Non-invasive prenatal testing using fetal DNA

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Running title: NIPT based on cell free fetal DNA

Abstract

Non-invasive prenatal diagnosis (NIPD) is based on fetal DNA analysis starting from a simple peripheral blood sample, which eliminates the risks associated with conventional invasive techniques.

During the pregnancy, the fetal DNA increases to approximately 3-13% of the total circulating free DNA in maternal plasma. The very low amount of circulating cell-free fetal DNA (ccffDNA) in maternal plasma is a crucial issue, and requires specific and optimized techniques for ccffDNA purification from maternal plasma. In addition, highly sensitive detection approaches are required. In recent years, advanced approaches for ccffDNA investigation have allowed the application of non-invasive prenatal testing (NIPT) to determine fetal sex, fetal rhesus D (RhD) genotyping, aneuploidies, micro-delections, and the detection of paternally inherited monogenic disorders.

Finally, complex and innovative technologies such as digital PCR (dPCR) and Next Generation Sequencing (NGS) (exhibiting higher sensitivity and/or capability to read the entire fetal genome from maternal plasma DNA) are expected to allow the detection of maternally inherited mutations causing genetic diseases in next future.

The aim of this review is to introduce the principal ccffDNA characteristics and their applications as source for current and novel NIPT.

Key Points

The ccffDNA is the source for non-invasive prenatal testing (NIPT) without risk to the fetus or the mother.

The current commercial kits for NIPT are able to detect the most common aneuploidies and micro-deletions.

Novel molecular strategies (such as dPCR and NGS) able to identify single point mutations causing genetic disorders could be useful for NIPT of maternally inherited monogenic diseases.

1 Introduction

Non-invasive prenatal diagnosis (NIPD) is based on the discovery, in 1997, of circulating cell-free fetal DNA (ccffDNA) within maternal plasma [1] and it is aimed to identify genetic abnormalities from the analysis of maternal blood during pregnancy. Currently, the first-trimester prenatal diagnosis requires invasive obstetric procedures, such as amniocentesis or chorionic villus sampling (CVS), carrying a potential miscarriage risk (with a frequency of approximately 0.5-1%) [2]. The ccffDNA discovery has permitted the development of safer and earlier testing procedures based on a simple maternal blood sample.

The non-invasive prenatal testing (NIPT) provides the determination of fetal sex [3], fetal rhesus D (RhD) genotyping [4], some pregnancy-associated conditions including preeclampsia [5,6], aneuploidies [7], and the identification of paternally inherited monogenic disorders [7-9]. Lately, significant advances have been investigated which would extend the potential applications to fetal whole-genome sequencing and maternally inherited mutations [10-14].

2 Circulating fetal DNA

In 1997, Lo et al. discovered the presence of fetal DNA in maternal blood, applying a simple and sensitive Y chromosome-specific PCR assay to detect circulating fetal DNA from women bearing male fetuses [1]. A year later, Lo et al. [15] demonstrated that fetal DNA concentration was very similar in maternal plasma and serum, but in serum a larger maternal DNA content is present, causing a less efficient detection of fetal DNA. For this reason, maternal plasma is preferred as the source of fetal DNA to be employed for diagnosis purposes. Furthermore, using quantitative real-time PCR (qPCR), they

determined the concentration of fetal DNA in maternal plasma as 3.4-6.2% of total circulating DNA, at early and late gestation, respectively [15].

While in the maternal blood different fetal cell types have been found (including trophoblasts, leukocytes, nucleated red blood cells and erithrocytes) [16], a high amount of ccffDNA is present in maternal plasma compared to fetal DNA extracted from the cellular component of maternal blood [17]. Therefore, ccffDNA is generally preferred for diagnostic purposes.

2.1 Amount and size of ccffDNA

Either there is a general agreement on the fact that the enrichment of ccffDNA in the blood is caused by (a) apoptosis of fetal hematopoietic cells [18], (b) transfer of fetal cell-free DNA through the placenta [19] and (c) trophoblast destruction [20], being this cause the most important.

In fact, the principal origin of fetal nucleic acids in maternal plasma is due to trophoblast breakdown as apoptotic fragments included in microvesicles [21] associated with continuous trophoblast turnover. These fetal cells quickly disappear after birth when the placenta is removed. A further cause of increased fetal DNA concentration is the placental expansion and the extension of the maternal-fetal interface associated to the progression of the pregnancy progresses [19,22].

As reported by Lo et al. [15], in maternal plasma fetal DNA reaches a mean of 25.4 and 292.2 genome equivalents/ml in early and late pregnancy, respectively, being the genome equivalent defined as the amount of a DNA sequence present in one diploid cell.

Fetal DNA comprises only a small portion of total cell-free DNA close to 10-20% in the last weeks of gestation [23], with a fetal DNA detection time spanning from 4-5 weeks of gestation until delivery [24].

The proportion of ccffDNA grows by 0.1% every seven days between the 10th and 21st gestational week; after the 21st week this increase is faster with a weekly 1% increase [25]. It should be underlined that the amount of circulating fetal DNA depends, in addition to the gestation period and the progression of the pregnancy, on other factors, such as presence of maternal diseases, body weight [26], aneuploidies [25] and twin pregnancies [27,28], The very low amount of ccffDNA in maternal plasma is of course critical issue, requiring highly specific and efficient techniques for its purification one hand and its sensitive detection on the other.

