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REVIEW ARTICLE

Noninvasive Prenatal Testing from Basic knowledge to Clinical Applications: Review of Literature; An Overview and Guidelines

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ABSTRACT

Cell-free fetal DNA (cffDNA) examination is an important non-invasive prenatal diagnostic tool playing an important part in screening of chromosomal or monogenic diseases in fetus. It includes fetal DNA recognition in maternal main bloodstream. We can examine it starting from four to six weeks of pregnancy. Invasive procedures are not manageable in some circumstances. Novel methods with noninvasive procedure are highly necessary at that time. Noninvasive techniques are preferred due to it is simple and easy technique. In addition, it is preferred due to its availability, via quantifiable detection or precise sequencing. Maternal plasma placenta derived cffDNA sequencing has progressed from a research project to implement it into clinical care. The clinical use of cffDNA sequencing for chromosomal aneuploidy screening has, by now, an international impact motivated by pregnant women's need for safer prenatal screening. NIPT only is able to test specific conditions, according to type of NIPT used.

INTRODUCTION

Circulating cell-free DNA (cfDNA) is a type of extracellular DNA found in biological fluids that comes from both healthy and sick cells[1]. Mandel and Metais identified cfDNA fragments in the circulation in 1948[2] as shown in **Fig1.** However, fifty-five years later after its detection, still cfDNA received little respect revered to the shortage in understanding about its configuration, function, biological role as well as its evolutionary roots. cfDNA is composed of degraded DNA remains (50 - 200 bp), it includes mostly the non-encapsulated DNA in the blood released in the circulation. cfDNA might be used to define numerous types of DNA spontaneously flowing in the circulation, involving circulating tumor DNA (ctDNA), cell-to free mitochondrial DNA (ccf mtDNA), and cell-free fetal DNA (cffDNA)[3].

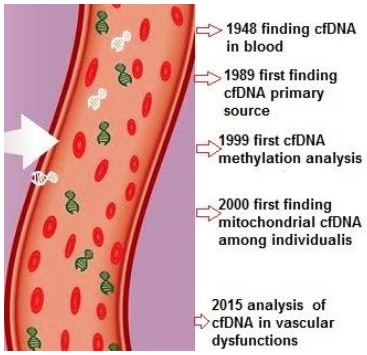

Fig. 1: Timeline of cfDNA discoveries

The finding of **(cf fDNA)** in maternal blood in 1997 used in prenatal screening paved the way for the marketing and expansion of noninvasive prenatal testing (NIPT) kits to detect fetal chromosomal abnormalities. For the past few years, there was a breakthrough especially in the diversity of tools has appeared focusing on the study of cfDNA by the means of (NIPT) assays. The outcome of those NIPT assays is a step ahead in reference to the old-style serum screening approaches[4].

Trisomy 21 (Down syndrome), the commonest prevalent chromosomal anomaly at birth, is routinely screened in many health systems. Screening tests available in the first trimester of pregnancy embrace ultrasound measurement of fetal nuchal translucency among other sonographic markers, as well as maternal serum measurement for beta human chorionic gonadotropin and pregnancy associated plasma protein. cfDNA in maternal plasma had lately been used as a first-line screening test or presented to females who have an intermediate or high likelihood (in liable screening) or a high chance (in secondary screening) following traditional **triple marker** screening test (combined test)[5]. The **triple marker-screening** test has a trisomy 21 detection rate (DR) of 90% and a false-positive rate (FPR) of 5% in singleton pregnancies. The main benefits of cfDNA screening that it have a bigger DR of 99.7% (95 percent confidence interval [CI], 99.1-99.9) and a substantially lower FPR of 0.04 percent (95 percent CI, 0.02-0.07), which agrees to a lot reduced

need for intrusive testing and thus a much lower miscarriage rate.

