UNIVERSITAT POMPEU FABRA

LABCO Quality Diagnostics

Cell-free DNA analysis in maternal blood, new accurate approach for non-invasive prenatal screening of chromosomal aneuploidies: review

Marina Sumarroca Bordas

Supervisor: Vincenzo Cirigliano

Treball de fi de grau. Biologia Humana 2011-2015

Facultat de Ciències de la Salut i de la Vida

Universitat Pompeu Fabra

INDEX:

1. Summary
2. Keywords and abbreviations
3. Introduction and background
4. Problem statement and objectives
5. Methodology
6. Non-invasive prenatal testing (NIPT)
    - 6.1. A brief history
    - 6.2. Description of the test
    - 6.3. Current target population
    - 6.4. Twin pregnancies
    - 6.5. Cost
    - 6.6. Limitations of the test
    - 6.7. Future perspectives and applicability
7. Conclusions
8. Acknowledgements
9. Bibliography

1. SUMMARY:

Prenatal screening and diagnostic tests for the detection of fetal chromosomal abnormalities are routine standard of care in pregnancy management. Traditional methods consist of a non-invasive combined test, which gives an individual risk, followed by an invasive procedure if necessary. Recently, non-invasive prenatal testing (NIPT) through the analysis of cell-free DNA (cfDNA) in maternal blood, has been shown to accurately detect common fetal autosomal trisomies during the first trimester of pregnancy. About 6-20% of cell free DNA circulating in maternal plasma are small DNA molecules coming from apoptotic trophoblastic cells. This approach, whose efficacy exceeds by far that of conventional screening, is based on sequencing and counting the cfDNA sequences originating from different maternal and fetal genomic regions. In pregnancies with aneuploid fetuses, the extra or missing copy of the affected chromosome would alter the proportional representation of this specific chromosome in the maternal plasma. This innovative approach is more sensitive and specific, offering a greater detection rate (DR) and a significantly lower false-positive rate (FPR), which would result in a reduction of invasive diagnostic procedures that carry a risk of miscarriage. CfDNA testing is gaining widespread acceptability. However, its high cost is actually still limiting its application to high- and intermediate-risk patients, identified as such by the conventional first-line method of screening. This project is based on a detailed bibliographic search of recent and relevant studies to review the current state of the art regarding the cfDNA screening test and its future perspectives and applicability in clinical practice.

2. KEYWORDS AND ABBREVIATIONS:

Non-invasive prenatal testing (NIPT), first-trimester combined screening (FTS), nuchal translucency (NT), maternal serum pregnancy-associated plasma protein-A (PAPP-A), free-β human chorionic gonadotropin (β-hCG), placental growth factor (PLGF), α-fetoprotein (AFP), cell-free DNA (cfDNA), cell-free fetal DNA (cffDNA), detection rate (DT), false-positive rate (FPR), fetal fraction (FF), chorionic villus sampling (CVS), trisomy 21 (T21), trisomy 18 (T18), trisomy 13 (T13).

3. INTRODUCTION AND BACKGROUND:

In the last three decades, a shift in birth trends has been reported, with more births in women in the "advanced maternal age" (35 years or older). These changing demographics highlighted the need of improving pre-natal screening protocols, as the risk for fetal aneuploidy increases together with maternal age.[1]

Fetal aneuploidy and other chromosomal aberrations have been reported to occur in 9 of 1000 live births.[2] Fetal aneuploidy is a genetic disorder caused by the presence of an abnormal number of chromosomes in the fetus. Chromosomal aneuploidies are major causes of spontaneous miscarriages, perinatal death and childhood handicap. The most common fetal aneuploidies found with significant incidence in the live born are trisomy 21 (T21), trisomy 18 (T18) and trisomy 13 (T13), leading to Down syndrome, Edwards' syndrome and Patau's syndrome respectively. Together with sex chromosome aneuploidies these account for up to 95% of chromosomal abnormalities detected in prenatal samples.[3]

Prenatal screening for fetal trisomies has been a part of routine prenatal care in developed countries over the past few decades. While in the 1970's the main method of risk assessment was by maternal age only, in the 1980's it was focused on a biochemical marker (Alpha-fetoprotein or AFP). Later, in the 1990's, the quad marker screen performed in the second trimester of pregnancy, which included more biochemical parameters, allowed a much higher detection rate, but it wasn't until 2000 that the emphasis shifted to the first trimester (**Figure 1**). It was reported that the majority of affected fetuses could be identified by what they referred to as the first-trimester combined test (FTS), which results in an individual risk for fetal aneuploidy.[4,5]

The first-trimester combined screening test is a non-invasive procedure that is usually performed between the 10th and the 14th week of pregnancy. It is the first-line conventional screening test nowadays, and it is based on sonographic findings together with the measurement of biochemical markers in maternal blood. Specifically, it combines maternal age, fetal nuchal translucency thickness (NT), maternal serum free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein A (PAPP-A). This test has been reported to detect about 90% of pregnancies carrying T21 fetuses at a fixed false-positive rate of 5%.[4–11]

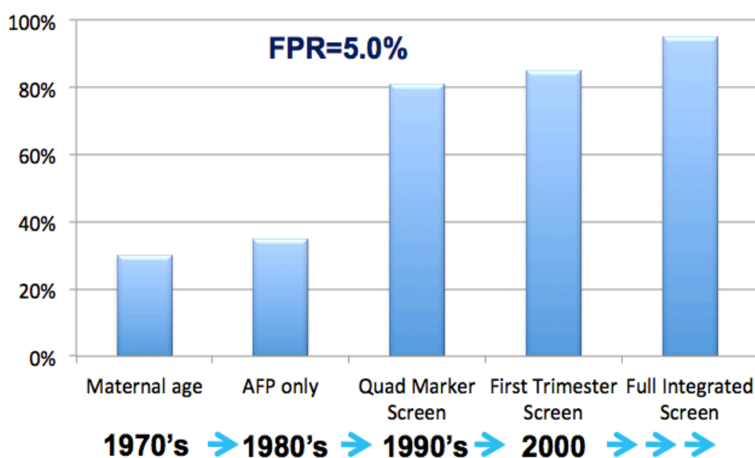

Figure 1. Evolution of trisomy screening. The X axis refers to different non-invasive traditional screening approaches while the Y axis indicates the DT. The DT has greatly improved over the years while the FPR is still fixed around 5%.

