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EXPERT OPINION

NIPT of Maternal Plasma-Originated cfDNA: Applications and Guide for the Implementation

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Abstract: The implementation of non-invasive prenatal testing (NIPT) in maternal plasma, based on cell-free DNA (cfDNA) analysis, has progressed over the last two decades and is now integrated into the Spanish National Health System. However, there remains significant heterogeneity in its indications, technical methodologies, and reporting standards, reflecting international variability. This guide, developed by experts from the Spanish Association of Prenatal Diagnosis (AEDP) and the Spanish Association of Human Genetics (AEGH), provides recommendations to standardize NIPT application. It addresses key aspects such as technical and analytical requirements, integration with invasive diagnostic methods, pre- and post-test genetic counseling, and legal considerations. Additionally, the guide discusses the detection of common aneuploidies, the limitations in identifying structural chromosomal abnormalities and rare variants, and the impact of biological and clinical factors on test performance. By establishing a minimum framework based on scientific evidence, this document aims to optimize NIPT implementation in a cost-effective manner, ensuring clinical validity and informed decision-making for both healthcare professionals and pregnant women.

Keywords: prenatal diagnosis, non-invasive prenatal test, cell-free DNA, functional guide

Introduction

Numerical chromosomal abnormalities and, in particular, trisomies 13, 18 and 21, are relatively common and their prenatal detection has been one of the main objectives of fetal medicine. Prenatal genetic diagnosis of fetal aneuploidies inevitably requires invasive testing, either a chorionic villus sampling or amniocentesis, both of which not only carry a risk of miscarriage but are also costly.[1,2] For these reasons, one of the main goals in the field of prenatal diagnosis has been to develop effective screening methods to define the group of pregnant women who would benefit most from these invasive tests.

Since its inception, screening efforts have primarily focused on detecting the most common chromosomal abnormalities (mainly trisomy 13, 18, and 21). Other chromosomal alterations, such as microdeletion or microduplication syndromes, can also lead to neurodevelopmental disorders and malformations associated with severe disabilities, affecting up to 1–2% of fetuses.[3] While diagnostic capabilities exist for many of these conditions, there is currently no efficient screening method to identify the high-risk group within the general population for invasive testing for these conditions.

Over the last 50 years, aneuploidy screening has evolved from the sole consideration of maternal age in the 1970s, with a detection rate (DR) for Down syndrome of 30% with a false positive rate (FPR) of 5%; to second-trimester serum biochemistry in the 1980s, with DR of 50–70% and FPR of 5%; and finally, in the 1990s, first-trimester combined screening (FTCS), achieving a DR of 90% for a FPR of 5%.[4–7]

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In the past decade, multiple validation and clinical implementation studies have demonstrated that cfDNA-based NIPT accurately detects a high proportion of trisomy 13, 18 and 21 fetuses with a much lower FPR. For singleton gestations, the DR is 99.7% (95% CI, 99.1–99.9%) for trisomy 21, 97.8% (95% CI, 94.9–99.1%) for trisomy 18, and 99.0% (95% CI, 65.8–100%) for trisomy 13, with a combined FPR of 0.13%.[8] In recent years, several studies have demonstrated the ability of this test to detect other aneuploidies, including sex chromosome aneuploidies, triploidies and even to identify other genome-wide alterations. This has led some laboratories to offer screening for numerical alterations of other chromosome pairs, as well as copy number variants (CNVs) of more than 3–7 megabases (Mb).[9–11]

However, the implementation of NIPT remains uneven, with differences in indications, methodologies, reporting standards and regulatory frameworks across health systems.[12] To address these differences, this guide, developed by experts from the Spanish Association of Prenatal Diagnosis (AEDP) and the Spanish Association of Human Genetics (AEGH), provides a structured framework for the integration and standardization of non-invasive prenatal testing (NIPT) in clinical practice.

In addition to outlining best practices, the document also examines the limitations of NIPT, particularly in the detection of structural chromosomal abnormalities, copy number variants (CNVs) and rare fetal aneuploidies.[13] It emphasizes the importance of interpreting results within a broader clinical context to ensure accurate assessment and appropriate follow-up.

In addition, this guideline provides science-based recommendations for the implementation of NIPT in Spain, taking into account public health policy, cost-effectiveness analysis and clinical benefit in different risk populations. By establishing a standardized framework for the use of cfDNA-based NIPT, the document aims to improve informed decision-making, optimize prenatal care and improve patient outcomes, while addressing key challenges related to test sensitivity and cost-effectiveness.

