Prenatal Diagnosis

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PRENATAL DIAGNOSIS

ORIGINAL ARTICLE

Extended Non-Invasive Prenatal Testing for Chromosomal and Monogenic Disorders by Cell-Free DNA Sequencing: A Blinded Comparison With Amniocentesis

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Received: 31 July 2026 | **Revised:** 6 October 2026 | **Accepted:** 9 October 2026

Keywords: amniocentesis | array-CGH | cell-free fetal DNA | clinical exome | iNIFS | prenatal diagnosis

ABSTRACT

Objective: To determine whether molecular integrated non-invasive fetal exome sequencing (iNIFS), using clinical exome sequencing of maternal-plasma cell-free DNA, concords per pregnancy with paired amniocentesis.

Method: Prospective, blinded, single center, paired sample diagnostic accuracy study in 641 consecutive pregnancies referred for clinically indicated amniocentesis at 16–18 weeks. Plasma cell-free DNA underwent low-pass whole-genome sequencing for aneuploidies and CNVs and, after short-fragment enrichment, clinical exome sequencing within a parent fetus trio design. The reference standard comprised QF-PC R, karyotyping, array-comparative genomic hybridization and, where indicated, trio clinical exome sequencing on amniotic-fluid DNA.

Results: iNIFS was reportable in 624/641 pregnancies (97.3%); 612 (95.5%) had complete paired data and formed the analysis population. The reference established a pathogenic or likely pathogenic finding in 107/612 (17.5%). iNIFS gave 106 true positives, 503 true negatives, 2 discordant positives and 1 discordant negative: sensitivity 99.1% (95% C I 94.9–99.8), specificity 99.6% (98.6–99.9), PPV 98.1% (93.5–99.5), NPV 99.8% (98.9–100.0), agreement 99.5% and Cohen's κ 0.98. The discordant negative was a maternally inherited variant, the class for which distinguishing maternal from fetal contributions is most challenging.

Conclusion: Molecular iNIFS showed high concordance with paired amniocentesis across chromosomal, copy-number and monogenic findings, w hile r etaining a n e xplicit n o c all r ate. M ulticentre v alidation a nd i nvasive c onfirmation of positive findings remain necessary.

1 | Introduction

Prenatal genetic diagnosis has long involved a compromise between safety and completeness. Amniocentesis and chorionic villus sampling give direct access to fetal or placental material and support the full repertoire of cytogenetic and molecular analyses, from karyotyping and chromosomal microarray to exome and genome sequencing, but carry a procedure-related risk of pregnancy loss estimated by contemporary meta-analyses at approximately 0.1% in experienced hands [1, 2], together with access and cost barriers that influence uptake.

Routine non-invasive prenatal testing has focused mainly on the common autosomal aneuploidies and a few recurrent microdeletions, although genome-wide approaches now also report the rare autosomal trisomies and segmental imbalances above approximately 7 Mb across all chromosomes [3]. It estimates

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Key Points

- What's already known about this topic?
  - Cell-free DNA screening is established for common aneuploidies but reports risk, not a diagnosis, and a positive result requires invasive confirmation.
  - Its extension to monogenic disorders and pathogenic CNVs rests on limited data with scarce paired invasive comparisons.
- What does this study add?
  - In 612 pregnancies with paired amniocentesis, molecular iNIFS detected 106 of 107 pathogenic findings, with two discordant positives.
  - Detection was complete for chromosomal and copy-number abnormalities; the only miss was maternally inherited.

risk rather than establishing a diagnosis, and a positive result still requires invasive confirmation, because the fetal fraction of cell-free DNA derives from the cytotrophoblast of the chorionic villi, which may not be representative of the fetus [4, 5]. Extension to the molecular tier, de novo monogenic disorders, pathogenic copy-number variants (CNVs) and recessive states have rested on limited data, and comprehensive interrogation of the fetal coding genome has until recently required invasive sampling [6, 7]. Deep sequencing of maternal plasma with parental genotypes and statistical analysis can recover fetal de novo and paternally inherited variants [8] and, in a parallel trio-exome approach, single-nucleotide variants, indels and large CNVs at high sensitivity [9]. Non-invasive diagnosis of single-gene disorders is not itself new, from achondroplasia in 2000 [10] to targeted testing for skeletal dysplasias by 2012 [11–13], but those assays targeted known variants within predefined regions [14]. Broader cfDNA screening for monogenic disorders has since proven feasible, from panels of dominant single-gene conditions in large clinical cohorts to genome-scale approaches covering conditions of heterogeneous etiology [15–18], as recently reviewed by Valovičová et al. [19] Direct prospective comparison against a paired invasive reference remains scarce.

We therefore compared molecular iNIFS against paired amniocentesis, the diagnostic reference standard, in consecutive pregnancies referred for clinically indicated amniocentesis, to assess its concordance for chromosomal, copy-number and monogenic findings.

