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סקירה ספרותית עדכנית:
בדיקת השימוש ב-DNA חופשי בסרום האמהי לצורך אבחון טרום לידתי בלתי פולשני

Use of cell free DNA for non-invasive prenatal testing
an up to date review of the literature

עבודת גמר לשם מילוי חלקי של הדרישות לקבלת התואר "דוקטור לרפואה"

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הוגשה לועדת עבודות גמר בתאריך:

עבודה זו מוקדשת להוריי, ד״ר יהודה ושלומית בן דוד,
שתמכו בי בעת כתיבת העבודה ולאורך כל תקופת לימודי הרפואה.

תודתי נתונה לד״ר יורי פרליץ שהנחה אותי בעבודה זו, על הערותיו והארותיו המפרים.

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1. Abstract

Ever since the introduction of amniocentesis in the early 1970's, prenatal diagnosis of fetal abnormalities has been gaining interest from pregnant women, clinicians, and society. Following the detection of cell free DNA (cfDNA) in 1997 and its use, as part of a noninvasive testing for detecting chromosomal abnormalities, a whole new field of opportunities has been discovered.[1] This method has revolutionized prenatal diagnosis, due to its high efficacy and sensitivity in detecting the common chromosomal aneuploidies, along with increasing evidence of its ability to detect a wider range of abnormalities in the era of prenatal testing and without any risk to the mother. This method has enormously developed since it was first introduced in 2011. Today, a total of 61 countries around the world have adopted this method into clinical practice as a non-invasive prenatal testing (NIPT) method. Initial studies demonstrated the high efficacy of NIPT for screening women in 'high risk' group, which caused different committees and clinicians to recommend it only to this group of women.[2] However, in the last few years there are growing number of studies which detected its high performance also in the 'low' and 'intermediate risk' groups of patients. Several drawbacks exist for using this method as a routine prenatal test for all pregnant women, one of these accounts for its high costs, still requiring further adjustments.[3]

In this work, we will present the different studies conducted in the field of cfDNA and its various applications into clinical practice. We also discuss the ethical complexity of using NIPT and its cost effectiveness both for the private market and to society. In addition to that we will aim to elucidate the public's opinion regarding this new advanced technological breakthrough.

Key words: Non-invasive prenatal testing (NIPT), cell-free DNA (cfDNA), prenatal diagnosis, aneuploidy.

2. תקציר

מאז תחילת שנות השבעים, דיקור מי שפיר לצורכי אבחון טרום לידתי, מהווה בדיקה חשובה שממקדת עניין הן מכיוון הרופאים המטפלים והן מכיוון הנשים ההרות. בדיקה זאת ובנוסף דגימת סיסי השליה היוו ומהווים עד היום את הבדיקות האמינות ביותר בתחום. ב- 1997 עם גלוי ואיתור חלקיקי DNA ממקור שלייתי בדם האם (cfDNA) ויישום הבדיקה לצורך בדיקת תקינות הכרומוזומים העובריים, פותחו והוכנסו יישומים שונים לבדיקה זאת תוך פתיחה של אופקים חדשים לאבחון הטרום לידתי. בדיקת הcfDNA- הפכה לבדיקה חשובה, בשל היותה בדיקה אמינה עם רגישות גבוהה לאבחנה של אי תקינות המטען הכרומוזומלי של העובר, ממצאים אחרים שקשורים לתקינות העובר וכל זאת מבלי להוות סיכון, או תופעות לוואי לאם או לעובר. הבדיקה עברה שינויים והתאמות מאז השקתה בשנת 2011 וקצב השימוש בבדיקה הולך וגובר בצורה חדה.

כיום, כשישים ואחת מדינות מאפשרות לנשים הרות לבצע בדיקה זו (NIPT) במהלך ההריון כחלק מהבדיקות הטרום לידתיות. העבודות הראשוניות בתחום הדגימו כי בדיקת זו הראתה תוצאות טובות מאד כבדיקת סקר בנשים הרות הנמצאות בקבוצת נשים עם סיכון גבוה ללידת תינוק עם בעיה כרומוזומלית. הבדיקה נמצאה כבעלת אמינות ורגישות גבוהים מאוד. ממצאים אלה הביאו ארגונים מקצועיים שונים להמליץ על ביצוע הבדיקה בקרב נשים שמצויות בסיכון גבוה, כגון נשים מבוגרות. בשנים האחרונות, נוספו עבודות שתמכו ביעילות הבדיקה גם בגילוי של נשים שאינם מוגדרות בסיכון גבוה. אחת הבעיות העיקריות שצפו בעת נסיון לאמץ בדיקה זאת כבדיקת סקר לכל הנשים ההרות, טמון במחירה הגבוה של הבדיקה. מחיר זה, צפוי כמובן לרדת בעתיד ואולי בכך תסלל הדרך להצבת בדיקה זו לכלל האוכלוסיה כחלק מבדיקות הסקר הקיימות.

בעבודתנו , אנו סוקרים את המחקרים השונים שפורסמו בנושא ,cfDNA ויישומה של הבדיקה בתחומי אבחון טרום לידתי שונים. כמו כן, אנו בוחנים את ההיבטים האתיים ונקודת המבט של ה"ציבור הרחב" על הבדיקה והשלכותיה השונים על תרשים הזרימה של הבדיקות הטרום לידתיות. כמו כן, אנו בוחנים את שיקולי העלות מול תועלת לפרט ולציבור הרחב.
בדיקה זאת שהחלה כעבודה מחקרית הולכת ותופסת מקום חשוב ומרכזי בתחום הבדיקות הטרום לידתיות שמבצעות נשים הרות ברחבי העולם.

3. Background on prenatal testing

Prenatal diagnosis of fetal anomalies and specifically of chromosomal abnormalities is an important part of modern obstetrics service. It refers to a complex of methods and tests designed to diagnose a disorder in the fetus before it is born. These disorders consist of Chromosome numerical changes (aneuploidy, polyploidy), large deletions and duplications, along with rearrangements. Common procedures include screening for presence of aneuploidies, and diagnostic testing such as chorionic villus sampling (CVS) and amniocentesis.

Aneuploidies are associated with significant fetal morbidity and mortality. Chromosomal abnormalities occur in 1 of 160 live births, with extra copies of chromosomes 21, 18, and 13 accounting for the majority of numerical alterations associated with the autosomal chromosomes. For trisomy 21, the prevalence is 1:800, whereas for trisomies 18 and 13 are 1: 6000 and 1:10,000 respectively. The majority of autosomal trisomies occur due to nondisjunction during maternal meiosis, which occurs in higher frequencies with advanced maternal age. [4]

Accurate detection of fetal disorders is achieved, using invasive methods such as chorionic villus sampling (CVS) or amniocentesis. These invasive methods enable to directly evaluate and analyze chromosomal abnormalities in the fetal DNA. Although these methods considered as reliable and feasible, they carry a considerable risk for both the fetus and the mother, including miscarriage and uterine infection. Therefore, different non-invasive screening methods were developed in order to distinguish between low risk and high risk pregnant women for fetal chromosomal abnormality. High risk women were then referred for the traditional invasive procedures which could give a definite fetal chromosomal diagnosis.[5]

Historically, invasive diagnostic tests were routinely offered to women 35 years of age and above. But this was proven eventually, to be a poor screening criterion, since the majority of children with Down's syndrome were still born to women younger than that age. To overcome the maternal age criteria, as the only criteria for chromosomal direct testing, different approaches were suggested, using noninvasive tests which present a better prediction of pregnancies complicated by aneuploidy. These non- invasive methods were then combined with maternal age, so for each pregnant woman, the risk for trisomy 21 could be calculated and high risk women were then referred for invasive testing. These new strategies have become widely available, and showed a significant decrease in the number of births of infants with Down's syndrome in the United States. The screening tests based on maternal serum test and sonographic examinations are performed during first and second trimesters of pregnancy. [6]

First Trimester screening

In the early 1990s, studies showed that an increased amount of fluid at the back of the fetal neck, referred to as nuchal translucency, were associated with fetal chromosomal abnormalities. In the following studies, the detection rate of trisomy 21, based on maternal age and the measurement of nuchal translucency, ranged from 72 to 77%.

An increased nuchal translucency has also been reported to be associated with other chromosomal abnormalities, including trisomy 18 and 13, 45, X0 monosomy and triploidy, as well as with numerous genetic disorders and fetal structural anomalies, especially congenital heart defects. Criteria have been established to ensure that a standard technique is used to accurately measure nuchal translucency, done between 11-13 weeks' of gestation. [6]

A combined test which consists of measurements of two biochemical markers, pregnancy-associated plasma protein A (PAPP-A) and hCG — with the measurement of nuchal translucency, increases the sensitivity of first-trimester screening for the discussed chromosomal abnormalities.

The combined screening tests allowed the detection of about 90% of trisomy 21 and 75% of cases of trisomies 18 and 13, with false positive rates of 5%.

It has been suggested recently, that the performance of the combined test in screening for trisomy 21 can be improved by the addition of three other tests: serum placental growth factor, α-fetoprotein (AFP) and fetal ductus venosus pulsatility index. The first two are serum blood tests and the third is a sonographic measurement.

First-trimester screening allows early evaluation and could enable high risk women to establish fetal chromosomal anomaly diagnosis early, using CVS or amniocentesis, so intensive genetic consultation and full fetal assessment could be completed before any decision is taken, including termination of pregnancy. [7]

Second Trimester Screening

In the early 1980s, measurement of maternal alpha- fetoprotein levels during the second trimester was introduced to refine estimates of the risk of having a child with Down syndrome. Later on, the additional measurement of biochemical markers — including human chorionic gonadotropin (hCG), unconjugated estriol, and, more recently, inhibin A — dramatically improved the rates of detection of fetal aneuploidy, making noninvasive screening a reasonable approach for screening women in the second trimester.

Typically, trisomy 21 is associated with high maternal levels of hCG and inhibin A and low levels of alpha-fetoprotein and unconjugated estriol. Testing for this combination of markers, which is referred to as quadruple screening, has a detection rate (DR) of 80% for trisomy 21 at a positive screening rate of 5%, with the detection rate being similar for trisomy 18.

The Quadruple test is best to be performed between 15-19 weeks' of gestation, as this is the optimal time for open neural tube screening.[6]

Sequential first and second trimester tests

To achieve higher detection rates of Down syndrome and further reduce the need for invasive testing, additional screening factors have been introduced.