Systematic Approach for the Development, Review, Acceptance, and Updating of the Guide

When evaluating this guide, the Board of Directors of the Spanish Association of Prenatal Diagnosis (AEDP) in collaboration with the Spanish Association of Human Genetics (AEGH), appointed a coordinator with extensive experience in the sector to coordinate and draft this working guide. Subsequently, a working group comprised of experts in genetics and prenatal diagnosis from both AEDP and AEGH was assembled. Once constituted (the authors of the guide), the group evaluated the main applications of cfDNA-based NIPT in prenatal diagnosis; these applications were collected based on various international guidelines. Additionally, a literature review was performed using indexed search tools such as PubMed to gather data on these applications and their integration into traditional workflows used in prenatal diagnosis. Subsequently, an initial document was generated, which underwent thorough review by all members of the expert group. This collaborative process involved incorporating relevant information and excluding what was deemed unnecessary by the group members, resulting in a consensus document that served as the basis for formulating recommendations on each topic. These recommendations were further reviewed and endorsed by the expert committee.

The AEDP and AEGH societies will disseminate the published guide to all their members and professionals in the sector. The guide will also be made available on the online platforms of both societies.

This guide is aimed at all professionals involved in prenatal diagnosis, including obstetricians, fetal medicine specialists, geneticists, and midwives. Its purpose is to provide updated information on non-invasive genetic screening for aneuploidy, to be applied in the clinical setting for both the target population (pregnant women and their fetuses) and for pre-test and post-test counseling.

After publication, an annual review of the guide is proposed to update it, if necessary. To this end, the entire working group will be consulted to discuss revisions to the points of interest.

Application Technology for cfDNA Study

Advancements in genomic analysis technologies have led to the development of several methods that allow the detection of fetal chromosomal alterations in maternal blood samples. These methods are based on the presence of small fragments (approximately 200 bp) of free placental-derived DNA circulating in maternal blood.

Most of the cfDNA found in plasma comes from hematopoietic cells.[14] However, during pregnancy, a small proportion is derived from the placental trophoblast.[15] The proportion of fetal-derived cfDNA in maternal plasma,

known as the fetal fraction, varies significantly due to factors such as gestational age, maternal body mass index, placental problems, sample collection, and storage conditions.

Measurement of the fetal fraction is an essential quality metric, as the ability to differentiate between fetal aneuploidy and euploidy decreases when the fetal fraction is low.[15] The efficacy of screening protocols is evaluated by considering the DR or sensitivity, FPR, positive predictive value (PPV) and no-result rate (NRR), which indicates the proportion of samples that do not yield a result.

In general terms, maternal blood cfDNA analysis techniques are based on massive sequencing of cfDNA, comprising both maternal and placental (fetal) DNA fragments. This process generates tens of millions of sequence reads that can be uniquely aligned and mapped against a human reference genome to determine their chromosomal location. Once mapped, the reads can be counted to determine chromosomal ploidy status. Anomalies such as trisomy or monosomy are identified by observing an increase or decrease, respectively, in the relative number of reads on the affected chromosome compared to euploid chromosomes.[16] The ability to analyze millions of reads and obtain results by complex counting and normalization algorithms allows very high sensitivity to detect aneuploidy in a given sample.

Validated Methodologies for the Study of Fetal Aneuploidies in cfDNA

Three approaches have been clinically validated for the performance of cfDNA analysis for the detection of fetal aneuploidy by NIPT:[17]

- Massively parallel shotgun sequencing (s-MPS) followed by DNA sequence counting. This widely used technique measures the relative abundance of whole chromosome cfDNA in maternal blood by analyzing all chromosomes.
- "Targeted" massively parallel sequencing (t-MPS) with counting of specific DNA sequences. This method is based on the enrichment of the sequences of the chromosomes of interest and measures the relative abundance of cfDNA of these chromosomes in maternal plasma.
- Single nucleotide polymorphisms (SNPs) analysis assesses the relative proportion of maternal and fetal genotypes and compares the patterns observed on specific chromosomes.

Most validation studies evaluate the performance of noninvasive aneuploidy detection in maternal plasma samples from pregnancies where the clinical diagnosis was established by amniocentesis, chorionic biopsy, live birth studies or neonatal phenotyping.[17]

The three approaches present high sensitivity and specificity for the detection of common aneuploidies (13, 18, and 21). However, methods relying on SNP analysis are necessary for detecting triploidies.