Another issue affecting diagnostic protocols is that circulating cell-free DNA in maternal circulation is highly fragmented. With respect to this point, it was found that the 85,5% of fetal DNA is shorter than 0.3 kb in early pregnancy (13 or 15 weeks of gestation) [28]. It constitutes the 28,4% of the < 0,3 Kb fraction in maternal plasma, increasing to 68,7% in the third trimester [28]. Chan et al. [29] and Li et al. [30] reported that 99% of fetus-derived DNA is shorter than 313 bp, whereas cell-free maternal DNA had a medium length of about 400-500 bp. In conclusion, while the length of ccffDNA is comprised, approximately, between 150-300 base pairs, the entire fetal genome is represented [29,30].

2.2 Stability of ccffDNA

The stability of ccffDNA is a key factor for obtaining an optimal DNA extraction, allowing better performance and greatly facilitating diagnostic analyses. While fetal DNA appears to be quite stable in the maternal plasma, it is promptly cleared after the birth, therefore eliminating the risk of its persistence into the next pregnancy, causing in this case confounding effects [31-33]. Stability of ccffDNA is reached with a particular care during purification and handling and a right storage procedure, which is recommended to be performed at -80°C [34]. Moreover, it has been found that the extraction efficiency is also

affected by the storage temperature of blood before plasma preparation, suggesting 4°C as the optimum temperature after sample collection [35]. In addition, in maternal plasma the fetal fraction can be reduced due to a maternal blood cellular lysis in the time-lapse between blood sampling and plasma preparation [34]. To overcome this drawback, treatment of blood samples with formaldehyde has been proposed, in order to prevent cell lysis and plasma DNase activity, leading to an increased fetal DNA recovery [36].

3 Technologies applied to prenatal diagnosis based on ccffDNA

3.1 Purification and enrichment strategies of ccffDNA

In respect to purification techniques suitable for ccffDNA extraction, several studies have compared different commercially available DNA extraction kits [40,41]. Two on the most frequently employed purification systems are QIAamp® Circulating Nucleic Acid Kit (Qiagen) and QIAamp® DSP Virus Spin Kit (Qiagen) [40,41]. In any case, in NIPT, the background due to circulating maternal DNA interferes with the sensitivity and detection of fetal genetic features, and therefore the ccffDNA concentration in maternal plasma becomes a critical limit, requiring enrichment strategies to increase ccffDNA or fetal cells from maternal plasma.

One possibility for the enrichment of the shorter fetal fragments (143 bp fragments for fetal DNA, in respect to 166 bp fragments for maternal DNA [37]) has been proposed based on gel size selection. This was applied to increase the sensitivity of paternal allele detection for β-thalassemia mutations [38]. Alternatively, Lun et al. [39] described a method to discriminate maternal and fetal molecules using 179 and 64 bp amplicons obtained with primers for zinc finger protein ZFX and ZFY genes. Size-based enrichment of fetal DNA is

not routinely applied to clinical protocols. Therefore, novel enrichment strategies are highly needed for increasing the proportion of ccffDNA in the DNA samples to be analyzed.

The importance of the enrichment step is demonstrated by the finding that the purification of circulating DNA fractions of lower molecular weight (100-300 bp) after agarose gel electrophoresis (associetd with increase of the fetal DNA fraction) improves the detection of point mutations and other fetal genotyping activity [42]. COLD-PCR (Co-Amplification at Lower Denaturation Temperature) has also been proposed as enrichment method, selectively amplifying minority alleles within a background of wild-type alleles [43]. Finally, the new frontier for fetal DNA enrichment might be based on the extensively studied mechanisms of epigenetic regulation such as DNA methylation. In this respect specific differentially methylated regions in maternal/fetal DNA have been identified and used as biomarkers for ccffDNA enrichment and NIPT of aneuploides [44], fetal trisomies [45], Down syndrome [46].

3.2 NIPD based on ccffDNA

In case of NIPD, molecular analysis techniques are required in order to detect fetal genetic alterations from ccffDNA. Some of the frequently proposed methods are well established, such as qPCR and PCR-based approaches (e.g. Nested PCR, PAP (pyrophosphorolysis-activated polymerization). Other experimental strategies, useful for NIPD, might be dPCR and NGS, which are both able to characterize very low amounts of fetal DNA, the crucial issue of non-invasive testing.

3.2.1 dPCR

The dPCR is a very sensitive method for DNA or RNA quantification without calibration curve. This technique is based on amplification of a single template using limited diluted samples, producing amplicons mainly derived from one template and detectable using different fluorophores [47].