Many structural abnormalities can be detected by ultrasound scan during the first trimester of pregnancy. Although these can be related to a wide range of chromosomal abnormalities, several markers related to trisomies 21, 18 and 13 have been described. Nuchal translucency (NT) is the most representative sonographic marker studied during the first trimester of pregnancy. It is usually measured by transabdominal ultrasound and it refers to the presence of fluid under the skin at the back of the fetal neck. In 1992, Nicolaides et al.[12] reported the association between the presence of fetal nuchal translucency of 3mm or more and an increased risk of chromosomal abnormalities. Additionally, they also observed that the risk of chromosomal abnormalities increases with the thickness of the nuchal translucency. Besides the three most prevalent trisomies (T21, T18 and T13), an increased NT value can be related to several other abnormalities and predicting adverse pregnancy outcomes including skeletal dysplasias, neural tube defects, pre-eclampsia and preterm births.[13,14]

As mentioned before, besides the structural and nonstructural sonographic findings, the FTS is also based on biochemical markers, which have reported to be altered in affected pregnancies.[15] The maternal serum concentration of β-hCG in trisomy 21 pregnancies is higher, and PAPP-A is lower compared to euploid pregnancies. On the other hand, both concentrations are decreased in trisomies 18 and 13.[6] As biochemical markers and sonographic findings are independent from each other, the combination of NT with these first-trimester biochemical markers is more effective than either method individually.[6] Finally, the calculated a priori risk based on maternal age is also added to this specific algorithm. All these measurements and observations shape the well-known and routinely established first-trimester combined test, whose advantage as a first-stage policy relies on its relative simplicity. The approach results in an individual risk: patients at a risk cut-off of 1 in 250 or more are considered as “high-risk” or “screen positive”, and patients with a risk smaller than 1 in 1000 are considered as “low-risk” or “screen negative”. Depending on local screening policies, those with the intermediate risk of 1 in 251 to 1 in 1000 might undergo a second-stage screening, which will readjust their first-stage risk.[4,6]

Recent studies have demonstrated that the performance of this test could be improved adding two additional markers: serum placental growth factor (PLGF), α-fetoprotein (AFP) and fetal ductus venosus pulsatility for veins (DV-PIV).[4,8]

After performing the first-trimester combined test, it is recommended that all pregnant women should consider undergoing an invasive diagnostic testing at a risk cutoff of 1:250.[4]

While the screening tests are designed to identify high-risk and low-risk pregnancies, invasive procedures are considered diagnostic tests. Amniocentesis and chorionic villus sampling (CVS) are both invasive procedures commonly performed for prenatal diagnosis. CVS can be performed at the 11th week of pregnancy while amniocentesis needs to be done after the 15th week.[16] Both tests carry procedure-related risks of miscarriage, and thus patients have to be given accurate information in order to take informed choices.[17] There is inconsistency in the recommendations from various official bodies regarding the report of these risks. The Royal College of Obstetricians and Gynaecologists (RCOG) states that there is a 1% risk of miscarriage from an amniocentesis and a 1-2% risk after a CVS.[18] The American College of Obstetricians & Gynecologists mentions that the procedure-related loss rate after mid-trimester amniocentesis is less than 1 in 300-500 and that the loss rate for CVS is approximately the same, and the Society of Obstetricians and Gynaecolgists of Canada states that the risk of pregnancy loss is unique to an individual and based on multiple variables, but ranges from 0,19% up to 1,53%.[17] The procedure of choice following the first-trimester combined screening is likely to be first-trimester CVS rather than second-trimester amniocentesis. Nevertheless, invasive techniques have been improving significantly and nowadays it is highly likely that the procedure-associated risks are much lower than the ones reported some years ago, especially in reference prenatal diagnostic units.

To summarize, the traditional protocol regarding prenatal screening and diagnosis is based on the first-trimester combined test, which differentiates pregnancies in high-, intermediate- and low-risk of fetal aneuploidy, followed by an invasive procedure in case of a high-risk pregnancy. This screening policy is in place in almost all European countries where all pregnancies have access to both the risk assessment and the invasive diagnostic procedure through the public health systems.

4. PROBLEM STATEMENT AND OBJECTIVES:

Actually, the established and traditional first-line screening method consists of the first-trimester combined test, which allows the detection of about 90% of the affected pregnancies at a FPR of 5%.[4–11] Although this approach is quite accurate and a FPR of 5% seems reduced and acceptable, it results in a substantial number of unnecessary

invasive diagnostic tests being performed, and thus, unnecessary procedure-related fetal losses.

In 2011, a new approach was introduced and since then it has been gaining widespread acceptability. It is a non-invasive test based on the analysis of cell-free DNA (cfDNA) in maternal blood using next-generation sequencing technology. Evidence suggests that it can detect more than 99% of cases of trisomy 21, 97% of trisomy 18 and 92% of trisomy 13, at FPRs lower than 0,1%, 0,2% and 0,2% respectively.[7,8,11,19–22] Compared to the first-line method of screening, this new approach offers higher detection rates and much lower FPRs. Thus, cfDNA testing appears to be a significant improvement over traditional multiple-marker screening, and in practice it would result in an important reduction in unnecessary diagnostic procedures. This would lead to a decrease in the number of pregnant women taking the procedure-related risk of miscarriage and also a decrease in the costs associated to the invasive tests.

Clinical studies have mainly included women identified to be at high- or intermediate-risk for aneuploidies assessed by maternal age and biochemical and/or sonographic findings.[5,9,10] Therefore, cfDNA testing was firstly introduced for patients at risk because of advanced maternal age (35 years or older at delivery), fetal sonographic abnormalities associated with increased risk for fetal aneuploidy, history of prior pregnancy with trisomy or a positive test result for aneuploidy risk from conventional prenatal screening tests.[23] More recently, data are being generated to demonstrate the same efficiency also in the general pregnancy population which will likely result in a more extensive use of cfDNA testing as a first- or second-line screening.[9,24,25]

However, although cfDNA testing as a primary screening strategy for the general population meets most of the criteria for an appropriate screening approach, it fails to reach the cost-effectiveness.

The objectives of this review are to describe and analyze the current situation regarding cfDNA testing, especially for trisomies 21, 18 and 13, and understand its potential future implementation as a routine screening test in clinical practice for the general population.

5. METHODOLOGY:

The aim of this project was to review recent publications and relevant studies in order to assess the current situation concerning NIPT and its future perspectives. A database search has been performed throughout May, using mainly PubMed and keywords such as “non-invasive prenatal testing”, “non-invasive prenatal testing”, “NIPT”, “cfDNA” and “cffDNA” among others. The majority of studies were published in “The New England Journal of Medicine”, “Prenatal Diagnosis”, “Fetal Diagnosis and Therapy” and “Ultrasound in Obstetrics and Gynecology”. The electronic search of the databases yield a large number of potential citations, but some of them were excluded after reviewing the title or the abstract. A total of 51 papers, reviews and previous studies were read in full text.

Only English-written papers published from 2012 onwards have been read to extract information about NIPT. On the other hand, as the classic screening methods have been well described since many years ago, older papers were accepted too to describe these approaches.