2 | Materials and Methods

2.1 | Study Design and Ethical Approval

This was a prospective, single-centre, paired-sample diagnostic-accuracy study conducted at the Department of Prenatal Diagnosis and Fetal Maternal Medicine, ALTAMEDICA, Artemisia S.p.A., Rome, Italy, between 1 January 2024 and 31 December 2025. Consecutive eligible women referred for clinically indicated amniocentesis were enrolled before either test result was known (Figure 1). Each pregnancy contributed one index test (molecular iNIFS on maternal plasma cfDNA) and one same-day reference test on amniotic fluid. The study is reported per STARD 2015 [20], approved by the Ethics Committee of Artemisia S.p.A. (2023-03), and conducted per the Declaration of Helsinki, with written informed consent from all participants.

2.2 | Participants

Eligible participants were women with a viable singleton pregnancy referred for clinically indicated amniocentesis at 16–18 weeks, in whom a maternal blood sample was obtained immediately before the procedure and sufficient amniotic fluid was available for all planned reference analyses. Exclusion criteria, all recognized confounders of cfDNA analysis, were multiple pregnancy or vanishing twin, maternal malignancy, solid-organ or hematopoietic transplantation, allogeneic transfusion within the preceding 6 months, and any known maternal chromosomal abnormality or constitutional CNV expected to invalidate cfDNA interpretation. Six hundred and forty-one pregnancies were enrolled; indications are summarized in Table 1, each assigned to a single category by the hierarchy shown, the highest applicable category applying where more than one was present.

2.3 | Index Test: Sample Handling, Library Preparation and Sequencing

Two 10 mL aliquots of maternal peripheral blood were collected into Streck Cell-Free DNA BCT tubes immediately before amniocentesis and processed within 24 h. Plasma was separated by double centrifugation (1600 × g then 16,000 × g, each 10 min at 4°C). cfDNA was extracted from 4 mL of plasma on the QIAsymphony SP using the QIAsymphony DSP Circulating DNA Kit (QIAGEN, Hilden, Germany); yield and fragment-size distribution were assessed on an Agilent TapeStation. Maternal and paternal genomic DNA (gDNA) were extracted from peripheral blood using the MagPurix system with the MagPurix Blood DNA Extraction Kit 200 (Zinexts Life Science Corp., Taiwan). The plasma cfDNA underwent two complementary analyses: low-pass whole-genome sequencing for chromosomal aneuploidies and segmental CNVs, and hybridization-capture exome sequencing for monogenic single-nucleotide variants and small insertions and deletions.

For the chromosomal analysis, an aliquot of the extracted cfDNA was prepared as a shallow whole-genome library with VAHTS reagents and MGI-compatible adapters (Vazyme, Nanjing, China), circularized into DNA nanoballs and sequenced on the MGI platform, in the configuration used for routine cfDNA aneuploidy screening.

For the monogenic analysis, a two-stage solid-phase reversible immobilization size selection was applied to the remaining cfDNA to deplete long fragments and recover molecules of approximately 90–160 bp [21], thereby raising the effective fetal fraction. Indexed libraries were prepared using the Vazyme Inherited Disease Clinical Exome workflow on the Automated Liquid Handling Workstation (Vazyme, Nanjing, China), enriched by hybridization capture with biotinylated probes, and sequenced paired end (2 × 100 bp) on an MGI T1 plus platform [22–24].

Reportability of the exome analysis was governed by quality thresholds prespecified before enrollment, all of which had to be