Since its clinical release in 2011, cfDNA-sequencing technology has transformed prenatal screening for fetal aneuploidy. The first report of cffDNA derived from placental tissue and discovered in maternal blood, was published by Dennis et al.[7] and primarily used to determine fetal sex[8]. cffDNA is used to screen for common autosomal aneuploidies among fetuses using next generation sequencing (NGS). Since then, a number of techniques that use cffDNA to screen for fetal aneuploidies have been developed, which are referred to as noninvasive prenatal screening (NIPS)[9].

DNA from both maternal and fetal sources is detected in the circulating cfDNA found in maternal blood. Maternal circulating cfDNA comes from all of the mother's organs, comprising solid tumors, and primarily derived from the hematopoietic system. cffDNA is produced predominantly from trophoblast cells in the placenta and signifies fetal DNA (placental DNA).

Fetal Fraction

The fetal fraction (FF) is the proportion of cffDNA in maternal plasma in relation to all circulating cfDNA **Fig.2.** FF is 10–15 percent at 10–20 weeks of pregnancy the most typical time for NIPS[10]. Because maternal and fetal cell-free DNA is not separated during NIPS, it is critical to comprehend FF in order to correctly understand NIPS results. Till now, a lot of research has been done on the FF, but it's comparatively disrupted, The FF, or the amount of cffDNA that belongs to the fetus, is thought to be a key constituent to guarantee the accurateness of NIPS[11].

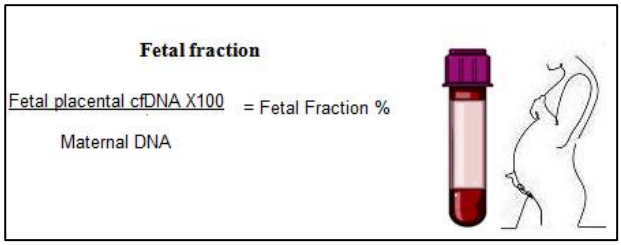

Fig. 2: Calculation of Fetal Fraction

Factors affecting cell-free DNA fetal fraction

Gestational age, mother BMI, placental mass and function, among other reasons known to alter FF. The FF level in the maternal blood is directly proportional with the fetus' gestational age. Several researches has found a strong link between maternal weight and fetal DNA: as maternal weight or Body Mass Index (BMI) rises, FF percentage in the maternal blood falls[12,13].

It has to put in consideration that because the sample size of the studies done is very small for consistent statistical examination, causes that already recognized to affect the FF of cfDNA did not attain global acceptance among numerous research. As a result, retrospective study was performed with the goal of enhancing and presenting some useful clinical statistics from a huge number of clinical cases by assessing the relationship between gestational age, maternal BMI, as well as maternal age[14]. According the study comprised 13,661 pregnant women who had singleton pregnancies and underwent NIPS. The cffDNA FF in maternal plasmas examined in relation to gestational age, maternal BMI, and maternal age for NIPS. The research concluded that the proportion of FF considerably grew with the rise in gestational age. On the other hand with the rising in maternal BMI, there was a noticeable decrease in FF. The fetal percentage was likewise negatively associated to maternal age[14].

Obesity and no-call results: when to test cell-free DNA and when to redraw

This part of the review will focus on maternal obesity in relation to FF and the ability to give successful NIPS. Obesity is one of the common problems that are widely spread among our Egyptian society and worldwide due to the unhealthy life style we are living in. Any increase in maternal weight, the FF of cfDNA declines. Because of the low FF, cfDNA screening for fetal aneuploidy has a greater rate of screen failures or **"no calls"** in overweight females. The best time to test depending on the weight of the mother is uncertain[15]. In spite of several scientific advancements, fractions of obese women who undergo cfDNA screening do not receive a result. The literature has documented a wide range of cfDNA failure rates, ranging from 0.0 percent to 12.2 percent. In up to 6.1 percent of cases, low FF (typically defined as a value of less than 4%) referred to it as cfDNA test failure (**Gil et al., 2017**)[6]. Various maternal and fetal variables, including gestational age, maternal obesity, enoxaparin medication, maternal autoimmune diseases, and fetal aneuploidy, affect FF[16]. One of the most common causes of low FF is maternal weight, which is inversely associated to the FF of cfDNA found in the circulation. A rise in maternal cfDNA levels in the presence of comparative decline in placental cfDNA concentration attributes to lower FFs among heavily overweight women. Apoptosis of adipose tissue may result in higher levels cffDNA in obese pregnant women[17]. Obese women's cfDNA, discharged heavily into the systemic circulation, could indicate extra inflammatory signals linked to metabolic abnormalities that usually occur among obese women during gestation period.