In particular, for each analyzed target, the sample reaction is partitioned into individual wells before the amplification step [47]. Then a PCR reaction is performed using fluorescent probes (such as in qPCR) and the end-point fluorescence is measured at the end of the amplification, in order to discriminate positive and negative events for each target [48]. In addition, the absolute quantification of target is calculated using statistical Poisson distribution [48]. In this way, dPCR permits to convert the exponential/analog results, typical for classical PCR to linear/digital signals, performing statistical analysis of the PCR reactions [47]. The precision and the reproducibility of this method depend on the high number of partitions of diluted sample, increasing the sensitivity and accurancy of the technique [49] and allows the application to different fields of diagnostics, such as the study of the genetic alterations of the fetus [49,50], the absolute quantification of ccffDNA [51,52], the analysis of the copy number variations usually found in tumorigenesis processes [53], the gene expression analysis of microRNAs involved in cell cycle, apoptosis, cell differentiation, cancer [54], viral load quantification [55].

3.2.2 Next Generation Sequencing (NGS)

NGS, massively parallel or deep sequencing are correlated terms indicating high-throughput DNA sequencing technology capable to analyze millions or even billions of

sequencing reactions at the same time [56]. NGS permits to sequence entire human genome, but also specific regions, such as coding or target genes, amplicons or RNA.

Different NGS platforms are now available, based on various technical principles, such as sequencing by synthesis, pyrosequencing, sequencing by ligation and ion semiconductor sequencing [57], but having some common characteristics. In particular, all NGS platforms require a library of small fragments obtained by chemical or enzymatic approaches. At the ends of the fragments, adapters are linked and used both for the clonal amplification (to obtain a measurable signal, e.g. fluorescence intensity or pH difference, allowing sequence determination) and for the sequencing reaction. The amplification generates DNA clusters, each originating from a single library fragment and optically read from repeated cycles of nucleotide incorporation. At the end of the sequencing, all generated reads are analyzed by bioinformatic tools and aligned to a known reference genome [56]. Recently, innovative NGS platform, based on single-molecule real-time sequencing (SMRT), has been developed using more sensitive detection system and able to sequence single DNA molecules without the clonal pre-amplification step, that can introduce errors during amplification reaction [58].

NGS still presents some limits for its application in NIPD, as sequencing to a depth sufficient to detect fetal DNA genotype is still quite expensive and time consuming. Nevertheless, it is considered the basis of commercially available NIPT, currently offered to detect fetal sex, common chromosomal aneuploidies and small insertions and deletions [59].

4 NIPT applications

Since the discovery of ccffDNA in maternal plasma [1], NIPT has been applied to different issues of great interest such as fetal gender determination and dection of X-linked disorders, fetal rhesus D genotyping, detection of aneuploidies, studies on monogenic disorders (Table 1).

4.1 Fetal gender determination and X-linked disorders

The first application of ccffDNA in maternal plasma was aimed at determining the fetal gender. This issue is extremely important when the mother is carrier of an X-linked disorder, such as Duchenne muscular dystrophy or hemophilia, because pregnancies with male fetuses are primarily at risk. In addition this issue is of interest for pregnant women at risk of conditions associated with ambiguous development of external genitalia (for example in the case of congenital adrenal hyperplasia) where early maternal treatment with dexamethasone can reduce the degree of virilization of female fetuses [60,61]. Furthermore, the value of cffDNA in maternal plasma, when looking at copy number of Y-specific sequence, has been reported as an indicator for preeclampsia [62]. In particular Zhong et al. demonstrated higher ccffDNA levels in plasma samples collected in the second trimester for pregnant women who later developed preenclampsia.

The fetal sex is routinely determined by ultrasound scanning (in the second-trimester) or amniocentesis or CVS (in the first-trimester), but for earlier diagnosis NIPT can be applied using ccffDNA in maternal plasma and approaches based on qPCR.

The most commonly used technology for detecting male fetus–specific DNA in maternal plasma is qPCR amplifying the single copy *SRY* (sex-determining region Y) gene located on chromosome Y as target gene [63], the single-copy sequence (*DYS14*) [64] and the

multicopy *DAZ* gene [65].

Devaney et al. [66] reported a systematic review including a PubMed based meta-analysis (January 1, 1997-April 17, 2011), identifying 146 publications used to determine the clinical validity of noninvasive prenatal sex determination based on ccffDNA analysis in maternal blood and urine, using PCR or qPCR. Despite the expected variability among the considered studies, the overall sensitivity (95.4%) and specificity (98.6%) of the employed technologies were high but when the analytical tests were performed using blood isolated before 7 weeks of gestation, they were found to be unreliable [66]. This conclusion has been confirmed in other studies [3,67].

Another paper by Breveglieri et al. [68] demonstrated that cffDNA, obtained during early gestational time periods and not suitable for detectation of SRY gene target by conventional qPCR, can be identified with a high accuracy and reliability employing Surface Plasmon Resonance (SPR)-based biosensors. In fact samples obtained from maternal plasma at early gestational age were able to generate a positive SPR signal, using a preamplification step generating PCR products which were then injected onto SPR-biosensor chip flow cells, allowing the identification of fetal sex with a high degree of accuracy after the 7th week of gestation.

However, we like to point out that NIPD for the identification of a female fetus based on null results expected using Y-chromosome-specific sequences may be the source of false negative results if the amount of male fetal DNA is too low to be detected by qPCR. In addition, this method could lead to false positive results if the sample is contaminated by male DNA, during the extraction from the maternal plasma.

