.0, the cfDNA from the placenta is abnormal in a non-mosaic form. MR values below 0.7 are suggestive of an aberration in a mosaic manner.[21] *Veriseq NIPT v2* supplies two different screening options: *Basic* mode shows aneuploidy for chromosomes 21, 18, 13, X, and Y; *Genome-wide* mode detects aneuploidy status for all chromosomes and partial deletions/duplications ≥7 Mb for all autosomes.

In this study, genome-wide mode was applied to all samples to heterogeneously investigate different combinations of aneuploidies, encompassing common trisomies (T21, T18 and T13), RAAs, CNVs ≥ 7 Mb, and SCAs.

A secondary data evaluation with an in-house-developed algorithm aided *Veriseq v2* estimation and, mainly, allowed an in-depth analysis for microdeletions <7 Mb with clinical relevance (Supplementary appendix 1 in Supporting Information S1). For any request of solely common trisomies and SCA screening, a decision tree was applied to derive basic calls, and any "Anomaly detected"-result beyond commons was reported as negative.

For inconclusive NIPT results, due to QC pass failure or borderline results, specimens were re-tested from residual plasma and/or a new blood sample, according to Illumina instructions. Only one result per patient was considered for the statistical evaluation, excluding those from the first analyses. When the second blood analysis also failed, a final report indicating insufficient FF or inconclusive result was generated and excluded from the statistical assessment; patients were addressed to genetic counseling and invasive procedures.

Non-Invasive Prenatal Testing results were reported as "NO Anomaly detected" or "Anomaly detected", later matched to diagnostic outcomes for concordance determined through several sources.

For abnormal NIPT results, the assessment of True Positive (TP) versus False Positive (FP) was based mainly on prenatal invasive techniques (chorionic villus sampling, CVS; amniocentesis; cytogenetic analysis on product of conception, POC), or postnatal karyotyping. A subgroup of TP with no karyotype available (likely TP) was assumed to be concordant when consistent with (1) any ultrasound abnormality highly suggestive of T21, T18, T13 or X0[22–25] and (2) adverse pregnancy outcome (miscarriage, therapeutic abortion, preterm birth) for common trisomies, SCAs and RAAs (Supplementary appendix 2; Table S5).

Negative NIPT results were considered confirmed (True Negative, TN) based on postnatal observations (physical exams, postnatal karyotyping) collected up to 24 months after delivery. False Negative (FN) cases were documented when the NIPT outcomes were discordant with pathological evidence from morphological ultrasound, karyotype from invasive diagnostics, or physical examination at birth (Supplementary appendix 2 in Supporting Information S1).

In case of inconsistency between the NIPT results and clinical observations (suspected false negatives and suspected sex discordance), an additional analysis on a second blood draw was offered.

All data collected were anonymized before being included in the study and informed consent for willing to be part in follow-up studies was obtained from all patients. The study was approved by the Institutional Review Board of Eurofins Genoma Group.

2.2 | Statistical analysis

Non-Invasive Prenatal Testing performance metrics (*sensitivity* and *specificity*, *Positive Predictive Value*, *PPV* and *Negative Predictive Value*, NPV) and descriptive statistics of patient demographics were performed using *MedCalc Statistical Software* version 18.2.1 (MedCalc Software bvba; http://www.medcalc.org; 2018). Statistical evaluations are described in more detail in Supplementary appendix 1 in Supporting Information S1.

3 | RESULTS

Among 72,137 pregnancy cases routinely presented for NIPT test from November 2019 to December 2021, 70,869 got a result at first attempt, while 1268 led to a "NO test result", with a first-pass failure rate of 1.7% (1268/72,137). After re-testing, 1014 failed samples were successfully reported, lowering the final failure rate to 0.35% (254/72,137). As a result, 71,883 patients were enrolled in the study, including 1763 multifetal pregnancies (1258 dichorionic, 215 monochorionic, and 290 vanishing twin pregnancies) (Table 1). In turn, 1011 (1.4%, 1011/71,883) received a positive outcome (Supplementary Table S1).