Furthermore, reduced levels of placental cfDNA in obese women occur as rebound to the dilution already present due to an augmented total blood volume. Moreover, women who weigh more than 100 kilograms have a greater than 50% chance of failing to have the NIPT test. Despite the fact that there is a well-established inverse association between maternal weight and FF, there is contradictory statistics on test failure rates and when is the best time to undertake cfDNA

screening in females suffering from obesity[18]. A published research reported that: of more than 14,000 women found no significant decrease in the estimated probability of test failure among females (10 and 20 weeks of pregnancy) and with a BMI equal to 35 kg/m2. Yet, another research reported that females between 8 and 16 weeks of pregnancy had a reduction in no call rates from 14.9 percent to 10.4 percent among obese females with BMI ranges moderate obesity having a BMI of (30 -35 kg/m2) to sever obesity having BMI equal to or more than 40 kg/m2 [19]. Additionally, there is no agreement as well as few data on the best time to wait for a second blood draw in pregnant obese female following a first no call result[20].

Hopkins and his colleagues conducted a study to determine the best time for first cell-free DNA testing depending on maternal BMI and the best time for retake cell-free DNA testing in situations of initial screen failure. It was clear that with each rise in maternal weight, the likelihood of a no call result occur due to a low percentage of FF. In females weighing less than 68 kilos, the percentage of a no call result due to a poor FF was 0.14 percent at 9 to 12 weeks, compared to 17.39 percent among females weighing more than 100 kilogram[15]. In another study, Hales and his colleagues stated that these findings could help women understand the significance of cfDNA aneuploidy screening. The fact that cfDNA is not a diagnostic test should be stressed during counseling. If there are no results or a poor FF percent additional testing or a diagnostic test may be recommended[21].

Clinical uses of NIPT, that is now in use, including the most recent ones.

NIPT framed the promising future for screening and diagnosis of a variety of chromosomal and genetic diseases, thanks to the advancement of modern laboratory technologies.

Gender determination of the fetus

The revealing of fetal sex was one of the first applications of NIPT in maternal serum. This is important in circumstances where the mother is an X-linked disease carrier. Duchene and Hemophilia are two instances of muscular dystrophies. Male embryos have a 50% probability of developing the atypical mutation and becoming symptomatic. Female fetuses, on the other hand, can be one or the other (normal or carriers). NIPT testing can offer solid evidence on fetal sex during early stages of gestation period. Referring to this, only male fetuses subjected to invasive testing to assure the existence of the mutation. This lowers the chances of abortion associated with invasive testing. NIPT is used as clue for pre-treatment for definite fetal gender-related circumstances, for example; Congenital Adrenal Hyperplasia, as early maternal treatment with dexamethasone can lessen the extent of virilization of a female fetus, because treatment has unfavorable side effects which can be escaped in pregnancies with male fetuses[22].

NIPT for numeric al aneuploidies

Among individuals (low risk and high risk), dependence on NIPT in screening for chromosomal abnormalities such as Trisomy 21, 18, and 13 has steadily increased. In some centers, the major use of NIPT amplified the screening rate of trisomies and resulted in a 77.2 percent and some other centers recorded 52.5 percent decrease in average monthly numbers of chorionic villus sampling (CVS) and amniocenteses needed, respectively[23]**.** NIPT detection rate (DR) for trisomy 21 was 99.7% in a study with females above the age of 35, this is a huge percent than that is given by the standard accessible screening tests; which comes in the form of (first-trimester Quad screen and the second trimester serum screen)[6,24].