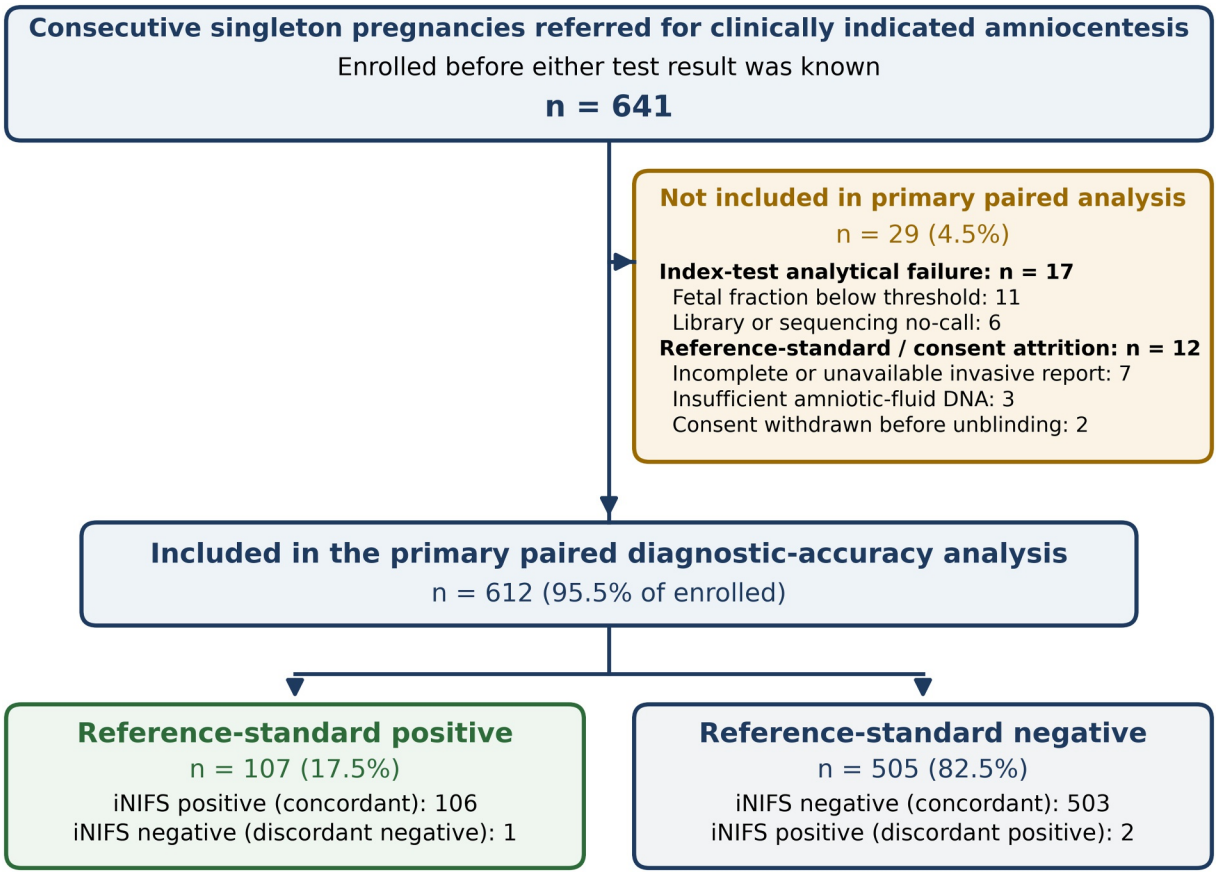


FIGURE 1 | Participant-flow diagram. Exclusions are positioned before the reference standard, denoting that the analytical failures of the index test arose independently of the invasive result.

TABLE 1 | Indications for testing in the 641 pregnancies enrolled in the study.

Indication for testing	*n*	%
Ultrasound-detected fetal anomaly	280	43.7
Advanced maternal age without another indication	128	20.0
Maternal request for diagnostic certainty	95	14.8
Familial or anamnestic genetic risk	92	14.4
Confirmation of a high-risk cell-free DNA aneuploidy screening result	46	7.2
Total	641	100.0

met: an effective fetal fraction $\geq$ 5.0% after enrichment, a unique mean on-target depth of $\geq$ 500x, $\geq$ 98.0% of the validated target region covered at $\geq$ 200x, a library complexity of $\geq$ 35 million unique molecules, and estimated sample contamination $<$ 0.5%. Failure of any mandatory metric produced a no-call, and no sample was resequenced for the primary analysis.

2.4 | Bioinformatic Analysis: Chromosomal Calls and Inference of the Fetal Genotype

Low-pass reads were aligned to GRCh37 (hg19) with Bowtie2. After GC correction and normalization of read depth in bins, whole-chromosome dosage was evaluated by z-score against a euploid reference set ($|z| \geq 3$), segmental CNVs were called with the Vazyme bioinformatic pipeline, optimized in-house [25, 26], combining a hidden Markov model with segmentation of the normalized read depth; this analysis did not use allelic information. Fetal fraction (FF) was estimated in these data with PREFACE [27], which predicts it from principal components of the autosomal read-depth profile using a model trained on male pregnancies in which FF was derived from Y-chromosome read depth, and is therefore applicable to both male and female fetuses.

Exome reads were aligned to GRCh37 (hg19) with BWA-MEM (v0.7.17); duplicate marking, base-quality score recalibration and indel realignment followed GATK best practice, and reads sharing a unique molecular identifier were collapsed into error-suppressed consensus reads. Variant calling used MUTECT2 in tumor-only mode, for its sensitivity to low-allele-fraction variants, with germline-aware filtering. Recurrent calls at identical positions across unrelated samples and within homopolymeric contexts were excluded through an artifact blacklist established during validation. FF was estimated from the exome data with SeqFF [28], which infers the fetal contribution from genome-wide read-count features without relying on sex-chromosome markers, and was used to set per-sample expectations for the plasma allele fractions described below.

