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Non-invasive prenatal testing (NIPT): limitations on the way to become diagnosis

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Abstract: With the discovery of existing circulating cell-free fetal DNA (ccffDNA) in maternal plasma and the advent of next-generation sequencing (NGS) technology, there is substantial hope that prenatal diagnosis will become a predominately non-invasive process in the future. At the moment, non-invasive prenatal testing (NIPT) is available for high-risk pregnancies with significant better sensitivity and specificity than the other existing non-invasive methods (biochemical and ultrasonographical). Mainly it is performed by NGS methods in a few commercial labs worldwide. However, it is expected that many other labs will offer analogous services in the future in this fast-growing field with a multiplicity of in-house methods (e.g., epigenetic, etc.). Due to various limitations of the available methods and technologies that are explained in detail in this manuscript, NIPT has not become diagnostic yet and women may still need to undergo risky invasive procedures to verify a positive finding or to secure (or even expand) a negative one. Efforts have already started to make the NIPT technologies more accurate (even at the level of a complete fetal genome) and cheaper and thus more affordable, in order to become diagnostic screening tests for all pregnancies in the near future.

Keywords: aneuploidy screening; massive-parallel DNA sequencing; next-generation sequencing (NGS); non-invasive prenatal testing (NIPT).

Introduction

Fetal aneuploidy and other chromosomal aberrations affect 9 out of 1000 live births, due to various mechanisms and advanced maternal age [1, 2]. In women identified by biochemical and/or sonographic screening to be at increased risk for such aneuploidies, prenatal diagnosis then involves invasive testing by chorionic villus sampling or amniocentesis. The gold standard for prenatal diagnosis of chromosome abnormalities is the conventional cytogenetic analysis using culture of fetal nucleated cells retrieved mostly by amniocentesis or chorionic villus biopsy. However, invasive testing carries a 1% risk of causing miscarriage.

The 1997 discovery of free fetal DNA in maternal plasma launched scientists' efforts to establish a reliable method for non-invasive prenatal diagnosis [3]. Several recent studies have demonstrated that the most effective screening method for trisomy 21, with a detection rate of more than 99% and false-positive rate of about 0.1%, is derived from examination of cell-free DNA (cf DNA) in maternal plasma [4–13].

Current prenatal diagnosis

Prenatal diagnosis is a medical procedure that employs a variety of techniques to determine the health and condition of an unborn fetus. The knowledge gained prevents an untoward outcome either for the fetus or the mother considering the fact that congenital anomalies account for 20%–25% of perinatal deaths. Specifically, prenatal diagnosis is helpful for:

- Managing the remaining weeks of the pregnancy
- Determining the outcome of the pregnancy
- Planning for possible complications with the birth process

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- Planning for problems that may occur in the newborn infant
- Providing parents the chance to "prepare" psychologically, socially, financially, and medically for a baby with a health problem or disability, or for the likelihood of a stillbirth
- Deciding whether to continue the pregnancy
- Finding conditions that may affect future pregnancies

There is a spectrum of techniques that are used for prenatal diagnosis, each one performed at a different level of invasiveness and sampling time. The available methods are presented in Table 1. A relatively reliable non-invasive screening approach is the combination of first trimester Pregnancy-associated Plasma Protein A (PAPP-A) and free β-subunit of Human Chorionic Gonadotropin (free-β-HCG) biochemical measurements with nuchal translucency that can detect, e.g., 88% of trisomies 21 with a 5% false-positive rate [14]. Prenatal diagnosis of chromosome abnormalities is performed by conventional cytogenetic analysis using in vitro culture of fetal nucleated cells retrieved by amniocentesis, chorionic biopsy or fetal blood sampling. The need for rapid prenatal diagnosis led to the use of the FISH assay; however, the cost of this procedure has limited its application in specific cases [3]. The quantitative fluorescent PCR (QF-PCR) test allows prenatal diagnosis of major numerical abnormalities of chromosomes 21, 18, 13, X and Y in a few hours after sampling [15]. Chromosomal microarray analysis is a technique that identifies chromosomal abnormalities, including submicroscopic ones that are too small to be detected by conventional karyotyping [16]. This analysis requires direct testing of fetal tissue and thus can be offered only with chorionic villus sampling or amniocentesis. This analysis is most beneficial when ultrasonographic examination identifies fetal structural anomalies. However, the potential of complex results and detection of clinically uncertain findings can result in substantial patient anxiety. This underscores the critical need for patient pretest and post test genetic counseling about the benefits, limitations and results of testing.

Cell-free DNA

Non-invasive prenatal testing (NIPT) uses fetal genetic material obtained from a maternal blood sample to detect

Table 1: Prenatal diagnosis testing.

Invasiveness	Test	Comments	Time
Non-invasive	Preimplantation genetic diagnosis (PGD)	During in vitro fertilization procedures, it is possible to sample cells from human embryos prior the implantation.	Prior to implantation
Less-invasive	Fetal cells in maternal blood	Based on the enrichment for fetal cells that circulate in maternal blood.	First trimester
Less-invasive	Cell free DNA in maternal plasma	Based on DNA of fetal origin circulating in the maternal blood. Testing could potentially identify fetal aneuploidy, rhesus status and gender of a fetus. Fetal DNA ranges from about 2–10% of the total DNA in maternal blood.	First trimester
Non-invasive	Ultrasound detection	Commonly *dating scans* starting from 7 weeks to confirm pregnancy dates and search for twins. The specialized nuchal scan at 11–13 weeks may be used to identify a higher risk for Down syndrome. Later *morphology scans* from 18 weeks may check for any abnormal development.	First or second trimester
Non-invasive	Fetal heartbeat	Evaluating the fetal heartbeat.	First or second trimester
Less invasive	Maternal serum screening	Including free β-hCG and PAPP-A (most often), α-fetoprotein, unconjugated E3, intact or β-hCG, inhibin-A [14].	First or second trimester
More invasive	Chronionic villus sampling (CVS)	Retrieving a sample of chorionic villus tissue and testing it by karyotyping or rapid molecular aneuploidy test. The procedure poses a significant risk of miscarriage, estimated at least 1%.	After 10 weeks
More invasive	Amniocentesis	Cells from the fetus floating in amnionic fluid can be separated and tested by karyotyping or rapid molecular aneuploidy test. Miscarriage risk of amniocentesis is commonly quoted as 0.06% (1:1600).	After 15 weeks
Less invasive	Embryoscopy and fetoscopy	Though rarely done, these involve putting a probe into a women's uterus to observe (with a video camera).	After 20 weeks
More invasive	Percutaneous umbilical cord blood sampling	Examines blood from the fetal umbilical cord to detect fetal abnormalities, provides a means of rapid chromosome analysis.	After 20 weeks

certain genetic conditions during pregnancy. Current literature often refers also to NIPT as non-invasive prenatal diagnosis (NIPD). This terminology may be misleading given that, at the time of this writing, the technology is recommended only as a highly specific screening measure, which requires follow-up diagnostic testing.

The first evidence for circulating nucleic acids in the peripheral blood was shown by Mandel and Metals in 1948 [17]. The identification of fetal cells from maternal blood by Bianchi et al. in 1997 [18] and ways to isolate them [19] have paved the way for NIPT until 1997, when the group of Dennis Lo reported for the first time the presence of circulating cell-free fetal DNA (ccffDNA) in the plasma of pregnant women [4].