Integrated screening is used to calculate a single adjusted risk of Down's syndrome after both the first- and second-trimester tests have been completed and is associated with a higher rate of detection of trisomy 21 ranging from 94%-96%, as compared with either first- or second-trimester screening alone. One of the major drawbacks of this approach is that results of first trimester are not revealed, and only the integrated result is provided, thus, not allowing the women the option for early diagnosis and early termination of pregnancy if needed.

Independent screening in both the first and second trimesters has been discouraged, since the positive screening rate is unacceptably high. To address these concerns, two sequential screening strategies, stepwise and contingent screening, were introduced.

In stepwise strategy the women is provided with the results of the first trimester, in case when the risk of aneuploidy is greater than a predetermined cutoff point, then the women is offered the option for invasive diagnostic testing. In case the risks for aneuploidy are low, she will precede to the second trimester screening.

Contingent sequential screening stratifies women according to the initial adjusted risk of Down's syndrome. Only women with an intermediate risk (e.g., between 1 case per 30 pregnancies and 1 case per 1500 pregnancies) will undergo the second-trimester screening. The advantage of this method is that results are readily available in the first trimester, along with higher detection rates (88–94% with 5% false-positive rate) .[8]

Additional first trimester and second sonographic markers

There are several commonly used markers evaluated by ultrasonography in the first trimester which may imply for aneuploidy. These consist of absence of a fetal nasal bone , tricuspid regurgitation determined by pulse wave Doppler ultrasound, and abnormal blood flow in the ductus venosus. The routine use of these markers can substantially increase detection, when this is carried out by specialized centers.[8]

Additional second trimester ultrasound markers can also improve aneuploidy screening. These usually rely on the detection of anatomical abnormalities. One approach is to measure three facial profile markers concurrently with the Quadruple test. These facial profile markers are nuchal fold thickness, nasal bone length, and prenasal thickness. The model predicted results are comparable with a first trimester combined test.

Findings reported to be useful in modifying aneuploidy risk include major malformations, increased nuchal fold thickness, short femur or humerus lengths, echogenic intracardiac focus, pylectasis, echogenic bowel and ventriculomegaly.

The genetic sonogram can be used for women who have received first trimester screening, second trimester screening, or both. The second trimester anomaly scan can be used simply to modify the maternal age-specific aneuploidy risk alone, as it is not a very effective screening test by itself. Using this to modify the risk assessment following other aneuploidy screening can improve detection rate. [6,9]

Multiple Gestations

Aneuploidy risks based on both NT and serum markers can be provided for twin pregnancies, despite poorer performance of the serum markers compared with singleton pregnancies.

Serum screening can be difficult to interpret in twin gestations, since the biomarker levels in a normal fetus can mask abnormal results in an affected fetus, and this screening is not applicable to higher-order multiple gestations.[9]

First trimester screening should take into consideration chorionicity. Monochorionic twins are assumed to be monozygotic with an identical risk for each fetus, while the majority of dichorionic twins are dizygotic and will be provided with separate risks for each fetus. First trimester serum markers require the use of gestation-specific and chorionicity-specific correction factors.

Second-trimester serum screening of twin gestations identifies about 50% of affected fetuses, at a 5% positive screening rate.

For triplets and higher multiplies, risks should be based on ultrasound markers alone.[6]

Diagnostic Testing for fetal Aneuploidies

Amniocentesis and CVS are commonly performed invasive procedures for prenatal diagnosis, in high risk pregnant women for fetal chromosomal anomalies.

A recently conducted meta-analysis, demonstrated 324 fetal losses in 42,716 women who underwent amniocentesis and 207 losses in 8,899 women who underwent CVS. The pooled risk of miscarriage prior to 24 weeks in women who underwent amniocentesis was 0.81% and 2.18% for those who underwent CVS. [10]

Prenatal diagnosis for chromosomal anomalies is still currently perceived as being highly desirable for many women.

Until recently, the only definitive and absolute test available for prenatal diagnosis of chromosomal abnormality was invasive and, as presented here, associated with a considerable degree of maternal discomfort and a risk to the pregnancy. Because of the potential risks associated with such invasive methods, a long-sought goal in prenatal diagnosis research had been to develop noninvasive and reliable methods that do not abounded such a risk.

In 1997 Lo et al, [1] detected circulating cell-free fetal DNA in the maternal plasma. This discovery has opened new area of research and changed the prenatal focus to methods of noninvasive prenatal diagnosis.[8]

4. Cell free DNA- origin and testing methods

Traditionally, prenatal diagnosis of fetal genetic status or aneuploidy has been dependent on the use of invasive diagnostic tests, chorionic villus sampling and amniocentesis, to obtain a sample of fetal genetic material. The invasive nature of these tests means that there is a small but significant risk of miscarriage and alternative sources of fetal material have long been sought in order to enable non-invasive methods of testing. [7]

The original focus of research for applying non-invasive testing rested on attempts for detecting fetal cells in the maternal circulation. However, this era of investigation was not successful, as it was found that isolating fetal cells had proven to be a complicated task, and it was questionable whether they are pregnancy specific. In 1997, Lo et al.[1] identified the presence of cell-free fetal DNA in maternal plasma. This was later found to be a misnomer as the origin of these fragments was found to originate from the placenta.

The placenta is a dynamic organ, with constant turnover of villous trophoblasts. There exists a physiological cycle in the placenta, beginning with cytotrphoblastic proliferation, followed by differentiation and fusion of these cells with the syncytiotrophoblast. Following that, further differentiation takes place and eventually apoptosis and extrusion, in the form packed apoptotic bodies containing DNA fragments results, as demonstrated in figure 1. Along with the apoptotic mechanism described as part of normal aging, accidental breakage or necrosis may also be responsible for extrusion of these DNA materials. [2]

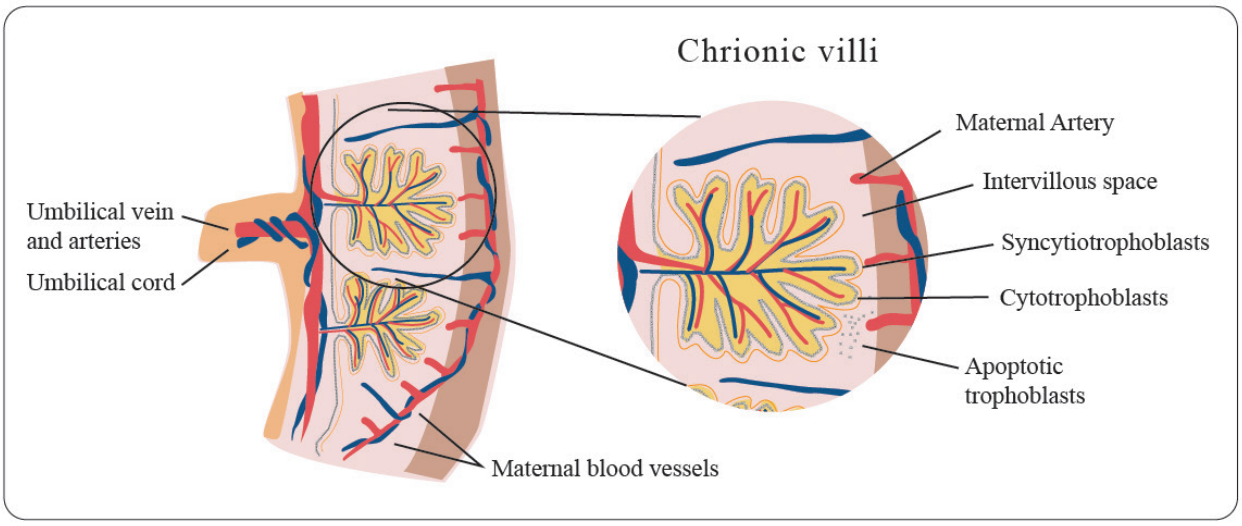

Figure 1: Mechanism of trophoblasts extrusion into maternal circulation. Image credit Liron Avrami, GDI

In support of these observations, linking DNA fragments to the placenta, later studies were able to detect cfDNA in cases in which only placental tissue is present, as in anembryonic gestations. Further evidence has been suggested from studies which demonstrated different genotype in the placenta as compared with the fetus. This is known as confined placental mosaicism (CPM). [2]

For practical considerations, the DNA which is isolated from maternal blood is a mixture of both fetus and maternal DNA. Fetal DNA was found to constitute 5-20% of all cfDNA in maternal blood, and can be detected as early as 5 weeks' gestation with a half- life of less than one day, making it pregnancy specific.

The procedure itself holds no risk to the developing fetus or the mother, as opposed to other diagnostic methods, except for minor adverse effects related to simple venous blood drawing.

Initially the key limiting factor in the development of specific prenatal tests based on cfDNA, has been the difficulty to differentiate between the genetic material from the fetus from that of the mother. As a result, the first clinical applications of NIPT have been restricted to the identification of alleles present in the fetus but not in the mother, either inherited from the father or arising de novo. These include fetal sex determination, paternally inherited single-gene disorders, or those arising de novo, such as achondroplasia. [11]

This perspective has rapidly evolved with the presentation of newly developed techniques for evaluating fetal free DNA fragments.

