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Invited critical review

Non-invasive prenatal testing using cell-free fetal DNA in maternal circulation

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ABSTRACT

The identification of cell-free fetal DNA (cffDNA) in maternal circulation has made non-invasive prenatal testing (NIPT) possible. Maternal plasma cell free DNA is a mixture of maternal and fetal DNA, of which, fetal DNA represents a minor population in maternal plasma. Therefore, methods with high sensitivity and precision are required to detect and differentiate fetal DNA from the large background of maternal DNA. In recent years, technical advances in the molecular analysis of fetal DNA (e.g., digital PCR and massively parallel sequencing (MPS)) has enabled the successful implementation of noninvasive testing into clinical practice, such as fetal sex assessment, RhD genotyping, and fetal chromosomal aneuploidy detection. With the ability to decipher the entire fetal genome from maternal plasma DNA, we foresee that an increased number of non-invasive prenatal tests will be available for detecting many single-gene disorders in the near future. This review briefly summarizes the technical aspects of the NIPT and application of NIPT in clinical practice.

Contents

1. Introduction

Prenatal testing aims to identify fetal chromosomal and genetic disorders prior to delivery. Prenatal testing includes both screening and diagnosis. Screening is typically offered to all pregnant women in the United States and many other women depending upon the region of the world they are from. Until now, prenatal screening has typically utilized maternal age, biochemical, and ultrasound markers instead of direct interrogation of the fetal genetic material, therefore, its performance has been suboptimal [1]. Positive screening results prompt further diagnostic testing, the majority of which involves invasive procedures (e.g., chorionic villus sampling and amniocentesis) to obtain

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fetal DNA for definitive diagnosis. However, these invasive procedures are associated with a small but real risk of miscarriage (i.e., 0.5–1%) [2].

For many decades, efforts have been made to find non-invasive approaches to interrogate fetal genetic features. The identification of cell-free fetal DNA (cffDNA) in maternal circulation in 1997 has created the possibility of non-invasive prenatal testing (NIPT) using fetal DNA in maternal plasma via a simple venipuncture [3]. In addition to maternal plasma, extracellular fetal DNA is also documented in other bio-fluids, such as peritoneal fluid and urine [4].

Maternal plasma DNA is a mixture of maternal and fetal DNA (Table 1). CffDNA represents a minor population in maternal plasma, which can be detected by real-time polymerase chain reaction (PCR) as early as gestational day 18 [5]. Although the exact origin and release mechanism of cffDNA in maternal blood is uncertain, a number of studies suggested that the placenta is the principle source of cffDNA. It is evidenced by the presence of cffDNA in anembryonic pregnancies [6] and the consistency between cffDNA and placenta tissue in epigenetic markers [7] and karyotypic abnormalities [8]. In contrast, the maternal DNA is speculated to be predominantly of hematopoietic origin according to a sex-mismatched bone marrow transplantation model [9].

Both maternal and fetal DNA molecules are short DNA fragments, and the fetal DNA is shorter than the maternal [10,11]. Early quantitative analysis of cffDNA using real-time PCR suggested that fractional concentrations of cffDNA accounted for 3–6% in maternal plasma and serum [12]. However, the recent application of more precise quantification techniques (e.g., digital PCR and massively parallel sequencing (MPS)) has demonstrated fractional fetal DNA concentrations as high as 10% on average in early pregnancy [13]. MPS also demonstrated that the entire fetal and maternal genomes are represented in maternal plasma at a constant relative proportion across the whole genome [11]. The absolute concentrations of cffDNA in maternal blood appear to increase as gestational age progresses [12].

Yu et al. performed a high resolution study (using MPS technique) and demonstrated that the clearance of circulating fetal DNA occurred in 2 phases, with different kinetics [14]. The initial rapid phase had a mean half-life of approximately 1 h, whereas the subsequent slow phase had a mean half-life of approximately 13 h. The final disappearance of circulating fetal DNA in normal pregnancies occurred at about 1 to 2 days postpartum. This rapid removal of cffDNA is essential for its use in prenatal genetic analysis because it ensures that fetal DNA from previous pregnancies will not be present as an interferant.

2. NIPT of paternally inherited features

The development of non-invasive prenatal testing (NIPT) technology has allowed for the detection of paternally inherited genetic features, derived from the father, which are not present in the maternal genome. The rationale is a qualitative analysis identifying the presence or absence of the paternal-specific DNA in maternal plasma.

