fetologue

Issue.5 January 2024

FGi RESEARCH INSTITUTE
FETAL AND GYNAE IMAGING

From The Editor's Desk

Welcome to this edition of Fetologue; your go-to resource for practice essentials of **cfDNA** test also known as **NIPT**. cfDNA test a popular prenatal screening test, must be implemented within the realms of evidence-based practice. We have attempted to present all the relevant information on this topic, in a concise and precise manner.
Stay ahead and stay informed!

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Prenatal screening for the traditionally screened **Common Autosomal Trisomies (CAT)** i.e trisomy 21, 18 and 13 is a routine practice in prenatal care. Currently the most common screening method is the combined first trimester screening (cFTS) using serum biochemistry and a detailed ultrasound scan.

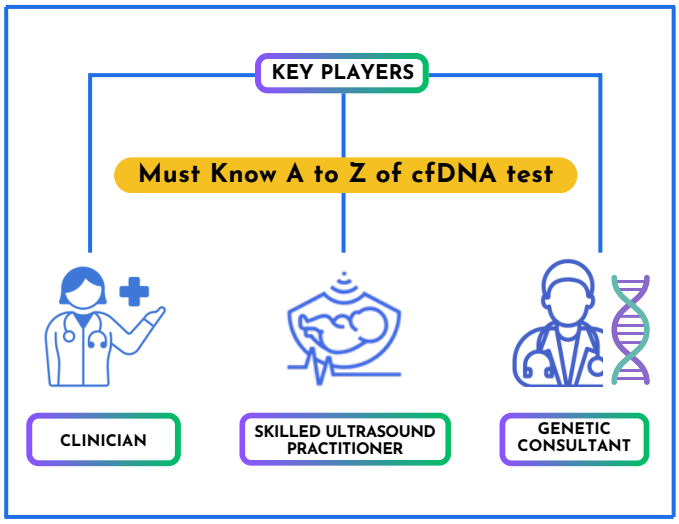
With the advent and increasing use of cell-free DNA (cfDNA)-based techniques to screen for the above aneuploidies, it is important to understand its application and clinical utility.

Synonyms

NIPT=Non-invasive prenatal testing. Term used by ISPD.

cfDNA Screening=cell-free DNA screening. Term used by ISUOG, FMF & SMFM.

The term cfDNA will be used in this newsletter, as it is conceptually more accurate.



Pre-requisite in India: PCPNDT Form F and Form G must be documented for cfDNA test, prior to performing the test at a registered centre.

Maternal blood
(draw 8 - 10 cc in K3EDTA vial. Should be processed within 36 hours after collection).

→ Maternal cfDNA + Fetoplacental cfDNA (From trophoblast)

→ Disappears from maternal blood: 16 minutes to 2 hours after delivery, maximum 2 days after delivery

Fetal fraction (FF) = Proportion of fetoplacental cfDNA, to the entire cfDNA in the maternal plasma. Expressed in %

Minimum threshold required: 4%

< 4%: Low fetal fraction
No-result on cfDNA test
Increase in false negative rate of cfDNA test

ISPD = International Society for Prenatal Diagnosis

ISUOG = International Society of Ultrasound in Obstetrics & Gynecology

ADVOCATING cfDNA test

A detailed ultrasound is mandatory before advising cfDNA test.

- Accurate dating, detect multiple pregnancy, and confirm viability.
- Note fetal anatomy, and aneuploidy markers.

Ideal time to perform cfDNA test is 12+ weeks onwards, after NT scan.

May be offered at 10 weeks, after counselling on the pros and cons of an early cfDNA test.

No upper limit of gestational age for performing cfDNA test*.

*Turnaround time of result, and further management in case of positive/no-result must be considered.

Detection rate of fetal structural abnormalities in the first trimester scan i.e NT scan is better (32% - 60%) when compared to earlier i.e < 11 weeks (17%).

Pre-test counseling is essential: Options, benefits, limitations, and incidental findings.

Provide sufficient easy-to-understand information to assist the couple in making a decision for prenatal testing.