It is essential to elucidate on the origin of the amounts of cell free DNA in maternal plasma (Figure 1). Circulating cell-free fetal DNA originates from the trophoblasts making up the placenta [20] and can comprise approximately 3–13% of the total cell free DNA in most of the samples [21]. Studies have shown that ccffDNA can first be observed as early as 4 gestation weeks and the amount of ccffDNA increases as the pregnancy progresses [22] but diminishes quickly after the birth of the baby, so that it is no longer detectable in the maternal blood approximately 2 h after birth. The fetal DNA is significantly smaller than the maternal DNA in the bloodstream, with fragments with a median size of 146 bp and makes its way into the maternal bloodstream via shedding of the placental microparticles into the maternal bloodstream [23]. Fetal ccffDNA is heavily diluted with maternal plasma and this "fetal fraction" is positively correlated with gestational age while preliminary evidence suggests that it is negatively correlated with maternal weight [7, 24, 25]. In general, the fetal fraction percentage has to be provided and to be estimated with real-time quantitative PCR [22, 26]: over 8% is considered satisfactory for most NIPT methods, while a 4–8% is considered marginal [11]. There are now research efforts ongoing for the extraction of the fetal DNA from the maternal plasma based on its size in order to distinguish it from the maternal DNA and/or accurately estimate it [23].

The limited amounts of cffDNA as well as the facts that it co-exists with maternal DNA and shares 50% identical sequences posed major challenges toward the development of NIPT. In 2008, two research groups applied the novel massive parallel sequencing (MPS) technology for the first time in maternal plasma in order to detect an overrepresentation of material from chromosome 21 in pregnancies affected with trisomy 21 (Down syndrome) [24, 27]. Another study then, demonstrated the ability of MPS of maternal plasma to detect fetal trisomy 21 with a near 99% sensitivity and specificity in high-risk pregnancies, defined by maternal age, family history or positive serum and/or sonographic screening tests [7]. The group then published an analysis from the same study demonstrating the detection of trisomy 18 (Edwards syndrome) at 100% sensitivity with a false-positive rate of 0.28%, and trisomy 13 (Patau syndrome) at 91.7% sensitivity with a false-positive rate of 0.97% [28]. The overall detection rate for trisomy 13, 18, and 21 was reported as 98.9% sensitivity with a false-positive rate of 1.4%. Bianchi et al. also examined the use of MPS in maternal serum of high-risk pregnancies, using a slightly different

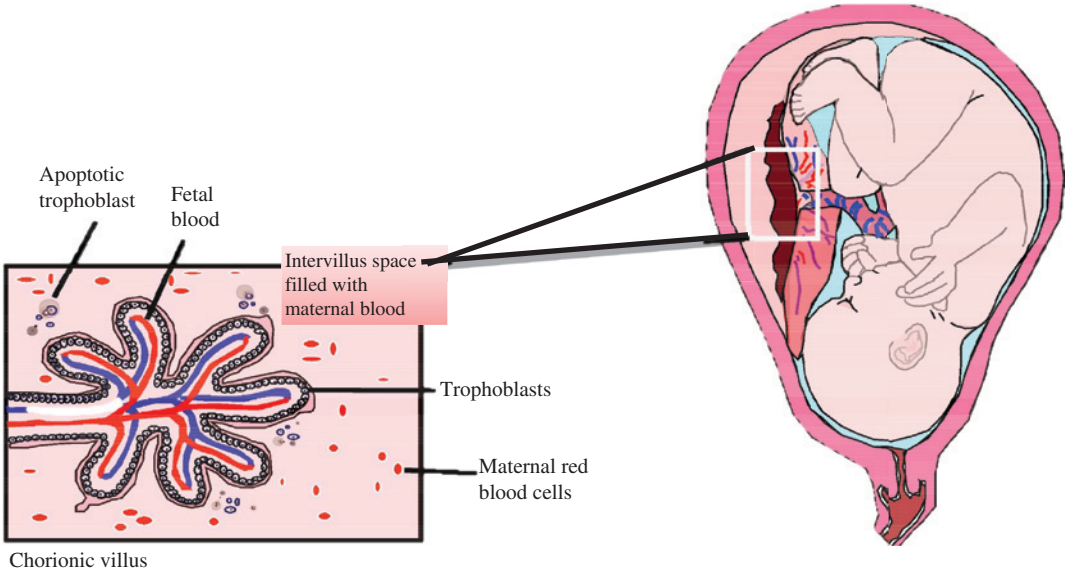

Figure 1: Origin of cell free DNA.

algorithm for analysis [12]. In this study, NIPT detected trisomy 21 with 100% sensitivity, trisomy 18 with 97.2% sensitivity, and trisomy 13 with 78.6% sensitivity – all with a specificity of 100%. They also reported monosomy X detection with 93.8% sensitivity and 99.8% specificity. Sparks et al. proposed sequencing analysis, for selected loci from specific chromosomes of interest in cell-free DNA from maternal as a more efficient option for NIPT [29]. A multicenter cohort study from Norton et al., evaluated the performance of chromosome-selective sequencing on chromosomes 21 and 18 in a population of women undergoing CVS or amniocentesis for any indication [11]. Using a predefined cut-off value of 1 in 100 (1%) for classifying a sample as high risk vs. low risk, the sensitivity and specificity for trisomy 21 were 100% and 99.97%. The sensitivity and specificity for trisomy 18 were 97.4% and 99.93%. For trisomy 13, Ashoor et al. reported 80% sensitivity and 99.95% specificity [10]. These studies validate NIPT as a reliable screen for trisomies 21, 13, and 18 and monosomy X in high-risk pregnancies. A blinded validation study from the group of K.H. Nicolaides in 2013, has demonstrated that ccffDNA testing in maternal blood using targeted sequencing of SNPs at chromosomes 13, 18, 21, X, and Y and use of the specific algorithm holds promise as an accurate method for detecting fetal autosomal aneuploidies, sex chromosome aneuploidies and triploidy in the first trimester of pregnancy [30].

The medical significance of the development of NIPT is of great importance as it could potentially be offered to all pregnancies (assuming that it will become cheaper in the future), it presents no risk of pregnancy loss and it provides a more effective prenatal diagnosis compared to currently-used invasive methods.

Available NIPT techniques

As mentioned earlier, the overall amount of fetal ccffDNA is small (<1 μg in 20 mL whole blood) and there are still no reliable routine methods to separate fetal from maternal ccffDNA, precluding fetal ccffDNA isolation prior to analysis [9, 31–33]. Thus, detection of fetal chromosomal copy number based on ccffDNA analysis requires polymerase chain reaction (PCR) amplification and analysis of the full (maternal+fetal) ccffDNA complement [21]. In most of the available methods, reactions can be prepared by massive parallel anchoring of numerous amplified molecules in a next generation sequencing (NGS) platform [24, 27]. Thus, the number of DNA molecules that can be sequenced and consequently the amount of information produced is dramatically increased when compared to "first-generation" Sanger sequencing methods [34, 35]. There are two different approaches in this concept: quantitative (either shotgun or targeted) and qualitative targeted single nucleotide polymorphism (SNP)-based methods [36]. A different approach originates from the epigenetics field: the methylation DNA immunoprecipitation (MeDIP) combined with real time qPCR is based on the identification of differentially methylated regions (DMRs) and their use in discriminating normal from abnormal cases [37]. All NIPT methods have several advantages and limitations that are analyzed in depth in the following section:

NGS NIPT approaches

Quantitative massively parallel shotgun sequencing (MPSS)

MPSS is an NGS-based technique that generates DNA sequence reads from all chromosomes non-specifically. This allows for tens of millions of short DNA fragments (typically 25–36 base pairs long) to be sequenced rapidly and simultaneously in a single run. Since ccffDNA size has been found considerable shorter (<200 bp) than that of maternal origin, no further fragmentation of the plasma-extracted DNA is needed. In general, in terms of costs and efficiency, the number of samples that can be sequenced simultaneously and the minimal numbers of reads so that results still possess statistical significance are vital issues. Too many reads will increase the costs significantly, as fewer samples can be multiplexed per run [32].