Demographic characteristics of the study cohort are summarized in Table 1. The mean age of the obstetric population was 38 years on average, with a median gestational age of 12 weeks and an overall median FF of 9%. Patient's personal choice or advanced maternal age mainly led the 88.8% of the obstetric population to present for NIPT in their first trimester of gestation.

After an active follow-up campaign, 143 positive cases (49 T21, 14 T18, 10 T13, 36 SCAs, 34 RAAs and CNVs) were excluded from the statistical evaluation due to the patient's denial to be part of the study or because no follow up available (*No outcome Available*, NA). Amid the remaining 868 results, 781 were confirmed (Supplementary Table S1).

3.1 | Detection of common trisomies and SCAs

Within 579 eligible samples positive for common trisomies, 440 were classified as Trisomy 21 (Table 2). Of these, 437 turned out to be TP, including 5 dichorionic twin pregnancies and 6 cases with a vanishing twin (Supplementary Table S2).

In four out of five dichorionic pregnancies positive for T21, one rather than both fetuses seemed to be affected, since data metrics indicated an aneuploid cfDNA contribution less than the whole fetal FF (i.e., MR < 0.7, on average) (cases #1–4, Supplementary Table S2). Conversely, in one case, data metrics for T21 hinted that both fetuses were impacted by the aneuploidy (case #5, Supplementary Table S2). The array-CGH confirmed the prediction that one twin versus both was affected.

Similarly, in five out of the six vanishing twin pregnancies (cases #2–6, Supplementary Table S2), the abnormal cfDNA contribution was lower than expected based on the overall FF, likely not from the ongoing fetus but rather from the placenta on reabsorption or a consequence of a CPM event.

In these cases, the array-CGH allowed to exclude the abnormality detected was at the expense of the ongoing twin, confirming

TABLE 1 Demographics of the study cohort.

Characteristics		
No. of eligible patients	71,883	
Singleton pregnancies	70,120	
Multifetal pregnancies	1763	
Dichorionic twin pregnancies	1258	
Monochorionic twin pregnancies	215	
Vanishing twin pregnancies	290	
		95% CI
Maternal age (year)		
Median (range)	38 (20–50)	38.0–39.0
25th percentile	35	35.0–36.0
75th percentile	41	41.0–41.0
Gestational age at sample collection (week)		
Median (range)	12 (9.4–28.2)	12.0–12.1
25th percentile	11.2	11.1–11.2
75th percentile	13	13.0–13.1
Trimester (*n*)		
First (up to 14 weeks)	63,832 (88.8%)	
Second (15–27 weeks)	8047 (11.2%)	
Third (28–40 weeks)	4 (<0.01%)	
Indications for NIPT		
Personal choice[a]	28,969 (40.3%)	
Advanced maternal age	36,661 (51%)	
Positive 1st trimester screening and/or abnormal ultrasound	5535 (7.7%)	
Prior pregnancy with fetal aneuploidy	575 (0.8%)	
Personal/Family history[b]	143 (0.2%)	
Fetal fraction (%)		**95% CI**
Median (range)	9 (2–26)	9.0–9.0
25th percentile	7	6.0–7.0
75th percentile	12	11.0–12.0

Abbreviation: CI, Confidence Interval.

[a]Personal Choice: pregnancies in which a clear medical indication was missing.

[b]Personal/family history refers to the presence of chromosomal abnormalities identified in one of the two parents and/or family history for chromosomal imbalances.

the prediction about the status of the vital fetus (Supplementary Table S2).

In singleton pregnancies, data analysis metrics can be helpful to hypothesize if chromosomal aberration is in a mosaic manner but not to discriminate between CPM and True Fetal Mosaicism (TFM). In fact, for one case with NIPT results of suspected mosaic T21 (MR = 0.4), invasive testing confirmed a low TFM (46, XX [80]/47, XX + 21 [20]) (data not shown).