- cfDNA test is a **screening test**. It cannot diagnose the presence or absence of a chromosomal abnormality. Hence majority of the laboratories report a result specific to a chromosome as low risk (usually <1 in 10,000) or high risk (>99%).
- cfDNA test does not test for all genetic syndromes.
- Availability of the result i.e turnaround time is laboratory-specific, and ranges from 10 - 14 days on average.
- Likely occurrence of "no-result", false positive or false negative results.

Detection rates

	cFTS (%)	cFTS (%)	cfDNA (%)	cfDNA (%)
	Singleton	Twins	Singleton	Twins
Trisomy 21	90	89	98.8	98.2
Trisomy 18	97	89	98.8	88.9
Trisomy 13	92	89	100	66.7
Triploidy	95	89	-	-
Monosomy X	-	-	97.6	-

ACMG statement 2016

DR by cfDNA test applies to:

- Down syndrome caused by trisomy 21, translocations, and trisomy 21 mosaicism.
- Edwards syndrome caused by trisomy 18 and trisomy 18 mosaicism.
- Patau syndrome caused by trisomy 13, translocations, and trisomy 13 mosaicism.

cFTS = combined First Trimester Screening

ACMG = American College of Medical Genetics and Genomics

cfDNA Test NOT RECOMMENDED

- As a substitute for a first or second trimester ultrasound.
- Prior to a detailed ultrasound.
- Triplets and higher-order multiple pregnancy.
- The presence of a fetal structural abnormality or soft marker suggestive of a chromosomal abnormality, wherein invasive testing must be recommended.
- NT > 3.5 mm. Peterson et al recommend an NT cut-off of 3.0 mm for invasive testing (AOGS 2019 – 522 fetuses, cohort analysis and literature review).
- High risk on cFTS.(cut-off 1 in 250), even in the absence of any fetal structural abnormality.
- Suspicion of inborn errors of metabolism, myoneuropathy, Fragile X syndrome or cystic fibrosis.
- Single-gene disorders such as skeletal dysplasia, cystic fibrosis etc (ACOG practice advisory September 2023)

OPTIONS: Primary vs selective screening with cfDNA test

Primary Screening using cfDNA test (Public-funded)	Contingent Screening using cfDNA test (Public-funded)	Case-by-case approach	Self-funded consumer choice
Netherlands Belgium	Denmark UK	Germany	Australia USA

Primary screening; cfDNA test is offered as an option to all women in the general obstetric population.

- The premise is cfDNA test has a better detection rate of CAT, when compared to the standard serum screening tests. However, for low-risk women a positive cfDNA test is more likely to be a false positive.
- SMFM does not recommend cfDNA testing for low-risk women.
- ISUOG statement 2017: "Using cfDNA in low-risk patients might be endorsed as a widely available option only when more data emerge and cfDNA costs decrease."
- ISPD: "It can be offered in primary or contingent screening models with context specific considerations in local health policy influencing decisions and implementation models."

Selective screening; includes contingency screening as well as offering cfDNA test to a "high-risk" group for aneuploidies.

Contingency screening

- cfDNA test is offered to the intermediate risk group.
- The presence of a fetal structural abnormality falls into the category of high-risk, regardless of the computed risk based on maternal age and other US markers of aneuploidy. This group will benefit from an invasive diagnostic test.
- The cut-off determining low/intermediate/high risk is set based on local/national policies and available resources.
- In India the cut-off for high risk on screening is 1 in 250 at most centres.

SMFM = Society for Maternal-Fetal Medicine

"High-risk" group based on the following: (SMFM statement).

- Maternal age > 35 years at delivery.
- Prior pregnancy with proven trisomy 13, 18 or 21.
- Parental balanced Robertsonian translocation with increased risk of fetal trisomy 13 or 21.

EXPANDED cfDNA test

Synonyms: NIPT-PLUS, extended NIPT.

The following are assessed in Expanded cfDNA test..