Method for cfDNA processing

The process of cfDNA evaluation, as described in **figure 2**, begins with five –to twenty five milliliters of maternal peripheral blood are collected into a blood tube containing EDTA. The blood sample is stored immediately at 4◦C. Plasma is in this way transported to the evaluating lab. In the lab it is prepared by using a 2- step centrifugation protocol. The whole-blood sample is initially centrifuged at 1600g for 10 min at 4◦C. The supernatant is then transferred to sterile 2.0-mL tubes placed on ice, which are then centrifuged again at 16000 g for 10 min at 4◦C. The final supernatant is then transferred to new tubes and then analyzed. [12]

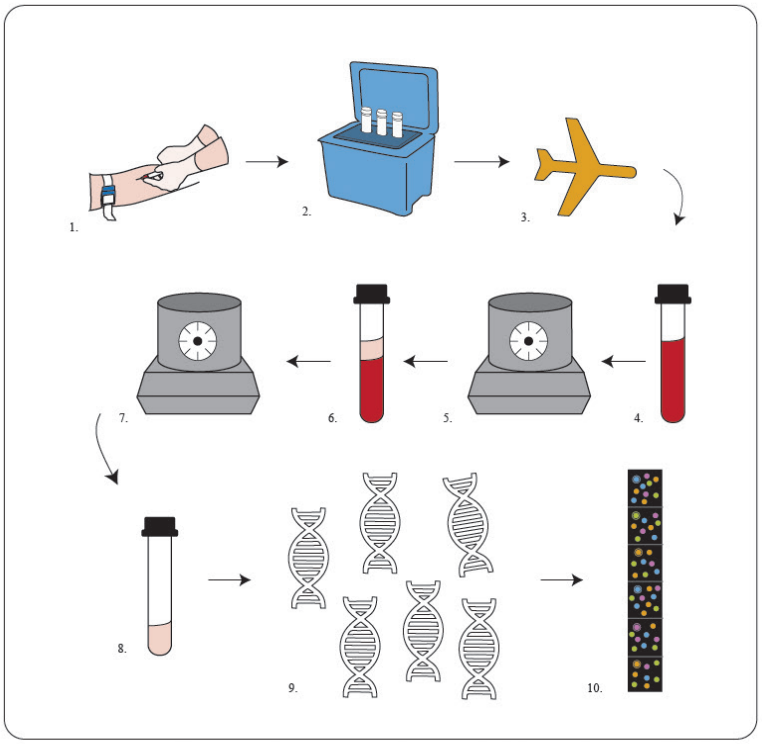

Figure 2: Diagram illustrating the process of cfDNA evaluation, from blood collection(1) to biochemical sequncing (10). Image credit Liron Avrami, GDI

There are currently 3 broad cfDNA testing methods available:

1. Shotgun Massive parallel sequencing (MPS)

2. Targeted MPS, which focuses on specific chromosomes of interest

3. Single nucleotide polymorphisms (SNPs), an approach that takes advantage of the differences between mother and fetus.

All approaches sequence the total cell-free DNA fragments, including that of maternal and fetal origin. Fetal DNA fragments are predominantly 143 bp in length, and maternal are predominantly 166 bp. Massive parallel sequencing allows to align each fragment being sequenced to a specific location on the genome. [13]

Shotgun MPS is an approach which allows to sequence millions of DNA fragments both from maternal and fetal origin. The method is based on counting large numbers of DNA fragments in the plasma collected. Afterwards, we are able to map each sequence to a specific locus on the chromosomes. If fetal trisomy is present, there will be a relative excess of counts for a given chromosome, as oposed to a deficit obtained in case of a monosomy. Large numbers of counts are necessary since in most cases the fetal fraction of cfDNA is low and the excess or deficit in the assigned DNA fragments are small. [14] The choice of bioinformatics platform for analysis is just as important as the technology used for genomic sequencing. Several approaches have been considered for this purpose, with the first presented by the z-score. This score allows to determine the difference in total cfDNA attributed to trisomy. The z-score reflects the number of standard of deviations the proportion of reads from a particular chromosome is above the mean. If the amount of a chromosome specific sequence exceeds the expected threshold in case of euploidy, the result is reported as positive for trisomy of that given chromosome.[15]

Targeted MPS is similar to that used with Shotgun, only it limits the sequencing only to the chromosomes of interest (e.g. 21, 18, 13, X and Y) and counts this subset .A patient-specific risk score can be generated by adjusting for the fetal fraction which then combines the results with maternal and gestational ages.[14]

The SNP approach allows to selectively amplify and then sequences 19,488 polymorphic loci on chromosomes 13, 18, 21, X and Y. This method relies on the DNA polymorphic differences between the mother and fetus.

Each product received is evaluated based on the hypothesis that the fetus has trisomy, monosomy or is euploid. After considering the positions of the SNPs on the chromosomes and the possibility that there may have been recombination, a maximum likelihood is calculated for each option. Results are presented as risk scores. [16]

The various cfDNA tests available should not be expected to be equivalent, one of the causes may be explaind by low fetal fraction which may show better efficacy when performed using counting methods as Shotgun and targeted MPS, involving greater depth of sequencing. When comparing s-MPS with t-MPS, the latter could potentially involve greater depth of sequencing for the chromosomes of interest while requiring considerably less total sequencing and thereby potentially lowering the costs.

The SNP method has several advantages. It can reduce the false positive results caused due to imbalances which are of maternal origin, and it may also identify additional haplotypes which may indicate undetected multiple pregnancies.

Other methods, such as digital polymerase chain reaction for detecting methylated DNA and epigenetic differences between fetal and maternal DNA, are currently under investigation for analyzing chromosomal aneuploidies using NIPT. [17]

The accuracy of NIPT performed can be affected by both biological and technical factors. A recent prospective analysis of cfDNA-based noninvasive prenatal screening in low-risk pregnancies showed false positive rates of 0.3%, 0.2%, and 0.1% for trisomies 21, 18, and 13, respectively - which were lower than those observed with standard screening tests. [3] The biological factors which may affect the false positive and false negative rates consist of fetal fraction, body mass index (BMI), placental mosaicism, multiple pregnancies, maternal copy number variations and coexistence of specific maternal tumors.

Fetal fraction (FF) is the amount of cfDNA divided by the total DNA. A strong positive correlation was found between z-score and fetal DNA fraction, making it easier to demonstrate an aneuploidy. Failure to measure the fetal fraction may be falsely reassuring.

BMI is the most significant factor found to affect the FF, and this was explained on the basis of larger serum volumes causing a dilution effect. Another contributing factor is the release of DNA fragments from adipose tissue, thus the total circulating free DNA is increased proportionally during pregnancy as a function of the maternal BMI. [18]

Placental mosaicism - Conventional cytogenic examinations have been able to document presence of confined placental mosaicism (CPM), observed in about 1% of invasive tests. This situation implies that a placental mosaicism is present, thus fragments obtained from the placenta do not necessarily represent in a correct manner the genetic material of the fetus. This was originally found by following a CVS with subsequent amniocentesis, presenting altered results. Therefore, it was provided that one of the causes of a false positive cfDNA NIPT could result from CPMs.

Multiple pregnancies - It has been demonstrated that NIPT can be used for the detection of aneuploidy in twin pregnancies. The quantity of cfDNA has been shown to be higher in twin pregnancies compared to singleton pregnancies. Zygosity can be determined from maternal plasma DNA sequencing and thus the fetal fraction from each twin can be estimated in dizygotic pregnancies. However, the accuracy may not be as high as for singleton pregnancies. [19]

Another possible biological cause of inaccurate NIPT results may be the presence of a ‘vanishing twin’. Cases of false-positive NIPT results in which the presence of a vanishing twin could be confirmed have been reported.

Furthermore, Curnow et al. estimated the theoretical incidence of a vanishing twin with a chromosome abnormality is 0.11%, which is in concordance with the false-positive rates reported in previous meta-analysis of NIPT. This emphasizes the importance of detailed ultrasound examination, particularly following discordant NIPT results. [20]

Maternal copy number variations - NIPT relies upon the analysis of cfDNA of which majority is derived from maternal DNA. This in fact can complicate the analysis and interpretation of NIPT results. The counting statistics of conventional Z-score chromosome- a wide analysis method can be affected by maternal copy number variants, leading to false positive NIPT results.

Two population factors contribute to the effects of maternal copy-number variants were identified. First, the distribution of copy-number- variant sizes varies according to chromosome length, with chromosomes 13 and 18 having higher population frequencies of large duplications than the smaller chromosome 21. Chromosomes with higher population burdens of copy-number variants — particularly the larger ones are more susceptible to false positive results. Second, the coefficient of variation of sequence reads for each chromosome modulates the effect of the size of copy- number variants on the probability of false positive results. For example, chromosome 13, which has the highest of the three examined coefficients of variation, is the most buffered from the effects of copy-number variants. [21]

Maternal mosaicism - can also be a source of false-positive results As a given example, it was found that 8.6% of the positive results for sex chromosomal aneuploidies detected using NIPT, was due to maternal mosaicism. Due to that, it is suggested that in some cases of positive NIPT it may be effective to perform maternal cytogenic analysis, in order to prevent unnecessary invasive procedures. [22]

Maternal tumors - another potential source of abnormal chromosome complements in maternal-derived cfDNA is from apoptosis of tumor cells existing in the examined mother. [19] Different tumors have been reported to influence cfDNA analysis results, with majority consisting of those tumors that are most common among childbearing women. Although, there were more hematologic malignancies than would be expected and no cases of malignant melanoma or cervical cancer were reported. A recent study using sequencing to analyze plasma cfDNA in patients with known cancers, found evidence of abnormal cfDNA patterns in more than 80% of metastatic solid tumor cases and 50% of localized cancers. The rates of detection varied widely by tumor type.[23] The use of genome-wide profiling has the potential to spot ‘tumor like’ genome-wide aneuploidy profiles and hence identifies pre symptomatic cancers in pregnant women. Maternal causes for false-positive NIPT results also highlight the importance of pre- and posttest genetic counseling. It should be made clear to patients beforehand whether they will later be informed of incidental findings observed. [19]

A further issue which clinicians have to deal with, when applying cfDNA for NIPT, is a situation when no results are received, also referred to as analysis failure. Data collected from a large meta-analysis reported an incidence of screening failure ranging from 0.5% to 6.2%. [3]

There are generally two reasons for result failure. (1) Preventable causes, which include problems of blood collection and its transport to the lab. These are further broken down to inadequate blood volume, hemolysis, incorrect labeling of tubes and delay in arrival to the laboratory. (2) Independent causes, including low fetal fraction (usually below 4%) and assay failure.

In several of the cfDNA studies a proportion of samples, 6.6%, were untested because of sample quality, possibly reflecting stringent standards required. Of greater concern were those where a cfDNA test was performed but failed because of insufficient fetal DNA or other technical reasons.

A meta-analysis discussing data related to blood collection and transportation of the samples reported a failure rate of 0.03% to 11.1%. In the discussed studies further details were given, defining the direct reasons for test failure, such as low fetal fraction reported in 0.5% to 6.1%. In cases where no results were reported due to low fetal fraction, repeating the sampling was successful in 66% of cases.

Timing of sample collection is highly important. Studies showed that there is an increase in total cfDNA over time, along the pregnancy (due to lysis of maternal cells), but that the absolute quantity of cfDNA remained constant.

Therefore, the proportion of cffDNA reduces over time making the time to processing a sample, a significant factor. [24]

Many studies support the finding that "test failure" due to "no results" are more common in cases of fetal chromosomal anomalies, with rates varying from 6.9% in trisomic pregnancies to 17.2% for sex chromosome aneuploidies. [25,26] Thus, emphasizing the need to repeat test when test failure is reported.

