

Relevance of Invasive Testing in Era of Non-Invasive Testing for Prenatal Chromosomal Abnormalities

Abhijeet KUMAR¹, Madhusudan DEY¹, Devendra ARORA¹

Delhi Cantt, India

ABSTRACT

Prenatal screening for chromosomal abnormalities has two components i.e. prenatal screening (maternal serum screening and cell-free fetal DNA screening) and prenatal diagnosis (chorionic villus sampling, amniocentesis, and cordocentesis). Prenatal testing in the past decade is evolving towards non-invasive methods to determine the chromosome abnormality disorders in the fetus without incurring the risk of miscarriage. Conventional tools for prenatal screening included maternal age, maternal serum markers, ultrasound marker (nuchal thickness), and their combinations. With the increased risk of screening test patients were offered diagnostic tests (chorionic villus sampling, amniocentesis, and cordocentesis). After the availability of noninvasive prenatal tests for commercial use in 2011, a great marketing drive is there to establish it as a master tool for prenatal testing. However various society guidelines i.e. ACOG, RCOG, and ISUOG have clearly stated that cell-free fetal DNA based noninvasive prenatal tests is a screening test, not a diagnostic test. In the succeeding paragraph, we will review current trends in the field of cell-free fetal DNA noninvasive prenatal tests and the relevance of invasive testing in the context of noninvasive prenatal tests. Noninvasive prenatal tests does not entirely replace invasive prenatal testing procedures. Positive noninvasive prenatal tests findings must be confirmed by diagnostic tests based on an invasive sample source, mainly chorionic villus sampling or amniocentesis due to false positive and false negative reports of cell-free fetal DNA based tests. Continuing research and development efforts are focused on overriding noninvasive prenatal tests limitations. Recent studies show that procedure-associated risks in the case of prenatal invasive testing are very low as compared to previous studies. Prenatal invasive testing will remain as the backbone of prenatal diagnostic testing until the limitation of noninvasive prenatal tests is overcome.

Keywords: Amniocentesis, Cell-free fetal DNA, Chorionic villus sampling, Invasive, Prenatal testing

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Introduction

Prenatal testing for chromosomal abnormalities has undergone an evolution from traditional invasive methods (amniocentesis or chorionic villus sampling (CVS)) to non-invasive methods i.e. maternal age, maternal serum screening, ultrasound, and cell-free fetal DNA (cffDNA) noninvasive prenatal tests (NIPT). Transabdominal amniocentesis was first re-

ported in 1877 but its use for genetic prenatal diagnosis started in the 1970s for high-risk pregnancies (1,2). CVS was first described by Mohr in 1968 (3). However, the introduction of ultrasound imaging guidance in the procedure led to a marked safety profile of CVS and amniocentesis (4). Due to a 1% to 2% risk of miscarriage associated with invasive procedure and advancement in next-generation sequencing (NGS) leads to the development of non-invasive prenatal testing based on maternal blood. Development of various screening test i.e. dual, triple, quadruple, combined, integrated, the contingent screen was based on maternal serum metabolites. The performance of various serum screening tests for trisomy 21 with a 5% false-positive rate is shown in table I (5).

Table I: Performance of serum screening and combined testing for trisomy 21

Screening methods	Detection rates (%)	False-positive rate (%)
Maternal age (MA) alone	30	5
Double test (HCG+ PAPP)	50-55	5
Triple test (HCG+AFP+UE3)	60-65	5
Quad test (HCG+AFP+UE3+INHIBIN)	65-70	5
MA+NT	70-75	5
Combined test	75-80	5

¹ Department of Obstetrics & Gynecology Army College of Medical Science and Base Hospital, Delhi Cantt, India

Address of Correspondence: Abhijeet Kumar,

Department of Obstetrics & Gynecology
Army College of Medical Science and Base
Hospital, 110010 Delhi Cantt, India
xyzabhi@yahoo.com

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ORCID IDs of the authors: AK: 0000-0001-8280-5053

MD: 0000-0003-3892-1529, DA: 0000-0002-8660-6737

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It was observed that intact fetal cells are present in maternal plasma in the late sixties (6). Later on, in 1969, Walknowska et al. demonstrated that this approach may have a contagion effect on the evolution of prenatal diagnosis (7). Seminal work by Lo, et al in 1997 demonstrated that during pregnancy, fetal DNA could be seen in the plasma of pregnant women (8). Continuous research has demonstrated the gestational variations and rapid clearance of circulating cell-free fetal DNA within two hours of delivery (9,10). Cell-free fragments derived from fetal DNA are shorter than those of maternal cell-free DNA, and the size distribution is typically lower than 150 base pairs (11-13). In clinical practice, NIPT is used for screening aneuploidy i.e. T21, T18, and T13. A recent meta-analysis found a 99.7% detection rate (DR) for Trisomy21 (T21), 97.9% DR for Trisomy18 (T18), and 99% DR for Trisomy13 (T13) with a false positive rate of 0.04%. This high sensitivity, specificity with a low false-positive rate of cffDNA based NIPT as compared to combined screening test makes cffDNA based NIPT as a better diagnostic tool than serum screening for prenatal aneuploidy (Table II).