Clinical outcomes were discordant for three pregnancies (FP) though data metrics were not suggestive of any mosaicism (Supplementary Table S3).

In two cases, no aneuploidy was detected by the NIPT test, but ultrasound abnormalities and the following invasive procedures revealed the presence of T21 (FN). Re-testing on a second blood draw at the time of amniocentesis confirmed NIPT-negative results (Supplementary Table S3).

For Trisomy 18, 93 out of 94 samples were confirmed to be TP (Table 2), including two vanishing twin pregnancies and one dichorionic twin gestation, with only one fetus appearing to be affected (Supplementary Table S2).

Only one T18 FP was reported. Based on data metrics, it was assumed that the aneuploidy was in a mosaic manner (Supplementary Table S3). However, both array-CGH and karyotyping on one hundred metaphases of amniotic fluid (AF) did not detect any mosaicism. No FN was recorded.

All 45 cases classified as T13 were singleton pregnancies. Upon invasive procedures, eight cases turned out to be FP (Table 2); data metrics were suggestive of the aneuploidy in a mosaic form. Among the discordant results, one case proved to be CPM by molecular cytogenetics performed on placental DNA at delivery (case# 3, Supplementary Table S3).

Overall performance in detecting common trisomies is detailed in Table 2: clinical sensitivity and specificity resulted in 99.65% and 99.98%, respectively, with a PPV of 97.9%.

Regarding SCAs, 173 samples returned "Anomaly-detected" calls, and 156 were confirmed by invasive tests (Table 3).

Among 17 FP, only three cases reported as X0 showed metrics ascribable to a mosaic rather than a full aneuploidy (cases #5, #10, #11, Supplementary Table S3). In addition, three FP cases led to sex discordance. For one X0 positive result (case #2), diagnostic testing on AF revealed a male with a duplication Yq11.21q11.223 (9.8Mb) and a deletion Yq11.223q12 (34.9Mb). The other two cases (case #1 45,X turned into 47,XYY, and case #16 47,XXY proved to be 46,XX) remain unresolved, and the origin of the detected aneuploidy is unknown.

For one case reported as 46,XX (FF = 5%), severe ultrasound cardiac abnormalities were found. The subsequent diagnostic confirmation revealed a 45,X fetus (case #14, Supplementary Table S3).

The overall observed sensitivity for SCA was of 99.36%, with specificity of 99.97%, and PPV of 90.17% (Table 3).

3.2 | Genome-wide detection of rare aneuploidies, copy number variants and microdeletions

About 65% (46,724/71,883) of enrolled pregnant patients were evaluated for RAA and CNVs ≥7 Mb, and 105 received a positive result (Table 4).

TABLE 2 NIPT performance for common trisomies (T21, T18, T13).

	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Trisomy 21	437	3	71,368	2	99.54% (98.36%–99.94%)	100% (99.99%–100.00%)	99.32% (98.02%–99.78%)	100% (99.99%–100.00%)
Trisomy 18	93	1	71,716	0	100% (96.11%–100.00%)	100% (99.99%–100.00%)	98.94% (94.21%–99.97%)	100% (99.99%–100.00%)
Trisomy 13	37	8	71,765	0	100% (90.51%–100.00%)	99.99% (99.98%–100.00%)	82.22% (67.95%–92.00%)	100% (99.99%–100.00%)
Overall	567	12	71,229	2	99.65% (98.74%–99.96%)	99.98% (99.97%–99.99%)	97.93% (96.41%–98.92%)	100% (99.99%–100.00%)

Note: Observed *Sensitivity*, *Specificity*, *PPV* and *NPV* are evaluated on pregnancies with known clinical outcomes ($N = 71{,}810$). No follow up cases for common trisomies excluded from the statistics ($N = 73$): T21 (49); T18 (14); T13 (10).

Abbreviations: CI, Confidence Intervals; FN, False Negative; FP, False Positive; NPV, Negative Predictive Value; PPV, Positive Predictive Value; TN, True Negative; TP, True Positive.

TABLE 3 NIPT performance for sex chromosome aneuploidies.