The first application for NIPT of paternally inherited features is a fetal sex assessment. In 1997, Lo et al. reported the use of conventional PCR to detect Y chromosome sequences (i.e., *DYS14*) in maternal plasma and serum which indicated a male fetus [3]. Since then, a number of large-scale clinical trials have further validated this approach for fetal sex assessment. A meta-analysis systematically reviewed 57 studies from 1997 to 2011 involving 3524 male- and 3017 female-bearing pregnancies [15]. The majority of these studies used real-time PCR to analyze Y chromosome sequences, such as *SRY* and *DYS14* in maternal plasma. The overall sensitivity was 95.4% and specificity was 98.6% for detection of a male fetus. This high detection accuracy has also been observed in more recent publications [16,17]. The traditional sonographic approach for fetal sex assessment can determine fetal sex accurately after gestational week 13 [18], whereas the cffDNA-based approach can be reliably conducted between gestational weeks 7 and 12 [15]. This is of particular value in certain clinical conditions. For example, in congenital adrenal hyperplasia, the pregnancy of an affected female fetus would prompt early dexamethasone administration to prevent virilization [19]. Due to its reliable performance, this method has been adopted into clinical practice by a number of countries for at-risk pregnancies [20,21].

Another application of NIPT of paternal inherited features is assessment of fetal rhesus D (RhD) status in RhD negative mothers. RhD is a surface antigen of red blood cells, which could activate the immune response in RhD negative individuals. Therefore, if an RhD negative mother is carrying an RhD positive fetus, there is a risk that the RhD antigen released from the fetus would enter the maternal circulation and cause alloimmunization to RhD positive fetuses in future pregnancies, leading to potentially severe hemolytic disease of the fetus and newborn [22]. In order to eliminate the risk of alloimmunization, it is suggested that all RhD negative mothers receive RhD immunoglobulin prophylaxis [23]. As a result, a proportion of RhD negative mothers, who carry RhD negative fetuses, would receive unnecessary prophylaxis treatment. A more logical strategy is to provide RhD immunoglobulin prophylaxis only to RhD negative mothers who carry RhD positive fetuses. This strategy would require the interrogation of fetal RhD status, ideally in a noninvasive manner. In 1998, Lo et al. [24] and Faas et al. [25] first reported the use of cffDNA to genotype the fetal *RHD* by detecting the presence of *RHD* sequences in RhD negative mothers when carrying RhD positive fetuses. A number of large-scale clinical trials have been conducted to further evaluate its performance, and a systematic review of these studies from 2006 to 2008 demonstrated high diagnostic accuracy (sensitivity = 99.5–99.8%, specificity = 94.0–99.5%) [26]. More recent publications also have confirmed this test's high accuracy [16,27]. Due to its reliable performance and noninvasive nature, this test has become clinically available in a number of countries [28].

Use of cffDNA has also been reported for paternity determination in forensics [29,30]. In this application, a panel of short tandem repeats, or SNPs, is analyzed in the maternal plasma DNA for comparison to the maternal and putative paternal genotypes. Concordance would indicate that the putative father is the biological father. Apart from qualitative analysis, paternally inherited sequences (i.e., paternal-specific alleles) could also be used quantitatively in a series of applications, such as fractional fetal DNA concentration estimation [31] and determination of twin zygosity [32].

Table 1
Characteristics of cell free and fetal DNA.

Features	Maternal	Fetal	References
Most likely origin	Hematopoietic	Placenta	[44,9]
Release kinetics	Absolute concentrations increase as gestational age progresses	1) Detectable as early as day 18	[5]
		2) Absolute concentrations increase as gestational age progresses	[12]
		3) Rapidly cleared following delivery (two phase kinetics)	[14]
Fractional concentration in maternal blood	~90%	~10% in early pregnancy	[13]
Size	Short DNA fragments (<200 bp) Larger than cell free fetal DNA molecules	Short DNA fragments (<200 bp) Smaller than maternally derived DNA molecules	[11,10]
Representation	Entire maternal genome	Entire fetal genome	[11]

3. NIPT of chromosomal aneuploidies

3.1. Challenges

An aneuploid fetus bears an abnormal number of chromosomes. NIPT of fetal aneuploidies in maternal plasma is technically more challenging than NIPT of paternally inherited features. Stringent quantitative analysis is required in order to identify the fetal chromosomal aberration due to the minor population of fetal DNA in maternal circulation. For example, if a pregnant woman carries a trisomy 21 (T21) fetus, the extra copy of chromosome 21 from the fetus would contribute additional 5% of chromosome 21 DNA sequences in a maternal plasma sample with fractional fetal DNA concentration of 10%.