Many validation studies excluded cases with mosaicism, complex karyotypes, and maternal samples with low fetal fraction. A summary of the no-result rate by methodology is shown in Table 1.

In cases where there is no result due to a low fetal fraction, repeating the analysis by taking a new sample may allow a result to be obtained, however, in up to one-third of cases it may fail again.

Alternative approaches for aneuploidy detection using cfDNA or cfRNA have been described, such as methods based on methylated DNA, cell-free RNA, or digital PCR, but the number of clinical validation studies is still limited.

Table 1 No-Result Rate by Methodology

Method	No Result	References
s-MPS	1–5%	Palomaki et al[18]; Bianchi et al[19]; Porreco et al[20]
t-MPS	3%	Norton et al.[21]
SNP	5–8%	Pergament et al[22]; Nicolaides et al[23]

Laboratory Recommendations

Quality standards for laboratories that issue reports on genetic testing (cytogenetic or molecular) in Europe are based on three systems: the Cytogenetic External Quality Assessment Service (CEQAS), the European Molecular Quality Network (EMQN), and the United Kingdom's National External Quality Assessment Service (UK NEQAS). These organizations collaborate to audit the standards upheld by participating laboratories, implementing external quality control programs for prenatal genetic testing for both monogenic disorders and cytogenetic testing.[24]

They have also developed evaluations for rapid aneuploidy detection tests using fluorescent quantitative PCR (CEQAS and UK NEQAS) or noninvasive prenatal diagnosis for sex determination (EMQN and UK NEQAS). The results from the participating laboratories, some outside the EU, highlighted the need for the establishment of external quality controls for tests to identify common aneuploidies in maternal plasma samples. Consequently, specific evaluation schemes for NIPT in cfDNA have recently been introduced.

A series of recommendations have been established regarding the presentation of test result reports. It is advised that the terminology used be clear, explicit, and not susceptible to confusion. Results should be reported as "high risk" or "low risk" for the analyzed chromosomes, along with the estimated fetal fraction percentage. If a specific cutoff for fetal fraction affects the test's diagnostic accuracy, it must be clearly stated, as different technologies vary in their sensitivity to this parameter.[25–29] Additionally, a minimum set of data to be included in the results report has been established (Table 2):

With the implementation of the Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017[30] which constitutes the Union regulatory framework for in vitro diagnostic medical devices, the use of such IVD-marked devices for conducting NIPT in cfDNA is established. This regulation also sets high quality and safety criteria for in vitro diagnostic medical devices in order to respond to the common safety concerns they raise, and is therefore recommended to be used in laboratories with a standardized Quality Management System.[31]

Statement: CE-IVDR-marked cfDNA testing is recommended in centers with Quality Management Systems in place through test accreditation.

Implementation Strategies for the Non-Invasive cfDNA Test

Initially, cfDNA analysis was introduced as a private healthcare product. Marketing strategies were aimed directly at potential customers, emphasizing the relief of anxiety, greater certainty in the identification of alterations, and the absence of risk to the fetus. Following this approach, testing spread rapidly throughout the USA, China, and Europe. The first European countries to adopt its implementation initiated programs to finance the tests through the public health system.[32]

Table 2 Minimum Data Set to Be Included in the Test Results Report

A minimum of two unique identifiers for the pregnant woman, including her full name and date of birth
Type of sample, date of sample collection and date of sample receipt
Gestational age established by ultrasound at the time of sampling
Issue date of the report
Test applicant's data
Methodology and test limitations
Fetal fraction
Results reported as high/low risk for aneuploidy
Recommendation for confirmation of results by invasive test, when appropriate
Individualized risks, positive and negative predictive values

Switzerland was the first country to offer such a program, refinancing the costs of non-invasive risk assessment for trisomies 13, 18, and 21 via the "compulsory health insurance program" in July 2015. As an immediate consequence, a 50% decrease in the number of amniocenteses among women with high-risk pregnancies was observed.[33]

Currently, seventeen European countries have integrated NIPT in cfDNA into their publicly funded prenatal care systems. However, regulations regarding the scope of testing, outcomes, counseling, funding, and inclusion criteria vary substantially from country to country and follow entirely different precepts.[12,32,34]

In Europe, usage rates are estimated to be about 15% in Italy, 10–35% in Switzerland, 20% in Norway, 30% in Germany, 51% in the Netherlands and 75% in Belgium. In most European countries, less than 25% of women undergo this test,[35] while in the USA and Australia, its use is more widespread. However, when analyzed in detail, there are few regulatory similarities and almost all models are unique.