Therefore other approaches are required for selectively detecting also female fetal DNA, such as assays based on epigenetic markers. Some genes have been identified displaying a differential methylation pattern in maternal blood cells and in fetal placenta [69,70], allowing to distinguish maternal from fetal DNA. For example, the *maspin* gene

(*SERPINB5*) promoter is unmethylated in the placenta but hypermethylated in maternal blood cells [69,71], while the RASSF1A gene (a tumor suppressor gene) is unmethylated in maternal cells and hypermethylated in the placenta [70].

Another strategy to detect fetal gender has been suggested by Tang et al. [72], who successfully identified female fetuses from maternal plasma using paternally-inherited tandem repeats (STR) located on the X chromosome.

Another aspect in NIPD of X-linked disorders is first the investigation of the maternal defect in maternal plasma and then the detection of disease status in male fetus. For example Tsui et al. [73] analyzed maternal mutations on 12 plasma samples obtained from 7 pregnancies carrying hemophilia risk using microfluidic dPCR. In particular, the relative mutation dosage, calculated by detecting the possible overrepression of the mutant or wild-type allele concentrations in heterozygous women carrying male fetuses, allowed to correctly determine the fetal genotype in all studied cases [73].

4.2 Fetal rhesus D genotyping

The development of a non-invasive method for the determination of fetal rhesus D (RhD) status in Rh- pregnant women, has been demonstrated of fundamental importance both to provide a prophylaxis (administrating anti-D immunoglobulins) in pregnancies at high risk for this condition, and to avoid risks (such as miscarriage, feto-maternal hemorrhage and sensitization) caused by conventional sampling methods (amniocentesis and CVS) [74].

NIPD of fetal RhD genotypes has been accurately performed using techniques based on PCR [75-77]. However, a small percentage of cases has been identified as false positive or negative, due to: (a) lack of fetal DNA in maternal plasma at early gestation; (b) low sensitivity of employed technology in detecting low quantity of fetal DNA [77].

It has been demonstrated that the RhD locus has many variants depending on the maternal geographical origin [78]. At present, in order to reduce the amount of false positive/negative results, large-scale clinical trials are being performed in the European Union taking in consideration racial differences and performing fetal RHD screening with high sensitivities (99.9%) PCR [79].

4.3 Aneuploidies

Diagnosis of fetal structural chromosomal anomalies have been routinely performed by ultrasound scanning or amniocentesis and CVS, but for earlier NIPT, sequencing technologies can be proposed.

NIPD has been successfully applied to the detection of the most common aneuploidies, in order to identify pregnancies at risk for trisomy 21 (Down syndrome), 18 (Edwards syndrome) and 13 (Patau syndrome) [80,81].

Currently, commercially-available NIPTs permit to disclose common chromosomal aneuploidies (trisomy 21, trisomy 18 and trisomy 13), sex chromosome abnormalities, triplody and are based on sequencing approach for gathering the genetic information of ccffDNA (Table 2). These approaches are, at present, available from the 9th-10th week of gestation and have been extended to allow the detection of microdeletions, microduplications and paternally inherited mutations causing genetic diseases or/and carrier status [90,91]. Unfortunately, these types of investigations are only considered as screening tests; therefore positivity should be confirmed using amniocentesis or CVS.

Another advanced technology applied to the analysis and characterization of aneuploidies for the accurate measurement of chromosomal aneuploidy using ccffDNA obtained from maternal plasma is dPCR [92,93]. For example, Lee et al. [93] applied the dPCR to the

trisomy 21 diagnosis on 877 maternal plasma samples introducing cut-off value and size selection method with 99,66% accuracy.

4.4 Monogenic diseases

The development of a simple, quick and cheap NIPT for monogenic disorders is of great interest due to their high incidence. However, the detection of fetal single point mutations from ccffDNA is extremely difficult because the maternal and fetal sequences are very similar; therefore the diagnosis of monogenic disorders remains limited to the detection of paternally inherited mutations. When the disease is autosomal dominant and the father carries the mutation or this occurs spontaneously *de novo* during oocyte or sperm formation, it can be easily detected by using the same approach employed in fetal sex or RhD status determination based on qPCR [91]. However, in case of an autosomal recessive disorder, this is significantly harder to be diagnosed, because the maternally inherited portion of the fetal genome and maternal DNA are identical. Therefore the development of strategies able to compare the mutant and the wild-type alleles at a quantitative level is necessary in order to determine whether the fetus has inherited the mutant or the normal one [94].

It is not surprising that NIPT needs to be highly sensitivite, since fetal DNA in maternal plasma is 20% of total DNA in the third trimester of pregnancy, but only 10% or less in the first trimester, when it would be useful for prenatal diagnosis [91]. Recently, despite the fact that many non-invasive approaches have been developed based on PCR technologies, mass spectrometry (MS), NGS, none of them has been approved and employed in the routine clinical practice (Table 3). This was mainly due to low sensitivity and reproducibility and the expensive and complex instrumentations requiring specialised staff [91].

Recently, advanced technologies including dPCR and NGS have allowed clinical application of NIPT implementation because of high sensitivity and capability to reveal the entire fetal genome from maternal plasma DNA. In the future, they are expected to detect also maternally inherited mutations [13,94,106,110].