1. Sex chromosome aneuploidies (SCA)–Some laboratories offer this on routine cfDNA test as well
2. Rare autosomal trisomies (RATs)
3. Copy number variants (CNVs)

ISPD 2023: "There is insufficient data to assess the performance and clinical utility of routine NIPT for RATs. NIPT for RATs is therefore not recommended for the routine care of unselected populations."

ISUOG 2018: "The detection rate for microdeletions has yet to be established and most national guidelines currently do not support testing for microdeletions on cfDNA. Screening for microdeletions also raises complex issues regarding pretest and post-test counselling."

SMFM: "Routine screening for microdeletions with cell-free DNA is not recommended."

Pretest counselling whilst offering expanded NIPT when indicated, is essential.

- Increased rates of false-positive results.
- Variable outcomes of conditions screened.

Sex chromosome aneuploidies (SCA)

"The Chromosome Abnormality Screening Committee on behalf of the Board of the International Society for Prenatal Diagnosis recommends that when prospective parents are offered NIPT, and SCA screening is available, they should have the option to separately accept or reject the sex chromosome analysis."

- There is an atypical number of sex chromosomes i.e X and Y, in a cell.
- The incidence of SCA is higher in the advanced maternal age group.
- Turner syndrome shows features on prenatal ultrasound and has an adverse prognosis when compared to other SCA.
- The phenotype of other SCA is variable and less severe and cannot be detected on prenatal US.
- There is insufficient data to accurately report DR for SCA, other than monosomy X.
- PPVs of 50% for 47, XXX, 66% for 47,XXY, and 83% for 47/XYY have been reported.

FMF = Fetal Medicine Foundation

Rare autosomal trisomies (RATs)

- RATs are chromosomal aneuploidies other than CATs i.e trisomy 21, 18, and 13, or SCA.

- True fetal trisomies of RATs are not compatible with life.
- Those that survive are fetal mosaics which have variable presentations ranging from near normal to severe anomalies.
- PPV of cfDNA test for RATs is 11.46%.
- When cfDNA test shows a high risk for RATs, and US is normal there is a 97% association with confined placental mosaicism (CPM).
- CPM refers to the occurrence of a chromosomal abnormality in the placenta but is not detected in the fetus.
- In the presence of CPM, there is the possibility of placental dysfunction, and hence an increased risk for fetal growth restriction and preeclampsia.
- For cfDNA test results of mosaicism in any of the imprinted genes (see algorithm) uniparental disomy is a possibility, and manifestations of this (FGR, macrosomia, visceromegaly) may not be evident until the third trimester.

Copy number variants (CNVs)

Synonyms: Segmental imbalances/structural aberrations.

- They include loss or gain of genetic material from segments of chromosomes in the form of microduplications, microdeletions and unbalanced translocations.
- They may be inherited from either parent or arise denovo and are independent of maternal age.
- CNVs fall into 5 groups: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign.
- Most prevalent microdeletion is 22q11.2, which causes Di George Syndrome.
- For CNVs > 5Mb, the detection rate is 90.9% with a FPR 5%.