After an initial amplification step and sequencing of the fetal and maternal ccffDNA mixture, the chromosomal origin of each DNA fragment is obtained by comparison of the sequence data from each fragment to the human genome sequence. This is followed most often by ratio comparisons of each chromosome sequence tag density over the median tag density of all autosomes (z-score analysis). Since a trisomic fetus has 50% more genetic material originating from the extra chromosome-of-interest, the proportion of DNA from that chromosome when compared with an assumed disomic reference chromosome is higher than in euploid pregnancies. Specifically, if the ratio of the number of sequence reads from a chromosome of interest to the number of sequence reads from the reference chromosome(s) exceeds a predetermined threshold, fetal trisomy is inferred and is reported as positive or high-risk for trisomy for that chromosome. This approach is referred to as "counting".

Taking into account that counting methods detect quantitative differences in the amount of fetal ccffDNA present and since fetal ccffDNA comprises a small proportion of the total ccffDNA, differences due to fetal trisomy are incremental. These methods do not have the ability to distinguish maternal from fetal ccffDNA. So, the ability to detect increased chromosomal dosage resulting from fetal trisomy, is directly related to the fetal ccffDNA fraction in maternal circulation [21].

At lower fetal fractions, the increase becomes marginal and amplification efficiency variation between chromosomes becomes significant. This is particularly important at early gestational ages, because the fetal fractions rise with increasing gestational age, and in women with high BMI, as fetal fractions are inversely proportional to weight [7, 24, 25]. To distinguish these minor differences with high confidence a large number of reads is required; because MPSS sequences all chromosomes, approximately 6.3 million uniquely-mapped reads from the entire genome are required to ensure sufficient, e.g., chromosome 21 counts for accurate copy number calls [27, 38–40]. Additionally, as only approximately 25% of MPSS-generated reads are uniquely mapped, approximately 25 million raw sequencing reads are required per sample to generate sufficient data for accurate analysis [9]. This is especially important if other chromosomes (13, 18, X, and Y) are considered in the analysis. Thus, this approach represents enormous redundancy considering that the clinically-significant chromosomes represent only ~14% of the genome; however, this doesn't preclude its future scaling up to detect aneuploidy of other chromosomes and to scan the whole fetal genome.

Quantitative targeted MPS

Targeted sequencing differs from whole MPSS by selectively amplifying and sequencing specific genomic regions of interest instead of random regions from all chromosomes. Thus, nearly all sequences are useful in assigning fetal chromosomal copy number, significantly reducing the total number of analyzed reads and increasing efficiency [29]. Selective sequencing allows for focused analysis of clinically important chromosomes, including chromosomes 13, 18, 21, X, and Y. Targeted sequencing followed by counting is subject to the same limitations as MPSS-based methods and shows similar sensitivities and specificities to those reported from MPSS-based approaches (there is though higher complexity in the bioinformatics processing of the data). The highest sensitivity reported is for trisomy 21 detection (100%), followed by trisomy 18 (98%) and trisomy 13 (80%) [11, 41].

Problems associated with quantitative methods

Despite the differences in amplification methods, all counting-based NGS methods use various bioinformatics and statistical methods to identify fetal chromosomal copy number. A common approach among some of these post-hoc bioinformatics statistical algorithms is the utilization of a single-hypothesis rejection approach. This method identifies cut-offs based on previously analyzed cohorts, detecting aneuploidies in samples that fall outside these predetermined cut-offs.

This is important at lower-to-intermediate fetal ccffDNA fractions. Indeed, in one study reporting an overall 98.6% trisomy 21 detection rate, isolated analysis of the samples containing 4%–8% fetal fraction demonstrated a detection rate of only 75% [7]. Also, commercially available ccffDNA-based NIPT tests that utilize counting methodologies typically do not routinely report X and Y chromosome copy number, but will report sex chromosome anomalies when detected.

When "no-call" rates for counting methods were reported, specifically excluding monosomy X from the analysis, this resulted in no-call rates from 0.8% to 5.8%; [7, 9, 11]; with inclusion of monosomy X this rate increased to >15% [12]. Inclusion of the major sex chromosome aneuploidies (45,X; 47,XXX; 47,XXY; 47,XYY) resulted in an overall no-call rate of 5% [42]. While the incidence of false-positives is estimated to be <1%, false-positive results have been reported for all counting methodologies [7, 10, 13, 28, 41, 42].

In both methods, the number of reads obtainable even using small bench top NGS platforms will be sufficient but the possibilities for high throughput are still rather limited. However, in larger platforms targeted sequencing can be a good alternative for the still rather expensive whole genome approach, as more samples can be sequenced simultaneously. One should, however, realize that when using a targeted approach, only the region(s) of interest can be studied.

Quantitative "counting" methods also show variation in the amplification efficiency of individual chromosomes, this is linked to guanosine-cytosine (GC) base content [24, 27]. This may alter the ratio of reads from the chromosome of interest to the reference chromosome(s), thus impacting identification of quantitative differences in sequence read number that would indicate fetal aneuploidy. Clinically, this translates to differences in the accuracy of fetal aneuploidy detection at different chromosomes. Indeed, sensitivities are the highest for trisomy 21 (98.6%–100%) and trisomy 18 (97.4%–100%) [7, 9, 11, 27–29, 43].

Chromosomes 13 and X, however, amplify with greater variability than chromosomes 21 and 18, demonstrating lower sensitivities: 80%–91.7% for trisomy 13 and 91.7%–94.4% for monosomy X [7, 28, 29, 42, 43]. Bioinformatic GC bias correction has improved sensitivity and specificity somewhat when detecting trisomy 13 and trisomy 18 [29]. However, this correction has not been reported to improve sex chromosome aneuploidy detection.

Qualitative SNP-based targeted sequencing

The inclusion of genotypic information, generally SNPs, allows for a more complex ccffDNA analysis, generating significantly more information than next generation methods that consider only the number of reads to identify fetal chromosomal copy number. SNP-based sequencing methods thus employ a more qualitative approach that allows for the identification of the specific maternal and fetal ccffDNA contribution to the sequence reads [44]. In addition to determining copy number, the method can also reconstruct haplotypes and potentially identify abnormalities that escape detection using counting methods, such as triploidy and uniparental disomy. Moreover, they allow the use of sophisticated data models that can flag samples that have insufficient data to generate an accurate result, and as such should be repeated [21]. To date, two described approaches incorporate genotype information:

Allele ratios

This first genotypic approach amplifies and sequences SNPs, counting the number of observed maternal and fetal alleles and generating an allele ratio between a chromosome-of-interest and reference chromosome to determine copy number [45]. The requirement for a reference chromosome also means this approach is incapable of detecting triploidy, and as this study only focused on chromosome 21, it is not clear whether this method will accurately detect copy number imbalances at other chromosomes. This method has not yet been developed commercially or validated in a clinical trial.