Early studies were focused on the elimination of interference from the background maternal DNA by using fetal-specific markers, such as epigenetic markers (i.e., fetal-specific hyper- or hypo-methylated DNA) [33–37] and RNA markers (e.g., *PLAC4* gene) [38]. However, these approaches are subject to a number of limitations. For example, the RNA-based allelic ratio approach is only applicable for fetuses that are heterozygous for the target SNPs [38]. Other limitations include lack of reproducibility, requirement for multiple markers, complex experimental and data analysis procedures, small numbers of participants, and/or less optimal diagnostic performance (relatively low sensitivity and specificity).

3.2. Single molecule counting

More recent studies have aimed to differentiate the subtle chromosomal dosage change with more precise single molecule counting techniques, such as digital PCR and MPS. In digital PCR, the maternal plasma DNA templates are diluted to a single molecule. The target loci are amplified and quantified to allow precise measurement of DNA molecules derived from candidate chromosomes [39,40]. The detection performance relies on the fractional fetal DNA concentration and the number of molecules used for analysis. For example, it is estimated that 7680 DNA molecules are needed to achieve a 97% detection accuracy for NIPT of T21 with a 25% fractional fetal DNA concentration [40].

MPS is a second generation sequencing technology, which allows single molecule counting in a high throughput manner. In 2008, two pilot studies demonstrated the feasibility of MPS as a powerful tool for NIPT of T21 [41,42]. In both studies, each analysis of MPS generated millions of sequence reads from maternal plasma DNA [41]. The sequence reads were then mapped to the human genome to identify their chromosomal origin. After that, the number of sequence reads of each chromosome was counted to calculate its genomic representation. The over-representation of chromosome 21 would indicate the presence of a T21 fetus. With a relatively small sample size, both these proof-of-concept studies demonstrated 100% sensitivity and specificity for T21 detection [41,42].

In order to increase the throughput and reduce the cost of MPS, strategies such as multiplex sequencing [13] and targeted sequencing [43] have also been employed. The goal of multiplex sequencing is to simultaneously sequence multiple patient samples in a single run. The goal of targeted MPS is to enrich the regions from the chromosome(s) under evaluation (e.g., chromosome 21, 18, and/or 13) before sequencing. Validation studies of different MPS strategies suggested that the performance of MPS in NIPT of aneuploidies depends largely on the total number of sequence reads (i.e., ≥2.3 million) and fractional concentration of fetal DNA (i.e., ≥4%) [44].

3.3. Clinical validation of MPS-based NIPT of aneuploidies

Several large-scale follow-up clinical studies using non-targeted or targeted MPS have further validated the clinical performance of MPS-based NIPT of T21, trisomy 18(T18), trisomy 13 (T13) and other chromosome aneuploidies. Studies with total participant numbers greater than 100 are listed in Table 2. T21 is the most common and clinically relevant type of chromosomal aneuploidy, followed by T18 and T13. Therefore the discussion in this section will focus on NIPT of these three trisomies.

The overall clinical performance of MPS-based NIPT for detection of T21 is more robust than that of T18 and T13. The relatively poor detection of chromosome 18 and 13 aneuploidies was speculated to be due to their relatively lower average GC content than chromosome 21 [41,45]. For T21, the sensitivity has been published to be 100%, except for one study that reported 98.6% [46]. The specificity has been reported to be 97.9% to 100%. When combining all the studies listed in Table 2, the overall sensitivity and specificity for detection of T21 are 99.64% and 99.96%, respectively, with only three false negative cases and nine false positive cases (Table 3). Notably, all three false negative cases were reported in one of the earlier studies in 2011. Most of these studies were prospective multi-center studies and focused on pregnant women at a high risk for fetal aneuplodies. The overall prevalence of these studies for T21, T18, and T13 is 1:25, 1:67, and 1:74, which is much higher than that in the screening population (1:500 for T21; 1:4000 for T18, and 1:7000 for T13) [6]. It is important to note that disease prevalence influences predictive values. For example, the positive predictive values (PPV) and negative predictive values (NPV) for T21 are calculated based on the average sensitivity of 99.64% and specificity of 99.96% derived from large scale studies listed in Table 2. At this near perfect sensitivity and specificity, the PPV is only 83.31% in the routinely screened population (prevalence 1:500) compared to 99.44% in the high risk population (prevalence 1:15).

