Positive predictive values and outcomes for uninformative cell-free DNA tests: An Italian multicentric Cytogenetic and cytogenomic Audit of diagnOstic testing (ICARO study)

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Abstract

Objectives: To establish the positive predictive values (PPV) of cfDNA testing based on data from a nationwide survey of independent clinical cytogenetics laboratories.

Methods: Prenatal diagnostic test results obtained by Italian laboratories between 2013 and March 2020 were compiled for women with positive non-invasive prenatal tests (NIPT), without an NIPT result, and cases where there was sex discordancy between the NIPT and ultrasound. PPV and other summary data were reviewed.

Results: Diagnostic test results were collected for 1327 women with a positive NIPT. The highest PPVs were for Trisomy (T) 21 (624/671, 93%) and XYY (26/27, 96.3%), while rare autosomal trisomies (9/47, 19.1%) and recurrent microdeletions (8/55, 14.5%) had the lowest PPVs. PPVs for T21, T18, and T13 were significantly higher when diagnostic confirmation was carried out on chorionic villi (97.5%) compared to amniotic fluid (89.5%) ($p < 0.001$). In 19/139 (13.9%), of no result cases,

a cytogenetic abnormality was detected. Follow-up genetic testing provided explanations for 3/6 cases with a fetal sex discordancy between NIPT and ultrasound. **Conclusions:** NIPT PPVs differ across the conditions screened and the tissues studied in diagnostic testing. This variability, issues associated with fetal sex discordancy, and no results, illustrate the importance of pre- and post-test counselling.

Key points

What's already known about this topic?

- The reported performance of cfDNA testing by NIPT laboratories is often based on incomplete follow-up.
- Data from cytogenetics laboratories can provide an alternative, independent, assessment on the positive predictive value (PPV), risk in cases when there is no result, and help explain contradictory fetal sex assignments.
- Prior cytogenetic laboratory studies assessing PPV from have been based on small numbers of cases.

What does this study add?

- PPV is higher when the diagnostic testing is based on CVS compared to amniocentesis, presumably because CVS includes cases with confined placental mosaicism.
- In a high proportion of no result cases, a cytogenetic abnormality may be present. Therefore, these do need to be considered high-risk pregnancies.
- Diverse disorders of sexual development can be present when fetal sex assignments based on ultrasound and NIPT are discordant.

1 | INTRODUCTION

Non-invasive prenatal testing (NIPT) based on cell-free DNA (cfDNA) circulating in maternal blood was first introduced in 2011 and is now used in clinical practice in many countries.[1] NIPT methods have evolved, reflecting technical and bioinformatic improvements. The chromosome targets of interest were extended from the common autosomal trisomies (defined as trisomies 13, 18 and 21 (T13, T18, and T21)) to include sex chromosome aneuploidies (SCAs) (monosomy-X (MX), XXX, XXY, XYY, and more complex karyotypes), panels of recurrent microdeletions, and other additional large genomic imbalances, which include large segmental imbalances (SI) typically ≥7 Mb, rare autosomal trisomies (RATs), and multiple aneuploidies.[2–8] Most of the professional societies (including those in Italy) have recognized NIPT in screening for the common autosomal trisomies but do not yet support testing for additional imbalances. However, testing for additional imbalances is variably included in NIPT panels in many clinical settings.[9]

Although NIPT for the common autosomal trisomies has been shown to have high sensitivity and specificity in validation studies, the testing is not considered diagnostic and follow-up confirmatory analyses of amniotic fluid cells (AFC) or chorionic villus sampling (CVS) is necessary. From a patient counseling standpoint, it is important to be able to present the probability that a positive result is a true positive (the positive predictive value, PPV) and this differs for each trisomy. Establishing the performance of the testing has been difficult for NIPT laboratories because they usually do not provide analysis of AFC or CVS and do not have direct access to medical records or neonatal evaluation. Therefore, studies from NIPT laboratories that assess PPV are often based on incomplete follow-up of test-positive cases.

Data from clinical cytogenetics laboratories can provide an alternative source of information about the relative performance of NIPT. Because professional societies emphasize the importance of confirming positive NIPT results through diagnostic testing, these laboratories can provide an independent assessment of the PPV of NIPT. However, thus far, there is only very limited data available from these laboratories.[10–12]

The purpose of this study was to determine PPVs through a large nation-wide survey of cytogenetic and cytogenomic diagnostic laboratories. We considered whether PPVs would differ between CVS versus AFC and also considered the NIPT methodology used. In this report, we present these findings.