Genotype analysis with maximum likelihood estimation

This second approach uses targeted amplification of SNPs followed by NGS and sophisticated informatics analysis to identify fetal chromosomal copy number [46]. This method differs from the quantitative targeted sequencing approach because it specifically targets SNPs instead of non-polymorphic regions and uses a genotype-based analytic method rather than a counting approach to detect fetal aneuploidy. The method employs a massively multiplexed PCR amplification targeting 19,488 SNPs in a single reaction [9]. By measuring polymorphic loci, this method succeeds to reveal multiple pieces of information (the number and identity of each allele) from each sequence read. It incorporates allelic information from the mother (and from the father, if available) to model a set of hypotheses that represent the different possible fetal genotypes (e.g., monosomy, disomy, or trisomy), and which take into account different genetic inheritance patterns and crossover locations for every possible copy number count. Bayesian statistics then assign a probability to each hypothesis and a maximum likelihood estimation analysis selects the most likely hypothesis and calculates the probability of that hypothesis being correct [46]. This unique approach allows the method to incorporate certain quality control metrics that flag questionable samples which would likely return incorrect calls using counting methods. This reduces the number of missed calls; up to date no false-positive results have been reported in studies utilizing this specific method [21]. To calculate a personalized risk score for each chromosome in each sample, the probability generated by the informatics approach, is used. Since the method analyzes the relative amount of alleles at polymorphic loci and does not utilize a reference chromosome, it is not subject to issues with amplification variation. Thus, it is expected to have consistent sensitivities across all regions interrogated; indeed, clinical data indicates sensitivities of >99% for trisomy 21, trisomy 18 and trisomy [21, 46]. It is also the only method that is capable of detecting triploidy [47]. The commercially available SNP NIPT test based on this methodology also routinely reports copy number for the X and Y chromosomes. Overall, this method shows an overall no-call rate of <6% for copy number calling at all five chromosomes implicated in most congenital abnormalities (13, 18, 21, X and Y) [21, 47].

Epigenetic-based NIPT approaches

MeDIP (methylated DNA immunoprecipitation) employs a 5′- methyl cytidine specific antibody to capture methylated sites and was originally used to study levels of DNA methylation [48]. Then, this methodology was employed by Papageorgiou et al. in 2009 with the aim of investigating and identifying differentially methylated regions

(DMRs) between placenta and female peripheral blood towards the development of a NIPT test [49]. They used MeDIP in combination with chromosome-specific high-resolution oligo arrays for the investigation of the methylation pattern of chromosomes 13, 18, 21, X and Y. More than 10,000 DMRs were identified for chromosomes 13, 18, 21, X and Y.

To provide chromosome dosage information, the ccffDNA has to be hypermethylated compared to the maternal DNA. This is crucial in order to achieve fetal specific methylation enrichment, which was the key to their study. They selected a subset of DMRs on chromosome 21 and after performing real-time quantitative PCR they were able to discriminate normal from trisomy 21 cases [37]. In addition to the evaluation of the fetal specific methylation ratio values at each site, they provided an equation by linear regression statistical analysis that incorporates the best eight DMRs and therefore increasing the discriminating power (Figure 2):

$D = -6.331 + 0.959X_{EP4} + 1.188X_{EP5} + 0.424X_{EP6} + 0.621X_{EP7} + 0.028X_{EP8} + 0.387X_{EP10} - 0.683X_{EP11} + 0.897X_{EP12}$ (where: D is the discriminating value, X is the methylation ratio value arising from real-time qPCR C_T raw data, Ep the 8 DMRs that can efficiently discriminate normal cases from trisomy 21). When $D>0$ the case is assigned as trisomy, when $D \leq 0$ the case is assigned as normal [37]. Then they studied 100 cases of pregnancy, which included 25 trisomy 21 pregnancies and the results demonstrated 100% sensitivity and 99.2% specificity [50].

The MeDIP assay can tolerate sample impurities – and thus, no prior sample purification is required – and is not affected by the amount of ccffDNA or fetal gender or the presence of informative polymorphic sites as it may happen to SNP-related methods [50, 51]. It can be applicable for low starting DNA templates, generating sufficiently enriched outputs, a development that renders possible its implementation with plasma samples [52]. Moreover, it is a technically robust methodology, available in most diagnostic labs, easy to use and affordable.

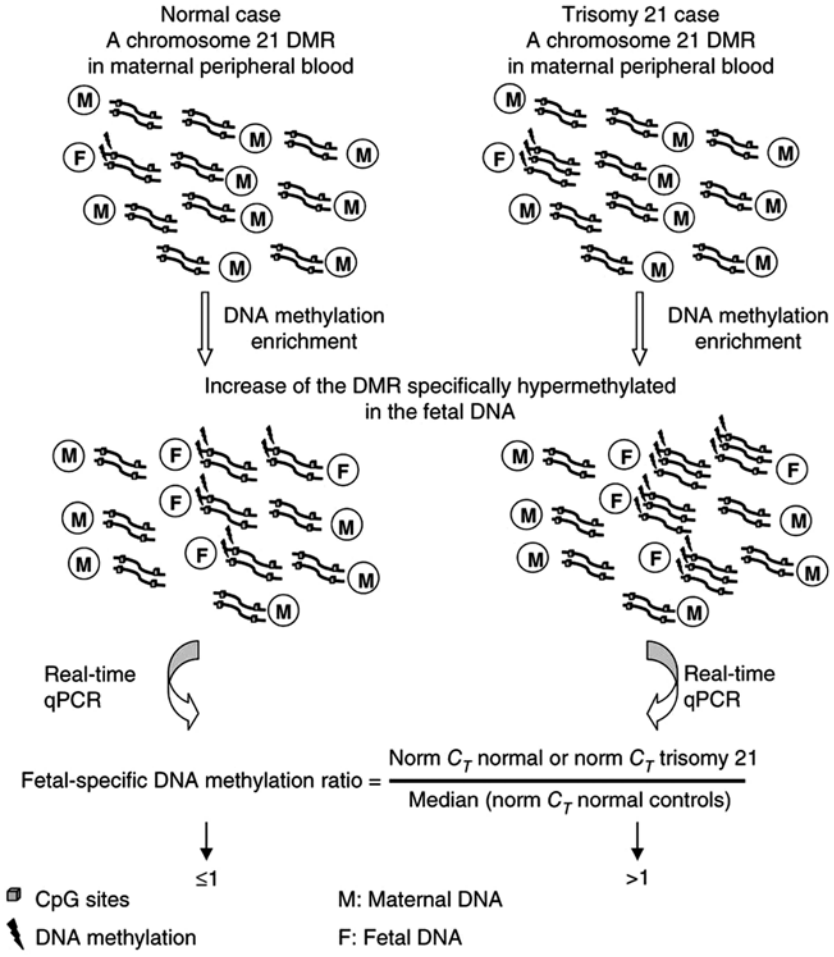

Figure 2: Schematic illustration of the fetal-specific DNA methylation ratio approach (reprinted after permission from [37]).

Nevertheless, the efficiency and performance of MeDIP greatly depends on determining the ideal combination of affinity reagents. This is very important, especially in regions with varying methylcytosine density such as the DMRs identified for the NIPT of common chromosomal aneuploidies [53]. However, an appropriate combination of DMRs should provide an accurate NIPT of normal and trisomy cases. Further refinements are anticipated with the inclusion of new DMRs in the algorithm, or removal of others due to the presence of copy number variations (CNVs) in their locus. In the future, it should be possible to expand this method for NIPT of aneuploidies of chromosomes 13, 18, 21, X. However, technical concerns have been raised already by other groups regarding the validity of this method [54] while others have suggestions to improve it [55].

A very recent interesting development in using DNA methylation for NIPT has been the implementation of sodium bisulfite DNA treatment in combination with NGS [35]. This chemical treatment of the DNA is associated with a high degree of DNA degradation in >90% of the template DNA [34]. So the accuracy and sensitivity of the test will be reduced because during pregnancy, the amount of fetal DNA in maternal plasma is already very low and further degradation will result in even fewer fetal DNA available for quantification [34, 35]. On the other hand, manipulations for removal of sodium bisulfite will bias the correct interpretation of the results. Additionally, the assay can only evaluate the methylation status of a specific and very limited number of genomic sequences; only those that include recognition site of a methylation-dependent restriction enzyme.