	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
X0	52	13	65,706	1	98.11% (89.93%–99.95%)	99.98% (99.97%–99.99%)	80% (68.23%–88.90%)	100% (99.99%–100.00%)
XXX	27	0	65,745	0	100% (87.23%–100.00%)	100% (99.99%–100.00%)	100% (87.23%–100.00%)	100% (99.99%–100.00%)
XXY	51	3	65,718	0	100% (93.02%–100.00%)	99.99% (99.99%–100.00%)	94.44% (84.61%–98.14%)	100% (99.99%–100.00%)
XYY	26	1	65,745	0	100% (86.77%–100.00%)	99.99% (99.99%–100.00%)	96.30% (81.03%–99.91%)	100% (99.99%–100.00%)
Overall	156	17	65,598	1	99.36% (96.50%–99.98%)	99.97% (99.96%–99.99%)	90.17% (84.73%–94.17%)	100% (99.99%–100.00%)

Note: *Sensitivity*, *Specificity*, *PPV* and *NPV* for SCA in the study cohort. The cohort size is adjusted for the proportion of cases for which SCA screening was requested ($N = 65{,}808$). Observed test metrics are evaluated on pregnancies with known clinical outcomes ($N = 65{,}772$). No Follow up cases ($N = 36$): X0 (18); XXX (6); XXY (7); XYY (5). The number of False Negatives calculated on existing data could be underestimated due the incomplete clinical information for low-risk cases retrospectively collected.

Abbreviations: CI, Confidence Intervals; FN, False Negative; FP, False Positive; NPV, Negative Predictive Value; PPV, Positive Predictive Value; TN, True Negative; TP, True Positive.

Rare autosomal trisomies (RATs) were identified in 69 samples with known outcomes, and 33 were confirmed, including one case of vanishing twin pregnancy with detected T15, affecting the fetus in reabsorption (Supplementary Table S2). The observed clinical sensitivity was of 99.99%, retaining high specificity (99.92%), despite the relatively modest PPV (47.83%), as shown in Table 4.

Among TP, 12 cases ended in spontaneous miscarriage, while six cases of the remaining 20 RATs which proceeded to invasive testing resulted in TFM (Supplementary Table S7).

An overview of chromosome abnormalities identified through expanded NIPT analysis is detailed in Supplementary Table S4. Trisomies 15, 7, and 22 were the most frequently encountered (Figure 1).

The highest FP incidence was for chr7 (11/12, 91.6%) (Supplementary Table S4). On average, T7 positive cases showed depressed MR values and metrics suggestive of aneuploidy in a mosaic manner, and in turn, all but one were discordant with diagnostic outcomes (Supplementary Table S3).

For chr16, the FP incidence was milder (6/9, 66.6%), while the lowest incidence was for chr 22 (4/12, 33.3%) and chr 15 (3/15, 20%) (Supplementary Table S4). For both T15 and T22, data metrics pointed toward a full aneuploidy rather than a mosaic, on average, regardless of the concordance with invasive diagnostic outcomes (Supplementary Table S3) and about 33.3% of pregnancies (5/15 for T15 and 4/12 for T22, respectively) resulted in early miscarriages (Supplementary Table S6). One case of T15 was confirmed by cytogenetic evaluation of the POC.

TABLE 4 NIPT Performance for rare autosomal aneuploidies (RAAs), segmental chromosomal abnormalities (CNV > 7 Mb) and microdeletions (<7 Mb).