2 | MATERIALS AND METHODS

2.1 | Study structure

This was a retrospective analysis of anonymized diagnostic test results collected through a collaborative study of 39 public and private cytogenetics and cytogenomics laboratories. Participating centres

adhered to the policies of the Cytogenetics and Cytogenomics working group of the Italian Society of Human Genetics (SIGU). Laboratories were located in Italian territory and in the Italian-speaking Swiss canton (Ticino). Eligibility criteria included any diagnostic investigation performed between 2013 and March 2020 on products of conception; CVS, AFC, or newborn blood. Data was requested for all cases with a high-risk NIPT result (which may have included variable criteria for defining a positive test result). Information was also gathered for cases without a result, which included samples with insufficient fetal fraction, those with results for only some chromosome abnormalities tested, failures, sample quality issues, and unknown reasons. Information on cases where there was discordancy between the fetal sex assigned by NIPT and that by ultrasound was also sought.

Laboratories were asked to complete an anonymized datasheet that included the following information: NIPT result, maternal and gestational age, number of fetuses at the time of NIPT, indication for NIPT, tissue analyzed in the diagnostic investigation, type of cytogenetic/cytogenomic analyses performed and results. Data was also collected on the NIPT testing technology/strategy, classified as either massively parallel shotgun sequencing (MPSS), digital analysis of selected regions (DANSR), single nucleotide polymorphisms-based (SNP-based), targeted massively parallel sequencing, or digital imaging and counting assay (DICA). A blank datasheet is available as a Supplementary material document.

The study was submitted and approved by TOMA laboratory Ethical Committee (#2020–29).

2.2 | Cytogenetic and cytogenomic analyses

Cytogenetics and cytogenomics testing were performed consistent with Italian guidelines (2013) that recommend applying karyotyping and/or chromosome microarray and/or other molecular genetic test considered to be diagnostic. Detailed description of diagnostic methods is reported in the Supplementary information.

PPV were calculated from tabulated data according to the type of diagnostic procedure, chromosome abnormality and NIPT strategy (targeted or genome-wide). Criteria for classifying high-risk results as true positive and statistical methods are provided in the Supplementary information.

For the purposes of this analysis, detection of a mosaic abnormal cell line through any diagnostic procedure was interpreted as a true positive test result. This included an abnormality confined to a direct preparation of CV (trophoblasts) and also the detection of an abnormality in a product of conception sample without other confirmatory information. For RATs that involved a chromosome where a clinically significant imprinting effect might be present in the disomic cells, we considered the detection of uniparental disomy (UPD) as a true positive result. A maternal chromosome abnormality identified as result of the NIPT was not considered a true positive result. Statistical methods are described in the Supplementary materials.

3 | RESULTS

The study included confirmatory diagnostic testing in 1327 pregnancies where there was an NIPT high-risk result (Table 1). Multiple samples from the same pregnancy were analyzed in 27 cases. Of the 1327 pregnancies, 1273 (95.93%) were singleton pregnancies, 23 (1.73%) twins, 3 (0.23%) vanishing twin, and in 28 (2.11%), the number of fetuses was unknown. The cfDNA testing methodology used was massively parallel shotgun sequencing (MPSS) in 45.29% (n = 601), digital analysis of selected regions (DANSR) in 15.60% (n = 207), single nucleotide polymorphisms-based (SNP-based) in 7.3% (n = 97), targeted MPS in 0.23% (n = 3), and digital imaging and

TABLE 1 Number of diagnostic tests performed according to high-risk cfDNA test results

High risk cfDNA result	All	CV	AFC	Product of conception	Newborn blood
T21	671[a]	275	393	2	1
T18	124[b]	44	78	2	0
T13	84[c]	15	68	1	0
MX	125[d]	11	108	0	6
XXX	45[e]	3	39	0	3
XXY	104[f]	16	81	0	7
XYY	27	2	23	0	2
RAT	47[g]	2	44	1	0
Segmental imbalance	29[h]	4	25	0	0
Multiple abnormalities	11	2	9	0	0
22q11.2DS	30	6	24	0	0
dup22q11.2	2	0	2	0	0
del1p36	8	0	8	0	0
del15q11.2	12	2	10	0	0
del5p	4	1	3	0	0
del17p11.2	1	0	1	0	0
XXXY	1	0	1	0	0
Triploidy	2	0	2	0	0
Total	**1327**	**383**	**919**	**6**	**19**

Note: Cases with studies on multiple samples.

Abbreviations: 22q11.2DS, 22q11.2 deletion syndrome; AF, amniotic fluid; CV, chorionic villi; del, deletion; dup, duplication; M, monosomy; RAT, rare autosomal trisomy; T, trisomy.

[a]Four with CV plus AF and one with CV plus POC.

[b]One with CV plus AF; two with CV plus product of conception.

[c]Six with CV plus AF.

[d]Five with CV plus AF and three with AF plus newborn blood.

[e]One with CV plus AF.

[f]One with AF plus newborn blood.

[g]One with CV plus AF and one with CV plus POC.