RATs and CNVs are also referred to as **atypical abnormalities.**

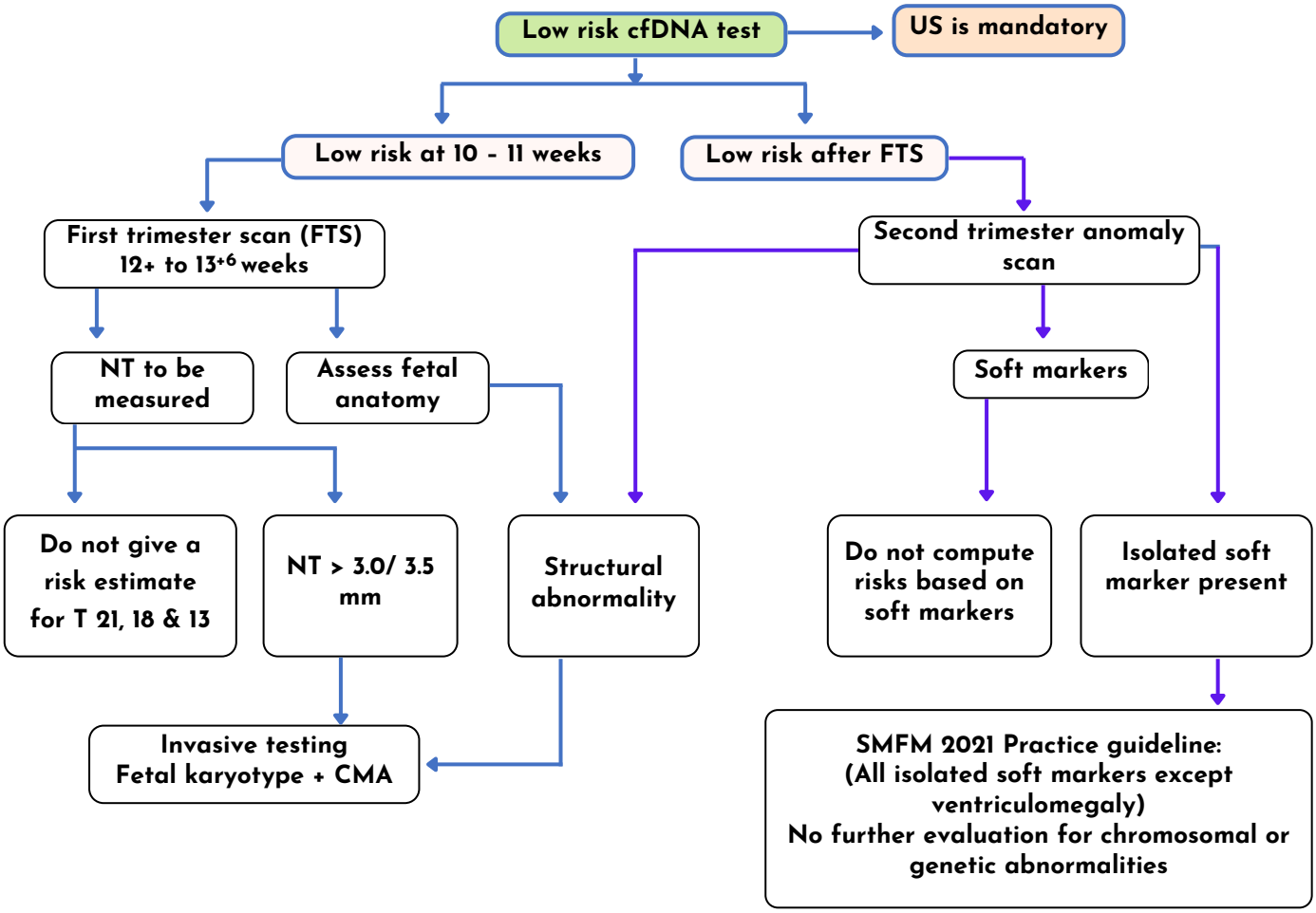
1. 90.2% of atypical abnormalities can be detected by invasive testing in women with:
   - High risk on cFTS
   - < 0.2 MOM serum markers (PAPP-A and ß-hCG)
   - Fetal structural abnormality
2. Clinically relevant micro deletions/duplications may be seen in 1 - 1.7% of structurally normal fetuses.

Low risk cfDNA test

Synonyms: Negative cfDNA, no aneuploidy detected.

The chance that the fetus does not have one of the common aneuploidies is > 99%, provided a detailed ultrasound has been performed prior to the cfDNA test, and fetal anatomy has been optimally assessed. However false negative is a small possibility.

If the cfDNA test has been performed between 10 – 11 weeks, it is mandatory to recommend the first trimester (NT) scan.