Non-invasive prenatal test in clinical practice

The American College of Obstetricians and Gynecologists Commitee on Genetics and the Society for Maternal-Fetal Medicine Publications Committee, have recommended that women be offered prenatal assessment for aneuploidy either by screening or invasive prenatal diagnosis regardless of maternal age; testing for ccffDNA is an option that can be considered as a primary screening test in women at increased risk of aneuploidy with the following criteria [56]:

- Maternal age 35 years or older at delivery
- Fetal ultrasonographic findings indicating an increased risk of aneuploidy
- History of a prior pregnancy with a trisomy
- Any positive test result for aneuploidy, including first trimester biochemical combined or sequential, integrated or quadruple screen, e.g., in combination with 2nd trimester AFP, uE3 and HCG α-test results [14]
- Parental balanced Robertsonian translocation with increased risk of fetal trisomy 13 or trisomy 21
- Follow-up confirmatory test for women with a positive first-trimester or second-trimester screening test result.

Pre-test counseling regarding the limitations of ccffDNA testing is recommended and should include a discussion with a clear statement that this test provides information solely for trisomy 21 and trisomy 18 and, in some laboratories, trisomy 13. The use of a non-invasive test should be an active, informed choice and not part of a routine prenatal laboratory testing. The family history should be reviewed to determine if the pregnant woman should be offered other forms of screening or prenatal diagnosis for a particular disorder. A baseline ultrasound examination may be useful to confirm viability, a singleton gestation, gestational dating, as well as to rule out obvious anomalies.

This technology can be expected to identify approximately 98% of cases of Down syndrome with a false-positive rate of <0.5%. Because false-positive test results can still occur, confirmation with amniocentesis or CVS is recommended. In general, referral for genetic counseling is suggested for pregnant women with NIPT positive test results. Patients also need to be aware that a negative test result does not ensure an unaffected pregnancy; false-negative test results can occur as well. In the high-risk population, a second-trimester ultrasound examination is suggested to evaluate pregnancies for structural anomalies. In patients in whom a structural fetal anomaly is identified, invasive diagnostic testing should be offered because a ccffDNA test can only detect major trisomies at the moment. Maternal serum α-fetoprotein screening or ultrasonographic evaluation for open fetal defects should continue to be offered.

At the moment, it should not be offered to low-risk women or women with multiple gestations because it has not been sufficiently evaluated in these groups. CcffDNA testing does not replace the accuracy and diagnostic precision of prenatal diagnosis with karyotyping of either CVS or amniocentesis material, which remain an option for women. Analogous guidelines exist from the American College of Medical Genetics and Genomics [47] and the International Society for Prenatal Diagnosis [57].

Available commercial NIPT testing

CcffDNA-based NIPT of aneuploidy by using NGS approaches is the first NIPT method that appeared in clinical practice and detects trisomy with high accuracy. Through the continuous development and improvement of algorithms for data sequencing and analysis, many commercial tests are now available and are presented in chronological order in the following text:

MaterniT21 PLUS (Sequenom Laboratories)

In October 2011, the MaterniT21 PLUS test appears in the market developed by Sequenom's Laboratories. It is a NIPT that detects chromosomal abnormalities for chromosomes 21, 18 and 13 in singletons and multiple gestations from a maternal blood draw [sex chromosomal aneuploidies (X, XXX, XYY, XXY) are reported in singleton pregnancies only]. With the recent introduction of their new "enhanced sequencing series", they can report also trisomies 16 and 22, and selected microdeletions as additional findings (22q11 DiGeorge, 5p Cri-du-Chat, 15q Angelman and Prader-Willi, 11q Jacobsen, 8q Langer-Giedion, 4p Wolf-Hirschhorn, 1p36). All of the information that they report is clinically relevant.

The technology used is massively parallel sequencing randomly in whole genome for more precise testing. By applying superior bioinformatic analysis to millions of sequence data points from chromosomes across the genome, it creates a highly accurate and distinct clinical picture based on direct results rather than mathematical models. And unlike limited targeted sequencing that restricts information it can evaluate multifetal gestations in the pregnancy, egg donors, IVF patients: essentially anyone at increased risk for fetal aneuploidy.

The test can be administered as early as 10 weeks' gestation and calls for two 10 mL blood draws in specific collection tubes and results are reported in approximately 7 working days. The MaterniT21 PLUS test sensitivity ranges from 99.1% for trisomy 21, >99.9% for trisomy 18, and 91.7% for trisomy 13, while maintaining a very low false-positive rate. Data from the validation studies are showed in Table 2 [7, 28, 42, 58]. The analytical performance of selective microdeletions/duplications and trisomy 16/22, was >99% regarding specificity, while sensitivity was 60–86% for 3–6 Mb deletions, 85–90% for 7–11 Mb deletions and >99% for trisomy 16/22 [30, 58]. Other studies relieved that the non-reportable rate for this test is 0.9% [11, 59, 60].

Results from the MaterniT21 PLUS test do not eliminate the possibility that other chromosomal abnormalities may exist in this pregnancy and a negative result does not ensure an unaffected pregnancy. While the test's results are highly accurate, not all chromosomal abnormalities may be detected due to placental, maternal or fetal mosaicism, or other causes. This test does not replace the accuracy and precision of prenatal diagnosis with CVS or amniocentesis. A patient with a positive test result should be referred for genetic counseling and offered invasive prenatal diagnosis for confirmation of test results.

Table 2: Results from validation studies for MaterniT21 PLUS prenatal test.

		Positive results	Sensitivity	Specificity
Clinical validation study				
1696 samples	DNA sequencing of maternal plasma to detect Down syndrome: international clinical validation study [7]	210 of 212 trisomy 21	99.1%	99.9%
1988 samples	DNA sequencing of maternal plasma reliably identifies trisomy 18 and 13 as well as Down syndrome [28]	59 of 59 trisomy 18	>99.9%	99.6%
		11 of 12 trisomy 13	91.7%	99.7%
2015 samples	DNA sequencing of maternal plasma to identify Down syndrome and other trisomies in multiple gestations [58]	8 of 8 7 of trisomy21 1 of trisomy13	Detection rate: >99.9%	
Y chromosome				
2017 samples	Accuracy of non-invasive prenatal fetal sex determination		Accuracy 99.4%	
Combined sex aneuploidies				
420 samples	Non-invasive prenatal detection of sex chromosomal aneuploidies by sequencing circulating cell free DNA [42]	25 of 26	96.2%	99.7%

Verifi prenatal test (VERINATA)

In March 2012, Verinata Health (owned since January 2014 by Illumina) began offering the Verifi prenatal test to healthcare providers in the US: only one tube (7 mL) of whole blood is needed and results are usually reported to the ordering physician within 3–6 working days. The Verifi test detects trisomies 21, 18 and 13 from a single maternal blood sample and is indicated for pregnant women with singleton gestation at 10+ weeks and at high-risk for fetal aneuploidy. Sensitivities are >99.9, 97.4 and 87.5% for trisomies 21, 18 and 13, respectively while specificities are all above 99.6% [12]. In July 2012, the Verifi prenatal test was expanded to include the detection of sex chromosome aneuploidies such as monosomy X (Turner), XXX (Triple X), XXY (Klinefelter), XYY (Jacobs) and to provide fetal sex. In October 2013, they offered a new NIPT test for women pregnant with twins through natural or assisted reproductive methods. This test can detect trisomy 21, trisomy 18, trisomy 13 and the presence of a Y. The verifi prenatal test uses 26 million tags per sample (while the MaterniT21plus 16.3 million [28], the Harmony 1.15 million [61] and the Panorama 6.47 million [46]).