Some countries have national policies in place and many others are in the process of developing them. Where a specific strategy has been chosen, there appear to be two models: universal screening or contingent screening for pregnancies classified as at high-risk by FTCS.

In this context, specific legislation has been enacted on the use of prenatal screening and/or cfDNA-based NIPT. In general, NIPT usually includes aneuploidies of chromosomes 13, 18, and 21 as well as sex chromosome studies and is performed either by default or based on the parents' desire to know the sex of the fetus. However, only a subset of countries has national guidelines on the reporting of sex pair aneuploidy and fetal chromosomal sex, and reporting practices in the case of a detected sex pair abnormality are variable.

In Spain, health policies vary according to each Autonomous Community, so there are currently different protocols for the application of the cfDNA study for the detection of pregnancies at risk of fetal aneuploidy. In the public health system of most autonomous communities, the cfDNA study is offered to pregnant women with high or intermediate FTCS risk, both in single and multiple pregnancies (up to 2 fetuses).

Different studies analyzed in a recent systematic review have demonstrated the superior performance of cfDNA-based NIPT over FTCS in all parameters (Table 3), including studies conducted in general-risk populations with singleton pregnancies. This has made it possible to establish a series of recommendations for the application of cfDNA in first and second-trimester gestations.[36]

In addition, these same studies conclude that, although there are fewer studies in twins than in singleton pregnancies, NIPT for T21 detection in twin pregnancies demonstrates screening characteristics equivalent to those of singleton pregnancies.

As previously mentioned, there is currently no common guideline in the contingent model implementation of a minimum cutoff point regarding the risk established by FTCS. However, there are studies in this regard that establish population models according to the risk established by FTCS, considering the detection rates of both screenings and the no-result rate of cfDNA-based NIPT failure (Table 4).[37]

Table 3 Diagnostic Performance of NIPT in cfDNA.[8,34]

	Detection Rate (95% CI)	False Positive Rate (95% CI)
	Singleton pregnancies	
Trisomy 21	99.7% (99.1–99.9%)	0.04% (0.02–0.07%)
Trisomy 18	97.9% (94.9–99.1%)	0.04% (0.03–0.07%)
Trisomy 13	99.0% (65.8–100%)	0.04% (0.02–0.07%)
	Twin pregnancies	
Trisomy 21	99.0% (92.0–99.9%)	0.02% (0.001–0.43%)
Trisomy 18	92.8% (77.6–98.0%)	0.01% (0–0.44%)
Trisomy 13	94.7% (91.4–99.97%)	0.10% (0.03–0.39%)

Table 4 Distribution of the High-Risk Population Based on the FTCS Results

Cut-off Point (1 in x)	Screening by NT, FHR, PAPP-A and ß-hCG			
	NIPT in cfDNA	DR T21	DR T18/13	IT
100	2.6%	86.7%	88.9%	0.52%
200	4.3%	90.1%	91.4%	0.54%
300	5.8%	91.5%	92.6%	0.56%
400	7.1%	92.5%	93.2%	0.56%
500	8.3%	93.2%	93.8%	0.57%
1000	13.4%	95.6%	95.1%	0.61%

Abbreviations: NT, nuchal translucency; FHR, fetal heart rate; PAPP-A, pregnancy-associated plasma protein A; ß-hCG, free ß-fraction of human chorionic gonadotropin; NIPT, non-invasive prenatal testing; cfDNA, cell-free DNA; DR, detection rate; T, trisomy; IT, invasive testing.

For its evaluation, scientific and socioeconomic criteria should be considered, and the cut-off point should be adjusted based on cost-efficiency and budget availability considerations. In 2016, a health technology assessment report was carried out for the implementation of NIPT in cfDNA for the detection of trisomies 13, 18 and 21. The authors concluded that the introduction of NIPT in cfDNA as a contingent test, for a risk cut-off point of 1 in 270 at delivery (equivalent to 1 in 250 at 12 weeks), is a non-cost-effective option whose main advantage over current screening is reducing pregnancy loss related to invasive testing. In contrast, they suggest that a cost-effective alternative would be to lower the risk cut-off to ≥1 in 500. This adjustment would lead to greater detection of Down syndrome cases at a 4% increased cost, assuming a reference a cost per NIPT of 550 €.[38]

However, considering the scientific evidence regarding the superior diagnostic performance of the cfDNA test compared to the FTCS, as presented in this work, along with the anticipated decrease in test costs, its cost-efficient implementation as universal screening is foreseeable in the near future. Nevertheless, depending on socioeconomic circumstances, intermediate risk thresholds, such as 1:1000, could be considered as part of a phased transition while maintaining the goal of universal screening.