A few recent studies have been performed using a routine screening population [47–49] or combination of high risk and screening populations [50]. Nicolaides et al. [48] demonstrated 100% sensitivity and 100% specificity of MPS-based NIPT performed on a screening population of 1949 cases at 11–13 weeks of gestational age. The prevalence of T21 in this study (1:244) is more representative of that in a routinely screened population (1:500). This study demonstrated that the performance of screening for T21 and T18 by MPS-based NIPT in a routine population is as robust as previously reported high-risk groups, paving the way, perhaps, for NIPT to replace traditional maternal serum screening.

3.4. Implementation into clinical practice

MPS-based NIPT for T21 has recently been launched by more than four commercial companies and several clinical laboratories in the United States, China, and Europe. At least four studies have been published to report the initial clinical laboratory experience in NIPT for fetal aneuploidy using maternal plasma [47,49–51].

Conventionally, the prenatal testing for fetal aneuploides is performed as a combination of screening and confirmation testing. Results from noninvasive screening that indicate increased risk are followed by invasive diagnostic procedures (e.g., chorionic villus sampling and amniocentesis) to obtain fetal DNA for definitive diagnosis. NIPT can be integrated into prenatal screening and diagnosis practice through three potential clinical approaches: 1) Replace the current maternal serum screening protocol with NIPT; 2) Add NIPT as an intermediate step after the screening and before the invasive diagnostic testing; or 3) Replace the invasive diagnostic testing. Which of these options is adopted will depend on multiple factors, such as clinical performance (sensitivity, specificity, PPV and NPV) and practical considerations (test availability, cost-effectiveness and turnaround time).

Professional societies, such as the American College of Obstetricians and Gynecologists (ACOG), the Society for Maternal Fetal Medicine (SMFM), the International Society for Prenatal Diagnosis (ISPD), the National Society of Genetic Counselors (NSGC), and the Society of Obstetricians and Gynecologists of Canada (SOGC) have recently published their statements/opinions on how to implement the MPS-

Table 2
Large-scale studies of NIPT of trisomies 21, 18, and 13.