Table II: Performance of cell-free fetal DNA noninvasive prenatal tests vs Combined test

Screening Methods	Detection rate (%)	False Positive Rate (%)
Combined test	85-90	5
Noninvasive prenatal tests	99.7	0.04
Combined Test	75	5
Noninvasive prenatal tests	97.9	0.04
Combined Test	75	5
Noninvasive prenatal tests	99	0.04

Discussion

Even with high detection rates of >99% and low false-positive rates, it is important to emphasize that cffDNA NIPT remains a screening test as per various society guidelines and those women with a high-risk result require invasive testing to confirm the findings of prenatal chromosome abnormality. The principal reason for cffDNA based NIPT being a screening test is due to its false-positive and false-negative results.

False-Negative Results of cffDNA based NIPT (the fetus is unaffected, but cffDNA testing indicates a chromosomal abnormality)

1. Confined placental mosaicism (CPM)

CPM is one of the main reasons for “false” NIPT results. Two (or more) cell lines with different chromosomal complements in a fetoplacental unit derived from a single zygote are termed as CPM. Mosaicism can be a result of cell division errors either mitotic or meiotic. In cases of early mitotic error will lead generalized mosaicism while late mitotic error may lead either mosaicism placenta or mosaicism fetus. In cases of mosaicism due to meiotic error develop from trisomic zygote which has been rescued in later stages by a mitotic error. If the

supernumerary chromosome is discarded it lead to uniparental disomy while if one of the original euploid set is discarded it leads to biparental disomy. The fetal cffDNA in the maternal circulation is placental cell (syncytiotrophoblast). The cffDNA test will provide results relevant to the placenta, which may be discordant with fetal tissue. CPM is observed during CVS as it contains both syncytiotrophoblast and mesenchyme and a normal karyotype is observed when amniocentesis is performed (14). Due to embryological fetoplacental discordance, cffDNA NIPT does not always reflect the fetal genome. Due to the statistical occurrence of true-negative, true-positive, false-negative, and false-positive results of the cffDNA mismatch of placental and fetal cell lines leads to clinical and analytical discordance.

2. Vanishing twins

In spontaneous abortions both cases with a normal and an abnormal karyotype placental cffDNA and total cffDNA increase (15,16). Each placenta in the dichorionic twin releases two individual fractions of cffDNA into the maternal circulation and these are measured during cffDNA based NIPT. An aneuploid vanishing twin continues to shed cffDNA weeks after fetal demise therefore cause a false-positive cffDNA NIPT result for the fetus that is alive (17).

3. Autosomal trisomies

Trisomy involving chromosomes other than chromosome 21, 18, 13, X, and Y are rare autosomal trisomies. In non-mosaic form, these are not compatible with life and therefore rarely been seen as results of invasive prenatal diagnosis. In mosaic form, rare autosomal trisomies have been associated with fetal growth restriction, fetal death, true fetal mosaicism, and uniparental disomy (UPD). Most of the cffDNA based NIPT methods centered around a comparison of targeted chromosomes with reference chromosomes will be effected either by a trisomy or monosomy or large copy number variation (CNV) in the reference chromosomes. Whole-genome sequencing and analysis can elucidate its etiology.

4. Fetal sex discordances

Noninvasive cffDNA based fetal sex determination has a sensitivity of 96.6% and specificity of 98.9% (18). Women who are carriers of sex-linked disorders any test for fetal sex determination must have the specificity of 100% to make a clinical decision. Following situation may arise:

a. The first scenario ultrasound shows male genitalia and karyotype and cffDNA indicates a female fetus. This may be due to an “XX male” due to a translocation of part of the short arm of the Y-chromosome, including the SRY gene, onto the X-chromosome [congenital adrenal hyperplasia, virilization of a female fetus such as an androgen producing tumor or exogenous androgens (19,20)].

b. The second scenario ultrasound and karyotyping results indicate a male fetus, but cffDNA reports female sex. This

may be due to a low fetal fraction, placental 46, XX/46, XY or 45, X/46, XY mosaicism a demise of a female cotwin.

c. The third situation is when ultrasound and karyotyping both indicate a female fetus, but cffDNA reports male fetuses. This may be due to placental 46, XY/46, XX mosaicism, the demise of a male cotwin, recent blood transfusion from a male donor, and history of transplantation with a male donor (21).

d. At last, if a karyotype and cffDNA testing report male fetuses but ultrasound shows female genitalia. This may be due to feminization as a result of Smith-Lemli-Opitz syndrome or by mutations in several sex developmental genes, for example, androgen receptor, 17 β -Hydroxysteroid dehydrogenase, steroid 5-alpha reductase, or SRY (22).