The Verifi prenatal test leverages the power of whole genome massively parallel sequencing (MPS) with a highly optimized algorithm to provide clear, informative results. The SAFeR method calculates a normalized chromosome value (NCV) for each chromosome, which significantly reduces data variation caused by GC content, sample-to-sample and run-to-run variations and other factors. By pre-determining the most optimal reference chromosomes in an empirical analysis, no post-hoc correction factors or patient-specific information are necessary.

Possible results for chromosomes 21, 18, and 13 are "aneuploidy detected", "no aneuploidy detected", and "aneuploidy suspected (borderline value)". This last classification category "aneuploidy suspected" is introduced to highlight borderline results where a false-positive result is more likely to occur. Both affected and unaffected cases may occur in this zone. The classification scheme for sex chromosome status does not employ the "aneuploidy suspected (borderline value)" category. Evidence of fetal cystic hygroma by ultrasound is no longer required to test for monosomy X, although it remains relevant to consider the verifi prenatal test in this setting due to the relatively high association of fetal aneuploidies with cystic hygroma [46].

Harmony prenatal test (ARIOSA diagnostics)

In May 2012, Harmony prenatal test was available on the market from Ariosa Diagnostics (owned now by Roche since December 2014). The test relied on a proprietary targeted multiplex MPS assay termed "digital analysis of selected regions" (DANSR) that analyzes short DNA fragments from specific chromosomes of the mother and the fetus circulating in maternal blood in order to provide accurate results. DANSR has been used to develop an algorithm, the fetal-fraction optimized risk of trisomy evaluation (FORTE), which combines both the maternal (or egg donor) age-related and gestation age-related risks and the proportion of cffDNA in the samples to provide an individual risk score for trisomy [9]. A higher percent of fetal DNA in the sample correlates with greater confidence in the results. The low proportion of ccffDNA within the maternal circulation can make quantification of fetal chromosome imbalances difficult and potentially inaccurate, however, the FORTE algorithm factors in the fetal fraction when calculating the risk of aneuploidy. When there is a high proportion of ccffDNA the difference between trisomic vs. disomic chromosomes is greater, making it easier to detect trisomy [9]. This approach was also followed by Ashoor et al. which included a cohort of 400 samples from pregnancies with known karyotypes: 300 euploid (normal), 50 with trisomy 18 and 50 with trisomy 21. Both these reports which used the DANSR/FORTE assay identified high degrees of accuracy [9, 10]. However, in these trials the test was only offered to high-risk pregnancies, but the future aim is to deliver this assay to all pregnancies as a highly accurate screening test for aneuploidies [62].

The Harmony test have been validated in pregnancies of at least 10 weeks gestation age and has been validated in singleton and in twin pregnancies, excluding twins from non-self egg donors. Fetal sex is reported and the risk of monosomy X, XYY, XXY, XXX are evaluated [11, 13]. Fetal trisomy is detected by counting approximately one-tenth as many ccffDNA specific DNA regions as in the other quantitative shotgun NGS approaches, therefore resulting in a much cheaper test. The test identifies more than 99% of fetuses with trisomy 21, 98% of fetuses with trisomy 18 and 80% of fetuses with trisomy 13. Fetal sex analysis provides accuracy greater than of 99% for male or female sex [9].

Panorama test (NATERA Inc.)

In 2013 Natera Inc. started offering its NIPT test (Panorama) for detection of fetal trisomy 21, 13, 18, monosomy X, triploidy and if requested, fetal sex in single pregnancies. The test analyses both maternal cells' DNA and fetal cell free DNA from maternal plasma and employs molecular biology and bioinformatics that are significant advances over traditional non-invasive screening.

Panorama measures the DNA in the maternal plasma, which contains DNA from both the mother and the fetus, but it also measures DNA that is exclusively from the mother. This test utilizes the mother's white blood cells ("buffy coat") to isolate and identify her DNA, and then uses this information to "subtract out" the maternal genotype, resulting in a more robust fetal genotype and thus higher accuracy even at fetal fractions as low as 4%. Up to 1/3 of all NIPT cases have <8% fetal fraction, which is often associated with decreased sensitivity. Panorama measures fetal fraction and adjust its algorithm accordingly to improve sensitivity even at low fetal fractions. The test examines hundreds of SNPs per chromosome for each sample (a total of about 20,000 SNPs). Then, it uses a complex, proprietary algorithm called next generation aneuploidy test using SNPs (NATUS) in order to determine the genetic status of the fetus in much greater detail than previously possible, especially in the cases of extra or missing chromosomes in the fetus [30].

The Panorama test can be performed as early as 9 weeks gestation. Father's saliva sample is helpful in 1–2% of cases to minimize the chances of failure, but is not required as it does not impact the accuracy of results. In validation studies, Panorama reported sensitivities of >99% and specificities of >99% when detecting the autosomal trisomies and fetal sex and 91.7% sensitivity with >99% specificity when detecting monosomy X (even in the presence of vanishing twin or maternal mosaicism). In addition the test can detect triploidy (while no other test can) and will report sex chromosome trisomies when seen (47 XXX, 47 XXY, 47 XYY) and it as well as provide a risk assessment on every report (low or high). A microdeletion panel (including 22q11.2 deletion syndrome, Cri-du-chat, 1p36deletion, Angelman, Prader-Willi) is also available to provide unparalleled scope among non-invasive prenatal screens.

In Table 3, the special characteristics of the four available US-based NIPT that have dominated the worldwide market are summarized [32, 63]. All these tests are performed in CAP-accredited and CLIA-certified commercial laboratories. None though has been cleared or approved by the U.S. Food and Drug Administration (FDA). Other companies from other countries have also started to offer analogous tests: Berry Genomics in China is offering Bambni test, LifeCodexx in Germany the PrenaTest (both aiming at the three common aneuploidies), etc.

Pre-analytical conditions

All tests are usually performed on maternal whole blood drawn in specific tubes: ccffDNA BCT™ tubes (Streck), since they have been proved to minimize increases in background DNA levels caused by temperature fluctuations or agitation that can occur during blood sample storage and shipping [55].

The risks, benefits, alternatives and limitations of the testing as well as a description of the population used for clinical validation should be explained to the pregnant woman after counseling and a signed informed consent for NIPT testing should accompany the sample. The family history should be reviewed to determine if the patient

Table 3: Comparison of available commercial non-invasive prenatal tests [32, 63].

	Sequenom Laboratories	Verinata Health (now Illumina)	Ariosa Diagnostics (now Roche)	Natera Inc.
Test name	Materni T21 PLUS	Verifi prenatal	Harmony prenatal	Panorama
Platform	Massively parallel shotgun sequencing with enhanced sequencing series	Massively parallel shotgun sequencing and SAFeR algorithm	DANSR technology (targeted sequencing) and FORTE algorithm	Next-generation targeted SNP-based aneuploidy testing and NATUS software
Conditions	Trisomies 13, 18, 21, sex chromosome aneuploidies and microdeletions	Trisomies 13, 18, 21, sex chromosome aneuploidies and fetal sex	Trisomies 13, 18, 21, option of testing for sex chromosome aneuploidies and fetal sex	Trisomies 13, 18, 21, monosomy X, triploidy, monosomy X and sex chromosome aneuploidies, fetal sex (optional), 22q11.2 Deletion syndrome and other microdeletions
Maternal blood requirements	Two 10 mL tubes	One 7 mL tube	One 10 mL tube	One 20 mL tube (+paternal saliva sample, optional)
Earliest sampling	10 weeks	10 weeks	10 weeks	9 weeks
Accuracy	>99%	100%	>99%	100%
Sensitivity	92–99%	87–99%	80–99%	92–99%
Cost (about)	$2700	$1500	$800	$1500
Turnaround time	7 days	3–6 days	8–10 days	7–10 days

should be offered other forms of screening or prenatal diagnosis for a particular disorder. Gestational age (GA) at the time of blood draw should be noted only in whole weeks as this is the kind of values employed from the databases for this analysis [64].