Inference of the fetal genotype is asymmetric with respect to the maternal genotype and determines the performance profile below. Where the mother is a homozygous reference, a fetal

alternate allele, whether de novo or paternally transmitted, appears against a null background at approximately FF/2, largely independently of fetal fraction. Where the mother is heterozygous, the fetal contribution is inferred only from the allelic imbalance in a signal she dominates: a non-transmitted maternal variant is expected at approximately $(1 - FF)/2$, a maternally inherited variant in a heterozygous fetus at approximately 50%, and a variant in a homozygous or hemizygous male fetus at approximately $(1 + FF)/2$ At a fetal fraction of 10% these correspond to about 45%, 50% and 55%, the separation widening only modestly as fetal fraction rises; this is the principal source of uncertainty for maternally carried variants. Observed allele fractions were interpreted relative to these expectations and to the matched maternal gDNA (Supporting Information S1: Figure S1).

For autosomal recessive conditions, maternal and paternal alleles were assessed jointly: a maternal allele at a dosage compatible with fetal transmission, with a fetal-specific paternal allele at approximately FF/2, suggested compound heterozygosity, whereas an excess of the maternal allele near $(1 + FF)/2$ suggested homozygosity (Supporting Information S1: Figure S1).The same expectations were applied to maternally carried X-linked variants in male fetuses. Interpretation intervals were established, before the present cohort, on a validation series of 60 pregnancies with matched maternal plasma cfDNA, maternal gDNA, paternal gDNA and invasive fetal DNA. The analysis was structured as a parent–fetus trio, interpreting the plasma-reconstructed fetal genome jointly with the maternal and, where available, paternal reference. Because the detectability of a fetal allele depends on the maternal genotype, the maternal reference is a precondition of calling rather than a later comparator. Pathogenicity also depends on inheritance: a loss-of-function allele arising de novo in a fetus with a concordant phenotype differs in implication from the same allele inherited from an unaffected parent. Paternal inheritance was therefore not an automatic exclusion but one element of an integrated assessment with variant classification, mode of inheritance and parental phenotype.

2.5 | Variant Classification and Reporting

Sequence variants were classified by the ACMG/AMP criteria [29], applying gene-specific ClinGen Variant Curation Expert Panel specifications where available, and CNVs by the ACMG/ClinGen technical standards [30]. Variants were reported only when pathogenic or likely pathogenic after re-curation against ClinVar, ClinGen and HGMD, and only such findings entered the primary endpoint; variants of uncertain significance were excluded from the 2×2 analysis and carrier states treated as secondary. Criteria depending on data unavailable at the non-invasive report, PS2 (confirmed de novo occurrence), PM3 (two alleles in trans) and PP1 (segregation) were applied provisionally and re-evaluated after the invasive result. Maternal CNVs, absence of heterozygosity and maternal or placental mosaicism were flagged as the principal biological sources of discordance between a plasma-derived and a constitutional genotype.

2.6 | Reference Standard: Amniocentesis

Transabdominal amniocentesis was performed at 16–18 weeks under continuous ultrasound guidance with a 20–22-G needle by an operator blinded to the index-test result. 15–20 mL of amniotic fluid was aspirated, the first 2 mL discarded to minimize maternal cell contamination, and divided between aliquots for direct extraction from uncultured amniocytes and for cell culture. Fetal DNA was extracted with the DNeasy Blood & Tissue Kit and parental DNA with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Maternal cell contamination was excluded in every case by QF-PCR against matched maternal genomic DNA.

2.7 | Definition of a Positive Result and Blinding

A pregnancy was reference-positive if the invasive analysis established at least one pathogenic or likely pathogenic fetal finding, chromosomal, copy-number, or monogenic, and index-positive if molecular iNIFS reported at least one such finding; carrier states did not enter this classification. Index-test analysts had access to the referral indication and fetal ultrasound phenotype, phenotype-driven filtering being intrinsic to trio-exome interpretation but not to the invasive result, and non-invasive calls were locked and time-stamped before the cytogenetic report. Assessors of the karyotype, QF-PCR and array-CGH, performed routinely in all pregnancies, were blinded to the index-test result. Targeted resequencing and trio clinical-exome confirmation on amniotic-fluid DNA could not be blinded, since the position interrogated was defined by the index-test report, but the assessment was binary, limiting bias.

2.8 | Analysis Population and Handling of Non-reportable Results

The primary analysis population comprised the complete paired cases with both an interpretable molecular iNIFS result and a complete invasive reference. Of the 641 enrolled, 29 (4.5%) did not meet this definition, leaving 612 (95.5%). Exclusions fell into two prespecified classes: 17 analytical failures of the index test, identified by quality thresholds from the plasma data alone, before and independently of the reference standard; and 12 reference-standard exclusions, an incomplete or unavailable invasive report, insufficient amniotic-fluid DNA, or consent withdrawn before unblinding. No exclusion was based on concordance between the tests, no sample was resequenced, and no result was imputed. A prespecified secondary analysis returned the index-test failures to the denominator as non-detections; reference-standard and consent exclusions cannot since no reference classification exists for them.