[h]One with CV plus AF.

counting assay (DICA) in 0.08% (n = 1). In 31.50% (n = 418), the cfDNA test technology was not available.

Table 2 summarizes the indications for NIPT testing for the cases with NIPT high-risk results. Over half (51.7%) were because of advanced maternal age, while only 5.3% were due to an abnormal first-trimester combined test and only 1.1% were because of an increased nuchal translucency, hydrops, or other ultrasound-identified abnormality. Median maternal age at the time of cfDNA test was 37.29 ± 4.40 years (information available in 1317/1327 cases) and the median gestational age at testing 12 ± 22 ± 1.59 weeks (data available in 772/1327 cases). Figure S1 depicts the geographic distribution of 1327 cases. Figure S2 shows the increasing trend of diagnostic confirmations of the NIPT result through the years.

3.1 | Common autosomal trisomies

PPVs for the various specific NIPT results are summarized in Table 3. Based on all types of confirmatory tests combined, the PPV for T21 was 624/671 (93%, 95% CI 91%–95%), for T18, 91/124 (73.4%, 95% CI 65%–80%), and for T13, 26/84 (31.0%, 95% CI 19%–38%). These three PPVs were significantly different from each other ($p < 0.0001$).

For all three common autosomal trisomies, PPV was significantly higher when the follow-up diagnostic test was based on CV compared to analysis of AFC. To further investigate the PPVs for CV confirmatory testing, we separately considered the results for studies on direct preparation (trophoblasts) versus long-term culture (mesenchyme). We also considered whether there was evidence for mosaicism, including for cases confirmed in AFC. Table 4 shows that PPVs for CV were similar regardless of whether the testing was based on trophoblasts or mesenchyme ($p > 0.05$). The numbers of mosaic cases were relatively low, regardless of the lineage analyzed. In CV, mosaicism was most common for T13 (in cytotrophoblasts 5/21 (23.8%) vs. 0/44 (0%) for T18 and 4/260 (0.77%) for T21; in mesenchyme 3/21 (14.3%) vs. 0/44 (0%) for T18 and 2/260 (0.77%) for T21). This chromosome also had the lowest PPVs (81.0% vs. 97.7% for T18 vs. 96.9% for T21 including mosaics; 57.1% vs. 97.7% for T18 vs. 95.4% for T21 excluding mosaics) (Table 4).

3.2 | Sex chromosome abnormalities (SCA)

The overall PPV for MX was 34/125 (27.2% 95 CI 20%–0.36%), which was significantly lower than for XXX, XXY, and XYY ($p < 0.0001$; Table 3). No significant difference could be demonstrated between the PPV when cases received CV as the confirmatory test versus AFC studies.

For XXX, XXY, and XYY considered individually, PPVs were similar regardless of whether the follow-up testing was based on trophoblasts or mesenchyme ($p > 0.05$) (Table 4). For MX including mosaic cases, the PPV was slightly higher (50%) when the testing was based on mesenchyme compared to cytotrophoblasts (44%) but this was also insignificant. Considering all SCAs combined, and including mosaic cases, the PPV was significantly higher (71%) for mesenchyme testing compared to AFC (51%) ($p < 0.05$) but the PPV for cytotrophoblast studies (68%) compared to AFC (51%) failed to reach

TABLE 2 Indication for cfDNA testing according to high-risk results

High risk cfDNA result	Unknown	Known	AMA (≥35 years)	Anxiety/low risk (<35 years)	FCT	Increased NT/hydrops	Abn US	Previous affected	Other	Total
T21	152	519	391	73	45	8	0	1	1	671
T18	29	95	68	12	9	0	2	0	4	124
T13	27	57	32	18	4	0	0	0	3	84
MX	36	89	48	37	3	0	0	0	1	125
XXX	9	36	21	12	2	0	0	1	0	45
XXY	23	102	48	27	3	1	1	0	1	104
XYY	10	17	9	6	1	0	0	0	1	27
RAT	12	35	25	7	2	0	0	0	1	47
Segmental imbalance	6	23	12	9	1	0	1	0	0	29
Multiple abnormalities	6	5	4	1	0	0	0	0	0	11
22q11.2DS	5	25	13	10	1	0	0	0	1	30
Other CNVs	7	20	13	6	0	0	0	0	1	27
Triploid	0	2	1	0	0	0	1	0	0	2
XXXY	0	1	1	0	0	0	0	0	0	1
Total (%)	322 (24.3)	1005 (75.7)	686 (51.7)	218 (16.4)	71 (5.3)	9 (0.7)	5 (0.4)	2 (0.2)	14 (1.1)	1327 (100)

Abbreviations: abn US, abnormal ultrasound; AMA, advanced maternal age; FCT, first trimester combined test; NT, nuchal translucency; 'Other' indications include IVF conception with, or without, egg donation and parent carrier of a balanced rearrangement. Other abbreviations see Table 1.