Statement: cfDNA-based NIPT is recommended in singleton or twin gestations with an estimated risk ≥1 in 500 established by the first trimester combined screening.

Coordination of Non-Invasive Test Indications with Invasive Diagnostic Procedures

NIPT is not a diagnostic test; therefore, high-risk aneuploidy results should always be confirmed by an invasive test, preferably amniocentesis in cases of trisomy 13 and 18. However, the choice of invasive test may vary depending on new ultrasound findings, in which an invasive test could be performed.

On the other hand, the design of the test presents limitations in detecting chromosomal alterations underlying other conditions that are detectable by identifying markers or ultrasound abnormalities. Thus, the implementation of the noninvasive prenatal approach involves its integration into workflows that include invasive testing and associated techniques for the identification of other genetically based alterations. In most cases, invasive testing includes a rapid diagnostic approach, usually QF-PCR (Quantitative Fluorescent Polymerase Chain Reaction) for the detection of aneuploidy in chromosomes 13, 18, 21 and sex pair, as well as to rule out possible maternal DNA contamination. The use of microarrays in cases with fetal ultrasound abnormalities is also well established[39] along with the more recent incorporation of exome sequencing.[40]

With all these considerations, a decision algorithm integrating both noninvasive and invasive approaches in the prenatal setting could be applied, as shown in Figure 1.

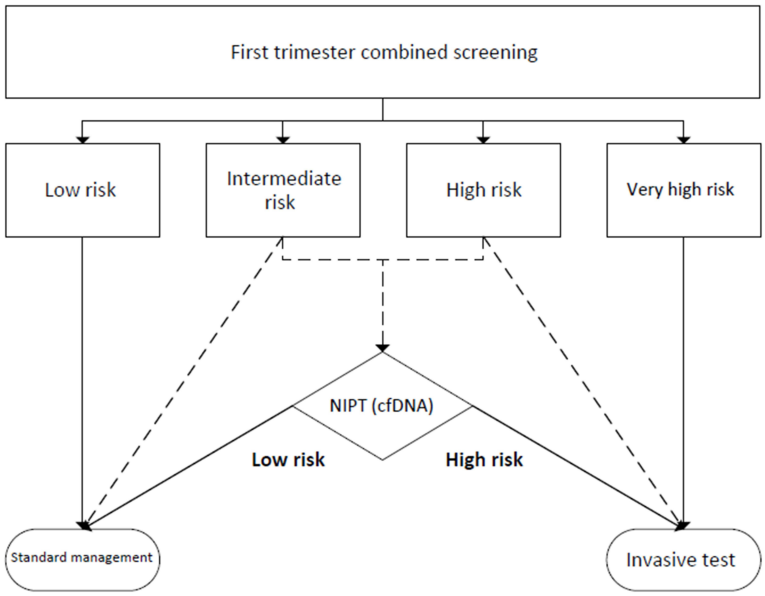

Figure 1 Contingent implementation algorithm of the cfDNA-based NIPT.

Statement: Invasive testing is recommended after a positive NIPT result, as well as in the presence of ultrasound abnormalities suggestive of genetic pathology.

Genetic Counseling in NIPT

Genetic counseling as defined in Order SSI/2065/2014, is the process aimed at informing a person about the possible consequences for themselves or their offspring of the results of a genetic test or screening, including its advantages and risks. It also involves advising them on possible alternatives resulting from the test. This procedure shall take place both before and after a genetic test or screening and even in the absence of such testing or screening.

Genetic screening, as defined by the law on biomedical research (Law 14/2007, BOE July 7, 2007), refers to systematic genetic testing conducted on a large scale and offered as part of a program to a population or a subset thereof to detect genetic traits in asymptomatic individuals. According to Article 54.1 of the same law, genetic screening aims to detect a disease or serious health risk in the participating individual or their offspring, with the aim of early treatment or access to preventive measures. To carry out the NIPT in cfDNA, the principles of justice, autonomy, beneficence, and non-maleficence must be respected. Therefore, pregnant women must provide written consent to participate in the test and undergo genetic counseling before and after the procedure.