2.9 | Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were computed against the paired reference standard with 95% Wilson score confidence intervals (CI); overall agreement and Cohen's κ were also reported. Categorical variables are expressed as frequencies and percentages, continuous variables as mean with range or median with interquartile range (IQR). Class-level sensitivity was computed separately for chromosomal abnormalities, pathogenic CNVs and monogenic findings, since the deconvolution logic of Section 2.4 predicts a different detection profile for each. Because the cohort was enriched for fetal pathology by referral, predictive values are reported as properties of this cohort and not extrapolated to an unselected obstetric population. Analyses used *R* version 4.3.2.

3 | Results

3.1 | Participant Flow

Of 641 enrolled pregnancies, molecular iNIFS returned a reportable result in 624 (97.3%, 95% CI 95.8–98.3). Six hundred and twelve (95.5%) had both an interpretable index-test result and complete paired invasive reference data and formed the primary analysis population. Of the 29 (4.5%) that did not contribute, 11 had an effective fetal fraction below threshold after enrichment, six a library or sequencing no-call despite adequate fetal fraction, seven an incomplete or unavailable invasive report, three insufficient amniotic-fluid DNA and two consent withdrawn before unblinding; thus, 17 (58.6%) were analytical failures arising before the reference standard was known and 12 reflected reference-standard or consent attrition (Figure 1). The mean fetal fraction, estimated from the exome data after size selection, was 14.2% (range 5.1–32.0), median 13.6% (IQR 10.8–17.1), and median gestational age at sampling was 17^{+1} weeks (IQR 16^{+5} to 17^{+6}). Referral indications are given in Table 1 and Figure 1.

3.2 | Diagnostic Classification

The invasive reference identified at least 1 pathogenic or likely pathogenic fetal finding in 107 of 612 pregnancies (17.5%, 95% CI 14.7–20.7). Molecular iNIFS classified 108 as positive and 504 as negative, giving 106 true positives, 503 true negatives, 2 discordant positives and 1 discordant negative (Supporting Information S1: Table S1). Overall, sensitivity was 99.1% and specificity 99.6%, with an overall agreement of 99.5% (609/612) and Cohen's κ of 0.98. Because discordance arises differently for dosage-based and sequence-based calls, performance was also assessed by class of finding (Table 2): PPV was 98.2% for chromosomal aneuploidies and 92.3% for pathogenic CNVs, each class including one discordant positive, whereas no discordant positive occurred among monogenic variants (PPV 100%). Sensitivity was complete for chromosomal aneuploidies and CNVs and 97.6% for monogenic variants, reflecting a single discordant negative.

3.3 | Distribution of Detected Findings

The 106 true positives comprised 54 chromosomal abnormalities, 40 monogenic disorders and 12 pathogenic CNVs (Supporting Information S1: Table S2); all 54 were concordant with the paired karyotype and QF-PCR, and all 12 CNVs with array-CGH. Separately, 92 fetuses (15.0%, 95% CI 12.4–18.1) were heterozygous carriers of an autosomal recessive disease allele and, not being affected, were reported as secondary findings and excluded from the diagnostic endpoint.

The 54 chromosomal abnormalities were all common autosomal trisomies, 43 trisomy 21 (79.6%), 7 trisomy 18 (13.0%) and 4 trisomy 13 (7.4%), with no sex-chromosome aneuploidies or other karyotypic abnormalities detected (Supporting Information S1: Table S3). The 12 pathogenic CNVs are listed with their cytogenetic band, genomic coordinates, size, and array-CGH confirmation in Supporting Information S1: Table S2 (Figure 2).

3.4 | Monogenic Findings

The monogenic findings are listed variant by variant in Supporting Information S1: Table S4, with transcript, HGVS description and mode of inheritance, and involved 28 distinct genes. Twenty-eight (68.3%) arose de novo, four (9.8%) were paternally inherited dominant alleles, six (14.6%) biallelic combinations underlying an autosomal recessive condition, and three (7.3%) maternally inherited X-linked variants.

Twelve findings involved RAS/MAPK-pathway genes, *PTPN11*, *RIT1*, *RAF1*, *SOS1*, *KRAS*, *BRAF* and *HRAS*, the largest group. A second group was skeletal and craniofacial: four *FGFR3* variants underlying thanatophoric dysplasia types I and II and achondroplasia, two *FGFR2* variants causing Apert syndrome, two *COL1A1* variants at the recurrent Gly257 residue, and one *COL2A1* variant causing achondrogenesis type II. A third comprised loss-of-function variants in chromatin and transcriptional regulators, *CHD7*, *KMT2D*, *NIPBL*, *ZEB2* and *EFTUD2*, where the prenatal phenotype is frequently non-specific. Six pregnancies were biallelic recessive genotypes, two in *CFTR* and one each in *DHCR7*, *CC2D2A*, *PAH* and *HBB*; the reportable result depended on identifying the paternally derived allele within the fetal fraction and inferring the fetal genotype by dosage. Three involved maternally inherited X-linked variants in *L1CAM*, *OTC* and *DMD*.