TABLE 3 PPV according to high-risk cfDNA test results and diagnostic tests

	TOTAL			CV			AFC			Product of conception			Newborn			
High-risk cfDNA result	HR cases (*n*)	TP (*n*)	PPV (%)	HR cases (*n*)	TP (*n*)	PPV (%)	HR cases (*n*)	TP (*n*)	PPV (%)	HR cases (*n*)	TP (*n*)	PPV (%)	HR cases (*n*)	TP (*n*)	PPV (%)	*p* Value CV versus AFC
T21	671	624	93.0	275	268	97.5	393	353	89.8	2	2	100.0	1	1	100.0	**<0.001**
T18	124	91	73.4	44	43	97.7	78	46	59.0	2	2	100.0	0	0	0.0	**<0.0001**
T13	84	26	31.0	15	12	80.0	68	13	19.1	1	1	100.0	0	0	0.0	**<0.0001**
MX	125	34	27.2	11	3	27.3	108	28	25.9	0	0	0.0	6	4	66.7	≥0.05
XXX	45	28	62.2	3	3	100.0	39	23	59.0	0	0	0.0	3	2	66.7	≥0.05
XXY	104	75	72.1	16	13	81.3	81	56	69.1	0	0	0.0	7	6	85.7	≥0.05
XYY	27	26	96.3	2	2	100.0	23	22	95.7	0	0	0.0	2	2	100.0	≥0.05
RAT	47	9	19.1	2	1	50.0	44	8	18.2	1	0	0.0	0	0	0.0	≥0.05
Segmental imbalance	29	7	24.1	4	1	25.0	25	6	24.0	0	0	0.0	0	0	0.0	≥0.05
Multiple abnormalities	11	3	27.3	2	1	50.0	9	2	22.2	0	0	0.0	0	0	0.0	≥0.05
22q11.2DS	30	6	20.0	6	2	33.3	25	4	16.0	0	0	0.0	0	0	0.0	≥0.05
dup22q11.2	2	0	0.0	0	0	0.0	2	0	0.0	0	0	0.0	0	0	0.0	NA
del1p36	8	0	0.0	0	0	0.0	8	0	0.0	0	0	0.0	0	0	0.0	NA
del15q11.2	12	1	8.3	2	0	0.0	10	1	10.0	0	0	0.0	0	0	0.0	≥0.05
del5p	4	1	25.0	1	0	0.0	3	1	33.3	0	0	0.0	0	0	0.0	≥0.05
del17p11.2	1	0	0.0	0	0	0.0	1	0	0.0	0	0	0.0	0	0	0.0	NA
XXXY	1	0	0.0	0	0	0.0	1	0	0.0	0	0	0.0	0	0	0.0	NA
Triploid	2	0	0.0	0	0	0.0	2	0	0.0	0	0	0.0	0	0	0.0	NA

Note: MX* = in 2 cases, counted as TP, the AF or newborn's blood showed another abnormality (47,XXX[11]/46,XX[89] and 47,XYY). Bold values are significantly higher PPV ($p < 0.05$) when the follow-up diagnostic test was based on CV compared to analysis of AFC.

Abbreviations: HR, high risk; NA, not applicable; PPV, positive predictive value; TP, true positive; Other abbreviations, see Tables 1 and 2.

significance ($p = 0.056$) (Table 4). MX cases were frequently mosaic in cytotrophoblasts (3/16 (18.8%, 95% CI 7%–0.43%)), mesenchyme (5/16 (31.3% 95% CI 14%–56%)), and AF (17/108 (15.7%, 95% CI 10%–0.24%)) (Table 4). Of the 34 cases considered to be TP, 7 had a structurally abnormal X-chromosome associated with partial deletion (Supplemental Table S1).

For sex chromosome abnormalities in which the follow-up diagnostic test was the newborn's blood, the PPVs were: MX 4/6 (66.7%), XXX 2/3 (66.7%), XXY 6/7 (85.7%), and XYY 2/2 (100%).

3.3 | Rare autosomal trisomies (RATs)

The overall PPV for RATs in cases receiving any type of confirmatory diagnostic testing was 9/47 (19.1%, 95% CI 10%–33%) (Table 3). TPs included 7 cases of true fetal mosaicism for T9 ($n = 2$), T20 ($n = 1$), and T22 ($n = 4$) and 2 cases of clinically relevant UPD (chromosomes 7 and 15). PPV was 4/7 (57.1%) for T22, 2/6 (33.3%) for T9, 1/4 (25%) for T20, 1/5 (20%) for T7, and 1/6 (16.7%) for T15; the PPVs for the remaining chromosomes were zero. Clinical follow-up of pregnancies with TFM was not available (Table 5). PPV for all chromosomes potentially associated with UPD (chromosomes 6, 7, 11, 14, 15, and 20) combined was 2/15 (13.4%).