5 Conclusions and future perspectives

In recent years NIPD has been becoming increasingly important, allowing the investigation of the fetal health status without risk for the fetus or the mother, although only with a predictive value which is expected to be affected by false-positive rates. In fact, in addition to technical problems, different biological factors can induce false-positive results in NIPT, such as fetal or maternal mosaicism, tumors, maternal duplication events [111]. For example, in order to reduce the false-positive rates in NIPT applied to fetal chromosome aneuploidies, Strom et al. designed a clinical assay based on massive parallel shotgun sequencing (MPSS), combining automation of all manual processes with bioinformatics and biostatistics tools [111].

While current commercial NIPT kits can detect the most common aneuploidies and micro-deletions, novel molecular strategies able to identify single point mutations causing genetic disorders are highly needed. These include NIPT for maternally inherited monogenic diseases. In the future, advanced technologies, as dPCR and NGS, could be very useful for translating NIPT from the laboratory research to clinical practice also in the case of autosomal recessive diseases caused by maternal mutated alleles.

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References

1. Lo YM, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350(9076):485-7.
2. Mujezinovic F, Alfirevic Z. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review. Obstet Gynecol. 2007;110(3):687-94.
3. Perlado-Marina S, et al. Overview of Five-Years of Experience Performing Non-Invasive Fetal Sex Assessment in Maternal Blood. Diagnostics (Basel). 2013;3(2):283-90.
4. Clausen FB, Damkjær MB, Dziegiel MH. Noninvasive fetal RhD genotyping. Transfus Apher Sci. 2014;50(2):154-62.
5. Rolnik DL, et al. Association between fetal fraction on cell-free DNA testing and first trimester markers for pre-eclampsia. Ultrasound Obstet Gynecol. 2018. doi: 10.1002/uog.18993.
6. van Boeckel SR, et al. Cell-free Fetal DNA and Spontaneous Preterm Birth. Reproduction. 2017. doi: 10.1530/REP-17-0619.
7. Skrzypek H, Hui L. Noninvasive prenatal testing for fetal aneuploidy and single gene disorders. Best Pract Res Clin Obstet Gynaecol. 2017;42:26-38.
8. Bustamante-Aragonés A, et al. Non-invasive prenatal diagnosis of single-gene disorders from maternal blood. Gene. 2012;504(1):144-9.
9. Hayward J, Chitty LS. Beyond screening for chromosomal abnormalities: Advances in non-invasive diagnosis of single gene disorders and fetal exome sequencing. Semin Fetal Neonatal Med. 2018. doi: 10.1016/j.siny.2017.12.002
10. Lee SY, et al. A new approach of digital PCR system for non-invasive prenatal screening of trisomy 21. Clin Chim Acta. 2018;476:75-80.
11. Kagan KO, et al. Principles of first trimester screening in the age of non-invasive prenatal diagnosis: screening for chromosomal abnormalities. Arch Gynecol Obstet. 2017;296(4):645-51.
12. Hudecova I, Chiu RW. Non-invasive prenatal diagnosis of thalassemias using maternal plasma cell free DNA. Best Pract Res Clin Obstet Gynaecol. 2017;39:63-73.
13. Perlado S, et al. Fetal Genotyping in Maternal Blood by Digital PCR: Towards NIPD of Monogenic Disorders Independently of Parental Origin. PLoS One. 2016;11(4):e0153258.
14. Hui WW, et al. Universal Haplotype-Based Noninvasive Prenatal Testing for Single Gene Diseases. Clin Chem. 2017; 63(2):513-524.
15. Lo YMD, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet. 1998;62(4):768-75.
16. Bianchi DW, et al. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci U S A. 1990;87(9):3279-83.
17. Lo YM. Fetal DNA in maternal plasma: biology and diagnostic applications. Clin Chem 2000:46(12):1903-6.
18. Sekizawa A, et al. Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. Prenat Diagn 2000;20(11):886-9.
19. Sekizawa A, et al. Evaluation of bidirectional transfer of plasma DNA through placenta. Hum Genet. 2003;113(4):307-10.
20. Jackson L. Fetal cells and DNA in maternal blood. Prenat Diagn 2003;23(10):837-46