This shows that NIPT can reduce the amount of pointless invasive tests. In the same time, guaranteeing a considerable reduction in missed instances. Trisomies 13, 18, and monosomy X, have much less detection rates (96.1 percent, 97.7%, and 90.3 percent, respectively) than trisomy 21**.** Although, laboratory expertise and tools has greatly improved still, several NIPT test suppliers are recording T18 and 13 DR that is similar to T21. The dependence of NIPT was limited to singleton pregnancies solitary[25]**.**

NIPT for structural aneuploidy

The advancement of NIPT applications has assisted by the emergence of novel analysis tools for instance deep sequencing, which is next generation sequencing. Deep sequencing stand for the process of sequencing a genomic area consequently done several times in order to find structural aneuploidies such as micro-deletions as well as duplications. In regions of Europe and the United States, this has incorporated in clinical practice. Deeper sequencing spots micro-deletions as tiny as 300-kilo bases (Kb), while micro-deletions or duplications larger than 3 megabytes (Mb) can be found with 99 percent sensitivity. 22q11 deletions (DiGeorge), 15q11-q13 deletion (Prader–Willi/Angelman), 4p deletion (Wolf Hirschhorn), and other clinically relevant partial aneuploidies have all been detected using this method[26].

NIPT for single gene disorders

Screening of single gene abnormalities and introducing NIPT for recognition of those abnormalities, had lately introduced into clinical practice. This includes illnesses that are autosomal dominant, recessive, or X-linked. Autosomal dominant cases, which caused by a particular allele, inherited from the father or developed spontaneously, are now more feasible to detect. It is now possible to detect from maternal blood, any of (existence or lack) of paternally inherited mutations, even the presence of new mutations for autosomal dominant illnesses to show either the fetus is the cause of the mutation or not. Maternal plasma samples can be tested for precise

mutations via restriction-digest PCR[27]. Defining of fetal Rhesus (Rh) D genotype among Rh D-negative mothers and detection of novel variations are two examples of such illnesses. Dissimilar kinds of skeletal dysplasia are caused by mutations in the FGFR-3 gene (fibroblast growth factor receptor)[28]. Huntington's disease, myotonic dystrophy, as well as early onset primary dystonia, are examples of autosomal dominant disorders where this strategy is applied.

Regarding autosomal recessive disorders if there is no mutation to reveal (paternal mutation) through cffDNA testing; this completely abolishes the chance of having an abnormal fetus especially in autosomal recessive disorders when both parents carry two distinct mutations. However, determining the inheritance of the maternal allele for autosomal recessive disorders (for instance congenital adrenal hyperplasia or spinal muscular atrophy) and X-linked (Duchenne muscular dystrophy and Becker muscular dystrophy), disease still needs NIPT. Due to the augmented levels of circulating maternal alleles, this is challenging and necessitates a calculation of the relative quantities of mutant and wild type alleles. Nevertheless, employing more innovative analysis tools like Relative Mutation Dosage (RMD) or Relative Haplotype Dosage Analysis (RHDO), this can be accomplished[29].

Hemolytic disease of the fetus and newborn

The key reason of infant hemolytic illness is antigen mismatch between fetal and maternal Rhesus D (Rh D) antigens. Rh D negative women make up about 15% of Caucasian women, 3% to 5% of black African women, and fewer than 3% of Asian women. Because the illness can be lethal to the newly born child and since available treatment, such as intramuscular immunoglobulin (Anti-D) or intravenous immunoglobulin, can be given to mothers at danger, precise, prenatal diagnosis is critical. In a study Rh D determination from newly born cord blood, with RHD (gene) exons 5 and 7 from cffDNA collected from maternal blood between 9 and 13 weeks of pregnancy showed a great level of specificity, sensitivity, and diagnostic accuracy (>90 percent). Results were compared to results acquired from fetal Rh D, droplet digital PCR and standard real-time PCR. Routinely determining fetal Rh D status from cffDNA in maternal blood enables prompt management high risk pregnancies while reducing Anti-D intake by approximately 25%[30].