The advice should be objective, avoiding bias and personal considerations (Table 5). The information should be provided both orally and in written form, with documentation maintained, as outlined in articles 4 and 48.3 of the Biomedical Research Act.

Sex Pair Aneuploidy

Currently, the detection rate for sex chromosome aneuploidies in cfDNA-based NIPT is high, with a sensitivity of 99.6% (95% CI: 94–100%) and a specificity of 99.8% (95% CI: 99%). The positive predictive value (PPV) with 95% confidence intervals (CI) varies for different chromosomal abnormalities: for XYY syndrome, the PPV is 74.5% (58–85%); for XXY syndrome (Klinefelter syndrome), it is 74% (59–84%); for XXX syndrome (Triple X syndrome), it is 54% (40–66%); and for monosomy X (Turner syndrome), the PPV is 29.5% (22–37%).[41] However, this performance may be somewhat lower for monosomy X, mainly due to the higher rates of both placental and maternal mosaicism for this aneuploidy, information that should be emphasized in counseling the pregnant woman.[42]

On the other hand, fetal sex determination is indicated in cases where there is a risk of having a child with a severe genetic disorder affecting a particular sex. While there are more cost-efficient methodological approaches than cfDNA-

Table 5 Recommendations Regarding Genetic Counseling for NIPT in cfDNA

Key points to address during pre-test genetic counseling:
Report the specific chromosomal alterations to be assessed.
Explain to which population it is advisable to offer it and at what gestational age.
Report the technique used and its limitations, as well as sensitivity, specificity, PPV, NPV data.
Report turn-around time.
Report the existence of other prenatal screening and diagnostic tests.
To inform about the current deadlines for the legal termination of pregnancy.
Inform about the recommendation to perform invasive diagnostic tests (chorionic villus sampling, amniocentesis) in case of a positive result.
Inform of the possibility of revocation of the test.
Key points to address during post-test genetic counseling
Offer an invasive prenatal test to confirm the result in case of a positive result.
Report situations that generate false positive results.
Recommend postnatal confirmation of the result in case of waiver of invasive test in case of a positive result.
Offer psychosocial support and multidisciplinary information.

based NIPT, such as real-time PCR, some cases still use NIPT for noninvasive fetal sex determination, depending on the availability of each approach.

Statement: It is recommended to offer detection of sex chromosome aneuploidy by cfDNA-based NIPT only if adequate pre- and post-test counseling is available.

History of Chromosomopathy

According to Gardner and Sutherland's book[43] the standard for genetic counseling regarding the risk of recurrence of numerical and structural chromosomal abnormalities ranges between 0.5 and 2%. This assessment considers chance recurrence, gonadal mosaicism and somatic-gonadal mosaicism.

More specifically, the risk of recurrence of *de ovo* trisomies has been estimated to increase by 1.6–1.8 times after trisomy 21. This risk is influenced by the age at which the first affected pregnancy occurred and is higher in younger women compared to older women.[44]

Warburton et al[45] estimated the risk of recurrence in various trisomies by analyzing subsequent cases identified by prenatal diagnosis in women with a previous prenatal diagnosis of trisomy, monosomy X, or triploidy. They reported a significantly increased risk of trisomy 13 or 18, XXX or XXY after a diagnosis of trisomy 21 (RR=2.3; 90% CI, 1.5–3.8). They also noted that following any of trisomy 13, 18 or 21, XXX or XXY, the risk of a different trisomy was significantly higher. The relative risk (RR) of recurrence of the same trisomy was highest for the least frequent trisomy, trisomy 13 (RR=9.5; 95% CI, 1.1–35.3), followed by trisomy 18 (RR=3.1; 95% CI, 1.0–7.2) and the lowest for the most frequent trisomy, trisomy 21 (RR=2.2; 95% CI, 1.6–2.9).

These data indicate that women who have had a trisomy 13, 18 or 21 have an increased risk of trisomies in the future based solely on their medical history.

Statement: It is recommended to offer cfDNA based NIPT screening in case of previous pregnancy with aneuploidy.