3.5 | Sensitivity by Variant Class

The reference-positive set decomposed into 54 chromosomal abnormalities, 12 pathogenic CNVs and 41 monogenic findings (total 107). Class-level sensitivity was thus 54/54 for chromosomal

TABLE 2 | Diagnostic performance of molecular iNIFS versus paired invasive testing by class of finding (n = 612; 95% CI, Wilson score method).

Class of finding	Sensitivity	Specificity	PPV	NPV
Chromosomal aneuploidies	100% (54/54) 93.4–100	99.8% (557/558) 99.0–100	98.2% (54/55) 90.4–99.7	100% (557/557) 99.3–100
Pathogenic CNVs	100% (12/12) 75.8–100	99.8% (599/600) 99.1–100	92.3% (12/13) 66.7–98.6	100% (599/599) 99.4–100
Monogenic variants (SNV/indel)	97.6% (40/41) 87.4–99.6	100% (571/571) 99.3–100	100% (40/40) 91.2–100	99.8% (571/572) 99.0–100
Overall	99.1% (106/107) 94.9–99.8	99.6% (503/505) 98.6–99.9	98.1% (106/108) 93.5–99.5	99.8% (503/504) 98.9–100.0

Note: Both false-positive calls fell in the dosage-based classes: a trisomy 16 signal attributed to confined placental mosaicism (chromosomal aneuploidies) and a low-level 22q11.2 duplication traceable to maternal mosaicism (CNVs). The single false negative was a maternally inherited X-linked nonsense variant in *DMD* (monogenic variants). Overall agreement was 99.5% (609/612; 95% CI 98.6–99.8) and Cohen's κ 0.98.
Abbreviations: CNV, copy-number variant; NPV, negative predictive value; PPV, positive predictive value; SNV, single-nucleotide variant.