Note:

- Isolated absent / hypoplastic nasal bone maybe associated with pathogenic CNV & sex chromosome abnormality.
- Isolated ventriculomegaly whether mild or severe is associated with pathogenic CNV (5% & 16%).
- For fetuses with long bones at -2 and 4 SD for gestational age, karyotyping, and CMA are recommended.
- When the finding is an isolated ARSA (aberrant right subclavian artery), the options depend on the prevalence in the specific population. The study by Bevilacqua et al have shown that where the incidence of 22q11.2 deletion is high, the PPV was 100% and NPV was 98% on targeted cfDNA. In the general population with a low prevalence, on a targeted cfDNA the expected PPV is estimated to be 12.2% at 99.5% specificity and 41.1% at 99.9% specificity, while the expected NPV is estimated to be >99.9%.

SMFM = Society for Maternal-Fetal Medicine

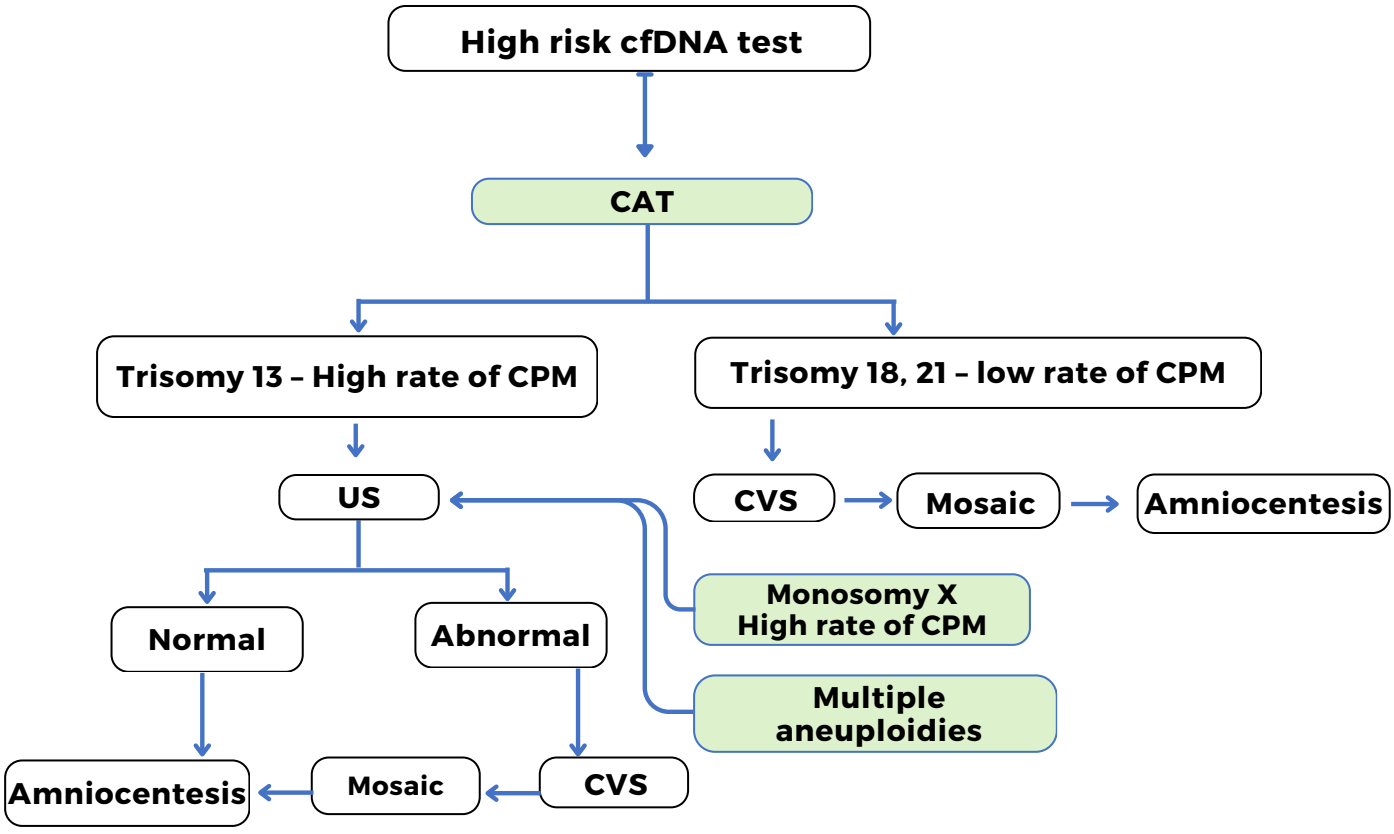
High risk cfDNA test

Synonyms: Positive cfDNA, aneuploidy detected.