3.4 | Segmental imbalances (SI)

Overall PPV of SI >7 Mb was 24.1% (7/29, 95% CI 12%–42%) (Table 3). In 89.7% (26/29) of the suspected SI, one segmental imbalance was identified (Table S2). In the three remaining cases, NIPT suggested two partial imbalances (implying an unbalanced translocation, unbalanced inversion, or ring chromosome); these were not confirmed by AFC analysis. In 2 out of 7 true positive cases, the imbalance was classified as likely benign because the variant was also present in an apparently normal mother or because the imbalances were found in the Database of Genomic Variants (DGV). In four unconfirmed cases (13.8%), supplementary investigations discovered another segmental imbalance. None of the imbalances were of the recurrent type typically seen in maternal cancer. Three fetal SI not detected by NIPT but discovered because of the provision of

TABLE 4 Common autosomal trisomies and SCAs: positive predictive value (PPV) according to high-risk cfDNA test result, diagnostic method (CVS direct and long-term or amniocentesis) and mosaicism

NIPT result		Cytogenetic analysis			PPV					
Type of positive result	N° positive results	Mosaic	Non-mosaic	Normal	Including mosaic	*p* Value (CV direct vs. CV culture)	*p* Value (CV vs. AFC)	Excluding mosaic	*p* Value (CV direct vs. CV culture)	*p* Value (CV vs. AFC)
Cytotrophoblasts (CV direct preparation)										
T21	260	4	248	8	96.9	1	< **0.001**	95.4	1	< **0.001**
T18	44	0	43	1	97.7	1	< **0.0001**	97.7	1	< **0.001**
T13	21	5	12	4	81.0	>0.05	< **0.0001**	57.1	1	< **0.01**
MX	16	3	4	9	43.8	1	>0.05	25.0	1	>0.05
XXX	4	1	3	0	100.0	1	>0.05	75.0	1	1
XXY	16	0	13	3	81.3	1	>0.05	81.3	1	>0.05
XYY	2	0	2	0	100.0	1	1	100.0	1	1
T21 + T18 + T13	325	9	303	13	96	>0.05	< **0.0001**	93.2	1	< **0.0001**
SCA	38	4	22	12	68.4	1	**0.056**	57.9	>0.05	>0.05
Mesenchyme (CV long-term cell culture)										
T21	260	2	249	9	96.5		< **0.01**	95.8		< **0.001**
T18	44	0	43	1	97.7		< **0.0001**	97.7		< **0.001**
T13	21	3	12	6	71.4		< **0.0001**	57.1		< **0.01**
MX	16	5	3	8	50.0		**0.074**	18.8		>0.05
XXX	4	1	3	0	100.0		>0.05	75.0		1
XXY	16	1	12	3	81.3		>0.05	75.0		>0.05
XYY	2	0	2	0	100.0		>0.05	100.0		1
T21 + T18 + T13	325	5	304	16	95.1		< **0.0001**	93.5		< **0.0001**
SCA	38	7	20	11	71.1		< **0.05**	52.6		>0.05
Amniotic fluid cells										
T21	393	9	344	40	89.8			87.5		
T18	78	2	45	31	60.3			57.7		
T13	68	1	12	53	19.1			17.6		
MX	108	17	11	80	25.9			10.2		
XXX	39	0	23	13	59.0			59.0		
XXY	81	2	54	25	69.1			66.7		
XYY	23	0	22	1	95.7			95.7		
T21 + T18 + T13	539	12	401	124	76.6			74.4		
SCA	251	19	110	122	51.4			43.8		

Note: Only cases of laboratories performing CVS analysis with direct preparation and long-term culture combined were considered. Bold values are significantly different PPVs ($p < 0.05$).

Abbreviation: See Table 1.

diagnostic testing were inherited from an apparently normal mother and one was de novo. All of them were classified as benign or likely benign. Of note, there were 6 FP cases with 5q- and 7q-, which are chromosome rearrangements typically associated with myeloid leukemias or myelodysplasia.

3.5 | Multiple abnormalities (MA)

MA showed a PPV of 27.3% based on 3 TP cases out of 11 high-risk results. The karyotype of the TP cases included an XXY in combination with an 18q terminal deletion ($n = 1$), an XXX with a T21 ($n = 1$),

and a T18 in combination with a pericentromeric variant of chromosome 12 ($n = 1$).

3.6 | Microdeletions

Overall, PPV for 22q11.2DS was 6/30 (20%). In one of the six confirmed cases (Tables 3 and 6), the deletion detected by CMA was atypical and interstitial, located within the 3 Mb A-D region. Studies on the parents showed a maternal origin of the microdeletion: arr [GRCh37] 22q11.21(20754422_21440514) × 1 mat. Parental studies were not routinely performed in unconfirmed cases.