5. Applications of cfDNA

Following the detection of cfDNA in maternal blood, described in 1997, initially it was only available for identifying DNA sequences which were not of maternal origin, but were either paternal or have arisen de novo in some autosomal dominant conditions. Examples include, Y -chromosome sequences used for fetal sex determination along with the detection of Rhesus D (RhD) blood-group. As advances in DNA sequencing technologies emerged, they allowed for accurate quantification of specific sequences in maternal plasma, and subsequently enabled use of non-invasive testing for conditions where the maternal cfDNA must also be accounted. These include conditions as common aneuploidies, autosomal recessive conditions, where the mother and father carry the same mutant alleles and some X-linked conditions. Recently cfDNA screening is also being extended by some laboratories to include microdeletion and micro duplication syndromes.

5.1. Fetal Sex Determination

With use of cfDNA we are currently able to detect the fetal sex, based on presence or absence of Y-chromosome sequences. This was the first NIPT technique developed for clinical application, as reviewed by Devaney et al. This detection is most clinically relevant for the management of pregnancies at risk of a serious X-linked condition, or congenital adrenal hyperplasia. The use of NIPT for fetal sex determination is now well established in a number of countries in Europe, and has shown to be cost-effective when offered from as early as 7th week of gestation. [27]

5.2. RhD Detection

Rh D detection is important for evaluating the risk of an Rh negative mother to develop the devastating disorder of hemolytic disease of the newborn. The process involves drawing of plasma from RhD negative pregnant women and this is then followed by PCR, with attempt to amplify at least two regions of RhD sequence . The use of cfDNA for this purpose allows avoiding an invasive test such as amniocentesis to determine fetal RhD status, which is often an event that can worsen the disease process. The technique is also beneficial in that it avoids the administration of anti-D if the fetus is RhD negative. A review of NIPT for fetal RhD genotyping showed high sensitivity rate-99.5–99.8% and specificity rate of 94.0–99.5%. A non-invasive fetal RhD genotyping service is now available in many countries, with several of them offering it as part of NIPT program. [27]

5.3. Single gene disorders

Despite several promising reports and great potential, the use of NIPT for the detection of common monogenic disorders has not yet been completely established as a part of common clinical practice. This may be explained by the fact that the number of families at risk of individual disorders is small, and also due to lack of available appropriate technical platforms. The studies conducted in this field, have been able to demonstrate the ability ofcfDNA to detect a variety of single gene disorders, including achondroplasia, thalassemia and cystic fibrosis.[24]

Current clinical use of prenatal diagnosis for monogenic disorders is limited to cases where the mother does not carry the mutant allele. For example, NIPT for achondroplasia has successfully been translated into clinical practice in the UK with full approvals and funding for use in the National Health Service. NIPT, to exclude the inheritance of paternal altered alleles, is also available for autosomal recessive disorders when the mother and father carry different altered alleles.

This technique is believed to be more suitable for conditions with a large number of possible mutations, such as thalassemia, but as to date, invasive testing is still required for definitive diagnosis when the paternal allele is detected in the maternal plasma.[28]

5.4. Common autosomal chromosome Aneuploidies

In 2008, the first proof of principle studies demonstrated that NIPT for Down syndrome was made possible using massive parallel shotgun sequencing. Since then, many studies have been conducted, and in the following three years the use of NIPT was made publicly available in the US and Asia.

Initially, published studies addressing the reliability of using cfDNA testing for the detection of trisomy 21, were carried out on plasma samples drawn prior to invasive prenatal diagnosis, testing only women who were considered as high risk for having this trisomy. More recently, studies have extended to evaluate both the “low” and “high risk” pregnancies, as samples were drawn as part of existing screening programs. The results presented in table 1 consist of a published meta-analysis and two recent cohort studies, [2,3,29] demonstrating the detection rate (DR) and false positive rates (FP) by using cfDNA for evaluating the presence of trisomy 21. The DR range from 94.4-100%, with pooled results of 99.3%, on the other hand, false positive rates ranged from 2.1% to 0%, with a pooled data of 0.05%.

Table 1- Studies demonstrating the detection of Trisomy 21 using NIPT

Study	Method	DR	FP
Chiu (a) (2011),	s-MPS	86/86 (100%)	3/146 (2.1%)
Ehrlich (a)(2011)	s- MPS	39/39 (100%)	1/410 (0.2%)
Sehnert.(a) (2011)	s-MPS	13/13 (100%)	0/34 (0%)
Palomaki (a) (2011-2012)	s-MPS	209/212 (98.6%)	3/1471 (0.2%)
Bianchi (a)(2012)	s-MPS	89/89 (100%)	0/410 (0%)
Sparks (a)(2012)	s-MPS	36/36	0/131
Jiang (a) (2012)	s-MPS	16/16 (100%)	0/887 (0%)
Lau (a)(2012)	s-MPS	11/11 (100%)	0/97 (0%)
Liang (a) (2013)	s-MPS	40/40 (100%)	0/372 (0%)
Ashoor (a) (2012-2013)	t-MPS	50/50	0/297
Norton et al. (a)(2012)	t-MPS	81/81(100%)	1/2887(0.1%)
Verweji(a)(2013)	t-MPS	17/18 (94.4%)	0/486 (0%)
Guex(a) (2013)	s-MPS	30/30 (100%)	0/146 (0%)
Stumm (a)(2014)	s-MPS	40/41 (97.6%)	0/430 (0%)
Porreco (a) (2014)	s-MPS	137/137 (100%)	3/3185 (0.1%)
Nicolaides (a)(2013)	SNP	25/25(100%)	0/204(0%)
Shaw (a) (2014)	s-MPS	11/11 (100%)	0/184 (0%)
Song (a)(2015)	s-MPS	2/2 (100%)	0/201 (0%)
Pergament(a) (2014)	SNP	58/58 (100%)	0/905 (0%)
Nicolaides (b) (2012, 2014)	t-MPS	8/8 (100%)	0/1941 (0%)
Song (b) (2013)	s-MPS	8/8(100%)	0/173 (0%)
Bianch (b)(2014)	s-MPS	5/5 (100%)	6/1904 (0.3%)
Comas (b) (2014)	t-MPS/SNP	4/4 (100%)	0/311 (0%)
Quezada.(b)(2015)	t-MPS	32/32 (100%)	1/2753 (0.04%)
Nortton (b)(2015)	-MPS	38/38 (100%)	9/15,803 (0.1%)
Zhang (b) (2015)	s-MPS	720/726 (99.17%)	61/122,000(0.05%)
Sum		**1799 /1810 (99.3%)**	**88/157,768 (0.05%)**

Abbreviations: s-MPS- Sequence massive parallel sequencing; t-MPS- Targeted massive parallel sequencing; SNP- Single nucleutide polymorphism; DR- Detection rate; FP- False positive rate

(a)- Either retrospective studies, using stored samples from pregnancies with known outcome, or prospective, using mainly samples from high-risk pregnancies undergoing invasive testing, or both.

(b)- cfDNA testing in routine screening for trisomies in the general population

Data collected from: Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. **Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated Meta-anlysis.** Ultrasound Obstet Gyenecology 2015;45:249-66;**;** Norton, ME, Jacobsson B, Swamy GK et al. **Cell-free DNA analysis for non-invasive examination of trisomy**. N Engl J Med 2015;372:1589–97**;** Zhang H, Gao Y, Jiang F, et al. **Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146,958 pregnancies.** Ultrasound Obstet Gynecol 2015;45:530–8.

In table 2, we present a total of 23 published studies reporting the detection and false positive rates of cfDNA analysis in screening for trisomy 18. In individual studies, the DR varied between 90.0% and 100% and the FPR varied between 0% and 0.3%. The pooled weighted DR and FPR were 96.8% and 0.047% respectively.

Table 2 Studies demonstrating the detection of Trisomy 18 using NIPT

Study	Method	DR	FPR
Chen et al (2011)	s-MPS	34/37	5/252 (1.98%)
Senhert et al. (2011)	s-MPS	8/8 (100%)	0/39(0%)
Palomaki et al (a)(2011- 2012)	s-MPS	59/59 (100%)	5/1688 (0.3%)
Bianchi et a l.(a) (2012)	s-MPS	35/38 (92.1%)	0/463 (0%)
Sparks et al (a) (2012)	s-MPS	8/8 (100%)	0/159 (0%)
Jiang et al. (2012)	s-MPS	12/12 (100%)	1/891 (0.1%)
Lau et al. (2012)	s-MPS	10/10 (100%)	0/98 (0%)
Liang et al (a) (2013)	s-MPS	14/14 (100%)	0/398 (0%)
Ashoor et al(a) (2012-2013)	t-MPS	49/50	0/297
Norton et al (a) (2012)		37/38(97.4%)	2/2888 (0.1%)
Song et al (b) (2013)	s-MPS	2/2 (100%)	1/1739 (0.1%)
Guex et al. (2013)	S-MPS	19/20 (95%)	0/156 (0%)
Stumm et al (a) (2014)	s-MPS	8/8 (100%)	1/463 (0.2%)
Porecco et al(a) (2014)	s-MPS	36/39 (92.3%)	0/3283 (0%)
Bianchi et al (b) (2014)	s-MPS	2/2 (100%)	3/1903 (0.2%)
Nicolaides et al (b) (2012, 2014)	t-MPS	2/2 (100%)	2/1947 (0.1%)
Norton et al (b) (2015)	t-MPS	9/10 (90%)	1/15,831 (0.0%)
Nicolaides et al (a)(2013)	SNP	3/3	1/939 (0.1%)
Shaw et al. (2014)	s-MPS	7/7 (100%)	0/188 (0%)
Pergament et al(a) (2015)	SNP	24/25 (96%)	1/939 (0.1%)
Quezada et al (b) (2015)	t-MPS	9/10 (90%)	5/2775(0.19%)
Song et al (2015) (a)	s-MPS	1/1	0/202
Zhang et al (2015)	s-MPS	167/170 (98.2%)	51/102,000 (0.5%)
Total		**555 /573 (96.8%)**	**78/164,513 (0.047%)**

Abbreviations: s-MPS- Sequence massive parallel sequencing; t-MPS- Targeted massive parallel sequencing; SNP- Single nucleutide polymorphism; DR- Detection rate; FP- False positive rate

Data from Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. **Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated Meta-anlysis.** Ultrasound Obstet Gyenecology 2015;45:249-66;**;** Norton, ME, Jacobsson B, Swamy GK et al. **Cell-free DNA analysis for non-invasive examination of trisomy**. N Engl J Med 2015;372:1589–97**;** Zhang H, Gao Y, Jiang F, et al. **Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146,958 pregnancies.** Ultrasound Obstet Gynecol 2015;45:530–8.