The likelihood that a fetus is affected is as follows:
Trisomy 21: 1 in 240
Trisomy 18: 1 in 650
Trisomy 13: 1 in 460

This must be confirmed by a diagnostic test i.e invasive test. **A pregnancy MUST NOT BE TERMINATED on the basis of a high risk cfDNA test report**.

Expert post-test counselling in the event of an inconclusive/ high risk cfDNA test

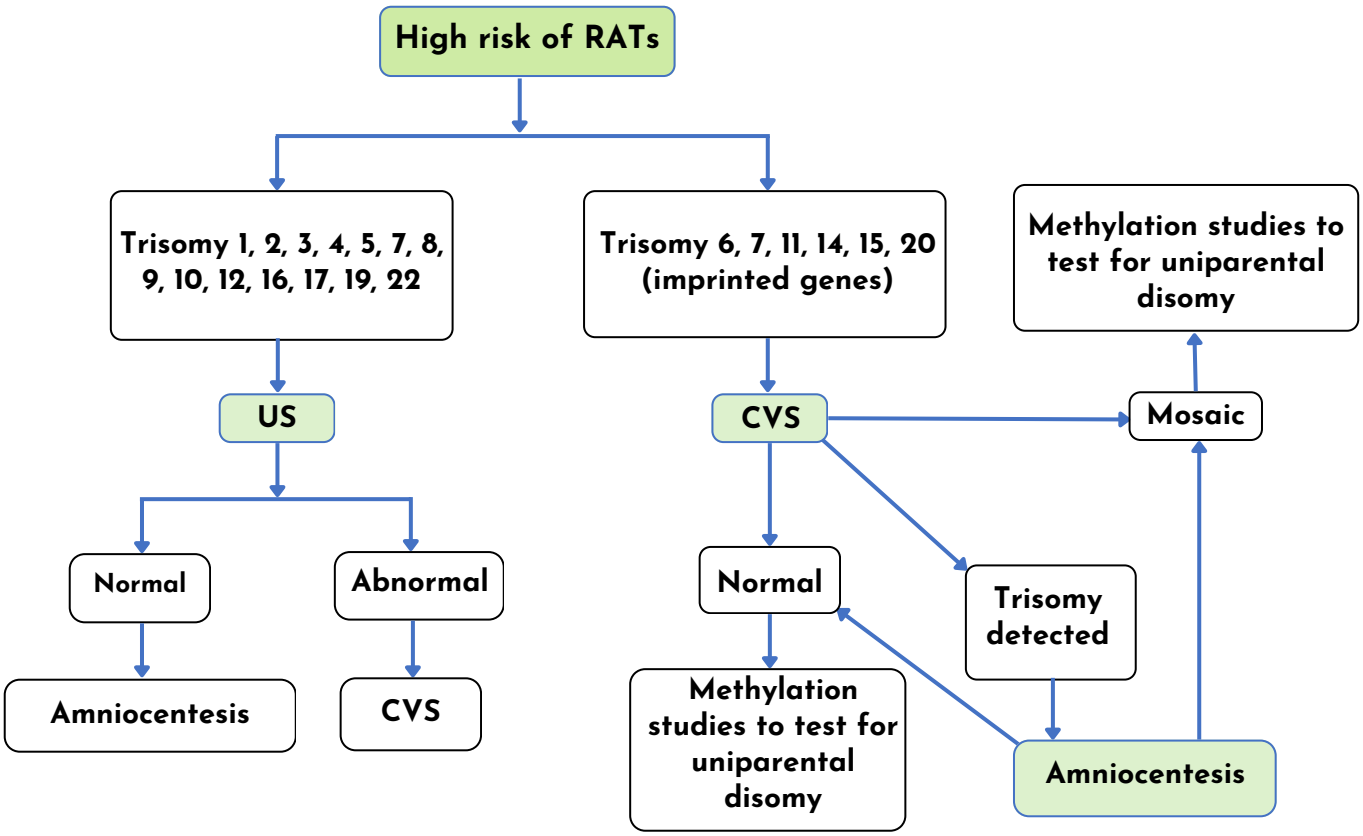


Monosomy x

Unaffected fetus: Perform karyotype of mother to look for mosaic turner syndrome. (8.6% postive sex chromosome aneuploidy on cfDNA test had maternal aneuploidy involving the X chromosome).

Multiple aneuploidies

Unaffected fetus: Consider work up for maternal malignancy (most common - liver, breast and lymphoma). PPV: 7.6% for maternal malignancy.



High risk for CNV results must be followed by prenatal diagnostic testing with chromosome microarray analysis, as well as maternal testing for the detected CNV.

No-Result

Synonym: No-call, test failure.

Ranges from 1 - 6.4%

Reasons:

1. Logistic issues: Challenges with collecting the blood sample, transporting the sample, inadequate sample, clotting of the blood etc. In such cases a repeat sample gives a 100% result.
2. Problems with the assay: 75% of cases obtain a result on repeat sampling.
3. Low fetal fraction (FF): Most common reason. On repeat sampling a result is obtained in 50% of cases.

Low fetal fraction (FF) - Causes

1. Small placental mass
   I. Early gestational age
   II. Low serum free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A)
   a. Impaired placentation
   b. Trisomy 18, trisomy 13, and triploidy (most common)

In two prospective studies which included 16,000 pregnancies trisomy 21 was seen in 23% of cases with low FF. Other studies showed that FF was not affected in trisomy 21 cases.

Low fetal fraction (FF) – Causes Continued..

2. Maternal weight