	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
RAAs	33	36	46,623	0	99.99% (89.42%–100.00%)	99.92% (99.89%–99.95%)	47.83% (35.65%–60.20%)	100% (99.99%–100.00%)
CNV≥7 Mb	20	16	46,656	0	99.99% (83.16%–100.00%)	99.97% (99.96%–99.99%)	55.56% (38.10%–72.06%)	100% (99.99%–100.00%)
Microdeletions[a] (CNV <7 Mb)	5	2	28,743	1	83.33% (35.88%–99.58%)	99.99% (99.99%–100.00%)	71.43% (37.40%–91.27%)	100% (99.99%–100.00%)

Note: *Sensitivity*, *Specificity*, *PPV and NPV* for anomalies other than commons in the study cohort. The cohort size is adjusted for the proportion of cases for which RAA and CNV ≥ 7 Mb screening was requested ($N = 46{,}724$). Observed test metrics are evaluated on pregnancies with known clinical outcomes $N = 46{,}692$. No follow up cases ($N = 32$): RAA (25); CNV ≥ 7 Mb (7). For microdeletions (CNV < 7 Mb): adjusted cohort size ($N = 28{,}753$); pregnancies with known clinical outcomes ($N = 28{,}751$). No follow up cases: Microdeletions <7 Mb (2). The number of False Negatives calculated on existing data could be underestimated due the incomplete clinical information for low-risk cases retrospectively collected.

Abbreviations: CI, Confidence Intervals; FN, False Negative; FP, False Positive; NPV, Negative Predictive Value; PPV, Positive Predictive Value; TN, True Negative; TP, True Positive.

[a]Investigated microdeletions: *22q11.2 (DiGeorge Syndrome)*, *5p- (Cri-du-chat Syndrome)*, *15q11.2 (Prader-Willi/Angelman Syndrome)*, *1p36 Deletion Syndrome*, *4p- (Wolf-Hirschhorn Syndrome)*, *11q23 (Jacobsen Syndrome)*, *8q24 (Langer-Giedion Syndrome)*, *and 17p11.2 (Smith-Magenis Syndrome)*.

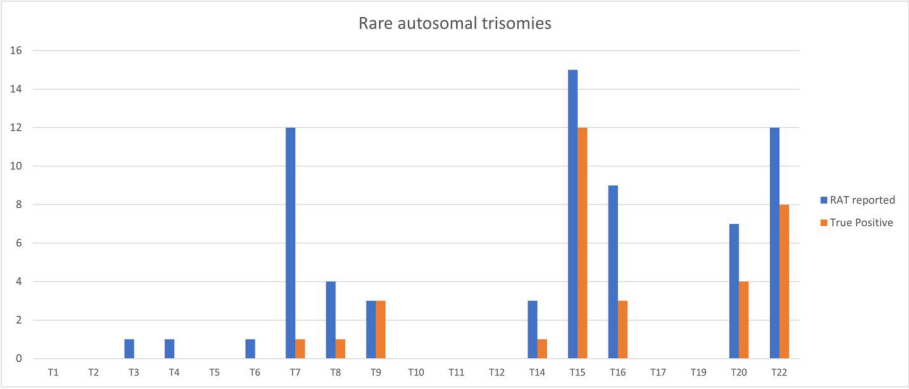

FIGURE 1 Number of rare autosomal trisomies reported per chromosome. Comparison between number of RATs reported (blue bar) and confirmed cases (True Positive; orange bar) per chromosome.

Regarding CNVs ≥ 7 Mb, among 36 positive calls, 20 were confirmed by invasive testing (Table 4) consisting of 4 duplications, 13 deletions and 3 complex CNVs. A list of detected CNVs ≥ 7 Mb is detailed in Supplementary Table S4. One out of the 16 FP was reported as a duplication 9p24.3p24.1 (8.6 Mb), but it was then traced back to the clinical history of the mother, suffering from Hodgkin's lymphoma (case #2, Supplementary Table S3). Interestingly, for a pregnant woman with a personal history as a carrier of a balanced reciprocal translocation (15; 20), *Veriseq v2* detected a duplication 15q22.31q26.3 (37.3Mb). Upon a secondary evaluation with the home-made algorithm, it was also identified a deletion 20q13.33 < 7 Mb, below the *Veriseq v2* limit of detection, unveiling a possible result of an inherited unbalanced translocation. The patient was reported for both segmental aberrations, and then confirmed with amniocentesis (Supplementary Table S4).