In table 3, we included 19 studies, testing the applicability of cfDNA test for the detection of chromosome 13 aneuploidy. The different studies showed variability in the DR, ranging from 80% to 100%, along with differing FP rates 0.9%-0% and with pooled values of DR and FP averaging 93% and 0.05%, respectively.

Table 3- Studies demonstrating the detection of Trisomy 13 using NIPT

Study	Location	Method	DR	FP
Chen et al. (2011)	China	s-MPS	25/25	3/264
Palomaki et al (a)		s-MPS	11/12(91.7%)	16/1688 (0.9%)
Bianchi et al (a) (2012)	USA	s-MPS	11/14 (78.5%)	0/485 (0%)
Jiang et al		s-MPS	2/2 (100%)	0/901
Liang et al. (a)(2013)	China	s-MPS	4/4 (100%)	1/408 (0.2%)
Ashoor et al. (a)(2012-2013)		t-MPS	8/10 (80%)	1/1939 (0.1%)
Guex et al (2013)		s-MPS	13/13	0/163
Song et al (b)(2013)		s-MPS	1/1 (100%)	0/1470 (0%)
Stumm et al(a) (2014)		s-MPS	5/5 (100%)	0/466 (0%)
Porecco et al(a) (2014)		s-MPS	14/16 (87.5%)	0/3306 (0%)
Bianchi et al (b)(2014)		s-MPS	1/1 (100%)	3/1913 (0.2%)
Hall et al (2014)		SNP	14/14(100%)	0/49 (0%)
Norton et al (b)(2015)		t-MPS	2/2 (100%)	2/11,183 (0.0%)
Nicolaides et al (a)(2013)		SNP	1/1 (100%)	0/228 (0%)
Shaw et al (2014)		s-MPS	3/3 (100%)	0/192 (0%)
Pergament et al (a) (2015)		SNP	12/12 (100%)	0/953 (0%)
Quezada et al (b)(2015)		t-MPS	2/5 (40%)	2/2780 (0.07%)
Song et al. (a) (2015)		s-MPS	1/1 (100%)	0/200
Zhang et al (2015)		s-MPS	22/22 (100%)	45/112,500(0.04%)
Total			**152/163(93%)**	**73 /141,088 (0.05%)**

Abbreviations: s-MPS- Sequence massive parallel sequencing; t-MPS- Targeted massive parallel sequencing; SNP- Single nucleutide polymorphism; DR- Detection rate; FP- False positive rate

Data from Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. **Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated Meta-anlysis. Ultrasound Obstet Gyenecology** 2015;45:249-66;**;**Norton, ME, Jacobsson B, Swamy GK et al. Cell-free DNA analysis for non-invasive examination of trisomy. N Engl J Med 2015;372:1589–97**;** Zhang H, Gao Y, Jiang F, et al. **Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146,958 pregnancies.** Ultrasound Obstet Gynecol 2015;45:530–8.

Generally, three broad strategies have been suggested for the clinical application of NIPT:

1. Primary cfDNA, routinely offered to all women in replacement of conventional screening methods

2. Secondary cfDNA, only offered to women with positive screening results using conventional methods such as the combined test.

3. Contingent cfDNA, which provides cfDNA to 15–20% of women with the highest combined test risks. [28]

Primary cfDNA- This strategy will maximize detection while ensuring that relatively few women will undergo invasive prenatal diagnosis. As such, it appears to be the best strategy. However, it is an expensive strategy, since it includes the largest number of tested women. Moreover, if it replaces a conventional first trimester aneuploidy screening test, the adjunctive benefits associated with routine first trimester scanning and the early maternal serum testing potentially may be lost. Although, cfDNA testing can be performed as early as 9 weeks' gestation, success rates are higher at later gestational age, while amniocentesis may be preferred over CVS for confirmation of abnormal results. On the other hand, the advantages of early reassurance, early diagnosis and, if required, a safer and less traumatic termination of pregnancy may be lost. [28]

Secondary cfDNA- Represents the most strict cutoff for using cfDNA. Its advantages rest on the substantial reduction in the number of amniocenteses or CVS procedures performed and thereby reducing iatrogenic fetal losses. A further consideration is that women will have additional indications for invasive testing, such as abnormal ultrasound findings or positive family history. Others may wish to know about other chromosome abnormalities detectable only through karyotyping or microarray analysis following invasive testing. [28]

Contingent- This strategy has been suggested, for example, by using using the Combined tests as initial selection. Thereafter women with intermediate risk will be offered to undergo NIPT and based on these results, it will be decided wether to proceed with invasive measures. [28]

In the beginning of the era, most studies have been conducted on higher risk groups.[3] Following the demonstration of the high efficacy of this approach, the American College of Obstetrcis and Gynecology (ACOG) published a committee opinion, recommending that routine screening use of cfDNA, should be applied only in women with high risk for fetal aneuploidies. These include: (1) maternal age 35 years old at delivery, (2) fetal ultrasound finding that indicates an increased risk of aneuploidy, specifically for trisomies 13, 18, and 21, (3) a history of previous pregnancy with a trisomy detectable by cfDNA screening (trisomies 13, 18, or 21), (4) positive test results for aneuploidy that include a first-trimester, sequential, integrated, or quadruple screen, and (5) parental balanced Robertsonian translocation with increased risk of fetal trisomy 13 or 21.[30]

As the use of NIPT began to rise, a few studies have been conducted in lower risk populations. These studies provided growing evidence that comparably good results can also be achieved in general obstetrical populations, making NIPT an a possible alternative to current first-trimester screening protocols. In the prospective multicenter Comparison of Aneuploidy Risk Evaluation (CARE) study, Bianchi et al, [31] collected blood samples from women carrying a singleton pregnancy, who were undergoing standard aneuploidy screening as is commonly performed in most centers in the United States (serum biochemical assays with or without nuchal translucency). The study was conducted in 21 different centers in the US, which used massive parallel sequencing in a blinded fashion, and compared the detection rates and false positive rates for trisomies 21 and 18 between the standard screening and cfDNA testing.

Results presented that for trisomies 21 and 18, the false positive rates with the use of cfDNA testing were significantly lower than those with standard screening (0.3% vs. 3.6% for trisomy 21 and 0.2% vs. 0.6% for trisomy 18). Also, the use of cfDNA testing was able to detect all cases of aneuploidies present (5 for trisomy 21, 2 for trisomy 18, and 1 for trisomy 13). The positive predictive values for cfDNA testing versus standard screening were 45.5% versus 4.2% for trisomy 21 and 40.0% versus 8.3% for trisomy 18. [31]

Recently, two large prospective studies were published, [2,29] aiming to confirm these observations supporting the use of cfDNA as part of general screening programs. The first study described, was carried out in centers around the US, Canada and Europe, the non-invasive examination of trisomy using cell-free DNA analysis (NEXT). [2]
In this Prospective, multicenter, blinded study conducted at 35 international centers, pregnant women presenting for aneuploidy screening at 10 to 14 weeks of gestation underwent both standard screening (with measurement of biochemical analytes with nuchal translucency) and cfDNA testing. Results demonstrated, that for trisomy 21, cfDNA testing identified 19 of 19 women with the trisomy, with 6 false positive results out of 19,994 low risk women. Among the 14,957 women for whom standard screening showed a risk of less than 1 in 270, cfDNA testing identified 8 of 8 women with trisomy 21, with 8 false positive results. The positive predictive value for cfDNA testing was calculated as 76% for women under the age of 35 years and 50% for those with a negative result on standard screening. For trisomy 18, cfDNA testing identified 9 out of 10 cases, while standard screening identified 8. Along with that, cfDNA testing had a false positive rate of 0.01% and a positive predictive value of 90.0%, as compared with false positive rate of 0.31 and a positive predictive value of 14% in standard screening. As for trisomy 13, cfDNA testing identified 2 out of 2 cases, while standard screening was able to identify only one. The false positive rates were 0.02% compared with 0.25% in the standard screening. [2]

The second study recently published, [29] a prospective, multicenter observational study with participants enrolled from 508 medical centers in China between 1 January 2012 and 31 August 2013, with a total of 146,958 women screened successfully. They were able to demonstrate high performance for DR and FP of trisomy 21, 99.17% and 0.05%, respectively. Along with that, they showed also high DR and FP rates for analysis of trisomy 18 and 13 in the screening of general population (as presented in tables 2 and 3). This study therefore demonstrated that NIPT was able to maintain high sensitivity and specificity also in large-scale clinical practice, for all three common aneuploidies. The authors also presented results which were comparable, if not better when compared to studies conducted on ‘high risk’ groups. [29]

When summing up all previoulsy presented reports, the results show that for trisomy 21 and other common aneuploidies, cfDNA testing represents a highly accurate screening option. However, maternal serum and nuchal translucency screening can identify risk for a broad array of abnormalities that are not detectable on cfDNA testing. The major issue presented, in the use of cfDNA for detection of the common aneupolidies is assessed in terms of its positive predictive value, as this measure also takes the low prevalence of the relevant conditions in the target population into account.

For instance, the PPV for trisomy 21 in the CARE-study was found to be 45.5%, meaning that in a general risk population more than half of positive NIPT results may generate false alarms. Thus, requiring further invasive measures for its diagnosis.[32]

The tremendous interest in NIPT prompted the International Society of Prenatal Diagnosis (ISPD) to release a position statement recognizing NIPT as the 'most effective method for screening for fetal trisomy 21 and trisomy 18', but acknowledged that the test is not a replacement for diagnostic testing using CVS or amniocentesis. [33]
The new studies in this field, already have presented much higher PPV for detecting trisomies 21, 18 and 13, thus bringing more hope for future application of cfDNA. [2,29]

Until recently meta-analysis studies which addressed the efficacy and utility of cfDNA for detecting the common aneuploidies, have all reached similar results, as were present in this review. But, a recently published intriguing meta-analysis which concluded the results of 41 previously conducted studies in this field, had demonstrated an even improved performances for NIPT, including DR of 99.3% for trisomy 21, 97.4% for trisomy 18 and 97.4% for trisomy 13, along with pooled specificity of 99.9% for all trisomies. Thus, adding further support to the ability to use NIPT as a tool for detection of the three common aneuploidies, in the near future.[26]

5.5 Sex Chromosomal aneuploidies

Table 4 summarizes the various studies addressing the detection of sex chromosomal aneuploidies (SCAs). Monosomy X detection rate was found to be as high as 90.9%, with a FPR of 0.2%. Data also demonstrates the ability of the test to detect the other SCA, including XXY, XYY and XXX.