5. Maternal factors

Most cffDNA based NIPT testing considers that a mother has a normal karyotype but this is not the case always. With advancing maternal age, a proportion of women have a small percentage of cells that have abnormal chromosomes due to age-related mosaic loss of a maternal X-chromosome which can cause a false-positive NIPT result for monosomy X that can be ruled out by karyotyping peripheral blood lymphocytes (23-25). Maternal cancer during pregnancy is rare (0.1%), hematologic malignancies, or solid organ tumors releasing cell-free tumor DNA into the maternal circulation have been described as a cause of false cffDNA testing results which can be detected by the whole genome-based NIPT (26). Autoimmune diseases such as systemic lupus erythematosus with anti-double-strand DNA antibodies and severe maternal vitamin B12 deficiency have been described in association with abnormal cffDNA profiles. Benign maternal de novo or inherited duplications or deletions represent another reason for false-positive and false-negative NIPT results (27). These variants translate to more or fewer cffDNA reads of the corresponding chromosome in the cffDNA assay (28). The sensitivity for false results due to maternal CNVs are highest for shorter test chromosomes and decrease with higher fetal fractions (29).

6. Statistical chance

Cut off for a positive test is often set at +3 standard deviation. By chance alone, 1 to 2 per 1000 normal karyotype fetuses will have false-positive results. In theory, all phenomena responsible for false-positive cffDNA-based prenatal test results can also cause false-negative cffDNA test results (30).

a. Low fetal fraction: Obesity, early gestational age, increased maternal age, ethnic variation, hemolysis in the sample due to storage, or maternal heparin use may cause low fetal fraction (31-33). Obesity has a higher number of adipocytes releasing maternal cffDNA into the blood and diluting the placental cffDNA leading to a low fetal fraction (34). The failure rate is 2.9% in unaffected pregnancies, 1.9% in T21, 8.0% in T18, and 6.3% in T13. The smaller placental size and fetal growth restriction are observed in T18 and T13 which may be

contributing to low fetal fraction and leading to false-negative cffDNA results.

b. CPM: In cases where there are normal syncytiotrophoblast and abnormal mesenchyme and fetus i.e. True Fetal Mosaicism type 5 (TFM 5) will cause false-negative cffDNA test results.

c. A normal vanishing twin will mask the presence of an affected aneuploid twin by a temporarily overrepresented cell-free fetal fraction. Theoretically, maternal deletions on the target chromosomes with a trisomic pregnancy can result in a false-negative result.

Our perspective

A prospective interventional study was performed between January 2019 and December 2019 out at this tertiary care Hospital. The study was approved by the ethical review committee of Army College of Medical science Delhi Cantt India. Written informed consent was obtained from all participants and informed consent for use of data was also taken. The study was conducted according to the International Conference of Harmonization/Good Clinical Practice (ICH/GCP) guidelines and the latest version of the Helsinki Declaration by World Medical. A total of 3500 pregnant women were counseled about 1st trimester combined screen between 11 to 14 weeks of period of gestation. 2500 women opted for combined screening. 136 women had high-risk screen positive (if T21>1:250 and T18/13>1:200), these women were counseled about aneuploidy risk, invasive testing, and NIPT was offered. 126 women opted for invasive testing (amniocentesis) and in this group, 2 cases of T21 were confirmed on karyotype and both pregnancies were terminated. 10 women opted for NIPT and all were negative for T21, T18, T13, and monosomy X (Figure 1). There was no procedure-related fetal loss. The diagnostic yield of invasive testing was 1.47%.

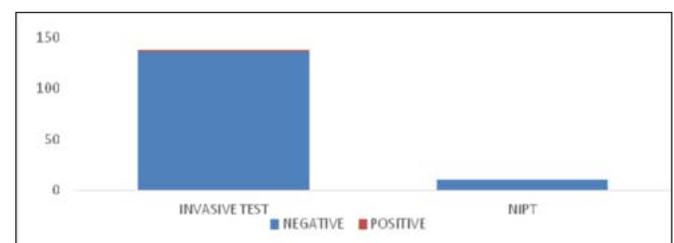


Figure 1: Prenatal invasive testing

Limitations of the study

1. The sample size was inadequate to achieve statistical significance.

2. All the high-risk patients underwent amniocentesis only hence true prevalence and complication of invasive testing could not be determined.