6. NON-INVASIVE PRENATAL TESTING (NIPT):

6.1: A brief history:

Over the last few decades, numerous attempts have been made to develop new improved non-invasive screening procedures to test for fetal aneuploidy. Initial research focused on the isolation of whole fetal cells from the maternal bloodstream, but this approach proved to be technically difficult and inconsistent, because just a tiny number of such cells can be retrieved – about 10 for every 200.000 billion of the mother’s–.[26] In the late 1990s, researchers discovered that when placental cells break down they released fetal DNA into the maternal bloodstream.[27] This cell-free fetal DNA (cffDNA) showed to be a much more easy way to access fetal DNA, as it represented about the 6-20% of the total cfDNA in a pregnant woman’s bloodstream.[5] cffDNA could be easily detected from the 7th week of gestation, which led to a wide range of possibilities regarding prenatal care. Detecting DNA sequences inherited from the father and absent in the mother led to the first clinical application of non-invasive prenatal diagnosis of fetal blood groups such as RhD-positive fetuses in RhD-negative women, which is a procedure performed routinely nowadays. Following applications included sex determination and paternity studies.[26,28]

Most recent technical developments such as next-generation sequencing (NGS) also referred to as massively parallel genomic sequencing (MPGS) or massively parallel sequencing (MPS) allowed researchers to accurately count maternal and fetal cfDNA fragments enabling determination of fetal chromosome copy number.[2,29–31]

Two groups published proof of principle studies regarding this new screening method in 2008; they both used massively parallel shotgun sequencing (MPSS) to analyze fragments across the whole genome. Specifically, when sequencing about 5 to 10-million short tags (25-36 base pair) and mapping them to individual chromosomes, after counting the number of tags that originated from each chromosome they observed that the proportion of tags coming from the affected chromosome was altered in case of aneuploidy.[2,30] This new approach was seen as an alternative to the traditional screening procedures, which rely on blood biomarkers and sonographic findings and have a false-positive rate of 5%.

It was not until 2011 that next-generation sequencing-based methods, commonly referred to as non-invasive prenatal testing (NIPT), was introduced commercially in the USA.[23,24] From that moment on, cfDNA testing has been gaining popularity and acceptance into clinical practice, although it is still not a first-line method of screening.

6.2: Description of the test:

NIPT is a non-invasive screening method performed to identify fetal aneuploidies by the analysis of cfDNA in the maternal bloodstream. As the placenta is not an impermeable barrier between the mother and the child, there is bidirectional traffic of DNA, proteins and blood cells between them during pregnancy. Small fetal DNA fragments in maternal blood, which are usually <150 base-pairs in length[5,32–34], are though to come from apoptotic trophoblastic cells that release their genetic material (**Figure 2**).[2,5,27,28,34] Thus, about the 6-20% of cfDNA in maternal plasma belongs to the fetus and represents an attractive source of genetic information accessible without any risk for the pregnancy (non-invasive testing).[5]

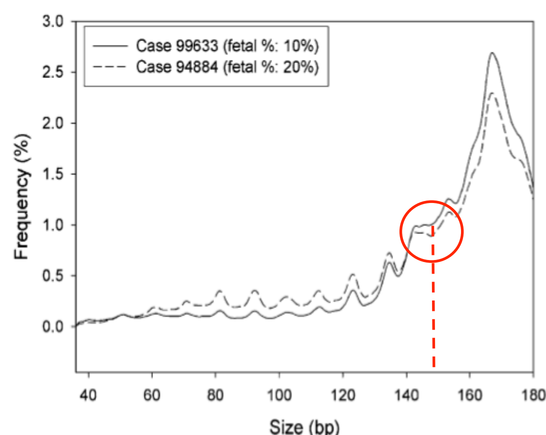

Figure 2. Size distributions of cfDNA in two maternal samples. The samples have different fetal DNA fractions; while the solid line represents a sample with a lower FF, the dashed line represents a sample with a higher FF. The red circle indicates the slightly increase of ≈149 bp DNA molecules' concentration, belonging to the fetus.[32]

NIPT has been shown to have a high degree of accuracy, with DT above 99% for trisomy 21, 97% for trisomy 18 and 92% for trisomy 13.[7,8,11,19–22,35] The high sensitivity and specificity of the test allows false-positive rates below 0,1% for trisomy 21, and about 0,2% for both trisomy 18 and trisomy 13. Therefore, the efficacy of this new screening approach exceeds by far that of conventional screening.[7–9,25]

Maternal peripheral blood samples are usually collected after 10 weeks of gestation to ensure enough fetal DNA is already present in the sample.[11,16,22] Cell-stabilizer solutions are needed in order to preserve the original proportion of cffDNA, as lysis of maternal cells in the sample causes an increase in total cfDNA over time, while the absolute quantity of cffDNA remains constant.[33]

Following plasma separation, a variety of methods can be used to efficiently extract cfDNA from the sample, including high throughput automated workstations.

The test is based on massively parallel sequencing (MPS) and three approaches have been developed:[5,28]

1. **Massively parallel shotgun sequencing (MPSS):** using this technology, millions of DNA fragments in plasma are randomly sequenced. Counting statistics follows the sequencing process.

2. **Targeted massively parallel sequencing (t-MPS):** this focuses the sequencing process to selected genomic regions. The sample is first enriched for regions of interest targeting, for example chromosomes 21, 18, 13, X and Y with chromosome specific probes which are hybridized and ligated before sequencing. It has the advantage of requiring less sequencing thereby reducing the cost. Counting statistics also follows this approach. Recent developments also led to the use of microarrays for detection instead of sequencing.[36]
3. **Single nucleotide polymorphism (SNP)-based approaches:** thousands of SNPs on the chromosomes of interest are selectively amplified by Polymerase Chain Reaction (PCR) and sequenced. Therefore, this approach looks for specific patterns through allelic measurements, and it is followed by investigation of SNP allelic ratios.[37]

For the first two approaches, the generated reads are first aligned – mapped – by standard postsequencing bioinformatics analysis in order to determine the chromosomal origin of each DNA fragment. Usually, only sequences that can be mapped just to one location in the reference genome with one, or no mismatches at all, are considered.[2,30] Then, the number of reads originating from each chromosome is tabulated and counted, which will lead to a specific value that indicates the percentage contribution of sequences mapped to each chromosome. Specifically, to get this value, the count of sequences belonging to a specific chromosome has to be divided by the total number of sequence reads generated in the sequencing run. Finally, to determine if the fetus is likely to present an aneuploidy, the Z-score has to be calculated. As this value refers to the number of standard deviations from the mean of a reference data set, it is expected to be greater in case of a trisomy than in euploid pregnancies. In other words, the Z-score can be converted into a probability that a certain chromosome differs from the normal diploid situation. Thus, results of the test are presented in terms of “positive” or “negative” regarding whether Z-scores exceed predefined thresholds.[2,5,7,16,30,38,39] The procedural framework for using massively parallel shotgun sequencing for NIPT is shown in **Figure 3**, and the results’ representation considering the Z-scores is shown in **Figure 4**.