Authors	Study design	Study population[a]	Technique	Case numbers		GA [b] (weeks)	Sensitivity (%)	Specificity (%)	Reference
				Aneuploidy	Total				
Chen et al.	Prospective and retrospective study Multi-center	High risk	MPS	T18: 37 T13: 25	289	13	91.9 (34/37) 100 (25/25)	98 (247/252) 98.9 (261/264)	[45]
Chui et al.	Prospective and retrospective study Multi-center	High risk	MPS	T21: 86	232	13	100 (86/86)	97.9 (143/146)	[13]
Enrich et al.	Prospective study Multi-center	High risk	MPS	T21: 39	449	16	100 (39/39)	99.7 (409/410)	[68]
Palomaki et al.	Prospective nested case–control study Multi-center	High risk	MPS	T21: 212	1683	15	98.6 (209/212)	99.8 (1468/1471)	[46]
Bianchi et al.	Prospective observational nested case–control study Multi-center	High risk	MPS	T21: 89 T18: 36 T13: 14	532	15	100 (89/89) 97.2 (35/36) 78.6 (11/14)	100 (443/443) 100 (496/496) 100 (518/518)	[69]
Ashoor et al.	Retrospective nested case–control study	High risk	Targeted MPS	T21: 50 T18: 50	397	11–13	100 (50/50) 98 (49/50)	100 (347/347) 100 (347/347)	[70]
Dan et al.	Prospective study Multicenter clinical experiences	High risk and screening	MPS	T21: 142 T18: 46	11,105	20	100(142/142) 100 (46/46)	99.99 (10,962/10,963) 99.99(11,058/11,059)	[50]
Jiang et al.	Prospective study Multicenter	NA	MPS	T21: 16 T18: 12 T13: 2	903	10–34	100 (16/16) 100 (12/12) 100 (2/2)	100 (887/887) 99.9 (890/891) 100 (901/901)	[71]
Lau et al.	Prospective study	High risk	MPS	T21: 11 T18:10 T13: 2	108	12	100 (11/11) 100 (10/10) 100 (2/2)	100 (97/97) 100 (98/98) 100 (106/106)	[72]
Nicolaides et al.	Prospective cohort study	Screening	MPS	T21: 8 T18: 2	1949	11–13	100 (8/8) 100 (2/2)	100 (1941/1941) 99.9 (1945/1947)	[48]
Norton et al.	Prospective cohort study Multi-center	High risk	MPS	T21: 81 T18: 38	3006	17	100 (81/81) 97.4 (37/38)	99.97 (2924/2925) 99.93 (2966/2968)	[73]
Palomakiet al.	Prospective nested case–control study	High risk	MPS	T18: 59 T13: 12	1971	15	100 (59/59) 91.7 (11/12)	99.7(1907/1912) 99.2(1943/1959)	[74]
Sparks et al.	Prospective study	High risk	Targeted MPS	T21: 36 T18: 8	167	18	100 (36/36) 100 (8/8)	100 (131/131) 100 (159/159)	[75]
Liang et al.	Prospective Multi-center	High risk	MPS	T21: 40 T18: 14 T13:4	412	15–39	100 (40/40) 100 (14/14) 100 (4/4)	100 (372/372) 100 (398/398) 99.75 (407/408)	[76]
Nicolaides et al.	Prospective	High risk	Targeted MPS	T21: 25 T18: 3 T13: 1	229	11–13	100 (25/25) 100 (3/3) 100 (1/1)	100 (204/204) 100 (226/226) 100 (228/228)	[77]

Abbreviations: GA: gestational age; MPS: massively parallel sequencing; NA: not available; T21: trisomy 21; T18: trisomy 18; T13: trisomy 13; XO: Monosomy X.
[a] High risk for aneuploidy is determined on the basis of one or more of the following: advanced maternal age, previous positive prenatal screen, fetal ultrasound abnormality, or prior pregnancy with fetal aneuploidy.
[b] Mean/median gestational age is shown except when range values are provided.

based NIPT for fetal aneuploidy into clinical practice [8,12,43,52]. These professional societies agree that there is evidence that NIPT is a safe and effective screening test for fetal aneuploidy in high-risk populations. It can also be used as a follow-up test for those patients who have a positive maternal serum screening test. It is recommended that patients with positive NIPT results should be counseled about confirmatory diagnostic testing.

Currently, NIPT is not recommended as a screening testing due to less evidence from the published data using a screening population. In the near future, once its clinical performance has been proven and the cost of MPS has been lowered, we predict that NIPT will likely replace the conventional maternal serum screening testing.

Table 3
Summary of overall performance of studies listed in Table 2.

	Case number		TP	TN	FP	FN	Sensitivity (%)	Specificity (%)
	Aneuploidy	Total						
T21	T21: 835	21,172	832	20,328	9	3	99.64 (832/835)	99.96 (20,328/20,337)
T18	T18: 315	21,068	309	20,737	16	6	98.10 (309/315)	99.92 (20,737/20,753)
T13	T13: 60	4444	56	4364	20	4	93.33 (56/60)	99.54 (4364/4384)

Abbreviations: T21: trisomy 21; T18: trisomy 18; T13: trisomy 13; TP: true positive; TN: true negative; FP: false positive; FN: false negative.

3.5. Regulatory requirements

With the rapid introduction of the MPS-based NIPT into clinical practice, it is important to consider how this testing will be regulated. Most recently, the College of American Pathologists (CAP) released the latest edition of the Laboratory Accreditation Program checklist on July 29, 2013. The new checklist includes a focus on next generation sequencing for maternal plasma to identify fetal aneuploidy. The CAP-accredited clinical laboratories which currently offer noninvasive prenatal testing based on cffDNA must meet the new checklist requirements, including requisition, quality control, quality assurance, and result reporting (http://www.captodayonline.com/for-prenatal-ngs-labs-new-accreditation-requirements-9135/).