Applying Whole exome sequencing (WES) for circulating cell-free DNA during pregnancy

The main reason of structural congenital anomalies (SCAs) in spite the presence of normal chromosome examination confirmed by karyotype or Chromosomal Microarray Analysis (CMA), is still a diagnostic mystery. Despite an in-depth diagnostic workup, prenatal identification of fetuses with SCAs having a doubtful genetic origin and whose particular genetic variant is uncertain or undiscovered by routine clinical diagnosis may go lacking a clinical diagnosis of their illness, may need to do whole-exome sequencing (WES)[31]. WES is a well-established tool utilized in postnatal and pediatric evaluation and genetic diagnosis. With the arrival of NGS or Massively Parallel Sequencing (MPS), WES may now be done in the antenatal period using cffDNA. In a study, 610 babies with abnormalities on ultrasonography, given WES. They discovered a diagnostic genetic mutation in 52 (8.5%) of the fetuses, as well as a variant of unclear significance in another 243 (9%) of the fetuses with potential clinical practicality. They summarized from this study that WES increased the detection of genetic disorders in fetuses with morphological abnormalities, however cautious during the processes of case selection should be taken into account[32].

Due to cost-effectiveness reasons, the present depth of sequencing used in clinical practice was insufficient to identify tiny copy number variants. Furthermore, the low fetal percentage is a vital cause in the failure of NIPT. If FF is low as 4%, the NIPT will not succeed to give a test result. As a result, in future studies, it will be significant to overcome the problems listed above in order to attain great detection rates concerning NIPT screening. Nowadays, just a few clinical studies testing the efficacy of noninvasive deletion detection is available. There is insufficient statistics to evaluate sensitivity, specificity, positive predictive value, and negative predictive value. Micro-deletion screening not recommended by any major national obstetric or genetic organization. A published retrospective study aimed to explore more about the clinical value of NIPT for detecting copy number variations gave a valuable conclusion regarding this issue[33].

cffDNA screening in twin pregnancies

In reference to the frequency of trisomy 21, which is higher in twin pregnancies than singleton pregnancies, and since combined screening in twin pregnancies has a lesser DR (75%) and higher FPR twins (9 percent of pregnancies and 7 percent of fetuses)[34]. These donates that cfDNA screening is highly needed in this situation. Especially due to high accidental abortion rates that occur after invasive prenatal procedures in twin pregnancies. High FPR is of special concern due to its coexistence with selective termination of pregnancy decision in cases fetuses with multiple congenital anomalies in the current pregnancy. In twin pregnancies, there is more doubt about the prognostic accuracy of cfDNA than in singleton pregnancies.

Screening accuracy regarding cffdna in twin pregnancies is expected due to a variety of genetic causes. One of these causes is that in dizygotic twins. Aneuploidy usually disturbs only one fetus. Yet, the cffDNA involvement of the two fetuses might differ by up to two fold[35]. Furthermore, when the fetus has trisomy 21, the fetal proportion of cffDNA in the

maternal blood is smaller in singleton pregnancies[36]. This is probably also correct in dizygotic twin pregnancies with aneuploidy, where a substantial influence from a normal co-twin could "cover" the afflicted fetus's low FF. As a result, a false-negative result obtained. This difficulty in using cfDNA in dizygotic twin pregnancies might clarify why di-chorionic twins have an advanced failure rate (nearly twofold) than mono-chorionic twins. Furthermore, in vitro fertilization (IVF) conception and high maternal BMI, both of which are more embraced in twin in comparison to singleton pregnancies, are known to be related with a lower FF in singleton pregnancies[10].