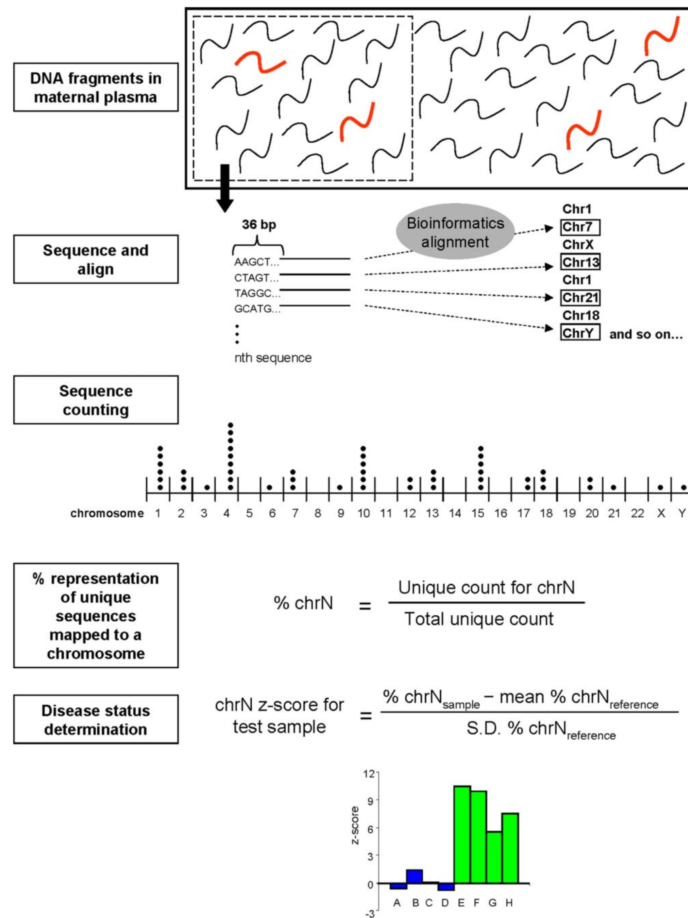

Figure 3. Schematic illustration of massively parallel shotgun sequencing for NIPT. CffDNA (red fragments) circulate in maternal plasma together with other maternal cfDNA molecules (black fragments). After obtaining the plasma sample, in this study the end of each DNA molecule was sequenced using the sequencing-by-synthesis approach. Then, each sequence was mapped to the human reference genome by bioinformatics analysis in order to assess their chromosomal origin. The number of sequences coming from each chromosome was counted and expressed as a percentage of all the sequences generated from the sample, referred to as % chrN for chromosome N. Finally, the formula shown was used to calculate Z-scores for each chromosome of the sample. In pregnancies with aneuploid fetuses, this value is supposed to be higher (cases E-H in green) than in pregnancies with euploid fetuses (cases A-D in blue).[30]

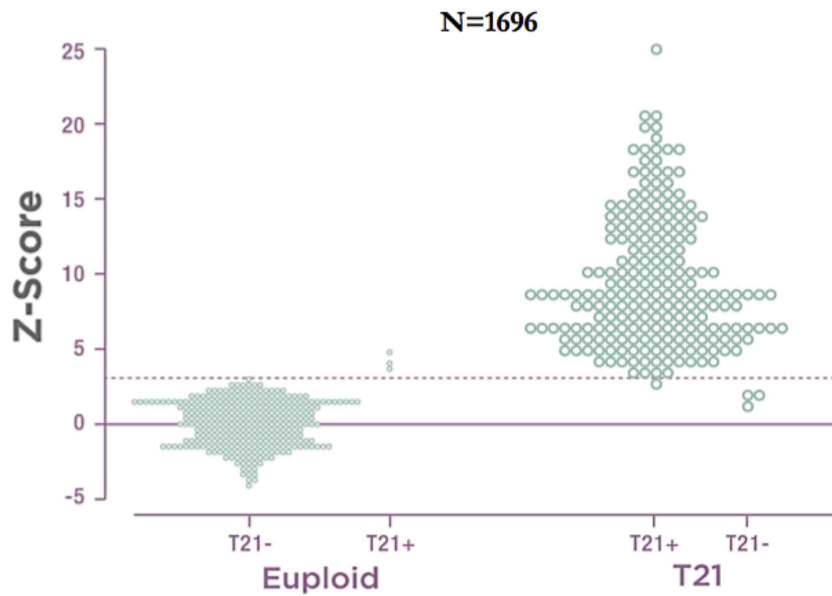

Figure 4. MPSS clinical interpretation regarding the obtained Z-scores. Euploid pregnancies have lower Z-scores than aneuploid pregnancies. All the pregnancies with a Z-score above the threshold are classified as "positive", with no distinction between extremely high values and those just above the cut-off.[40]

Although MPSS has been reported to be a feasible tool for NIPT, it could be further improved by bioinformatics (postsequencing) strategies. For example, the theoretical copy number variations are known for some regions, so the number of sequences that come from there could be adjusted in order to obtain an even tighter reference range for euploid pregnancies. Furthermore, sequences with some mismatches could be also counted, as they may represent a polymorphic difference between the sample and the reference genome thereby increasing the number of usable counts. Moreover, there is a significant CG bias caused by the sequencing process, which should also be considered as it can interfere the mapping process of the generated reads. Due to the lower percentage of GC in chromosomes 13 and 18, the detection of such aneuploidies has been shown to be more challenging. This was reported by Chen *et al.*, who achieved a low sensitivity rate of 73% for T18 and 36% for T13[41], but the development of different algorithms to correct this bias allowed drastic improvements in the detection rates.[2,5,29–31]

To summarize, the ratio from particular chromosomes or regions of interest can be compared with reference values, and small changes in the representation of

chromosomes can be detected with statistically significant power, allowing an accurate identification of fetal chromosome copy number (**Figure 5**).[28]

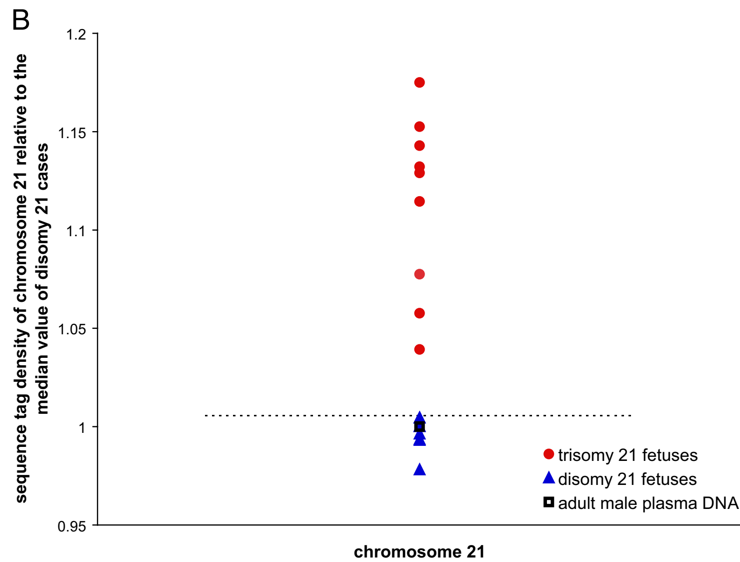

Figure 5. The overrepresentation of the affected chromosome in maternal blood is a good indicator of fetal aneuploidy. This figure shows the chromosome 21 sequence tag density in trisomy 21 fetuses compared to the chromosome 21 sequence tag density of disomy 21 fetuses. The dashed line indicates the upper boundary of the 99% confidence interval, which was calculated from all disomy 21 samples.[2]