In contrast to non-invasive testing, invasive testing still holds value as it provides diagnostic value and to a major ex-

tent overcome the above-mentioned limitation of non-invasive testing. One major disadvantage associated with invasive testing is 1-2% risk of miscarriage, based on an old study in which the actual pregnancy loss rate was 1.7% in the study group and 0.7% in the control group (35). Rates of fetal loss following CVS were 1.9% in 2009 (36). A recent meta-analysis found a procedure-related risk for amniocentesis of 0.11%, with an actual pregnancy loss rate of 0.81% in the study group and 0.67% in controls, and a procedure-related risk for CVS of 0.22%, with an actual pregnancy loss rate of 2.18% in the study group and 1.79% in controls which was also endorsed by ACOG (37). A population-based study consisting of 150 000 women found no increased risk of fetal loss with either amniocentesis or CVS in 2016 (38).

Why invasive testing superior to cffDNA NIPT in the current context?

1. Abnormal cffDNA-based NIPT results have to be confirmed by a diagnostic test i.e. invasive testing either by amniocentesis or CVS to obtain a chromosomal analysis. Culture report of amniocytes obtained by amniocentesis is considered superior to CVS as amniocytes are true representative of the fetal genome while CVS culture both cytotrophoblast which is representative of placental unit and mesenchyme which represent fetal genome. Mosaicism in amniotic fluid cells is much uncommon than in chorionic villi. The cffDNA-based prenatal testing is offered from the 9th week, abnormal cffDNA results confirmation with an amniocentesis will delay the result as amniocentesis is performed after 16 weeks. It might lead to pregnancy termination without confirming cffDNA-based NIPT results due to patient anxiety. This scenario can be avoided by doing CVS, which is usually done between weeks 11 and 14 of gestation. However, CVS has got its limitation, i.e. possible detection of CPM type 3 mosaicism confined to the placenta leading to a false-positive result, and TFM type 5 leading to a false-negative result if the only cytotrophoblast is analyzed (39). Also, mosaicism between cytotrophoblast and mesenchyme can be detected (CPM types 1 and 2 and TFM types 4 and 5) making a second invasive procedure, amniocentesis, necessary to ascertain the fetal karyotype. Grati et al. have noted that the rate of mosaicism for trisomy 21, trisomy 18, trisomy 13, was, respectively, 2%, 4%, 22%, and 59% of abnormal CVS cases (14). The anomaly was confirmed by amniocentesis in, respectively, 44%, 14%, 4%, and 26% only. These data can be used instead of the risk that amniocentesis will be required as a second invasive test when CVS is chosen to confirm an abnormal cffDNA NIPT result.

2. Due to the phenomenon of fetoplacental discrepancies, cffDNA NIPT does not always reflect the fetal genome.

3. Cytotrophoblast cells (STC), as well as mesenchymal cells (LTC), should always be analyzed together for accurate interpretation of CVS reports. If results of either or both are mosaic or if there is a discrepancy between the 2 cell layers,

amniocentesis, and FISH analysis of amniocytes are diagnostic, to confirm whether it is CPM or TFM.

4. cffDNA NIPT is not considered diagnostic as the “fetal DNA” detected is placental in origin i.e. placental cytotrophoblast and syncytiotrophoblast cells and cffDNA test provide results relevant to the placenta which may be discordant with the fetal genome (40).

5. Amount of genetic material available in NIPT is small and cannot be used for exon sequencing, whole-genome sequencing which is required to prognosticate if a couple had the previous baby affected with a genetic disorder or his pregnancy complicated by soft markers or sonographic abnormalities and also in case of stillbirth to elucidate syndromic association and cause of death.

6. The cost of cffDNA based NIPT at present is quite high as compared with invasive testing.

Conclusion

To sum up, due to the multitude of mechanisms and reasons for abnormal results of cffDNA-based tests even after the introduction of NGS platforms. Before any pregnant women are undergoing cffDNA based testing patient history is taken, ultrasound to confirm viability, number of fetuses, and rule out vanishing twins, and genetic counseling should be done. False-positive and false-negative results are due to fetoplacental biology and not a failure in the actual test. Elucidating the placental origin of cffDNA will lead to a better understanding of the advantages and limitations of cffDNA-based prenatal screening by patients. If cffDNA-based tests indicate a trisomy, confirmatory testing in the form of invasive testing is needed before offering a conclusion.

It is clear that at present cffDNA based NIPT remains a screening test for aneuploidy at this juncture. cffDNA based NIPT is widely used as a screening test for aneuploidy worldwide due to its non-invasive nature and high sensitivity and specificity. Invasive testing such as amniocentesis and CVS are increasingly safe with low rates of pregnancy loss and are essential diagnostic tools in case of abnormal cffDNA results evaluation. Advancement and research continuum may allow to further decrease false-positive and false-negative results of cffDNA NIPT by improving identification of chromosomal abnormalities of placental and maternal origin in near future till that time invasive testing will remain as the backbone of prenatal diagnostic testing.

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