TABLE 5 PPV of rare autosomal trisomies high-risk cfDNA results by chromosome

Type of RAT	n° HR results	n° TP		PPV (%)
		n° TFM	n° UPD	
T2	1	0	0	0
T3	1	0	0	0
T4	1	0	0	0
T7	5	0	1	20
T8	2	0	0	0
T9	6	2	0	33.3
T10	2	0	0	0
T15	6	0	1	16.7
T16	11	0	0	0
T19	1	0	0	0
T20	4	1	0	25
T22	7	4	0	57.1
Total	47	7	2	19.1

Abbreviations: HR, high-risk; PPV, positive predictive value; RAT, rare autosomal trisomy; T, trisomy; TFM, true fetal mosaicism; TP, true positive; UPD, uniparental disomy; Other abbreviations see Table 1.

3.7 | Sex discordances

In addition to a review of the confirmatory test results in NIPT high-risk pregnancies, we reviewed the cytogenetic and cytogenomic results in cases referred because of a discordance between the sex reported by NIPT and that seen by ultrasound (Table 7). Of the six cases, an explanation was established for 4 cases, either through cytogenetic testing (two cases), retrospective review of the clinical case records (1 case), or additional molecular genetic testing (1 case).

3.8 | Inconclusive cfDNA test results

An abnormal karyotype was detected in 19 out of 137 diagnostic investigations (13.9%; 95% CI 9%–21%) with a no result after two consecutive blood draws (Table S3). The rate was 7.7% (3/39) in cases with low fetal fraction (FF), which included 1 case of T18, 1 case of T21, and 1 case of mosaic T9. There were 2 (13.3%) abnormalities in 15 cases with only partial results (missing sex chromosome results) with one mosaic MX and one XXY subsequently identified by diagnostic testing. In 20 cases with sample quality issues, 3 abnormalities (15%) were present including one T13, one XXY, and one microdeletion of maternal origin. Of the 63 cases without a result for unknown reasons, 11 (17.5%) showed abnormality with one T21, one T13, one XXX, 2 RATs, three SI, one triploidy, and two balanced translocations.

In this population of 137 without an NIPT result, the reason for cfDNA testing was an increased First trimester Combined Test (FCT) in 14.6% (20 cases), AMA 43.1% ($n = 59$), anxiety/personal decision (<35 years) 24.8% ($n = 34$), others 2.9% ($n = 4$), and unknown in 13.9% ($n = 19$). Compared with cases with a high-risk NIPT result, those without a result showed a significantly higher incidence of cases undergoing cfDNA testing because of an abnormal first-trimester combined screening test (20/137, 14.6% vs. 71/1332, 5.3%, $p = 0.00005$) and personal decision (<35 years) (34/137, 24.8% vs. 219/1332, 16.4%, $p = 0.01816$) (Table 2).

TABLE 6 Positive predictive value (PPV) according to high-risk cfDNA test results and type of cfDNA tests

High-risk cfDNA result	Total			Targeted			Genome-wide			Unknown			*p* Value targeted versus genome-wide
	n	TP	PPV (%)	*n*	TP	PPV (%)	*n*	TP	PPV (%)	*n*	TP	PPV (%)	
T21	671	624	93.0	161	153	95.0	287	261	90.9	223	210	94.2	0.17
T18	124	91	73.4	24	20	83.3	61	43	70.5	39	28	71.8	0.35
T13	84	26	31.0	20	4	20.0	27	9	33.3	37	13	35.1	0.50
MX	125	32	25.6	24	7	29.2	53	16	30.2	48	11	22.9	1.00
XXX	45	28	62.2	14	6	42.9	18	13	72.2	13	9	69.2	0.19
XXY	104	75	72.1	30	17	56.7	43	30	69.8	31	28	90.3	0.37
XYY	27	26	96.3	10	10	100	5	5	100	12	11	91.7	NA
22q11.2DS	30	6	20.0	12	4	33.3	11	1	9.1	7	1	14.3	0.37
All	1210	908	75.0	295	221	74.9	505	378	74.9	410	311	75.9	1.00

Abbreviations: PPV, Positive Predictive Value; T, trisomy; TP, True Positive; Other abbreviations see Table 1.

TABLE 7 Cases with sex discordancy between NIPT results and ultrasound findings

Case	NIPT result	Ultrasound assignment	Cytogenetic/cytogenomic results	Explanation
1	M	F	AFC 46,XY	Sequencing studies identified a likely hemizygous pathogenic variant in the androgen receptor gene (*AR*, *313700, Xq12) in the fetus. Therefore, the couple was counselled about the likely presence of androgen insensitivity syndrome.
2	M	F	AFC 45,X	Turner syndrome; possible placental mosaic 46,XY/45,X in cytotrophpoblast
3	M	F	AFC 46,XX	Case review indicated an earlier twin pregnancy; vanishing twin
4	M	F	Newborn blood 100 cells 46, XY	Unknown
5	M	F	Newborn blood Karyotype and FISH 46,XY	Unknown
6	F	M	AFC QF-PCR normal male AFC CMA normal male AFC 45,X[5]/46,XY[45]	Turner syndrome mosaic

Abbreviations: AFC, amniotic fluid cells; CMA chromosome microarray; QF-PCR, quantitative fluorescence polymerase chain reaction.