One of the key factors that impact the reliability of NIPT is the proportion of fetal DNA in the sample or fetal fraction (FF) (**Figure 6**).[7,16,21,25,28,32,33]

Sample	Fetal DNA content	↑ chr21 (%)	↑ chr21 (fold)
CVS	100%	50%	1.5
Maternal plasma	~ 10%	5%	1.05

Figure 6. The proportion of fetal DNA affects the detection of T21 using cfDNA in maternal plasma. While in a purely fetal sample (i.e.CVS) the increment of chromosome 21 sequences in T21 is 50% (1.5 fold), in a sample with 10% of fetal DNA, the laboratory has to be able to discriminate only a 1.05 fold increment. Thus, NIPT can only be performed with a method that can accurately measure very small decreases or increases in the chromosome or region of interest.

The FF must be calculated when performing NIPT to ensure the reliability of the result. This is usually achieved by detecting and quantifying paternally inherited DNA sequences, which are absent in the mother. For male pregnancies, the presence of cffDNA can be confirmed if Y-chromosome sequences are found in the sample. An alternative approach applicable to both male and female pregnancies is the analysis of Single Nucleotide Polymorphisms (SNPs) which allow quantification of paternally inherited markers.[42]

Moreover, there is a fetal marker independent of paternal inherited sequences, called RASSF1A, which has been shown to be hypermethylated in the placenta and hypomethylated in maternal blood. Thus, a digestion of the hypomethylated maternal signal with restriction enzymes allows the amplification and quantification of the hypermethylated RASSF1A by PCR.[33]

A FF of about 4% is generally considered as the minimum required for a reliable result.[7,16,28] If it is insufficient, the greater amount of maternal cfDNA might mask fetal aneuploidies, leading to false-negative results of the test. The FF increases during pregnancy, and other biological factors, such as gestational age, maternal weight and number of previous gestations, have also been shown to impact the amount of fetal DNA in maternal blood.[16,25,28]

To summarize, cfDNA testing offers a higher sensitivity and specificity at a much lower FPR than the standard screening based on NT measurement and biochemical analytes.[11,22,43] It represents a highly accurate screening option, which besides the most common trisomies, is able to detect some sex chromosome anueploidies that cannot be identified by standard screening.[22] On the other hand, the main advantage of the standard first-trimester screening is its simplicity and low cost. It should also be taken into account that maternal serum and nuchal translucency measurements are able to identify other abnormalities that cfDNA testing is unable to detect; incremental values of NT are associated with other genetic aberrations, as well as altered values concerning the biochemical markers.[25]

6.3: Current target population:

As mentioned before, the majority of the studies regarding NIPT have been performed with women identified to be at high- or intermediate-risk for aneuploidies by conventional screening methods.[7,9,43]

More recently is has also been shown that the test can perform with the same efficiency in the general population, outperforming conventional first-trimester screening (**Figure 7**).[5,9,24,25] Concerns remain in the relation to its possible wide application in the general population due to its high cost, especially in countries where screening programs are well established and supported by public health systems.[7,9,44]

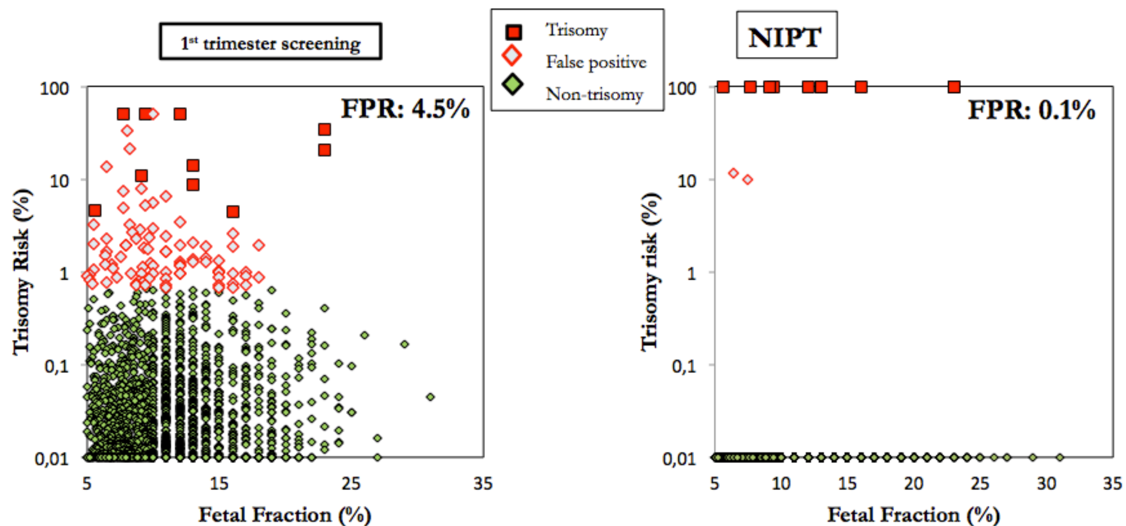

Figure 7. Comparison between 1st trimester traditional screening and NIPT. Both graphics refer to the same number of patients: 10 trisomies (red squares) and 1.939 unaffected pregnancies (green rhombuses). Red rhombuses represent the number of false-positive results, which is much greater in the first-trimester screening test (FPR=4,5%) than in NIPT (FPR=0,1%). This would lead to a high number of unnecessary invasive diagnostic tests being performed. This retrospective study was the first performed in general population, showing that NIPT's accuracy exceeds that of conventional screening tests.[9]

However, nowadays cfDNA testing is considered an appropriate approach just for patients with both singleton pregnancies and twin pregnancies with the most common indicators being: advanced maternal age (35 years or older), fetal ultrasound abnormalities associated with a risk for fetal aneuploidy, a history of a previous pregnancy with trisomy, a parental balanced Robertsonian translocation and a positive test result from conventional prenatal screening tests.[23]

6.4: Twin pregnancies:

The increased use of assisted reproductive techniques worldwide and the increased maternal age at the first pregnancy also increased the incidence of multiple pregnancies. Multiple pregnancies have an increased risk of miscarriage resulting from

invasive testing and the risk for aneuploidies is higher than in singleton pregnancies. Therefore, NIPT would be the ideal option in these situations even if its application presents some technical difficulties. The contribution of cfDNA of each fetus in maternal blood can vary a lot, and in the majority of affected cases the aneuploidy is present in only one of the fetuses. Consequently, if the FF from the healthy fetus is higher than the FF from the affected fetus, the test could provide false-negative results. This could explain the lower detection rate reported in twin pregnancies. To avoid this problem, it has been suggested that the lowest fetal fraction should be used to estimate the risk for aneuploidies, instead of the total fetal fraction.[5,22,45]