No FN were found, resulting in high sensitivity (99.99%) and specificity (99.97%) and a relatively low PPV = 55.56%, as shown in Table 4.

Among the 28,753 patients undergoing GW-NIPT for microdeletion <7 Mb, five out of seven samples with positive calls were later confirmed with amniocentesis: 3 cases of del(22) (q11.2) (2.46Mb, on average), 1 del(15) (q11.2q12) (3.7 Mb) and 1 del(1) (p36.33p36.23) (6.8 Mb) (Supplementary Table S4). Two FP were reported as a del(22) (q11.21) (600Kb), and a del(5) (p15.31p15.32) (552Kb), while NIPT failed to detect a del(4) (p16.3p16.1) (Supplementary Table S3). Observed clinical sensitivity and specificity were 83.33% and 99.99%, respectively, despite the rarity of these conditions (Table 4).

4 | DISCUSSION

This study demonstrated the GW-NIPT elevated reliability, with clinical sensitivity and specificity in line, or even exceeding, those of previous studies,[20,26] reinforcing its feasibility, and providing the chance to reconsider its utility in clinical practice.

A standardized protocol was uniformly applied to the wide cohort of unselected patients retrospectively collected with the aim to: (1) evaluate NIPT test performance in detecting common trisomies, RAAs, SCAs, CNVs (≥7 Mb), and microdeletions (<7 Mb) by the paired-end-based *Veriseq NIPT solution v2*, along with an in-house-developed algorithm for data analysis; (2) show how the integrated use of all data metrics can be useful for outcomes interpretation, and to predict the origin of aneuploidies in twin and vanishing twin pregnancies; (3) reconsider the clinical utility of GW-NIPT in clinical practice.

Overall, among 71,883 patients presented to NIPT, a total of 752 received positive results for common trisomies and SCAs than matched for clinical outcomes, including 440 T21, 94 T18, 45 T13, and 173 SCAs.

Results demonstrated high accuracy for common aneuploidies and assessed the PPV at 97.9% within the range posed by recent publications. PPVs for T21 (99.3%) and T18 (98.9%) exceeded those of previous studies on routine clinical cases.[27,28] As a comparison, the TRIDENT-2 study[20] applied to the Dutch general obstetric population revealed lower PPVs for both T21 (96%) and T18 (98%).

However, PPVs can be influenced by different indications for testing.[27,29] For instance, the older the mother, the more common trisomies, SCAs, and RAAs are likely.[30] In this study, a significant proportion of patients come to NIPT at advanced maternal age; therefore, our study cohort should be considered, although unselected, as a mixed-risk population and PPVs may be overestimated.

Consistent with the above publications, PPV for T13 (82.2%) was depressed compared with those of other common trisomies. That could be related to the relatively high number of FPs, maybe due to its propensity to be associated with CPM.[31]

Placental mosaicism can also be a cause of NIPT false negatives[32] as suggested by two discordant outcomes among the study cohort. Despite ultrasound findings and following array-CGH unveiled a full T21, second blood draws collected at the time of amniocentesis confirmed negative NIPT results from first samplings. Data analysis metrics returned no indication of any aneuploidy, FF was high (Supplementary Table S3), and low TFM for T21 was excluded by invasive procedures; therefore, a mosaicism involving normal cells in the placenta (fetus-placental discrepancy) was assumed as the plausible reason for the discordant outcomes.

In our laboratory, we routinely offer screening for fetal SCAs as strongly recommended by the ACMG society.[15] However, one of the issues concerning SCA testing is the difference in PPV among its common types (X0, XXX, XXY, and XYY), with usually a lower PPV for X0.[33] Similarly, in our study, the relatively low PPV for monosomy X (80%) heavily influenced the overall PPV for SCAs to 90%, possibly due to its involvement not only in CPM but also in maternal mosaicism.[34] Moreover, for one X0 FP (#2; supplementary table S3), then resulted male with a dup(Y) (q11.21q11.223) and a del(Y) (q11.223q12), this complex profile may have caused chrY missed-call and, in turn, the discordant result.