Table 4. Studies reporting on cfDNA analysis for the screening of sex chromosome abnormalities. Monosomy X has been separated from the other sex chromosomal aneuploidies**.**

		Monosomy X		other SCA	
Study	**Method**	**DR**	**FP**	**DR**	**FPR**
Sehnert (2011)	s-MPS	2/2 (100%)	0/45 (0%)		
Bianchi (2012)	s-MPS	15/20 (75%)	1/462 (0.2%)	8/9 (88.8%)	0/453 (0%)
Jiang (2012)	s-MPS	3/3 (100%)	1/899 (0.1%)	3/3 (100%)	0/896 (0%)
Lau (2012)	s-MPS	8/8 (100%)	0/100 (0%)	1/1 (100%)	0/99 (0%)
Guex (2013)	s-MPS	15/15	0/161 (0%)	5/5 (100%)	0/156 (0%)
Liang	s-MPS	5/5 (100%)	1/407 (0.2%)	3/3 (100%)	0/896 (0%)
Mazloom (2013)	s-MPS	17/21 (94.4%)	1/390 (0.3%)	8/8 (100%)	0/382 (0%)
Samango-Sprouse (2013)	SNP	11/12 (91.6%)	0/175 (0%)	3/3 (100%)	0/172 (0%)
Song et al. (2013)	s-MPS	2/3 (66%)	0/1737 (0%)		
Porreco et al (2014)		9/9 (100%)	11/3269 (0.3%)	6/6 (100%)	5/3263 (0.15%)
Nicolaides (2012, 2014)	t-MPS	43/47 (91.5%)	0/125 (0%)	9/9 (100%)	1/107 (0.93%)
Nicolaides (2013)	SNP	2/2 (100%)	0/227 (0%)		
Hooks (2014)	t-MPS	26/27(0.9%)	2/387 (0.005%)	7/7 (100%)	0/380 (0%)
Shaw (2014)	s-MPS	3/3 (100%)	0/192	1/1 (100%)	0/191 (0%)
Pergament (2015	SNP	9/10 (90%)	1/955 (0.1%)		
Song (2015)	s-MPS	0/0	1/203 (0.04%)	0/1 (0%)	0/202 (0%)
Total		**170/187 (90.9%)**	**19/9531 (0.2%)**	**54/56 (96.4%)**	**6/7197 (0.08%)**

Abbreviations: SCA- sex chromosomal aneuploidies; s-MPS- Sequence massive parallel sequencing; t-MPS- Targeted massive parallel sequencing; SNP- Single nucleutide polymorphism; DR- Detection rate; FPR- False positive rate

Data from Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. **Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated Meta-anlysis.**

Abbreviations: SCA- sex chromosomal aneuploidies; s-MPS- Sequence massive parallel sequencing; t-MPS- Targeted massive parallel sequencing; SNP- Single nucleutide polymorphism; DR- Detection rate; FPR- False positive rate.

Data from Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. **Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: Updated Meta-anlysis**. Ultrasound Obstet Gyenecology 2015;45:249-66;; Norton, ME, Jacobsson B, Swamy GK et al. **Cell-free DNA analysis for non-invasive examination of trisomy.** N Engl J Med 2015;372:1589–97; Zhang H, Gao Y, Jiang F, et al. **Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146,958 pregnancies.** Ultrasound Obstet Gynecol 2015;45:530–8.

Sex chromosome aneuploidies, including monosomy X (45,X), Klinefelter syndrome (47,XXY or 48,XXYY), Triple X syndrome (47,XXX), and 47,XYY, with a combined prevalence of 1: 500 represent a more common finding than the major trisomies. Although most cases of sex chromosome aneuploidies are generally mild without intellectual disability, some have a well-established phenotype that can include physical abnormalities, learning delays and infertility.

It may therefore be of interest to some parents that these conditions could be diagnosed prenatally with the option of pregnancy termination. However, the traditional methods of screening for trisomies, including maternal age, maternal serum biochemical testing and ultrasound examination of the fetus, are not effective in detecting sex chromosome aneuploidies, except cases of Turner syndrome presenting with cystic hygromas. The introduction of cfDNA analysis in maternal blood has now made it possible to screen for these conditions as part of general screening programs.[3]

Currently, a small number of studies, with a combined total of 177 singleton pregnancies with fetal monosomy X and 56 with other sex chromosome aneuploidies reported that cfDNA analysis achieved a DR of 90% for X monosomies,and 93% for the other sex chromosome aneuoplidies, and a combined FPR of 0.37% for both.[3] It is believed that the routine screening for sex chromosome aneuploidies may be questionable with a limited clinical utility, given the variability in phenotype of and the fact that most of these newborn will have no major clinical disorders despite the chromosomal abnormality. Therefore, cultural and societal factors likely will play a role in parental decision to continue or to terminate a pregnancy because of a sex chromosomal aneuploidies for which the phenotypic outcome cannot be predicted. Also, emphasizing the importance of pre-screening consultation. [32]

5.6. Detection of other chromosomal aneuploidies

Some studies have presented the ability of using NIPT for detection of rare autosomal aneuploidies. Initially the first laboratory reported on the detection of trisomy in chromosomes 16 and 22, while others have expanded this to include a larger set of autosomal trisomies. The clinical relevance of detecting these aneuploidies is questionable. In the first trimester, this may identify pregnancies at high risk for spontaneous fetal loss but in the second trimester the clinical significance of a positive result is yet to be clarified, as most positive results will reflect a confined placental mosaicism, but only few could reflect either rare instances of true fetal mosaicism or may be associated with a risk for clinically significant uniparental disomy.

Also, the great phenotypic varibility among neonates with the same genotype, further increases the debate regarding the use of NIPT for detecting these aneuploidies. [3]

We believe that more studies addressing the application of NIPT for this matter are still required, in order to establish the detection and false positive rates for those aneuploidies. This will allow both, physicians and the public, a better understanding before considering to screen for these less prevalent conditions.

5.7. Use of cfDNA in Multiple pregnancies

Mainly due to increased use of assisted reproductive therapy in recent years, and the increasing age of gravida, the incidence of multiple pregnancies has risen all over the world. It has been demonstrated that the risk for aneuploidies and the risk of miscarriage from invasive testing are higher in twin pregnancies than in singletons, emphasizing the necessity for an adequate non- invasive prenatal testing. [34]

However, the use of cfDNA in twins is more complex as compared with singleton, due to the fact that in dizygotic twins only one fetus is likely to have aneuploidy when detected, and the contribution of cfDNA of the two fetuses into the maternal circulation can vary by nearly two-folds. Therefore, if the fetal fraction of the affected fetus is below the threshold of 4%, necessary for successful cfDNA analysis, but there is a high contribution from the normal co twin, the total fetal fraction can be considered satisfactory, and a false negative test may result. In order to avoid these possible mistakes, laboratories began to assess the lower of the two fetal fractions rather than the total, when addressing cfDNA in twins. However, an inevitable consequence of such a policy is that the no-result rate in twins is likely to be higher than in singleton pregnancies.

Currently, five studies have assesed the ability to use cfDNA testing in multiple pregnancies. A total of 758 twin pregnancies with known outcome, demonstrated satisfactory results, with detection rates of 95% for trisomy 21, 86% for trisomy 18 and 100% for trisomy 13, at an FPR of 0%. There are today, sufficient data to suggest that the false positive rates and the detection rates are very close to those of singletons pregnancy. It has also been noted that in twin pregnancies, women who had underwent IVF therapy showed increasing incidence of failure rates as compared to those who conceived naturally. [34]

5.8. Detection of fetal Sub-chromosomal abnormalities

Conditions such as microdeletions and duplications, may lead to more severe physical and/or intellectual impairments compared to the common chromosomal aneupoloidies. Also, the risk of these sub-chromosomal abnormalities is independent of maternal age.

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Clinically relevant microdeletions and duplications occur in 1-1.7% of all structurally normal pregnancies. In younger women, the risk for a clinically significant deletion exceeds the risk for Down syndrome. Because some infants with sub- chromosomal abnormalities may benefit from early therapeutic intervention, prenatal detection is important for optimal management. Therefore, with the introduction of NIPT along with whole genome sequencing, there was great hope that this will provide the solution.

Recently, several studies that used shot- gun or whole-genome sequencing reported the detection of sub- chromosomal microdeletions and micro-duplications. However, these approaches were limited by the requirement for exceptionally high sequence reads, and interpretation was complicated by the identification of variants of unknown clinical significance.[35]

Additional studies were able to accurately detect microdeltions of 22q11.2, 1p36, cri-du-chat, and Prader-Willi/ Angelman by using a SNP-based NIPT approach with low positive predictive value. Some laboratories also have currently extended cfDNA screening to include microdeletion and microduplication syndromes.

But, it is important to remember that for many of these disorders, there are alternative molecular mechanisms reported other than copy number changes and not all cases are therefore detectable, thus directly affecting on the false negative results of these assays. We believe that the screening and detection of women at high risk for these sub-chromosomal abnormalities using cfDNA technology, may be of great benefit in the future, but still there are lacking evidence of test accuracy, specificity, and sensitivity before offering it for all prenatal testing.

Recently published study by Lo et al, [36] concluded that PPV of 0.55 is demonstrated when attempting to detect microdeltion or microduplications syndromes with a copy number variation of above 6 Mb. This finding may explain the ACOG's recent recommendation that routine cfDNA aneuploidy screening should not be expanded to include microdeletion syndromes at the moment.[30]

5.9. Relation to pregnancy complications

As was previoulsy presented, the extrusion of cfDNA is closely linked to placental morphogenesis, therefore it was hypothesized that conditions which affect the placenta can directly impact its levels in maternal circulation. These include conditions that are linked to pregnancy complications, as preeclampsia, preterm labor, intrauterine growth restriction (IUGR) and others.