Quality assurance

The clinical use of non-invasive prenatal testing to screen high-risk patients for fetal aneuploidy is becoming increasingly common. Guidelines on the use of this testing in clinical practice have been published; however, data on actual test performance in a clinical setting are lacking, and there are no guidelines on quality control and assurance. The different non-invasive prenatal tests employ complex methodologies, which may be challenging for health-care providers to understand and utilize in counseling patients, particularly as the field continues to evolve. How these new tests should be integrated into current screening programs and their effect on health-care costs remain uncertain.

Most of the described technologies for genetic analysis of cffDNA have been patented or exclusively licensed to a small number of companies in the US. Patenting can have both positive and negative effects on the availability of tests, clinical adoption and patient access to NIPT. Perceived or real intellectual property barriers could reduce market competition, limit quality assurance and improvement mechanisms, decrease availability of alternative or cheaper tests and reduce eventually the cost effectiveness of NIPT. However, it is assumed that soon these technologies will be offered by a plethora of academic and medical institutions around the world using in-house NGS or other NIPT methodologies or as reagents to be sold separately (e.g., the recent CE-IVD Premaitha Iona test reagent).

American College of Medical Genetics and Genomics (ACMG) recommends compliance with the standards and guidelines for clinical genetics laboratories. Considering the nature of the methods used, NIPT is subject to the same internal and external quality control requirements as those for clinical molecular laboratory tests (accreditation). Quality control should assess the entire test process, including pre-analytical, analytical and post-analytical phases. Appropriate internal quality controls, e.g., stabilized plasmas with different levels of ccffDNA percentages and at different maternal ages should be constructed and analyzed periodically. Until external proficiency testing schemes sponsored by professional or regulatory organizations are available, alternative methods for proficiency testing preferably by using an interlaboratory comparison method should be required. Test performance characteristics should be available to patients and providers assessing testing, e.g., the National Accreditation bodies [47].

Other NIPT purposes

Besides the main use in detection of trisomies 21, 18 13 and monosomy X or other sex chromosomal aneuploidies (XXX, XYY, XXY), NIPT can be used for the determination of other fetal characteristics:

Fetal rhesus D determination

The rhesus D antigen is the protein product of the *rhesus D* (*RHD*) gene. A person is rhesus D positive if his/her genome has at least one copy of *RHD*. A person is rhesus D negative if his/her genome does not have any functional *RHD*.

Rhesus D incompatibility describes the situation when a rhesus D negative woman is pregnant with a rhesus D positive fetus. In a rhesus D negative woman with prior rhesus D antigen sensitization, preformed anti-rhesus D antibodies may cross the placenta and harm the fetus, resulting in hemolytic disease of the newborn. Because the genome of a rhesus D negative woman does not contain *RHD*, when *RHD* sequences are detected in maternal plasma, such sequences are likely to be from the fetus and are inherited from the father.

This rationale formed the basis for the development of a circulating fetal DNA test for non-invasive fetal rhesus D status determination. The clinical use of the non-invasive test for fetal rhesus D status assessment was launched soon after the discovery of circulating fetal DNA [65, 66]. When a fetus is tested to be rhesus D positive, the pregnancy would need to be closely monitored and anti-D immunoglobulin is typically administered later in pregnancy. On the contrary, when a fetus is tested to be rhesus D negative, the pregnancy may be managed like other uncomplicated pregnancies. Due to the different consequences in terms of management, efforts have been made to improve the reliability of the non-invasive fetal rhesus D testing and real-time qPCR methods have being applied with success.

Fetal sex

The positive detection of DNA sequences from chromosome Y in a maternal plasma sample suggests the presence

of a male fetus. The analysis of chromosome Y sequences in maternal plasma for fetal sex determination has been performed by many groups. It seems that fetal sex assessment is less reliable when performed before the seventh week of gestation. The use of real-time qPCR achieved better accuracy than conventional polymerase chain reaction methods [26, 66, 67].

NIPT for fetal sex determination would be offered to couples if the mother is a carrier of an altered gene for a serious X-linked condition, or both parents are carriers of alterations for congenital adrenal hyperplasia (CAH).

Duchenne muscular dystrophy (DMD) is an X-linked single gene disorder, which means a male fetus has a 50% chance of inheriting the condition from the mother. However, in this instance the woman would first need to have a genetic test herself to determine if she is a carrier of DMD. If the woman was found to be a carrier of DMD, then NIPT could be offered to determine the fetal sex. If NIPT shows the fetus is male, there is a 50% chance that it will inherit the condition, so an invasive test for DMD can be performed to determine if the male fetus is affected. Haemophilia is another example of a X-linked single gene disorder: if circulating fetal DNA analysis excludes a fetus as being male, an invasive prenatal diagnostic procedure could be avoided.

In addition, non-invasive fetal sex determination has been found to be useful in the prenatal management of congenital adrenal hyperplasia. 21-Hydroxylase deficiency is the commonest form of congenital adrenal hyperplasia [68]. One of the manifestations of 21-hydroxylase deficiency is the overproduction of androgens, resulting in virilization of female fetuses which can be avoided with the administration of steroids during early pregnancy. However, steroid administration is not necessary for male fetuses. Thus, knowing the sex of the fetus early in the course of the pregnancy allows clinical decisions to be made regarding the necessity for steroid treatment [69]. Prenatal fetal sex assessment would also facilitate the management of fetuses shown to have ambiguous genitalia on ultrasound.

Single-gene disorders

Lun et al. addressed this challenge with the use of a quantitative approach [70]. This type of prenatal diagnosis is generally performed in the context of a woman who is a carrier for a mutation associated with a single gene disorder in a heterozygous state. One method used is digital PCR which relies on the ability to perform each reaction with an average of one template molecule. Digital PCR can be performed by the spatial separation of PCR reactions in capillary systems. Lun et al. designed digital PCR assays to detect and quantify the number of mutant and normal alleles originating from the disease locus in maternal plasma. The relative amounts between the mutant and normal alleles obtained after PCR reactions of successive dilutions of the genetic material are compared and hence, the approach is termed relative mutation dosage (RMD). If the fetus is also heterozygous for the mutation, it should contribute additional molecules of the mutant and normal alleles – but in equal amounts – to maternal plasma. Thus, the combined (maternal and fetal) mutant alleles should be of similar quantity as the combined number of normal alleles. On the contrary, if the fetus is homozygous for the normal allele, it would only contribute additional molecules of the normal allele into maternal plasma, resulting in an overrepresentation of the normal allele relative to the mutant allele. Similarly, when the fetus is homozygous for the mutant allele, there will be an over-representation of the mutant allele relative to the normal allele. The use of this method has been reported for the NIPT of thalassaemia, sickle-cell anemia and hemophilia [71].

However, it is necessary to look beyond technology development and address issues such as costs, service regulation and stakeholder needs. Until now, research specifically addressing the ethical and psychosocial concerns and issues for service delivery for NIPT for single-gene disorders has been very limited, although some work is now emerging [72–74]. Concerns have been raised regarding the potential ease of access and hence "routinization" of testing, with health professionals and service users feeling strongly that NIPT should be offered through existing specialist services such as genetics units, to ensure appropriate pre-test and post-test counseling [72, 73, 75]. The development of policy and guidelines will be critical to ensure high quality and equitable service provision. In addition, costs will have a major impact on how testing for single-gene disorders is implemented. Hall et al. have raised concerns that the costs of setting up tests for individual disorders may limit which conditions are tested for and how many laboratories are able to offer tests [76]. To promote equity of access and standardized service delivery, it will be important to seek formal approval for new tests, explore cost-effectiveness and encourage further development.