21. Alberry M, et al. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. Prenat Diagn 2007;27(5):415-8.
22. Sandovici I, et al. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. Reprod Biomed Online. 2012 Jul;25(1):68-89.
23. Lun FM, et al. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem. 2008;54(10):1664-72.
24. Illanes S, et al. Early detection of cell-free fetal DNA in maternal plasma. Early Hum Dev. 2007;83(9):563-6.
25. Zhou Y, et al. Effects of maternal and fetal characteristics on cell-free fetal DNA fraction in maternal plasma. Reprod Sci 2015;22(11):1429-35.
26. Vora NL, et al. A multifactorial relationship exists between total circulating cell-free DNA levels and maternal BMI. Prenat Diagn. 2012;32(9):912-4.
27. Attilakos G, et al. Quantification of free fetal DNA in multiple pregnancies and relationship with chorionicity. Prenat Diagn. 2011;31(10):967-72.
28. Bischoff FZ, Lewis DE, Simpson JL. Cell-free fetal DNA in maternal blood: kinetics, source and structure. Hum Reprod Update. 2005;11(1):59-67.
29. Chan KC, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem. 2004;50(1):88-92.
30. Li Y, et al. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clin Chem. 2004;50(6);1002-11.
31. Angert RM, et al. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and thirdtrimester samples. Clin Chem. 2003;49(1):195-8.
32. Lo YM, et al. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet. 1999;64(1);218-24.
33. Hui L, Vaughan JI, Nelson M. Effect of labor on postpartum clearance of cell-free fetal DNA from the maternal circulation. Pren Diagn. 2008;28(4);304-8.
34. Ordonez E, et al. Evaluation of sample stability and automated DNA extraction for fetal sex determination using cell-free fetal DNA in maternal plasma. Biomed Res Int. 2013: 2013:195363.
35. Hidestrand M, et al. Influence of temperature during transportation on cell-free DNA analysis. Fetal Diagn Ther. 2012;31(2):122-8.
36. Zhang Y, et al. Effect of formaldehyde treatment on the recovery of cell-free fetal DNA from maternal plasma at different processing times. Clin Chim Acta 2008;397(1-2):60-4.
37. Chan KC, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem. 2004;50(1):88-92.
38. Li Y, et al. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. JAMA 2005; 293:843-49.
39. Lun FMF, et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. Proc Natl Acad Sci USA 2008;105:19920-25.
40. Clausen FB, et al. Improvement in fetal DNA extraction from maternal plasma.

Evaluation of the NucliSens Magnetic Extraction system and the QIAamp DSP Virus Kit in comparison with the QIAamp DNA Blood Mini Kit. Prenat Diagn 2007;27(1):6-10.
41. Stray J, Zimmermann B. Isolation of Cell-Free DNA from Maternal Plasma. Methods Mol Biol. 2019;1885:309-323. doi: 10.1007/978-1-4939-8889-1_21.
42. Li Y, et al. Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma--case report. Prenat Diagn. 2004;24(11):896-8.
43. Li J, Makrigiorgos GM. COLD-PCR: a new platform for highly improved mutation detection in cancer and genetic testing. Biochem Soc Trans. 2009;37(Pt 2):427-32.
44. Papageorgiou EA, et al. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. Am J Pathol. 2009; 174:1609-18.
45. Tong YK, et al. Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. Clin Chem. 2010;56(1):90-8.
46. Ioannides M, et al. Inter-individual methylation variability in differentially methylated regions between maternal whole blood and first trimester CVS. Mol Cytogenet. 2014; 7(1):73.
47. Pohl G, et al. Principle and applications of digital PCR. Expert Rev Mol Diagn. 2004 Jan;4(1):41-7.
48. Majumdar N, et al. Poisson Plus Quantification for Digital PCR Systems. Sci Rep. 2017 Aug 29;7(1):9617. doi: 10.1038/s41598-017-09183-4.
49. Pinheiro LB, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem. 2012;84:1003-11.
50. Parkin B. Rare Variant Quantitation Using Droplet Digital PCR. Methods Mol Biol. 2019;1881:239-251. doi: 10.1007/978-1-4939-8876-1_18.
51. Hindson BJ, et al. High-Throughput Droplet Digital PCR System for Absolute quantitation of DNA copy number. Anal Chem. 2011;83:8604-10.
52. D’Aversa E, et al. Non-invasive fetal sex diagnosis in plasma of early weeks pregnants using droplet digital PCR. Mol Med. 2018; 24(1):14.
53. Lodrini M, et al. Using droplet digital PCR to analyze MYCN and ALK copy number in plasma from patients with neuroblastoma. Oncotarget 2017;8(49):85234-51.
54. Zhao G, et al. Droplet digital PCR-based circulating microRNA detection serve as a promising diagnostic method for gastric cancer. BMC Cancer 2018;18:676.
55. Rutsaert S, et al. Digital PCR as a tool to measure HIV persistence. Retrovirology 2018;15:16.
56. Behjati S, Tarpey PS. What is next generation sequencing? Arch Dis Child Educ Pract Ed. 2013;98(6):236-8.
57. Mardis ER. Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet. 2008;9:387-402.
58. McCarthy A. Third generation DNA sequencing: pacific biosciences' single molecule real time technology. Chem Biol. 2010;17(7):675-6.
59. Manegold-Brauer G, et al. What does next-generation sequencing mean for prenatal diagnosis? Biomark Med. 2014;8(4):499-508.
60. Forest M, Morel Y, David M. Prenatal treatment of congenital adrenal hyperplasia. Trends Endocrinol Metab. 1998;9(7):284-9.
61. Hyett JA, et al. Reduction in diagnostic and therapeutic interventions by non-