In contrast, the observed PPV for other SCAs (XXX, XXY, and XYY) was more accurate (>95%), emphasizing the importance of reporting SCA testing performance for each type separately rather than overall.

Biological confounders, such as CPM, low-level fetal mosaicism,[35,36] vanishing twins[37] and maternal genomic contribution (malignancy; sex chromosome mosaicism),[38,39] all constitute a limit for any NIPT test, and it is even more duly to take into account for GW-NIPT.

In this study, expanded NIPT identified 69 RATs among the eligible cohort for GW-analysis (0.15%, 69/46,724), in line with the TRIDENT-2 study (0.18%).[20]

The most frequently encountered RATs were T15 (15/69; 21.7%), T7 (12/69; 17.4%) and T22 (12/69, 17.4%).

RATs are usually associated with either fetal or more frequently placental mosaicism.[11,40,41] Twenty positive results for RATs that proceeded to invasive confirmation revealed six cases of TFM (Supplementary Table S7).

In contrast, T7 showed the highest FP incidence (91.6%), maybe because of the high propensity to be involved in CPM[40,42] as well as T13. *Kleinfelger* et al. 2020,[41] suggested screening RAA conditions only for those trisomies most often involving the fetus (T8, T9, T22) or those with known clinical implications (T14, T15, T16 and T12). Screening these RAAs only, in fact, may depress the FP rate because the mostly detected rare aneuploidies are frequently confined to the cytotrophoblast with no fetal involvement (T3 and T7), contributing to lower the PPV for these rare conditions. However, CPM can be associated with intrauterine growth restriction (IUGR) and with the risk of different negative outcomes (abnormal placentation, impairing placental function, and fetal development) depending on the chromosome involved[40,43] and requiring careful monitoring of placental functionality. Identification of these adverse conditions strictly associated with CPM before clinical manifestation could allow better management of ongoing pregnancies, enhancing the clinical utility of expanded NIPT.

Since CPM can also be associated with uniparental disomy (UPD)[43,44] every positive result involving imprinted chromosomes such as chr 7 and chr 15 was tested to rule out UPD.[45] In this study, among the 11 samples tested, all returned a negative result (Supplementary Table S3). However, in our clinical experience with GW-analysis in over seven years, we found two cases of UPD15, proving that FP results for imprinted chromosomes could be the consequence of a trisomy rescue that would otherwise remain unnoticed.[8]

In addition, 12 cases resulted in early miscarriage, including about 33% of T15 and T22. Some chromosomal aberrations and many rare aneuploidies are not viable; therefore, an additional potential of GW-NIPT could be to provide an underlying etiology for early pregnancy losses when no POC is available, and the pregnancy tissue remains in situ at the time of sampling.[46,47]

This study evaluated the feasibility of non-invasive prenatal detection of fetal CNVs in routine practice, observing high performance despite low PPV (55.5%). It demonstrated the usefulness of integrated data analysis with a secondary algorithm, especially for small imbalances missed by *Veriseq v2* but revealing the real genetic condition of the fetus. That was for the inherited unbalanced

translocation (15; 20) described, for which it was the secondary evaluation that unveiled a deletion of chr.20 (<7 Mb).

Recently, *Rafalko* et al. 2020[21] demonstrated that PPVs can be influenced by the MR, suggesting its utility in identifying NIPT results more likely to be discordant.

The lower the MR, the more likely the sample is FP, and this correlation becomes more valuable for those chromosomes often implicated in mosaicism events with less fetal involvement or confined only to the placenta. Our findings seem to confirm this correlation. For instance, the average MR of FP cases for T13 and T7 was <0.5, and X0 FPs were associated with a median MR ≤ 0.6. Therefore, MR values could be used to identify a priori those results that are more likely to be discordant with the genetic status of the fetus, helping to define a more accurate and refined MR-based PPV for the abnormal NIPT results. As a future perspective, it would be useful to deeply explore the MR-PPV correlation, especially for RAAs and CNVs.