Despite these difficulties, a prospective study concluded that screening for trisomies 21, 18 and 13 by cfDNA is also feasible in twin pregnancies, although the failure rate might be higher and the detection rate might be lower than in singleton pregnancies.[45]

6.5: Cost:

Screening tests need to be judged, not only by their sensitivity and specificity in clinical practice but also by their cost-effectiveness. Nowadays, the cost of cfDNA testing, which is around 1000 US$ or 600-800€, is the main reason that prevents its implementation as a first-line screening test in the general population.[5,7,9,22,30,46]

A recent study, focused on Down syndrome, evaluated three different approaches: a primary strategy in which cfDNA testing was offered to all patients, a contingent strategy in which cfDNA testing was offered to the high-risk group identified by the current first-line screening method, and a hybrid strategy in which cfDNA testing was offered to the patients that were 35 years or older and to younger patients who were identified to be at high-risk after the first-trimester traditional screening. They concluded that the primary strategy was significantly more costly than the other strategies, and that the contingent strategy at a risk cut-off of 1/1000 was the lowest-cost alternative.[46]

In conclusion, it seems that the primary cfDNA testing at present is not a cost-effective approach. Its integration in a contingent model (following conventional screening for intermediate risk patients) seems more feasible especially in countries where screening programs are well established. In this case, the high-risk population at a certain risk cut-off, which is suggested to be 1:10-1:50 as identified by the traditional screening methods, would undergo directly an invasive procedure. Intermediate risks up to 1:1000-1:1500 without ultrasound abnormalities would undergo NIPT, and low risk pregnancies would only go through the normal follow up. This would increase the

detection rate of Down syndrome up to 97% reducing the FPRs to almost 0,1% only applying NIPT to about 20% of the population. [7–9,22,35,44]

6.6: Limitations of the test:

Despite the high reliability offered by NIPT, discordant negative and positive findings are still possible. In many cases these could be explained by biological reasons, such as low FF, confined placental mosaicism, maternal mosaicism, maternally derived genetic aberrations (**Figure 8**), undetected tumors and vanishing twins.[10,16,19,38] Technical errors are also a possible cause for discordant results, although infrequent with current laboratory set-ups.

For all these reasons NIPT still has to be considered a screening method, not diagnostic, with all high risk or positive results to be confirmed by diagnostic (invasive) procedures before taking any decision on pregnancy outcome.[9,11,19,21] Invasive testing should also be performed if a high fetal NT is reported, even if NIPT gives low-risk results, as it can be associated with other chromosomal abnormalities besides T21, T18 and T13.[11]

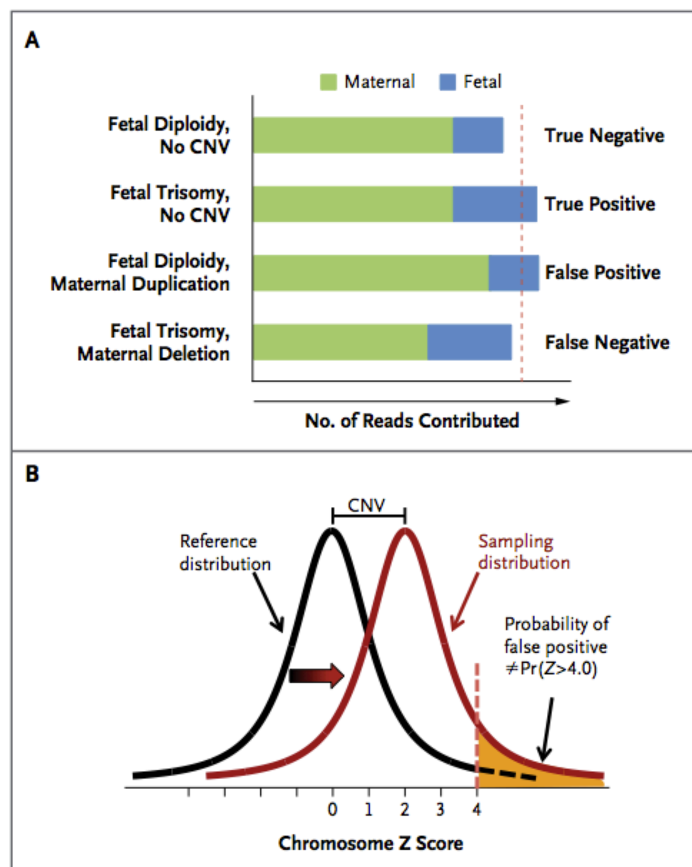

Figure 8. This figure illustrates the role of maternal copy-number variants in false-positive results. In **A**, four different outcomes of the test, depending on the amount of maternal and fetal DNA, are represented. A false-positive result can be caused by a maternal copy-number variant. Also, as it can be seen, cffDNA is contained in a smaller proportion in maternal plasma. The red vertical dashed line indicates the threshold between a negative and a positive test result. In addition, **B** represents the effect of a maternal copy-number variant on the probability of obtaining a false-positive result, as it shifts the whole sampling distribution to the right.[38]

Finally, some published studies face some limitations that should be considered. One of the latest studies published in 2015, demonstrating that cfDNA testing performs better than standard first-trimester screening, reported the false-positive rate of cfDNA testing to be 100 times lower than that of standard screening.[25] However, they were comparing cfDNA testing just with standard first-trimester screening; if they had compared it with integrated first- and second-trimester screening, which includes more parameters like inhibin A or alpha-fetoprotein, the difference would have probably been smaller. Moreover, the study recently published by Bianchi et. al.[24], whose purpose was to compare the false-positive rates of detection of fetal trisomies with the use of standard screening and cfDNA testing, is just focused in the USA, where the first-trimester screening procedures are less extended and less strictly and accurately performed than in Europe. Therefore, the DT of the traditional first-trimester screening tests was lower than the one observed in other countries, leading to an overestimation of the increased efficiency of NIPT.

6.7: Future perspectives and applicability:

At present, NIPT is routinely performed in pregnancies at risk, but it is still not a first-line screening method offered to the general population mainly because of its high cost.[46] There is evidence that this innovative screening approach identifies trisomies accurately at a low false-positive rate in routinely screened first-trimester general population. Nicolaides et al.[9] reported that the accuracy of NIPT is also applicable to all pregnant women in the general population, where the prevalence of trisomies is lower. This finding suggests that the accuracy of the test does not depend on the prevalence of the disease in the study population; it depends on the assay precision and the fetal fraction. Therefore, although NIPT can potentially be used in universal screening for fetal aneuploidy in all singleton pregnancies, the main limitation of its widespread application is its associated cost. If sequencing becomes more affordable in a near

future, the non-invasive screening test could be offered as a routine test to all the pregnant population.