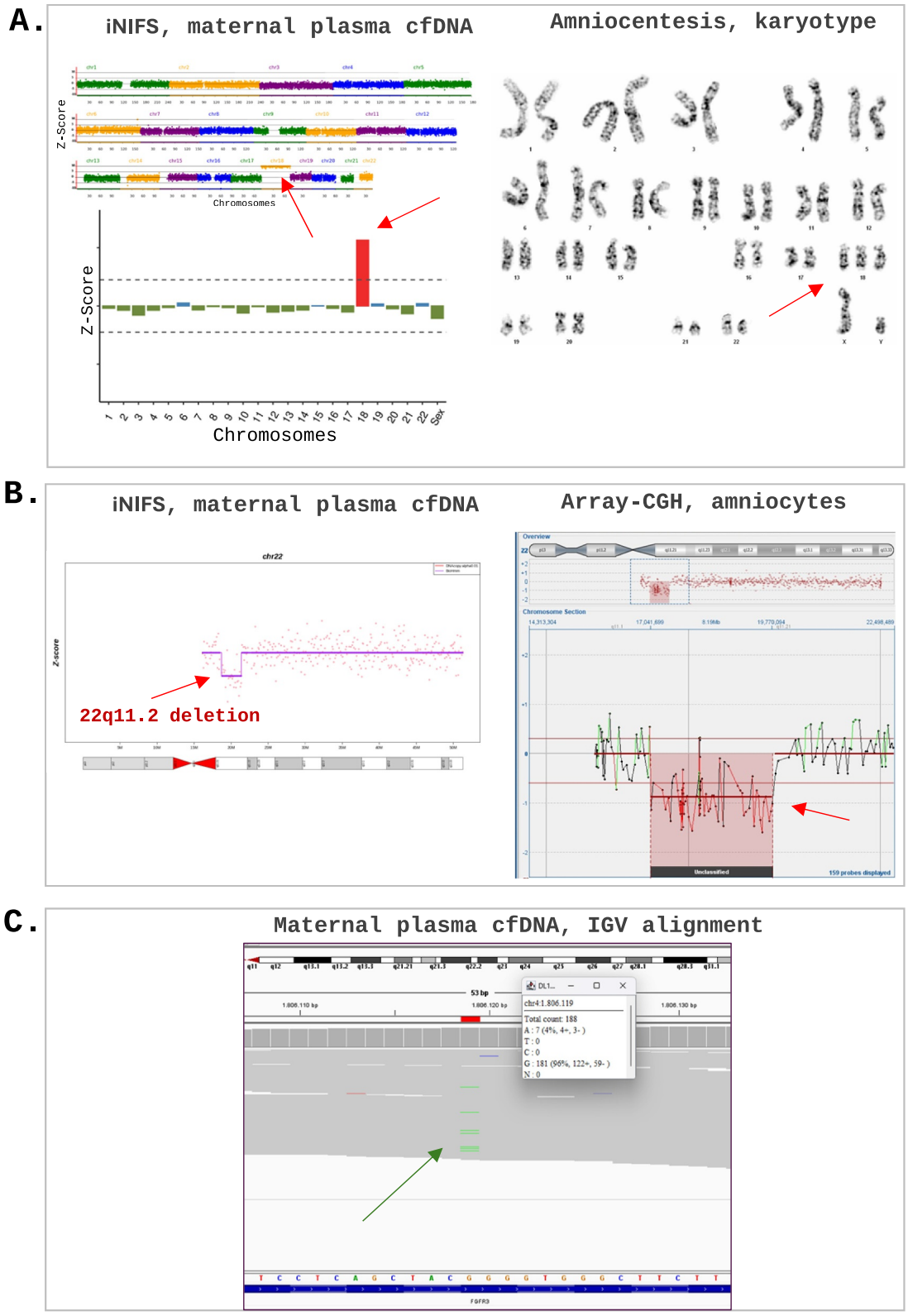


FIGURE 2 | Concordance between cfDNA-based iNIFS and confirmatory diagnostic testing. (A) iNIFS: genome-wide Z-score profile across all chromosomes (top) and per-chromosome Z-scores (bottom), with the chromosome 18 Z-score exceeding the positive-call threshold (red bar); right, karyotype on cultured amniocytes confirming trisomy 18. (B) iNIFS: chromosome 22 Z-score profile showing a 22q11.2 microdeletion (left); right, array-CGH on amniocytes delineating the deleted interval (shaded). (C) De novo *FGFR3* variant detected in maternal plasma cfDNA: IGV alignment of the variant-supporting reads (green arrow).

abnormalities (95% CI 93.4–100), 12/12 for pathogenic CNVs (75.8–100) and 40/41 for monogenic findings (97.6%, 87.4–99.6).

The only discordant negative result, an X-linked, maternally inherited nonsense variant in DMD, occurred in the variant class for which distinguishing maternal from fetal contributions is intrinsically most challenging.

3.6 | Discordant Results

Three of the 612 paired cases were discordant (0.5%); both discordant positives are most plausibly attributable to the tissue of origin of the analyte rather than analytical error. First, a low-level 22q11.2 duplication signal in plasma accompanied a normal amniotic-fluid result, with maternal low-level mosaicism documented independently in maternal leukocyte DNA. In the second, a trisomy 16 signal accompanied a normal amniotic-fluid karyotype and array-CGH; placental trisomy 16 is almost invariably constitutional, arising from a meiotic error, and a euploid fetal karyotype in this setting reflects trisomy rescue, leaving the trisomic cell line confined to the placenta [31]. The discordant negative was DMD NM_004006.3:c.8713 C > *T*, p.(Arg2905Ter), a maternally inherited nonsense variant identified by the invasive trio exome; at a fetal fraction of 5.3%, the plasma allelic imbalance remained below the prespecified interpretation interval. No reference-positive finding fell outside the index-test capture content, so this miss reflects plasma deconvolution rather than assay content.

4 | Discussion

In 612 pregnancies with a paired invasive reference, molecular iNIFS identified 106 of 107 pathogenic or likely pathogenic fetal findings, with two discordant positives. Detection was complete for chromosomal abnormalities and pathogenic CNVs; the only miss, maternally inherited, was the expected consequence of how the fetal genotype is reconstructed from plasma.

A fetal allele absent from the maternal genome appears against a null background and is recoverable at a low fetal fraction, whereas a maternally carried allele can be inferred only from a small imbalance in a signal the mother dominates. The performance profile is therefore structured by inheritance rather than variant type.

The composition of the monogenic yield reflects the same logic clinically. Twenty-eight of the 41 findings arose de novo, conditions that carrier screening cannot anticipate, that no family history would prompt, and for which karyotype and chromosomal microarray are normal by definition. Twelve involved the RAS/MAPK pathway, the category most often underlying increased nuchal translucency, cystic hygroma and effusions and least tractable to cytogenomics; a further nine involved *FGFR3*, *FGFR2*, *COL1A1* and *COL2A1*, where recurrent gain-of-function substitutions suit a hotspot-aware plasma assay. The technique thus occupies niche cytogenomics cannot reach.

A recent systematic review of non-invasive monogenic testing reported a merged positive predictive value of 98.9% (range 66.7%–100%) for de novo and paternally inherited pathogenic variants [19], closely matching the 98.1% observed here across the pathogenic and likely-pathogenic yields. That value is comparable to non-invasive screening for trisomy 21 and appreciably higher than for trisomy 13, the rare autosomal trisomies or subchromosomal imbalances [3, 19], consistent with a variant read against a defined background carrying a lower discordant -positive burden than a copy-number call inferred from sequencing depth.