Copy Number Variants. Microdeletions and Microduplications

The 22q11.2 microdeletion syndrome, while rare, stands as the most common pathogenic CNV identified prenatally and is a major cause of congenital heart defects and neurodevelopmental delay. Its prevalence ranges from 1 in 990 to 1 in

2148 gestations.[46] Reviews performed thus far indicate that the sensitivity and specificity of CNV screening generally fall below those observed for more common trisomies and sex aneuploidies. Dar et al prospectively evaluated the reliability of SNP-based cfDNA in detecting microdeletions within the 22q11.2 region.[11] The reported sensitivity and specificity were 75% and 99.84%, respectively, with a PPV of 23.7% and a NPV of 99.98%. This study concluded that cfDNA-based NIPT for 22q11.2 microdeletion syndrome can detect most pathologic cases with a low false positive rate. However, they suggested that to accurately assess the detection performance of this alteration, it would be more appropriate to focus on a particular CNV (eg the 22q11.2 microdeletion), rather than attempting to detect various potential CNVs simultaneously.

On the other hand, there is currently insufficient evidence to assess the risk associated with the presence of CNVs smaller than 7Mb. A recent study of a series of pregnancies, some with fetal anomalies, found that the majority of clinically relevant CNVs were larger than 7Mb.[47] There is limited data from studies with gestational follow-up that evaluate the presence of CNVs smaller than 7Mb, and the available sensitivity and specificity information remains scarce. The cohorts in these studies are heterogeneous and many contain fetuses with ultrasound abnormalities, suggesting that estimates are likely influenced by verification bias, thereby complicating the generalization of findings.

Statement: The study of cfDNA-based NIPT for the detection of CNVs other than 22q11.2 deletion is not recommended based on current evidence.

Rare Fetal Aneuploidies

Rare fetal aneuploidies in the field of noninvasive prenatal genetic diagnosis are those involving chromosomes other than pairs 13, 18, 21 and sex chromosomes. Typically, these aneuploidies are not compatible with life unless they are present in mosaic. As such, there is insufficient clinically relevant evidence to recommend determining their risk by NIPT in cfDNA. Mosaicism identified in chorionic villi occurs in 1–2% of pregnancies,[48] with the vast majority being confined to the placenta (MCP). The rare cases of mosaicism confirmed by amniocentesis, however, are associated with a wide range of phenotypic consequences.[49] Preliminary data shows that MCP may be associated with placental insufficiency, fetal growth restriction or other adverse perinatal events; however, this application remains experimental and may have an adverse outcome.

Additionally, the detection of rare autosomal trisomies (RATs) by non-invasive prenatal testing (NIPT) represents an additional challenge in prenatal genetics, as it increases the risk of uniparental disomy (UPD), a condition with significant clinical implications.[50] UPD occurs when both copies of a chromosome are inherited from a single parent, potentially leading to imprinting disorders if the chromosome contains imprinted genes. This risk is particularly high if the trisomy results from a meiotic error and undergoes trisomy rescue, a process that can result in a uniparental disomic state. For example, trisomy 15 mosaicism often results from a maternal meiotic error, increasing the likelihood of Prader-Willi syndrome due to maternal UPD15. In contrast, trisomies of mitotic origin carry little or no risk of UPD, highlighting the importance of distinguishing between these origins in clinical assessment. Given the different risks associated with different chromosomes - such as those involving chromosomes 7, 14, 15 and 20 - genetic counselling needs to be expanded to include UPD testing where appropriate.[51] Without proper evaluation, undiagnosed cases of UPD could lead to unforeseen developmental and health consequences. The potential for UPD further complicates decision-making following an abnormal NIPT result, underscoring the need for more refined guidelines and a comprehensive approach to prenatal genetic counselling.

Statement: There is insufficient evidence to recommend the study of cfDNA-based NIPT for screening of rare fetal aneuploidies (non-13, 18, 21 and sex pair), irrespective of prior risk.

Biological Circumstances of the Pregnant Woman

Several factors can alter the NIPT result, leading to false positives or negatives or complicating the acquisition of results. These factors include maternal neoplasms, mosaicism,[52] organ transplantation, maternal transfusions, autoimmune diseases, body mass index or the presence of a vanishing twin.

Maternal Neoplasms

Tumor processes often have chromosomal abnormalities, leading to the presence of DNA from these cells in maternal plasma. This presence can result in false-positive or uninformative cfDNA-based NIPT results. Malignant neoplasms in pregnant women are relatively rare but occur in approximately 1 in 1000 cases.[53]

Several studies have reported the identification of maternal cancer cases from NIPT results in cfDNA.[54] This circumstance can contribute to up to 15% of false positives in NIPT.[55] Consequently, if the result suggests the presence of aneuploidies in multiple chromosomes despite a normal invasive test result, it would be advisable to consider the presence of malignant tumors, mainly hematological neoplasms such as leukemia or lymphoma.[54] In such cases, pregnant women should be informed during the genetic counseling and their referral for further assessment should be considered.