Nevertheless, nowadays the widespread uptake of NIPT into routine clinical practice is likely to follow a contingent strategy, meaning that the test is contingent on the results of the traditional combined test at 11-13 weeks' gestation.[43,46] As only an additional 5-10% of cases are detected compared to the traditional screening protocols, it is better to perform this less costly traditional approach first. This strategy retains the advantages of first-trimester screening, as well as an increased DT and a decreased FPR, but at a considerably lower cost than offering NIPT to the whole population.[8] As mentioned before, pregnancies who were identified to be at a risk ≥1:50 in the combining test should directly undergo invasive testing; pregnancies at a risk between 1:51 and 1:1500 should be offered NIPT, and pregnancies at lower risk would not need to undergo further tests. Therefore, the intermediate-risk population would be given a more accurate risk by a non-invasive test, that would readjust their first-stage risk, which would lead to less invasive procedures being performed.

In clinical practice, NIPT is able to efficiently identify a risk group that requires further investigation by invasive testing, but as its sensitive and sensitivity is not 100%, it should not be considered a diagnostic test to replace CVS or amniocentesis in high-risk pregnancies.[9,11,19,21]

Analysis of sex chromosomes aneuploidies has been routinely included in NIPT. These aneuploidies are often found accidentally when analyzing pregnancies that screened positive for autosomal trisomies, and they are often caused by fetal mosaicism. The inclusion of these parameters in a screening test for the general population should be prudent, as they drastically increase the FPR.[5,22]

Finally, the feasibility of other potential applications of NIPT, such as analysis of microdeletion and duplication syndromes or sequencing the whole fetal genome, requires further validation before clinical implementation. It may be inappropriate to offer pregnant women screening for rare conditions just because it is feasible; parents should be given enough and detailed information about the implications of the test by an expert, and finally, the incorporation of the evaluation of these conditions could result in an increased FPR.[5,10]

7. CONCLUSIONS:

The traditional pre-natal care protocol – combined test followed by CVS or amniocentesis – has offered pregnant women the choice to know if their fetus carries a genetic aberration for many years. But recent advances in cfDNA-based non-invasive prenatal screening have led to screening techniques with significantly better test-performance characteristics than the previous approaches. Non-invasive prenatal testing based on the analysis of cell-free fetal DNA in maternal blood far exceeds the efficacy of traditional screening methods, as it yields results with a higher detection rate of about 99% and a substantially smaller false-positive rate of about 0,1%. NITP can be seen as the perfect solution to a long-standing challenge, as women are willing to get information about their fetus, but they are even more willing not to harm the fetus. As cfDNA testing lowers the FPR, the number of invasive procedures, performed after first-trimester screening tests to the high-risk population, would be smaller, therefore reducing an unnecessary risk of miscarriage.

Although some private obstetrical practices in the United States are already offering cfDNA testing as a first-line screening option, the test is expensive, and thus, its uptake into routine clinical practice is likely to be contingent on the results of the combined test, rather than being the first-line method of screening. This less costly protocol improves the performance of screening for fetal aneuploidy but it also retains the advantages of the combined test, such as early detection of major defects and a wide range of complications, which leads to an optimal pregnancy management and early intervention. Currently, NIPT is not economically viable as a primary screening approach, but as the costs of sequencing might keep decreasing over time, it could be offered to all pregnant women in the near future.

8. ACKNOWLEDGEMENTS:

The author wants to acknowledge Dr. Vincenzo Cirigliano (Molecular Genetics, Labco Diagnostics) for directing and guiding this review, and providing the needed bibliography and orientation, Dr. Mari Paz Cañadas (Molecular Genetics, Labco Diagnostics) for her support and advice, and Dr. Mariano Sentí (Department of Experimental and Health Sciences, FCSV) for tutoring the review.

9. BIBLIOGRAPHY:

1. Ferguson-Smith, M. a & Yates, J. R. Maternal age specific rates for chromosome aberrations and factors influencing them: report of a collaborative european study on 52 965 amniocenteses. *Prenat. Diagn.* **4 Spec No,** 5–44 (1984).

2. Fan, H. C., Blumenfeld, Y. J., Chitkara, U., Hudgins, L. & Quake, S. R. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 16266–16271 (2008).

3. Cirigliano, V., Voglino, G., Adinolfi, M. Non-invasive screening and rapid QF-PCR assay can greatly reduce the need for conventional cytogenetic analyses in prenatal diagnosis. *Reproductive BioMedicine Online.* **11,** 2–4 (2005).

4. Wright, D., Syngelaki, a., Bradbury, I., Akolekar, R. & Nicolaides, K. H. First-trimester screening for trisomies 21, 18 and 13 by ultrasound and biochemical testing. *Fetal Diagn. Ther.* **35,** 118–126 (2014).

5. Benn, P., Cuckle, H. & Pergament, E. Non-invasive prenatal testing for aneuploidy: current status and future prospects. *Ultrasound Obstet. Gynecol. Off. J. Int. Soc. Ultrasound Obstet. Gynecol.* **42,** 15–33 (2013).

6. Nicolaides, K. H. Screening for fetal aneuploidies at 11 to 13 weeks. *Prenat. Diagn.* **31,** 7–15 (2011).

7. Wright, D., Wright, a. & Nicolaides, K. H. A unified approach to risk assessment for fetal aneuploidies. *Ultrasound Obstet. Gynecol.* **45,** 48–54 (2015).

8. Nicolaides, K. H., Syngelaki, a., Poon, L. C., Gil, M. & Wright, D. First-Trimester Contingent Screening for Trisomies 21, 18 and 13 by Biomarkers and Maternal Blood Cell-Free DNA Testing. *Fetal Diagn. Ther.* (2013). doi:10.1159/000356066.

9. Nicolaides, K. H., Syngelaki, A., Ashoor, G., Birdir, C. & Touzet, G. Noninvasive prenatal testing for fetal trisomies in a routinely screened first-trimester population. *Am. J. Obstet. Gynecol.* **207,** 374.e1–374.e6 (2012).

10. Chitty, L. S. Use of Cell-free DNA to Screen for Down's Syndrome. *N. Engl. J. Med.* 150401140035006 (2015). doi:10.1056/NEJMe1502441.

11. Gil, M. M., Quezada, M. S., Bregant, B., Ferraro, M. & Nicolaides, K. H. Implementation of maternal blood cell-free DNA testing in early screening for aneuploidies. *Ultrasound Obstet. Gynecol.* **42,** 34–40 (2013).

12. Nicolaides, K. H., Azar, G., Byrne, D., Mansur, C. & Marks, K. Fetal nuchal translucency: ultrasound screening for chromosomal defects in first trimester of pregnancy. *BMJ* **304,** 867–869 (1992).

13. Souter, V. L. & Nyberg, D. a. Sonographic screening for fetal aneuploidy: first trimester. *J. Ultrasound Med.* **20,** 775–790 (2001).