invasive determination of fetal sex in early pregnancy. Pren Diagn. 2005;25:1111-6.
62. Zhong XY, Holzgreve W, Hahn S. The levels of circulatory cell free fetal DNA in maternal plasma are elevated prior to the onset of preeclampsia. Hypertension Pregnancy, 2002;21(1):77-83.
63. Shah VC, Smart V. Human chromosome Y and SRY. Cell Biol Int. 1996;20(1):3-6.
64. Lo YMD, et al. Detection of single-copy fetal DNA sequence from maternal blood. Lancet 1990;335(8703):1463-4.
65. Stanghellini I, et al. Quantitation of fetal DNA in maternal serum during the first trimester of pregnancy by the use of a DAZ repetitive probe. Mol Hum Reprod. 2006;12(9):587-91.
66. Devaney SA, et al. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. JAMA 2011;306(6):627-36.
67. Wright CF, et al. Non-invasive prenatal diagnostic test accuracy for fetal sex using cell-free DNA a review and meta-analysis. BMC Res Notes. 2012;5:476.
68. Breveglieri G, et al. Y-chromosome identification in circulating cell-free fetal DNA using surface plasmon resonance. Prenat Diagn. 2016;36(4):353-61.
69. Chim SSC, et al. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proc Natl Acad Sci USA, 2005;102(41):14753-8.
70. Chan KC, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem. 2006;52:2211-8.
71. Bellido ML, et al. MALDI-TOF mass array analysis of RASSF1A and SERPINB5 methylation patterns in human placenta and plasma. Biol Reprod. 2010;82(4):745-50.
87.
72. Tang NL, et al. Detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma. Clin Chem. 1999;45(11):2033-5.
73. Tsui NB, et al. Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. Blood 2011;117(13): 3684-91.
74. Lo YM, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med. 1998;339(24):1734-8.
75. Scheffer PG, van der Schoot CE, Page-Christiaens GC, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. BJOG. 2011 Oct;118(11):1340-8.
76. Kolialexi A, Tounta G, Mavrou A. Noninvasive fetal RhD genotyping from maternal blood. Expert Rev Mol Diagn. 2010 Apr;10(3):285-96.
77. Clausen FB, Damkjær MB, Dziegiel MH. Noninvasive fetal RhD genotyping. Transfus Apher Sci. 2014;50(2):154-62.
78. Tax MG, Van Der Schoot CE, Van Doorn R, Douglas-Berger L, Van Rhenen DJ, Maaskant-vanWijk PA. RHC and RHc genotyping in different ethnic groups. Transfusion. 2002;42(5):634-44.
79. Clausen FB. Lessons learned from the implementation of non-invasive fetal RHD screening. Expert Rev Mol Diagn. 2018 May;18(5):423-431.
80. Mennuti MT, et al. Cell-free DNA screening and sex chromosome aneuploidies. Prenat Diagn. 2015;35(10):980-5.
81. Skrzypek H, Hui L. Noninvasive prenatal testing for fetal aneuploidy and single

gene disorders. Best Pract Res Clin Obstet Gynaecol. 2017; 42:26-38.
82. Palomaki GE, et al. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study Genet Med. 2012;14:296-305.
83. Swanson A, Sehnert AJ, Bhatt S. Non-invasive Prenatal Testing: Technologies, Clinical Assays and Implementation Strategies for Women's Healthcare Practitioners Curr Genet Med Rep. 2013;1(2):113-21.
84. Nicolaides KH et al. Prenat Diagn. Validation of targeted sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of aneuploidy of chromosomes 13, 18, 21, X, and Y. 2013;33(6):575-9.
85. Ryan A et al. Validation of an Enhanced Version of a Single-Nucleotide Polymorphism-Based Noninvasive Prenatal Test for Detection of Fetal Aneuploidies. Fetal Diagn Ther. 2016;40(3):219-23.
86. Bianchi DW, et al. Fetal sex chromosome testing by maternal plasma DNA sequencing: clinical laboratory experience and biology. Obstet Gynecol. 2015;125(2):375-82.
87. Taneja PA, et al. Noninvasive prenatal testing in the general obstetric population: clinical performance and counseling considerations in over 85 000 cases. Prenat Diagn. 2016;36(3):237-43.
88. Stokowski R et al. Clinical performance of non-invasive prenatal testing (NIPT) using targeted cell-free DNA analysis in maternal plasma with microarrays or next generation sequencing (NGS) is consistent across multiple controlled clinical studies. Prenat Diagn. 2015;35(12):1243-6.
89. Juneau K et al. Microarray-based cell-free DNA analysis improves noninvasive prenatal testing. Fetal Diagn Ther. 2014;36(4):282-6.
90. Bianchi DW. From prenatal genomic diagnosis to fetal personalized medicine: progress and challenges. Nat Med. 2012;18(7):1041-51.
91. Ferrari M, et al. New trend in non-invasive prenatal diagnosis. Clin Chim Acta 2015; 451(Pt A):9-13.
92. Evans MI et al. Digital PCR for noninvasive detection of aneuploidy: power analysis equations for feasibility. Fetal Diagn Ther. 2012;31(4):244-7.
93. Lee SY, et al. A new approach of digital PCR system for non-invasive prenatal screening of trisomy 21. Clin Chim Acta. 2018;476:75-80.
94. Traeger-Synodinos J. Real-time PCR for prenatal and preimplantation genetic diagnosis of monogenic diseases. Mol Aspects Med. 2006;27(2-3):176-91.
95. Galbiati S, et al. COLD-PCR and microarray: two independent highly sensitive approaches allowing the identification of fetal paternally inherited mutations in maternal plasma. J Med Genet. 2016;53(7):481-7.
96. Mauger F, How-Kit A, Tost J. COLD-PCR Technologies in the Area of Personalized Medicine: Methodology and Applications. Mol Diagn Ther. 2017;21(3):269-83.
97. Guissart C, et al. Non-invasive prenatal diagnosis of monogenic disorders: an optimized protocol using MEMO qPCR with miniSTR as internal control. Clin Chem Lab Med. 2015;53(2):205-15.
98. Phylipsen M, et al. Non-invasive prenatal diagnosis of beta-thalassemia and sickle-cell disease using pyrophosphorolysis-activated polymerization and melting curve analysis.