Mosaicisms

Mosaicism refers to a genetic alteration where an individual has two or more cell populations that differ in their genetic complement. Placental mosaicism is caused by an error in cell division that occurs very early in fetal development. The presence of placental mosaicism can lead to false-positive or false-negative NIPT results.[56]

Additionally, some pregnant women may exhibit chromosomal mosaicism. For example, advanced age may lead to a gradual and preferential loss of the X chromosome that is inactivated, presenting mosaic monosomy X in peripheral blood. This mosaicism could potentially alter the results of cfDNA.[57]

Organ Transplantation and Maternal Transfusions

In the case of transplants from a male donor, NIPT can misidentify a female fetus as a male fetus due to the release of male cfDNA from the transplanted organs.[58] Pregnant women with a history of bone marrow or organ transplantation from a male donor should be informed of the possibility of erroneous results and offered alternative aneuploidy screening tests.

Similarly, pregnant women who have received blood transfusions from male donors should undergo NIPT at least four weeks after the transfusion, to avoid misidentification of a female fetus as male.[59]

Autoimmune Diseases

Autoimmune diseases have been associated with a higher no-result rate in pregnant women undergoing NIPT, primarily due to a lower fetal fraction.[60–62] This occurs because the inflammatory responses characteristic of autoimmune diseases may increase the percentage of maternal cfDNA in the bloodstream, thereby decreasing the proportion of fetal cfDNA.

Body Mass Index

The relationship between high body mass index and a higher no-result rate in cfDNA-based NIPT due to a low fetal fraction is well known. However, there is currently no consensus regarding the cutoff point at which the risk of a no-result in cfDNA NIPT increases. Two recent reviews recommend addressing this point during pre-counseling sessions with pregnant women who have a high body mass index, given the increased likelihood of not obtaining a result associated with this circumstance.[63,64]

Vanishing Twins

Vanishing twins refer to gestational losses resulting from the spontaneous reduction of one or more fetuses, typically occurring during the first trimester of initially multiple pregnancies.[65] This phenomenon is often attributed to aneuploidies.[66] The presence of a vanishing twin can complicate the interpretation of cfDNA-based NIPT results, especially in pregnancies with discordant abnormalities. In dizygotic twins, each fetus may contribute different amounts of cfDNA to the maternal circulation, and fragments from the deceased co-twin, likely abnormal, may persist in maternal blood for several weeks after its demise.[67] Currently, there are insufficient data to fully evaluate the performance of cfDNA in such cases. Some studies suggest that while cfDNA NIPT can successfully detect common autosomal aneuploidies in conceptions affected by a vanishing twin, it may be associated with a higher false-positive and no-result rate.[66]

Feto-Placental Biological Circumstances

Although inconsistently, conception by in vitro fertilization has been described as a potential risk factor for a lower fetal fraction or failure to obtain a conclusive result after cfDNA analysis, especially with targeted analysis.[68]

Conclusions

This functional guide presents a series of recommendations resulting from a comprehensive literature review carried out by a collaborative working group comprising members of the Spanish Association of Prenatal Diagnosis (AEDP) and the Spanish Association of Human Genetics (AEGH). NIPT is a screening test for common aneuploidies with proven clinical utility, which is applied in Spain as a contingent test in most of the autonomous communities, although in a heterogeneous manner. The objective of this work is to establish a minimum framework for action based on the latest scientific evidence. This does not imply any restriction concerning the inclusion of other indications that are adapted to the socioeconomic circumstances of the health services and the individual needs of the patients.

Hence, this document outlines key aspects to be addressed in genetic counseling, its integration alongside other prenatal genetic diagnosis tools, and the establishment of a safe methodological environment based on cost-efficiency principles. These considerations should always be contextualized within the clinical, biological, and sociocultural circumstances of the feto-maternal environment.

The rapid clinical and technological evolution associated with genetic studies requires investment to provide the necessary resources and training. The current application of cfDNA-based NIPT, along with its potential for detecting submicroscopic rearrangements such as microdeletion and duplication syndromes, as well as rare fetal aneuploidies, whether in a contingent strategy with FTCS or as a potential future universal screening, make this test one of the pillars of prenatal genetics.

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Disclosure

The authors report no conflicts of interest in this work.

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