14. Huang, T. *et al.* First trimester screening for Down syndrome using nuchal translucency, maternal serum pregnancy-associated plasma protein A, free-β human chorionic gonadotrophin, placental growth factor, and α-fetoprotein. *Prenat. Diagn.* n/a–n/a (2015). doi:10.1002/pd.4597.

15. Brambati, B. *et al.* Serum PAPP-A and free β-hCG are first-trimester screening markers for down syndrome. *Prenat. Diagn.* **14,** 1043–1047 (1994).

16. Song, Y. *et al.* Non-invasive prenatal testing for fetal aneuploidies in the first trimester of pregnancy. *Ultrasound Obstet. Gynecol.* **45,** 55–60 (2015).

17. Akolekar, R., Beta, J., Picciarelli, G., Ogilvie, C. & D'Antonio, F. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. *Ultrasound Obstet. Gynecol.* 16–26 (2014). doi:10.1002/uog.14636.

18. Guideline summary for amniocentesis and chorionic villus sampling. *Royal College of Obstetricians and Gynaecologists (RCOG).* London (UK); 8 (2010).

19. Cheung, S. W., Patel, A. & Leung, T. Y. Accurate Description of DNA-Based Noninvasive Prenatal Screening. *N. Engl. J. Med.* **1,** 150401140035006 (2015).

20. Nicolaides, K. H., Wright, D., Poon, L. C., Syngelaki, A. & Gil, M. M. First-trimester contingent screening for trisomy 21 by biomarkers and maternal blood cell-free DNA testing. *Ultrasound Obstet. Gynecol.* **42,** 41–50 (2013).

21. Quezada, M. S., Gil, M. M., Francisco, C., Oròsz, G. & Nicolaides, K. H. Screening for trisomies 21, 18 and 13 by cell-free DNA analysis of maternal blood at 10-11 weeks' gestation and the combined test at 11-13 weeks. *Ultrasound Obstet. Gynecol.* **45,** 36–41 (2015).

22. Gil, M. M., Akolekar, R., Quezada, M. S., Bregant, B. & Nicolaides, K. H. Analysis of Cell-Free DNA in Maternal Blood in Screening for Aneuploidies: Meta-Analysis. *Fetal Diagn. Ther.* 156–173 (2014). doi:10.1159/000358326.

23. Futch, T. *et al.* Initial clinical laboratory experience in noninvasive prenatal testing for fetal aneuploidy from maternal plasma DNA samples. *Prenat. Diagn.* **33,** 569–574 (2013).

24. Bianchi, D. W. *et al.* {DNA} Sequencing versus Standard Prenatal Aneuploidy Screening. *N Engl J Med* **370,** 799–808 (2014).

25. Norton, M. E. *et al.* Cell-free DNA Analysis for Noninvasive Examination of Trisomy. *N. Engl. J. Med.* 150401140035006 (2015). doi:10.1056/NEJMoa1407349.

26. Allison, M. Genomic testing reaches into the womb. *Nature Biotechnology* **31,** 595–602 (2013).

27. Dennis Lo, Y. M. *et al.* Presence of fetal DNA in maternal plasma and serum. *Lancet* **350,** 485–487 (1997).

28. Thung, D. T., Beulen, L., Hehir-Kwa, J. & Faas, B. H. Implementation of whole genome massively parallel sequencing for noninvasive prenatal testing in laboratories. *Expert Rev. Mol. Diagn.* **15,** 111–124 (2015).

29. Liao, C. *et al.* Noninvasive prenatal diagnosis of common aneuploidies by semiconductor sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 7415–20 (2014).

30. Chiu, R. W. K. *et al.* Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 20458–20463 (2008).

31. Lo, Y. M. D. Non-invasive prenatal diagnosis by massively parallel sequencing of maternal plasma DNA. *Open Biol.* **2,** 120086 (2012).

32. Yu, S. C. Y. *et al.* Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 8583–8 (2014).

33. Lench, N. *et al.* The clinical implementation of non-invasive prenatal diagnosis for single-gene disorders: Challenges and progress made. *Prenat. Diagn.* **33,** 555–562 (2013).

34. Jeon, Y. J. *et al.* The feasibility study of non-invasive fetal trisomy 18 and 21 detection with semiconductor sequencing platform. *PLoS One* **9,** e110240 (2014).

35. Gil, M. M., Giunta, G., Macalli, E. a., Poon, L. C. & Nicolaides, K. H. UK NHS pilot study on cell-free DNA testing in screening for fetal trisomies: factors affecting uptake. *Ultrasound Obstet. Gynecol.* **45,** 67–73 (2015).

36. Juneau, K. *et al.* Microarray-Based Cell-Free DNA Analysis Improves Noninvasive Prenatal Testing. *Fetal Diagn. Ther.* **36,** 282–286 (2014).

37. Dar, P. *et al.* Clinical experience and follow-up with large scale single-nucleotide polymorphism-based non-invasive prenatal aneuploidy testing. *Am. J. Obstet. Gynecol.* **211,** 527.e1–527.e17 (2014).

38. Coe, B. P. *et al.* Copy-Number Variation and False Positive Prenatal Aneuploidy Screening Results. 1–7 (2015). doi:10.1056/NEJMoa1408408.

39. Srinivasan, A., Bianchi, D. W., Huang, H., Sehnert, A. J. & Rava, R. P. Noninvasive Detection of Fetal Subchromosome Abnormalities via Deep Sequencing of Maternal Plasma. *Am. J. Hum. Genet.* 1–10 (2013). doi:10.1016/j.ajhg.2012.12.006.

40. Palomaki, G. E. *et al.* DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet. Med.* **13,** 913–20 (2011).

41. Chen, E. Z. *et al.* Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma dna sequencing. *PLoS One* **6,** 1–7 (2011).

42. Sparks, A. B., Struble, C. a., Wang, E. T., Song, K. & Oliphant, A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from

maternal blood: Evaluation for trisomy 21 and trisomy 18. *Am. J. Obstet. Gynecol.* **206,** 319.e1–319.e9 (2012).

43. Song, K., Musci, T. J. & Caughey, A. B. Clinical utility and cost of non-invasive prenatal testing with cfDNA analysis in high-risk women based on a US population. *J. Matern. Fetal. Neonatal Med.* **7058,** 1–6 (2013).

44. Kagan, K. O., Wright, D. & Nicolaides, K. H. First-trimester contingent screening for trisomies 21, 18 and 13 by fetal nuchal translucency and ductus venosus flow and maternal blood cell-free DNA testing. *Ultrasound Obstet. Gynecol.* **45,** 42–47 (2015).

45. Bevilacqua, E. *et al.* Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies. *Ultrasound Obstet. Gynecol.* **45,** 61–66 (2015).

46. Evans, M. I., Sonek, J. D., Hallahan, T. W. & Krantz, D. a. Cell-free fetal DNA screening in the USA: a cost analysis of screening strategies. *Ultrasound Obstet. Gynecol.* **45,** 74–83 (2015).