We cannot deny that cffDNA testing also have some advantages. In twin pregnancies, the possible benefits of noninvasive prenatal testing are far more than in singletons, with a lesser necessity for invasive testing and a lesser chance of inducing accidental abortion as a result. Several maternal – fetal societies on the other hand advise against using cffDNA in twin pregnancies and advocate for bigger forthcoming studies. Regarding that important issue, a prospective multicenter research conducted to determine the accuracy of cffDNA in screening for the three most frequent trisomies among twin pregnancies. The result of this research was pooled with results from other published research to get the most accurate approximation of screening routine. The traditional screening routine of cffDNA in maternal serum for the diagnosis of fetal trisomies in twin pregnancies was evaluated in this prospective multicenter-blinded investigation. The research was conducted in six fetal medicine institutes across England[37]. The screening performance and test failure rate of cffDNA utilizing next generation sequencing were the key outcomes of this research. Trisomy 21 and trisomy 13 did not show false-positive or false-negative results, however trisomy 18 had one false-negative and one false-positive result. Regarding Twin Pregnancy The separate FF for each twin is lower in dizygotic twin pregnancy than in singleton pregnancy, which poses a technical problem. This could result in reduced cffDNA detection and a higher test failure rate among twins. In dizygotic twins, to depend on NIPT for extracting cfDNA, it is almost impossible to establish which twin is defective. Lately, there has been accumulating evidence that NIPT can be utilized in twin pregnancies and that its routine in detecting Trisomy 21 is similar to that of singleton pregnancies. However, confirmatory intrusive testing would be mandatory before proceeding in pregnancy management[39].

Cell-free DNA noninvasive prenatal screens to ACMG recommendations

The American College of Medical Genetics and Genomics (ACMG) released a situation statement in 2016 that included precise testing laboratory guidelines as referred to in **Table 1**[40].

Table 1: Guidelines for laboratories that conduct cffDNA tests.

First recommendation [Common Aneuploidy]	To help care providers reaching a decision a, labs must offer readily visible and obviously specified (DR), (SPEC), (PPV), and (NPV) for disorders being screened
Second Recommendation	Except autosomal aneuploidies affecting chromosomes 13, 18, and 21, the ACMG does not advocate using NIPS to screen for autosomal aneuploidies.
Third Recommendation	On NIPS reports, all labs must put a clearly apparent fetal fraction.
Fourth Recommendation	When reporting NIPS results, labs must state the cause for the no-call.
Fifth Recommendation [Sex Chromosome Aneuploidy]	When reporting data for each sex chromosomal aneuploidy, labs include immediately identifiable and extremely noticeable DR, SPEC, PPV, and NPV .
Sixth Recommendation	DR, SPEC, PPV, and NPV of each copy number variant examined must be stated in lab requests and in the pre-test counseling data.
Seventh Recommendation	When providing lab data, laboratories must straight away give identifiable and highly visible DR, SPEC, PPV, and NPV for every copy-number variant screened to help and caregivers in taking decisions and understanding results.
Eighth Recommendation	As soon as giving positive test results, labs propose patient-specific PPV.

*Specified detection rate (DR), specificity (SPEC), positive predictive value (PPV), and negative predictive value (NPV), non invasive prenatal screening (NIPS).

Recommendations

- More exploration is desirable to figure out in what best way to use this novel technology:
  - Post-marketing surveillance is required to determine how well patients and care givers comprehend cfDNA tests and its influence on the families.
  - More studies is wanted to establish which counseling strategies and educational aids improve patient knowledge and reduce the morbidity

associated with false positive and false negative test results.

- Before taking irreparable action, for example terminating a pregnancy, odd results need to be validated with diagnostic testing via CVS or amniocentesis.
- If a patient's background risk is low, the likelihood that she will have an afflicted foetus – the positive predictive value; is reduced. A positive test is more likely to be a false positive in low-risk women and for rare illnesses. As a result, cfDNA testing not suggested for low-risk women.
- Genetic counseling services play a significant role in patient care by giving data. To ensure optimal treatment for patients, the Society of maternal and fetal medicine advises that customers offer acceptable payment for these services.

SUMMARY &CONCLUSION

Since the established cffDNA validity as good screening tool for nearly a decade now, maternal plasma cfDNA sequencing has progressed from a research project to be implemented into clinical care. The clinical use of cfDNA sequencing for chromosomal aneuploidy screening has by now has an international impact, motivated by pregnant women's need for safer prenatal screening. As it is was done with old-style normal serum screening, cffDNA test findings should be provided with a positive predictive value or patient-specific risk to avoid false positives with screening for rare illnesses due to micro deletions.

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