Both discordant positives arose in dosage-based calls rather than single-nucleotide variants: a low-level 22q11.2 duplication traceable to documented maternal mosaicism, and a trisomy 16 signal most consistent with confined placental mosaicism. Maternal mosaicism is a recognized source of discordant positive results in non-invasive testing; Wang and colleagues similarly attributed two discordant positive KMT2D calls to low-level maternal leukocyte mosaicism [31]. Such mosaicism is intrinsically less frequent for single-nucleotide variants than for whole-chromosome or segmental events [3, 32], so the absence of any discordant -positive single-nucleotide call reflects the analyte's biology and underlines the value of matched maternal genotyping and dosage modeling within deconvolution [17].

The single discordant negative, a maternally inherited nonsense variant, illustrates the one detection gap this and comparable assays share: sequencing-based non-invasive testing recovers de novo and paternally inherited alleles most readily, whereas maternally transmitted variants must be resolved against the maternal background and remain the least tractable class [19]. The trio design mitigates but does not remove this, duo or trio interpretation being the principal determinant of reliable classification [16, 19]. Outstanding questions concern disorder selection, counseling and the predictive value expected outside a referral cohort. Recent data extending cfDNA screening for autosomal dominant conditions to low-risk pregnancies [33] and prospective work combining aneuploidy and monogenic screening with increased detection [18] indicate the direction of travel; but large multicentre implementation studies with prospective confirmation remain necessary before molecular iNIFS can be offered outside a diagnostic pathway [19].

Amniotic fluid served as the analytical reference standard. A positive plasma call can arise from confined placental mosaicism, a vanished co-twin, maternal mosaicism, an unrecognized maternal condition, or sequencing artifact at low fetal fraction; amniocentesis directly samples fetal-derived cells and resolves each of these. Chorionic villus sampling can also be appropriate for confirmation in many cases, because the villous mesenchymal core, unlike the cytotrophoblast from which cell-free DNA derives, shares its inner-cell-mass origin with the fetus; therefore, best-practice guidelines recommend separate analysis of the mesenchymal core in this setting. Several limitations apply. First, the cohort was assembled by referral for invasive testing and is enriched for fetal pathology (43.7% referred for an ultrasound-detected anomaly; reference-positive rate 17.5%), so predictive values reflect this pre-test probability and must not be extrapolated to an unselected population, where the same sensitivity and specificity would yield a lower PPV. Second, the assay interrogates the clinical exome, so a negative result does not exclude a monogenic disorder outside that content, nor repeat expansions, imprinting defects, or balanced rearrangements. Third, 17 pregnancies (2.7%) had an analytical failure of

the index test, and performance depends on obtaining a reportable result, which in turn dependent on fetal fraction and sequencing depth; some pregnancies will require invasive testing regardless. Fourth, index-test analysts had access to the ultrasound phenotype, a departure from complete blinding that may have favored the index test. Fifth, this single-centre study was performed by the laboratory that developed the assay, and numbers within disease categories are small, so class-level estimates carry wide confidence intervals: for CNV detection, the interval ranges from, 75.8% to 100% despite all 12 being detected.

5 | Conclusion

In pregnancies referred for invasive testing, molecular iNIFS showed high concordance with paired amniocentesis across chromosomal, copy-number and monogenic findings, while retaining an explicit no-call rate. Two thirds of the monogenic findings (28/41) arose de novo, variants that carrier screening cannot anticipate, that no family history would prompt, and for which karyotype and array-CGH are normal by definition; this is the segment of the pathway the technique occupies distinctively. The remaining third was inherited: four paternally transmitted dominant alleles, six biallelic recessive genotypes and three maternally inherited X-linked variants (Supporting Information S1: Table S4). Across the whole yield, the proportions are more modest since 66 of the 106 confirmed findings were chromosomal or copy-number abnormalities already accessible to conventional cytogenomics. Molecular iNIFS therefore extends the existing pathway rather than displacing it, and confirmatory invasive testing remains necessary to resolve both the biological ambiguity of a plasma-derived sample and the evidentiary gaps that constrain variant classification at the non-invasive report. Multicentre external validation is the next necessary step.

Acknowledgments

The authors thank the women and families who participated in this study, and the laboratory and clinical staff of the Department of Prenatal Diagnosis and Fetal Maternal Medicine, ALTAMEDICA, for their technical assistance.

Funding

The authors have nothing to report.

Ethics Statement

This study was approved by the Ethics Committee of Artemisia S.p.A. (reference number 2023-03, approved on 2 November 2023) and was conducted in accordance with the Declaration of Helsinki.

Consent

All participants provided written informed consent for the additional maternal blood sample, for the genomic analysis of the parental samples, and separately for the reporting of secondary carrier findings.

Conflicts of Interest

All authors are employees of ALTAMEDICA, Artemisia S.p.A., which offers the index test (molecular iNIFS) as a clinical service. This constitutes a potential competing financial interest, which the authors declare.

Data Availability Statement

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

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Supporting Information

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