Preeclampsia has been one of the most well studied examples. Initial reports, after the discovery of cfDNA, claimed that in preeclamptic placentas, oxidative stress leads to increased trophoblast apoptosis and shedding of syncytiotrophoblast microparticles, leading to increased release of cfDNA into the maternal circulation. More relevant were the reports that higher cfDNA levels, compared with normal pregnancies, have been noted prior to the onset of clinical symptoms. These were found to occur in a bi-modal pattern, with levels increasing above controls between 17 and 28 weeks of gestation, followed by a second rise around three weeks prior to the development of clinical preeclampsia. These results, led to increasing studies in this field, with a recently presented study showing results to be inconclusive, and that the use of cfDNA for the early detection of preeclampsia is questionable.[37]

Early studies have also believed that an increase in circulating cfDNA concentrations was associated with the increased risk of preterm labor and the incidence of IUGR. However, these observation have also been excluded, as one of the more promising studies in this field, anzalyzed stored maternal plasma from 1,949 pregnant women obtained during prospective first-trimester combined screening. The authors failed to show a link between elevated levels of cfDNA and the risk for prenatal complications in these women, leaving more work for future studies.[38]

6. Public view

As would be expected for any new technology introduced into clinical practice, initially there was an uncertainty regarding patients and physicians acceptance of NIPT as a prenatal test done routinely. The specific indications, and the right algorithm and protocols for any test are a prerequisite for any screening or diagnostic test, especially any prenatal test. Several studies addressing the public's opinion on the NIPT test had been conducted before the test was even available for public use. Most of the studies were based on questionnaires regarding the usefulness of the test. These studies concluded, that majority of population see NIPT as a satisfactory and excellent noninvasive test with reliable results on fetal aneuploidy and hence of fetal wellbeing.

Also, most women did not correlate the test with the idea of having the "optimal baby" born. The majority of the population considered the noninvasive test as a safe and convenient test with no related side effects that can give an optimal data reassuring low risk for Down syndrome when it is negative. The most important point from the public's point of view was a minimal or no risk for miscarriage. [39] These results were further emphasized in a subsequent study conducted in Hong Kong,[40] suggesting that majority of women would prefer to uptake a non- invasive test, such as NIPT, which carries no risk to the fetus or the mother, while willing to accept uncertainty regarding the test's sensitivity and reliability. [40]

Of great concern from the public point of view were the costs of the new adopted technology, with comparison to the currently available prenatal screening methods. [39]

Overall, before the introduction of NIPT in common clinical practice, it was accepted as a positive advancement in the prenatal care area, especially due to the fact that it offers an early and more accurate approach as compared with current screening tests, thereby allowing the patients more confidence, and reducing the need for risky invasive procedures, associated with anxiety and risk for miscarriage. Despite that, women were concerned that the introduction of NIPT will pressure the population to perform prenatal testing, once it becomes a routine test. Nevertheless the advantages of the new test outweighed the presented concerns, as the majority claimed that they will take this test if presented to them in the future, including half the women who currently declined any other screening programs.

Following the introduction of clinical application of cfDNA for NIPT in 2011, there had been growing interest in the public's opinion and response to such tests. The first qualitative study to describe the perceived clinical utility of DNA-NIPT for the detection of trisomy 21 in the fetus among pregnant women, attempted to demonstrate the reasons and motivations behind the women's decision to uptake this test. [41] A number of reasons were outlined, including the aim to reduce the uncertainty associated with risk probability- based results from serum and ultrasound based –Down syndrome screening, the understanding that NIPT counteracts the risk from childbearing especially at advanced age, perceived predictive accuracy and absence of risk of harm to the fetus. On the other hand, high-risk women explained to have accessed DNA-NIPT in order to get a clearer idea of their risk.

This group perceived serum and ultrasound based –Down syndrome screening as an unnecessary and confusing procedure because of its varying, protocol-dependent detection rates. While, those women not deemed high-risk, in contrast, under went NIPT for psychological assurance and in order to reduce anxiety even following negative result from serum and ultrasound based –Down syndrome screening. There was also evidence that the source of information about DNA-NIPT influenced women's decisions to access testing. Some women were more knowledgeable with regard to NIPT than others before their first obstetric visit. The study also highlighted the fact that DNA-NIPT was currently used by those who could afford to pay, as 90% of the women had come from higher income groups.[41]

As the test began to gain popularity, increasing public interest grew, even among women who did not take a previous prenatal testing, with the majority willing to pay more than they do for current prenatal testing.[42]

Immediately after the introduction of NIPT into clinical practice, it has generated extensive changes in the patterns of clinical diagnosis as reported in the United States. NIPT had become well accepted among high risk women there, becoming the preferred first trimester screening program among this group. With reports presenting that already in the initial year of its use, it had decreased the number of combined first trimester screens by almost 50%. More important clinically, had been the fact that it profoundly reduced the number of diagnostic procedure performed. As was expected by studies which predicted the effects of NIPT before it was clinically introduced, this method had decreased the number of CVS procedures by 76%, whereas amniocentesis had decreased by 54%.[43] In light of recent studies and the potential for broadening of recommendations regarding NIPT by national and international societies, indicating that NIPT is effective in screening of low-risk patient populations, it is important to evaluate the uptake of NIPT in this population.

As mentioned previously, there is a growing evidence for the use of cfDNA not only for the detection of common aneuploidies, but also for addressing other possible disorders, including the sex chromosomal aneuploidies, complications of pregnancy and also the possibility for screening microdeletions and microduplications. This has raised a great debate and concern as to the public's preferences for extent, for using NIPT. A study conducted in Amsterdam, Netherlands,[42] demonstrated that majority of women believe that NIPT should be used for screening severe mental retardation disorders and life threatening conditions, including neuromuscular disorders. Whereas only minority of women requested that the test be made available for any disorder, or disorders which manifest later in life, such as the risks for developing breast or ovarian cancer. But along with that, there was an agreement among most study participants that the decisions on which conditions to be screened, should rely on the couples' preferences. However, women seemed aware that testing for a broad range of conditions may complicate the decision-making process beyond what most couples are able to comprehend. There was also a positive response towards the use of NIPT for identifying sex chromosomal aneuploidies. The authors presented a further interesing fact, emplying that the majority of women did not think that the test should attempt to detect also risks for pregnancy complications.[42]

As of today, NIPT is offered in over 60 countries throughout six continents, with the recent market report showing that North America accounts for 64.5% of global NIPT revenue, followed by Europe. In the US, clinical uptake of NIPT appears to be high, and this is expected to rise as clinicians and the population will gain more knowledge and experience with such technology. [44]

We believe that the capabilities and extent of use of cfDNA will expand in the following years, and will present an even greater challenges to clinicians who offer the test and to patients who must decide if this information is of value to them, their partners, and their families. Therefore it should be up to general committees to decide while considering the public's opinion, clinical efficacy and costs, to which extent this technology should be offered and used.

7. Costs and effectiveness

Prenatal screening for Down syndrome is a standard clinical test available in many countries and has been employed over many years. Screening for less common aneuploidies, such as trisomy 18 and trisomy 13 is often included as well. The prenatal screening methods have evolved over the past several decades, and in recently NIPT using cfDNA emerged.[2]

NIPT which uses cfDNA offers tremendous potential as a screening tool for fetal aneuploidy. Early attempts to detect trisomic fetuses using cfDNA required the use of multiple placental DNA, or RNA markers, which made the screening test time consuming and expensive. In recent years, number of groups have validated a technology known as massively parallel genomic sequencing, which uses a highly sensitive assay to quantify millions of DNA fragments in biological samples in a span of days and has been reported to accurately detect trisomy 13, trisomy 18, and trisomy 21, as early as the 10th week of pregnancy with results available approximately 1 week after maternal sampling. The initial implementation of NIPT has primarily been in pregnant women classified as ‘‘high risk’’ based on maternal age, or other risk factors. ACOG issued a statement in 2012, that supported NIPT as an option only in ‘‘high risk’’ women. [30]

Several studies evaluated the costs and effectiveness of conducting NIPT as part of the prenatal work up programs. Most of them found that there was an economic advantage in offering NIPT to women at high risk for fetal Down syndrome. One of these studies, performed in Australia,[45] compared the costs and benefits of current practice of first-trimester screening with a testing pathway incorporating NIPT. A model was applied in 32,478 singleton pregnancies screened between January 2005 and December 2006, adding Medicare rebate data as a measure of public health system costs. It showed that the introduction of NIPT could reduce the number of invasive diagnostic tests and the number of procedure-related fetal losses and increase the cost by 9.7% over two years. Since then, numerous clinical studies have validated the performance of NIPT in ‘‘average risk’’ or ‘‘low risk’ women and professional groups have supported NIPT as an option in any pregnant woman, regardless of age or risk. The primary barrier to adopt NIPT in the general pregnancy population appears to be the cost. [45]

In 2013, a study conducted in the Netherlands, [46] addressed the attitude among pregnant women regarding NIPT and quantified their willingness to pay for such a test. The method was applied by handing out questionnaires to pregnant women, who received counselling for first- trimester screening, in two hospitals and nine midwife practices in the Netherlands. A total of 147 women completed the questionnaires, with 81% stating that if NIPT was available for them, they would choose to have it, and more surprisingly, 57% of women who elected not to undergo first trimester screening in their current pregnancy said they would take the NIPT test, if it would be available. The willingness to pay for NIPT correlated with age and income, but not with education. The average cost a woman was ready to pay was slightly higher than the current average cost of first trimester screening in the country (which was 150 Euros), and some women were prepared to pay even much more. [46]

In this section we would like to assess the costs and benefits along with the optimal method for employing the use of cffDNA in the general population.

In the US current list prices range from $500 to $2100 per test. Despite the cost, studies have shown that universal NIPT screening, which include replacement of the conventional maternal screening, by NIPT is cost effective when viewed from a societal perspective. But, still most decision makers actually use narrower perspectives such as governmental or payer perspective. Universal NIPT is not cost effective when viewed from these narrower perspectives. NIPT-based screening policies might be acceptable if they were used in a select subset of pregnancies rather than applied universally to all preganancies. Indeed, studies have shown that the selective use of NIPT among higher risk women (contingent NIPT) is less costly than universal NIPT.

Contingent NIPT strategies are less costly than universal NIPT screening because relatively small subsets of "high-risk" patients are referred for NIPT testing. Because it is applied to a small subset of pregnancies, contingent NIPT has the potential to reduce costs relative to universal NIPT screening with little loss of accuracy. Thus, contingent NIPT may be a cost-effective alternative to universal NIPT and MSS.[47]

The risk cutoff of the primary screen is a key design factor for contingent NIPT policies because it affects both screening performance (sensitivity and specificity) and downstream costs. Thus, it is important to determine the best cutoff point of the primary screen to optimize the overall cost-effectiveness of a contingent screening policy.