3.9 | Test methodology

There was only sufficient data to compare targeted (including SNP-based) with genome-wide methodologies. Additionally, we limited this analysis to those areas of testing that were most commonly included in NIPT test panels (common autosomal trisomies, SCAs and 22q11.22DS). Table 5 summarizes these results. No significant difference could be demonstrated between the two test methods, for each specific chromosome abnormality or for all abnormalities combined.

4 | DISCUSSION

This is the largest collection of laboratory confirmatory diagnostic testing aimed at verifying PPVs of cfDNA screening and presents the clinical cytogenomic findings in cases with no result and discordant fetal sex results. In this study, overall PPVs were consistent with previous studies where genetic confirmation was based on cytogenetic and cytogenomic testing (Table S4).[10–12] Results were also broadly consistent with reports from NIPT laboratories.[14–17] This large dataset also allowed us to further assess performance for rarer disorders, to investigate differences for the type of diagnostic testing performed, and to review uninformative testing and to compare different test methods.

We found that PPVs for T21, T18, and T13 were high when the confirmatory test was based on cytotrophoblasts or mesenchyme. Confirmation through CV could include some cases where there is a confined placental mosaicism and the fetus is unaffected. Consistent with this, we observed that the PPVs for these three chromosomes were significantly lower when the confirmation was based on AF. This pattern was seen either including, or excluding, mosaic cases. It was significantly lower for SCAs combined (including mosaic cases) when the confirmatory test was based on mesenchyme (Table 4). A high concordance between cell-free DNA and CVS trophoblasts can be expected because both tests are based on the analysis of the same cell lineage.[18–20] However, high concordance was also seen with mesenchyme, despite the distant developmental relationship between cytotrophoblasts and mesenchyme, differentiation pathways, and functional roles of these lineages.[21] It should be noted that in actual clinical practice, confirmation is not based only on cytotrophoblasts or only on mesenchyme but on the analysis of both (with reflex analysis of AF cells in cases with suspected mosaicism). PPVs for CV confirmation are therefore expected to lower in actual practice, particularly for aneuploidies where mosaicism is common.

CV confirmation has been accepted as being sufficiently reliable for NIPT results positive for T21 because T21 CPM is very rare.[22,23] In contrast, for T18 and T13, confirmation through AF cells has been advocated because of higher rates of CPM. Our observations should not be interpreted as indicating that CVS is preferred over AF cells when confirming all NIPT positive results. It is well established that, in general, AF cell analysis is the most reliable indicator of the true karyotype. We have previously pointed out, based on CV cytogenetics, that analysis of only one cell line and in particular, cytotrophoblasts, can result in false discovery of mosaic aneuploidy, relative to diagnosis based on AF.[24] Consistent with this, we found NIPT PPVs were lowest for RATs, MX, and T13 when confirmatory studies were based on AF. The observation of differences in PPV depending on follow-up testing has important implications for women with positive NIPT results. The choice of CVS versus amniocentesis follow-up needs to take into consideration the specific abnormality identified by NIPT, presence of ultrasound findings, and diagnostic test accuracy as well as timing of the testing.[25]

In this study, the overall PPV for RATs was 19.1%. This included cases with a clinically significant UPD, discovered secondarily to the NIPT result. This PPV was higher than previously reported (4%–6%) in a study where the genome-wide approach was applied as nationwide first-tier non-invasive prenatal screening.[26–28] Most of the confirmed RATs in our study showed a mosaic fetal karyotype, which is typically associated with a high level of uncertainty due to the

unpredictable distribution of the abnormal cell line in fetal tissues and therefore uncertain clinical effects. Confirmation of a RAT, even in AF cells, is not necessarily indicative of an abnormal phenotype.[28] Our survey did not seek phenotype information for these pregnancies. Moreover, non-mosaic RATs identified by NIPT would be expected to spontaneously abort and generally, not come to our attention. To adequately assess the clinical utility of detecting each specific RAT, unbiased and fully comprehensive outcome data is needed, preferably collected through a blinded randomized control trial. This should include an assessment of the proportion of abnormal cells in cases with, and without, adverse outcomes.[28–31]