Prenat Diagn. 2012;32(6):578-87.
99. Lo YM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med. 2010;2(61):61ra91.
100. Breveglieri G, et al. Postnatal and non-invasive prenatal detection of β-thalassemia mutations based on Taqman genotyping assays. PLoS One. 2017;12(2):e0172756.
101. Lun FM, et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. Proc Natl Acad Sci U S A. 2008;105(50):19920-5.
102. Li Y, et al. Size fractionation of cell-free DNA in maternal plasma improves the detection of a paternally inherited beta-thalassemia point mutation by MALDI-TOF mass spectrometry. Fetal Diagn Ther. 2009;25(2):246-9.
103. Zhong XY, Holzgreve W. MALDI-TOF MS in Prenatal Genomics. Transfus Med Hemother. 2009;36(4):263-72.
104. Papasawa T, et al. Arrayed primer extension for noninvasive prenatal diagnosis of beta-thalassemia based on detection of single nucleotide polymorphisms. Ann N Y Acad Sci. 2008;1137:302–8.
105. Yi P, et al. PCR/LDR/capillary electrophoresis for detection of single-nucleotide differences between fetal and maternal DNA in maternal plasma. Prenat Diagn. 2009;29(3):217-22.
106. Camunas-Soler J, et al. Noninvasive Prenatal Diagnosis of Single-Gene Disorders by Use of Droplet Digital PCR. Clin Chem. 2017. doi: 10.1373/clinchem.2017.278101.
107. Nectoux J. Current, Emerging, and Future Applications of Digital PCR in Non-Invasive Prenatal Diagnosis. Mol Diagn Ther. 2017. doi: 10.1007/s40291-017-0312-x.
108. Hahn S, Hösli I, Lapaire O. Non-invasive prenatal diagnostics using next generation sequencing: technical, legal and social challenges. Expert Opin Med Diagn. 2012;6(6):517-28.
109. Chiu RW, 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. 2008;105(51):20458-63.
110. Wong FC, Lo YM. Prenatal Diagnosis Innovation: Genome Sequencing of Maternal Plasma. Annu Rev Med. 2016;67:419-32.
111. Strom CM, et al. Improving the Positive Predictive Value of Non-Invasive Prenatal Screening (NIPS). PLoS One. 2017;12(3):e0167130. doi: 10.1371/journal.pone.0167130. eCollection 2017.

APPLICATION	METHOD/TECHNOLOGY	REFERENCES
Fetal gender X-linked disorders	PCR; real-time PCR	64-67
	methylation-specific PCR	69-71
	microfluidic digital PCR	73
	SPR-based biosensors	68
Fetal rhesus D genotyping	PCR-based methods	75-78
Aneuploidies	NGS based-technology	80,81
	digital PCR	92,93
Monogenic Disorders	PCR-based variants	13, 94-101, 104-107
	Mass Spectrometry	102,103
	Massive Parallel Sequencing	108,109

Table 1. Possible NIPT applications.

NIPT	GENETIC TEST METHOD	TESTING	EARLY GESTATIONAL AGE	REFERENCES
Sequenom MaterniT21®PLUS	Massively Parallel Shotgun Sequencing (MPSS)	Trisomy 21, 18, 13; Sex chromosome aneuploidies; Microdeletions	Nine weeks	82,83
Natera Panorama®	single nucleotide polymorphisms	Trisomy 21, 18, 13; Sex chromosome abnormalites; Microdeletion; Triplody; Gender Fetus	Nine weeks	84,85
Illumina® Verifi Plus	Massively Parallel Shotgun Sequencing (MPSS)	Trisomy 21, 18, 13; Sex chromosome abnormaliites; Microdeletion; Triplody	Ten weeks	83,86,87
Ariosa Diagnostics Harmony™	Digital Analysis of Selected Regions (DANSR)	Trisomy 21, 18, 13; Sex chromosome aneuploidies; Microdeletion; Monosomy X; Fetal sex	Ten weeks	83,88,89

Table 2. Examples of commercially-available NIPT kits.

TECHNOLOGY	REFERENCES
qPCR	91,94
COLD-PCR	95,96
MEMO qPCR	97
Pyrophosphorolysis-Activated Polimerization (PAP)	98
Digital Relative Mutation Dosage (RMD)	101
Relative Haplotype Dosage (RHDO)	99
Genotyping Assay	100
MALDI-TOF mass spectrometry	102,103
Array primer single-base extension	104
PCR/LDR/capillary electrophoresis	105
Digital PCR	106,107
NGS	108,109

Table 3. NIPD for monogenic diseases.