The Danish Fetal Medicine Society state 90 kgs, and the Gynecologists of Canada state 110 kgs as the weight above which, there is a failure of the test. Results from a study which included around 1500 patients showed that low FF was present in 10.5% samples of women > 110 kg. At < 60 kgs the % of low FF was 0.2%.

3. Autoimmune diseases in the mother.

4. Other reasons for test failure

- Black or South Asian racial origin - 2-fold.
- Dichorionicity - 3 fold.
- Parous women - Risk of test failure is 37% lower than in nulliparous women.
- IVF conception - 4-fold .
- Use of low molecular-weight heparin or enoxaparin.
- Large regions of homozygosity for a single/many chromosomes.

Test failure is unrelated to the method used for cfDNA analysis.

Management of test failure

- Analyse the cause of no-result.
- Where the cause can be circumvented eg: early gestational age, resampling may be offered.
- In cases of obesity, IVF pregnancies etc an alternate aneuploidy screening (if not already done) may be offered.
- In cases suspicious of low placental mass due to a chromosomal aneuploidy, offer invasive testing.
- In cases where the failure is due to large regions of homozygosity, offer invasive testing.

DISCORDANT RESULTS on cfDNA test

This includes the false positives and false negatives. These occur because the circulating cfDNA is derived from the cytotrophoblast of chorionic villi in the placenta, and hence is not always representative of the fetus.

False positive cfDNA test

cfDNA testing indicates a chromosomal abnormality, but the fetus does not have the abnormality. cfDNA test is methodically correct but clinically incorrect. The false positive rate for CAT is approximately 0.1 – 0.2%. It is higher for RATs and CNVs.

Causes:

1. Confined placental mosaicism
2. Maternal copy number variations
3. Vanished twins/co-twin demise
4. Maternal malignancy

1 – 2/1000 euploid fetuses may have a false-positive result by chance alone.

Hence a high-risk cfDNA test result must be followed with invasive diagnostic testing.