The PPV for SI was consistent with previous studies.[26–28] We found that 2/7 confirmed SI were benign because the imbalance was present in the genome of an apparently normal mother. In addition, in 13.8% (4/29), the high-risk results were attributable to a genetic imbalance present only in the maternal genome and not in the fetus. This is likely an under-estimation as not all cases underwent parental investigations. A non-mosaic, or high proportion, imbalance present in the mother can often be recognized by a high cfDNA involvement that is incompatible with the fetal fraction. NIPT can be expected to identify maternal somatic cell 5q-, 7q-, 11q-,13q-, 20q- deletions (and also T8 and T13), which can be attributable to myeloid leukemias, myelodysplasias, and myeloproliferative disorders and may be present before any overt disease is evident.[32] At this time, false-positive NIPT results for such regions do need maternal follow-up and monitoring although the overall benefit of using genomewide NIPT to purposefully discover results consistent with maternal malignancies or pre-malignant conditions has not yet been demonstrated and is controversial.[26,28,33–36] This study also confirmed that NIPT can detect sub-microscopic deletions such as those at 22q11.2, including interstitial regions that may not be amenable to confirmation with the currently commercially available FISH probes.[20]

Cases where there was a no result constituted a heterogenous set of cases where there was low fetal fraction, cases without a sex chromosome result, poor-quality samples, and unknown reasons. In aggregate, 19/137 (13.9%) had a cytogenetic abnormality detected by follow-up diagnostic testing. This high frequency can be partially explained by cytogenetic abnormalities known to be associated with a small placenta and therefore low fetal fraction (e.g., digynic tripoidy, t18, t13). Some may have also had poor cfDNA quality due to fetal death around the time of testing. It is also possible that this group could include cases where NIPT laboratories had borderline data and were reluctant to assign a positive or negative call. Regardless of the reasons, our observations indicate that this group of pregnancies does need to be considered high risk and additional clinical and laboratory test evaluation is indicated.

The frequency of cases where there is a discordancy between the fetal sex assigned by NIPT and ultrasound appears to depend on test methodology because widely different rates have been reported.[14,37] Many cases are attributable to clerical, ultrasound or laboratory error and are resolved without invasive testing.[37] Cases that are referred for cytogenetic or cytogenomic testing therefore probably represent high-risk situations in which the cause for the discordancy is unresolved. Our observations are consistent with follow-up gathered by an NIPT laboratory that indicated that diverse disorders of sexual development can be present in these pregnancies.[37] Our study did not investigate the presence of maternal chromosome abnormalities that can account for false-positive results for SCA.[38,39]

A comparison of the PPVs for all chromosome abnormalities combined indicated no significant difference between targeted and genomewide methodologies. This analysis was not sufficiently powered to determine whether some techniques might be better than others in detecting specific abnormalities, for example, small microdeletions, or MX, where maternal imbalances are an additional confounder in test interpretation.

Determining NIPT PPVs through the results of cytogenomics laboratories has some limitations. Those cases with abnormal ultrasound findings may have either received earlier diagnosis through CVS, or spontaneously or electively terminated prior to the time of amniocentesis. Conversely, those case without ultrasound evidence of abnormality may have continued without any further testing. Italian guidelines strongly recommend diagnostic confirmation of positive NIPT results[40,41] and therefore we believe most cases should have received a diagnostic test. Non-viable aneuploidies may have spontaneously aborted prior to any confirmatory testing. We counted all mosaic cases (including some that may have been confined to the placenta) as true positives and also included cases with a clinically significant UPD as true positives, even though direct confirmation of trisomy was not found. Finally, information on maternal chromosome abnormalities (including sex chromosome mosaicism, small copy number variants, and malignant or pre-malignant somatic cell chromosome imbalances) was not assessed.

In summary, our findings show that cfDNA testing has PPVs that differ across the conditions screened and also PPVs are dependent the methods used for follow-up diagnostic testing. This variability and the issues associated with fetal sex discordancy and no results illustrate the importance of adequate genetic pre- and post-test counselling. Additional systematic collection of data from diagnostic testing and comprehensive information on pregnancy outcomes is important to further refine PPVs and thereby improve patient counselling.

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CONFLICT OF INTEREST

Daria De Siero and Francesca Malvestiti are full-time employees of TOMA laboratory, Impact Lab. At the time of the study development, data analysis and drafting manuscript, Francesca Romana Grati was the coordinator of the SIGU cytogenetics and cytogenomics working group, full-time employee of TOMA laboratory, Impact Lab and consultant for Menarini Silicon Biosystems; now she is a full-time employee of Menarini Biomarkers Singapore (MBS). Lorena Miele and Cecilia Corti are full time-employees of Unilabs Ticino laboratory. Laura Cardarelli and Elisabetta Lippi are full-time employees of RDI Lab, Lifebrain. Peter Benn is a consultant for and holds stock

options in Natera, Inc. and an Advisory Board member for Menarini Silicon. Remaining authors have no COI.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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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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