Overall, our findings show compelling evidence that, besides technical improvements, relying on all metrics for a critical interpretation of NIPT outcomes can partially overcome test sensitivity to biological confounders responsible for discordant results. These metrics are not exclusive to singleton gestations but also helpful for an accurate early screening in twin pregnancies by predicting whether one of both fetuses is affected or hypothesising the origin of the aneuploidy in case of co-twin demise.

The internal algorithm became exclusive for microdeletion detection, prompting GW-analysis beyond the limits of *Veriseq v2*. Despite the low prevalence of this condition, the wide cohort of this study and the algorithm resolution enabled the collection of seven microdeletions. Four out of the total were suspected del(22) (q11.2), related to DiGeorge Syndrome, and in turn, three proved to be TP, confirming it as the most prevalent among microdeletions.[48,49] Children with inherited microdeletions may exhibit a different phenotype from their parents because of the variable penetrance of CNVs. For this reason, we chose to report a del(22) (q11.2) later recorded as FP, despite it being expected to be of maternal origin due to the high intensity versus the small size of the signal (0.6Mb), and therefore a more likely discordant result.

Considering together the observed performance for each class of fetal abnormalities, clinical sensitivity ranged from 99.65% for common trisomy to 83.33% for microdeletions, while retaining the same high specificity (99.98%), and suggesting elevated genome wide reliability.

However, retrospective studies always have limitations due, for instance, to bias in classifying concordant/discordant results and lost to follow up. Consistent with previous studies,[29,50] sensitivity and specificity were estimated by assuming that "Likely TP" cases were all concordant. To mitigate a possible overestimation of test performance, we considered the ultrasound confirmation reliable only when highly suggestive of common trisomy or X0,[22–25] while miscarriage was the inclusion criterion for RAAs positive cases, with the anomaly detected as the most reasonable cause of the pregnancy loss.[46]

Moreover, excluding 143 positive cases from the statistics, we are not able to estimate how the observed test performance would differ if properly categorized in the study.

Another limitation is that most NIPT negative cases were considered TN without diagnostic confirmation but relying on postnatal physical examination up to 24 months from delivery, with no further communication of discordant results, consistent with other studies.[27,29,50–52]

Complete clinical outcomes on a cohort as wide as reported were unrealistic to be performed retrospectively since low-risk patients may be less likely to have diagnostic testing. The FN recording was based on cases self-reported to the laboratory, and we documented four discordant results (0.005%). Although clinicians may be more likely to report discordant outcomes immediately, we encouraged records of any inconsistency with the NIPT result for a reasonable period after birth. Nevertheless, the number of FN cases could be underestimated, especially for small CNV-driven syndromes and SCAs, which are not easily identifiable. Even though incomplete diagnostic information for low-risk results may limit conclusions regarding specificity, the high percentage (85.9%) of the overall positive cases with known follow up allowed reasonable confidence in some aspect of performance calculation, not for common trisomies solely. However, sensitivity and specificity for SCAs and microdeletions should be treated as estimations, though realistic, that need to be confirmed with prospective follow up studies relying on complete diagnostic information.

5 | CONCLUSION

Prenatal screening results are a complex combination of biological factors and bioinformatics algorithms and should be dealt with case-by-case, considering any adverse pregnancy outcome, ultrasound findings, and anamnestic data.

With this large-scale study, we demonstrated that expertise in data metrics evaluation and the design of proper algorithms for a more careful analysis could reduce discordant outcomes. Consequently, high clinical sensitivity to any fetal condition in the general obstetric population is yielded without affecting specificity, even when widened to microdeletions. In turn, this high accuracy in detecting whole genome fetal aberrations, along with aware and careful pre- and post-counseling, reinforces the clinical utility of expanded NIPT in pregnancies management.

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CONFLICT OF INTEREST STATEMENT

All authors are current or former employees of Eurofins Genoma Group.

DATA AVAILABILITY STATEMENT

Additional supporting information may be found online in the supporting information section. Research data are not shared.

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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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