False negative cfDNA test

- cfDNA testing indicates no chromosomal abnormality, but the fetus has a chromosomal abnormality. cfDNA test is methodically correct (i.e., detecting those placental cells of the mosaicism which are euploid) but clinically incorrect (i.e. the fetus itself is aneuploid).

- False negative rate is 0.01% (1 in 10,000).
- The cfDNA test result is determined by the relative proportion of cells with normal and abnormal karyotypes within the villi. When there is a mosaic if there is high proportion of cells with normal karyotype in the villi, as compared to the cells with the abnormal karyotype, the NIPT test result is negative i.e low risk.
- Studies analysing false negative NIPT results have shown that some of these cases (3.5% – 6%) have ultrasound abnormalities.
- Opstal et al have shown that the estimated risk of missing trisomy 13, 18 and 21 on cfDNA test is 3.5%. However, fetuses with trisomy 13 and 18 will show abnormalities on subsequent ultrasound examinations or have an intrauterine demise.
- The major contributing factor is mosaicism, with the chromosomal aberration being present in the fetus and mesenchymal core of the villi but the cytotrophoblast is chromosomally normal. If the fetal fraction is low, a mosaic of < 30% is expected to be missed by cfDNA test.

67% - No obvious biological or technical explanation for the discordant result either false positive or false negative.

The Z score in cfDNA test

The z score is obtained from a comparison of the patient's sample with a control group of normal euploid i.e. diploid samples. This is then evaluated against a predefined threshold which is set at +/- 3.

If the Z score of the patient's sample is > 3 or < - 3, the test is reported as high-risk which warrants an invasive diagnostic testing. If the Z score is between -3 and + 3 the test is reported as low risk.

Many studies have assessed the positive predictive value (PPV) vis a vis Z score.
Three groups were defined based on the predefined threshold i.e. + 3 and an optimal cut-off which varied in different studies.

Studies	Z score based groups	Inference
Ma et al (2023)	> 3 to < 6, > 6 to < 10 > 10	PPV 50% 84.6% 87.95%
Tian et al (2018)	≥ 3 to < 5 ≥ 5 to < 9 ≥ 9	Higher accuracy

In all the studies higher the Z score, higher the PPV.

- The PPV improves with a higher Z score above cut-offs of 7.5, 4.9 and 9.1 for Trisomy 21, 18 and 13 respectively (Yang et al. 2023).
- Whilst an invasive diagnostic test is mandatory in all high-risk NIPT results regardless of the optimal cut-off, looking into the z scores may help lessen the anxiety in those cases where the Z score is > 3 and < the optimal cut-off value.

- The Z-score interpretation has been evaluated for Trisomy 21, 18 and 13. The accuracy of Z- score for other chromosome aneuploidies has not been extensively studied.

cfDNA Test in TWIN PREGNANCY

- Results are pregnancy specific and not fetus specific. Hence diagnostic testing when recommended must be performed for both the fetuses.
- The lower FF, and not the total FF must be considered.

- cfDNA test should not be offered for at least 6 – 8 weeks after documentation of a vanishing or a co-twin demise.

- There is insufficient data for screening of RATs, SCA and CNVs in twin pregnancies.

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KEY POINTS

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- In India cfDNA test must be performed at registered centres, and after documenting PCPNDT Form F and Form G.
- Ultrasound is mandatory before advising the test.
- Best time to perform cfDNA test is upwards of 12 weeks after the NT scan.
- Pre-test and post-test counselling is essential.
- The detection rate for the common trisomies i.e 21, 18, and 13 is around 99% with a false positive rate of 0.1 – 0.2%.
- cfDNA is not recommended as a routine for the detection of atypical chromosomal abnormalities i.e rare autosomal trisomies and copy number variants.
- When including testing for sex chromosomal abnormality by cfDNA, patients should be informed about the same.
- High risk cfDNA pregnancy must undergo invasive testing for final diagnosis, and a pregnancy should never be terminated on the basis of a high risk cfDNA report.
- False negatives may occur in 0.01% cases.
- In twins the cfDNA result is pregnancy specific and not fetus specific.

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