Limitations

The clinical use of NIPT to screen high-risk patients for fetal aneuploidy is becoming increasingly common.

Several studies have demonstrated high sensitivity and specificity and there is hope that these tests will result in a reduction of invasive diagnostic procedures as well as their associated risks. Chiu et al. identified that if referrals for amniocentesis or CVS were based on NIPT results, approximately 98% of the invasive diagnostic procedures could be avoided [5]. Guidelines on the use of this testing in clinical practice have been published. The different non-invasive prenatal tests employ complex methodologies, which may be challenging for healthcare providers in counseling patients, particularly as the field continues to evolve. How these new tests should be integrated into current screening programs and their effect on healthcare costs, need further study [77].

There is widespread agreement that these tests are not yet ready to replace invasive diagnostic procedures because of many limitations. The main is that they currently detect only a subset of the chromosomal abnormalities that are uncovered by using the standard invasive procedures. The sensitivity and specificity of NIPT varies per tested chromosome and with bioinformatics corrections. This is due, at least in part, to differing GC content. The result of the test does not eliminate the possibility of other anomalies of the tested chromosomes like mosaicism, deletions or duplications. Therefore, when fetal anomalies are detected, invasive diagnostic testing and cytogenomic microarray analysis are more likely to detect these chromosomal imbalances than NIPT and may be a better testing option. Concerning aneuploidy of other chromosomes, molecular or congenital anomalies including neural tube defects are not excluded with NIPT. This is why maternal AFP testing should still be offered at 15–20 weeks gestation in order to screen for open neural tube defects even when NIPT is performed. NIPT does not screen for single-gene mutations at the moment. Sex chromosomal aneuploidies are not reportable usually for known multiple gestations. In some rare cases, false-positive or negative results may occur due to mosaicism in the fetus, placenta or mother, or due to a vanishing twin. It is likely that in real clinical practice the percentages of false-negative or positive may be higher than reported in carefully controlled, small clinical trials. NIPT is less reliable if pregnancy is <12 weeks and so far, it is impossible if pregnancy is <10 weeks. In a small number of pregnancies, due to too little DNA from the fetus in the maternal blood, the NIPT test cannot be performed, neither on pregnancies in which the mothers has had a prior bone marrow transplant. Thus NIPT is not a perfect test and it does not replace the accuracy obtained with diagnostic tests, such as chorionic villus sampling (CVS) or amniocentesis and currently it does not offer other genetic information than regarding the main numerical aneuploidies [66].

Additionally, they have largely been validated in high-risk populations. Indeed, ACOG recommends testing for high-risk women, stating that more research is required to determine how these tests perform in low-risk populations [47]. Perhaps most significantly, the effects of mosaicism (fetal or placental) or other events such as trisomy rescue are unknown, and discordant results have already been reported. There is lack of outcome data for low-risk populations. Studies examining the use of NIPT in screening low risk populations have included between 289 and 2049 patients. So far, ccffDNA testing is not recommended for low-risk women.

Preliminary data available on twins demonstrate accuracy in a very small cohort, but more information is needed before use of this test can be recommended in multiple gestations. Limited data are currently available on the use of NIPT in twins and higher-order pregnancies. When there has been an early demise of a co-twin ("vanishing twin") results may be inaccurate [57].

Furthermore NIPT is not able to distinguish specific forms of aneuploidy. For example, NIPT cannot determine if Down syndrome is due to the presence of an extra chromosome (trisomy 21), a Robertsonian translocation involving chromosome 21, or a high-level mosaicism. In cases where mosaicism is present (including confined placental mosaicism) results may be inaccurate [78]. Identification of the mechanism of aneuploidy is important for recurrence risk counseling and emphasizes the importance of confirmatory testing following NIPT: in many studies this would be CVS. However, CVS analyzes a small sample of placenta tissue and it is possible that the incidence of placental mosaicism may be higher [77].

The studies on NIPT also assume a normal maternal karyotype. In very rare occasions low-level maternal mosaicism or the presence of a maternal solid tumor will result in variations in the maternal contribution to circulating ccffDNA, which may also impact NIPT results.

Currently, it takes longer for NIPT test results to be returned than for test results on biochemical maternal serum analytes. Providers should keep this in mind when offering patients NIPT if timing is important for reproductive decision making. In most cases, NIPT is offered between 10 and 20 weeks gestation, which allows sufficient time for follow up of positive test results.

Biologic factors such as a mother's obesity expressed by a high body mass index and early gestational age are associated with reduced available cell-free fetal DNA: in a small percentage of cases, a cell free fetal DNA result will not be able to be obtained. Uninformative test results due

to insufficient isolation of cell-free fetal DNA could lead to a delay in diagnosis or eliminate the availability of information for risk assessment [77].

There might be reasons to be concerned about the use of cell-free fetal DNA testing for sex selection, especially in areas where gender imbalance is already widespread. Even if there are laws against sex selection, it would be relatively easy to obtain a blood sample and also relatively easy to send it out of the country and e-mailed a result back. It is certainly worrisome. Companies that offer testing will have to think about how they're going to determine whether the samples are being used for things that are actually illegal in other countries; it may be their obligation to ensure that they're not contributing to illegal behavior.

Last but not least, is the fact that the majority of the published literature on the performance of NIPT includes authors affiliated with or funded by commercial laboratories currently offering NIPT on a clinical basis or by companies that are financially investing in the NIPT field [77].

Conclusions

NIPT is one of the most fascinating research areas in molecular medicine of the last decade; however, as with most new technologies, there is room for refinement. The technology will become cheaper, more cost-effective and therefore, even comparable to the biochemical screening tests. Large studies are needed to explore the possibility whether the addition of the cheap PAPP-A 1st trimester screening (without NT) to NIPT could obliterate the need for a definite diagnosis with karyotype in either negative or positive results. NIPT technology is perhaps only a few steps away from an eventual whole genome (or whole exome) fetal sequencing from non-invasively isolated ccffDNA [79]. Where or if it becomes possible to do whole fetal genome analysis in a clinical setting routinely, it will open up the possibility that people can get information about the fetus that is well beyond a handful of fetal conditions, such as trisomies. This raises the concern that people will be faced with a huge amount of information about which there might be a lot of uncertainty and lack of clinical significance. Moving from a limited set of conditions to potentially any kind of human trait that has a major genetic component could really change the way people think about pregnancy and prenatal testing. After having reached the point of diagnosing the fetal diseases in such an early time point, the next step that it could be considered is the prenatal treatment in utero with the least invasive procedures (e.g., with gene editing new available tools).

NIPT should be offered in the context of informed consent, education, and counseling. Patients whose results are abnormal, or who have other factors suggestive of a chromosome abnormality, should receive genetic counseling and be given the option of standard confirmatory diagnostic testing [47].

Developments in proteomics to detect multiple novel biomarkers could also provide a cheaper screening alternative to NGS approaches in NIPT but will most likely display a reduction in sensitivity. However, new biomarkers can only be used for screening purposes, whereas NGS directly identifying fetal DNA can provide – maybe in conjuction with ccffDNA size ratio and epigenetic approaches – real NIPD that could potentially replace current invasive prenatal diagnosis techniques. With the continuous decline in the costs of all the aforementioned tests, NIPD of fetal aneuploidy is an exciting area of research that could become a clinical reality for all pregnancies in the near future.

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