A study recently conducted in the US, [47] compared the use of contingent NIPT to conventional maternal serum sampling and universal NIPT using three different economic perspectives: societal, governmental, and payer. This was done using a simulated population designed to represent the general population of women in the United States. The study concluded that from a societal perspective, universal NIPT is a cost-effective alternative to MSS and contingent NIPT. But when viewed from government or payer perspectives, contingent NIPT was more cost-effective relative to conventional maternal serum sampling but was both more costly and less effective than universal NIPT. In these cases, the choice of policy depends on the willingness to pay for additional detections. Adopting universal NIPT would cost $203,088 for each additional case detected from a government perspective and $263,922 for each additional case detected from a payer perspective. [47]

Another group compared the use of NIPT to first trimester screening, as both can be performed in early stages of preganancy. They found that NIPT was able to identify 15% more trisomy cases than first trimester screening, as well as significantly reducing the amount of invasive procedures, and as a consequence leading to 656 normal fetuses being saved annually. The analysis also demonstrated that at a NIPT unit cost of $453 or less, it will be more cost savings over first trimester screening. The conclusion was that at this unit cost, NIPT is clearly the dominant screening strategy since the overall costs are lower with additional clinical benefits.[48]

A recent study, [49] analyzed the economic value of replacing conventional fetal aneuploidy screening approaches with NIPT in the general pregnancy population. Their results demonstrated that replacing conventional screening with NIPT would reduce healthcare costs if it can be provided for $744 or less in the general pregnancy population.

Of the 13,176 affected pregnancies undergoing screening, NIPT detected 96.5% of cases, compared with 85.9% by conventional approaches. NIPT reduced invasive procedures by 60.0%. The number of procedure-related euploid fetal losses was also reduced by 73.5% in the general screening population. These finding led the authors to conclude, that universal application of NIPT would increase fetal aneuploidy detection rates and can be economically justified. Moreover, offering this test to all pregnant women is associated with substantial prenatal healthcare benefits.

Published prices vary between different countries which currently offer NIPT. In the US, test prices range from $795 to over $3,000, with inconsistent insurance coverage, while invasive procedures like amniocentesis or CVS are nearly always covered by public or private insurance. In Europe, NIPT prices range from €631–858 and in the UK range from £400–900. While NIPT prices in Hong Kong are approximately 4,500 to 8,000 Hong Kong Dollars (US$580 to $1,000), and 3,500 real (US$1492) in Brazil. Prices in many low- or middle-income countries are not yet available or published. [49]

We conclude, that there is an agreement among most researchers that NIPT can be offered to all women undergoing prenatal testing in terms of its efficacy and benefits, which are clear and proved. However, it is an expansive method for screening at the current time for the majority of population, especially those in low-income countries, where the governments generally cannot afford to subsidize it. **Therefore, there is a need for technological innovation directed at low-resource settings in order to make NIPT comparable to, or cheaper than, maternal serum screening, so it will be affordable and available to as many women as possible.**

8. Ethical Aspects

Since the first detection of cfDNA in maternal serum, there has been tremendous progression in the field, expanding the use and availability of NIPT as part of the prenatal testing. As a result, clinicians and the public have to deal with the new era, with regard to the ethical dilemma accompanied wide use of NIPT.50 In this section we aim to raise the ethical aspects which need to be discussed and considered when offering such technologies to patients. The ethical challenges that become apparent with regard to NIPT include: - Inequity of access, full informed consent and logical protocols, social effects and the possibility of performing tests for the "wrong" reasons. [51]

Inequity of access

NIPT initially has been introduced as a strictly commercial product, raising the ethical concern, of making NIPT available only to those who can afford to pay. In this case, two types of inequity of access may occur: inequity of access to the NIPT technology itself, and, in some instances, preferential access to related services, typically in the form of access at an earlier gestational age, which means that patients who are able to afford NIPT, have access to a more effective test, and can proceed with diagnosis at an early stage of pregnancy. Early access to publicly funded services after private NIPT means that the health care system implicitly enables different care services to different patients on the basis of their economic privilege. For instance, NIPT may facilitate preferential access in the form of longer counselling sessions or pregnancy termination at an earlier gestational age.[52] It is therefore clear that further clinical and funding policy on NIPT are needed, and that policy should consider the principle of equity when determining whether NIPT should be part of the general population health service.

Informed Consent

Prenatal testing is grounded on the principle of informed choices, which insists that each woman should have the opportunity to obtain as much information as she needs to take her decisions regarding the pregnancy. Information should be provided in a comprehensive and easily understandable manner, and accompanied by the opportunity to ask questions for clarification.[53] This ethical issue is been questioned with the use of NIPT on a wide base. This is due to the fact, that a single test of blood drawn may have wider consequences as opposed to the genetic counseling and explanations that the patients receive, as opposed to the combined and integrative tests that are done. These two step- screening tests allow the physician more time to provide between the tests and results about the challenges and possible outcomes of further testing. It is also important to remember that only minor number of patients will have a positive screening result, whereas all patients need full information as part of the blood drawing test. [54]

If cfDNA testing were to become relatively inexpensive and routine, women offered the test as part of their prenatal care, might give consent to the test, as to any other request for blood sampling without the necessary knowledge needed. Blood sampling is often taken as a routine paradigm of a minimum-risk procedure and patients give consent to the procedure without much or any counseling. Therefore, concern has been raised that offering cfDNA testing on a wide scale basis, would undermine informed consent and risk trivializing a procedure that might compel exceptionally difficult decisions to be made, with lifelong consequences. A further ethical concern, is the ability to provide each patient with adequate information.

It is of concern that due to time constraints, inadequate clinician knowledge, or insufficient effort to inform the parents, the information will not be adequately provided to the patients, thus not enabling them to make an informed decision. [51]

It has often been observed that in current prenatal screening programs, test uptake is not always on the basis of adequate understanding, there is indeed reason for concern that the ease and safety of NIPD testing will make this even more difficult to achieve. This concern is reinforced by the finding that health care professionals seem inclined to the view that a less stringent standard of informed consent would be sufficient for NIPD testing. [54]

Social issues

The social aspects that have to be considered if and when NIPT test becomes available to the general pregnant women, will focus on the availability of the test to all women end especially to the low socioeconomic group. Screening is specifically offered to allow the population, to avoid having a child with a serious disorders or disabilities and to try to have children without these conditions instead. When this occurs, parents of a child with a known chromosomal abnormality for example, or perhaps even the child himself may feel not welcomed, and as a burden on the family and the society. This further may turn selective abortion into a public health dilemma, placing pressure on the mother carrying the affected fetus. [51] Given current termination rates for pregnancies found to have aneuploidy, it is reasonable to expect that as prenatal testing options expand, the number of people with the tested-for conditions will decrease, especially when new testing options, such as NIPT, remove existing barriers to termination, such as gestational age. As the number of people with disabilities decreases, it is likely that acceptance, support, and resources afforded to these people may also decrease as they (and their families, friends, and advocates) become 'less visible'. [51]

Performing the test for the 'wrong' reasons

A future public concern, is the possibility to perform NIPT to determine whole genome of the fetus with the knowledge of possible traits and features. Until recently, couples have been encouraged to consider the risk to the fetus of any invasive procedure when deciding about prenatal screening or testing. If this 'barrier' of risk to the fetus is removed, the use of testing in pregnancy is expected to be higher, including those considered to be low risk population for chromosomal abnormality. The problem with this type of testing is complex. On one hand, information on the genetic basis of the fetus may be of benefit to health providers, for instance allowing to provide the newborn with more adequate treatment. Though, we cannot ignore the fact that the parents may take advantage of this possibility, and may even use their right for autonomy to terminate the current pregnancy due to a minor established genetic defect. Also, when considering the possibility of whole genome sequencing, we have to consider the fact that many of the conditions which may arise may not be adequately treatable. This may be due to costs and benefits, or in some cases that have yet to be found a cure. Therefore, knowledge of such clinical conditions may be of any benefit to the parents in case they would like to proceed with the pregnancy.[55] The above data again emphasizes the importance of clinician counseling especially in the field of prenatal testing, in order to provide the patients with necessary knowledge, required for them to reach properly informed decisions.

9. Discussion

- Prenatal testing is a growing field attracting both physicians and patients for many years. It was intended to diagnose the in-uterus growing fetus and to avoid the major fetal anomalies. Amniocentesis was the first and still considered as the gold standard test in prenatal diagnosis. This procedure was then followed by CVS as an earlier method for prenatal testing. Later on, in order to prevent the complications of these invasive procedures, different non- invasive tests were introduced, including use of serum markers along with ultrasonographic measurements. As time passed, different approaches were attempted in order to increase the effectiveness of such prenatal testing, and with the discovery of cfDNA, in 2011, it seems that the solution has been found. In this study we focused on cfDNA which has been proven as an exquisite non- invasive method for prenatal testing. Its efficacy has been proven to be higher than that of the current methods used for prenatal screening for the detection of the common aneuploidies. It also has been proven to show excellent results in the detection of other aneuploidies including the sex chromosomal aneuploidies. Additionally, we reviewed other possible benefits achieved by conducting this test, such as the detection of subchromosomal abnormalities and other pregnancy complications. Though, currently technological data is lacking, and we believe that there is still work to be done for improving the efficacy and clinical application regarding this field.

- We also presented one of the major disadvantages of NIPT, namely, the high costs of the test. Following the different studies addressing this issue, we believe that today the cost maybe quite high for the private consumer, but as a society perspective, it may actually be cost-effective when applied appropriately, as compared with the current screening and detection methods. The major progress that had been made in NIPT in such short time, looks promising that a solution to these high costs may very well be found in the near future.

- We expect that in the next few years, further widespread adoption of NIPT will take place, leading to a further reduction in invasive sampling for prenatal genetic diagnostic testing. With reduction in costs and technological improvements, the resolution and scope of this method will keep on progressing. With further advancements, clinicians will have to deal with more complex ethical issues including informed consent and patients which will attempt to exploit this method. Also, the importance of pre and post-testing counseling will play a major role also in the future.

- **In conclusion, cfDNA testing is a highly effective form of prenatal aneuploidy screening test, which can allow early detection or on the other hand, reassurance for the future parents regarding their growing fetus. With the different studies presented in this review, we suggest that efforts should be aimed to transforming this method to be the primary screening method for all pregnant women. Despite the major advances in this field, there still is room for much more to discover regarding the use of cfDNA, and this study is just the tip of the iceberg, as this exciting new technology is still evolving as we speak.**

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