4. Genome-wide fetal profiling

Deciphering the fetal genome sequence from maternal plasma DNA is another milestone in NIPT. In 2010, Lo et al. first demonstrated the feasibility of genome-wide fetal profiling by deep sequencing of maternal plasma DNA on the basis of parental genomic scaffolds [11].

The paternally and maternally inherited portions were deduced respectively, by using corresponding parental genotyping information.

In this proof-of-principle study, the deduction of the paternally inherited portion targeted the paternal-specific alleles. As fetal DNA only accounts for a minor population in maternal plasma, deep sequencing is required in order to generate sufficient statistical confidence to rule-in or rule-out the presence of a paternal-specific allele. By deep sequencing up to 65-fold, 93.9% of paternal-specific alleles had been identified in the maternal plasma DNA with a fractional fetal DNA concentration of 11.43%.

Deduction of the maternally inherited portion is more challenging, as the maternal portion per se is identical to the maternal DNA background. In principle, the maternal genome should have one pair of haplotypes, namely haplotypes I and II. In this regard, with a fractional fetal DNA concentration of 10%, there would be 5% overrepresentation of haplotype I in maternal plasma, if haplotype I had been passed to the fetus. Therefore, the deduction of the maternally inherited portion is to identify the overrepresentation of the transmitted maternal haplotype, which entails precise quantitative analysis. In order to achieve such a goal, Lo et al. developed a method named "relative haplotype dosage analysis" (RHDO) [11]. In Lo's proof-of-principle study, the maternal haplotypes were deduced based on genotypes of the parent-offspring trio by using SNPs that were maternally heterozygous and paternally homozygous. The maternal plasma DNA reads aligned to two maternal haplotypes were accumulated respectively, and compared to each other. The allelic accumulation and comparison continued along the maternal haplotypes until a statistically significant classification had been drawn by sequential probability ratio test (SPRT). As a result, segments of maternal haplotypes were sequentially interrogated to reveal the maternally inherited portion. In Lo's study, over 7000 RHDO classifications had been made across the whole genome with error rate less than 0.2% using a consecutive-block algorithm, in which two consecutive RHDO segments with the same haplotype classification were required in order to conclude a switch in haplotype inheritance.

Recently, another three publications further validated the feasibility of fetal genome profiling in maternal plasma DNA by using the concept introduced in Lo's study [53–55].

5. NIPT of monogenic diseases

Monogenic diseases affect approximately 1 in 100 live births, which are caused by a defective gene [56]. Monogenic diseases are typically sub-classified into 3 main categories: autosomal dominant, autosomal recessive, and X-linked.

The NIPT of certain autosomal dominant diseases, in which father carries the mutation, resembles the qualitative analysis used in fetal sex or RhD status assessment. Examples included NIPT of Huntington disease [57–59] and myotonic dystrophy [60]. A similar strategy could also be applied to NIPT of another group of autosomal dominant diseases, which are mainly caused by de novo mutation, such as achondroplasia [61,62].

The NIPT of autosomal recessive diseases is considered as challenging, since the maternally inherited portion of fetal genome is identical to the maternal DNA background. Therefore, quantitative comparison between the mutant and wild-type allele is required in order to identify whether the fetus had inherited the mutant. For example, assuming that the fractional fetal DNA concentration is 10%, for autosomal recessive diseases in which both parents are heterozygous carriers with the same mutation, the fetus would become affected if it inherited both mutations from the parents. In this situation, the ratio between mutant and wild-type allele would become 1.22:1 (Table 4). The ratio would become 1:1 if the fetus is a heterozygous carrier, and become 0.82:1 if the fetus is normal. For X-linked diseases, mostly mother carries a recessive mutation, and a male fetus would become affected if it receives the mutation from the mother. Therefore, the NIPT of fetal sex could serve as a first-tier screening test as described above. However, further interrogation of mutation inheritance in male fetuses would require quantitative analysis. In this situation, the ratio between mutant and wild-type allele would become 1.11:1 if carrying an affected male fetus, and become 0.9:1 if carrying a normal male fetus (Table 4).

Table 4
Mutant and wild-type ratio in autosomal recessive and X-linked diseases.

Disease	Maternal genotype	Fetal genotype	Maternal plasma DNA (fe% = 10%)		
			M (copies per 100 GE)	W (copies per 100 GE)	M:W ratio
Autosomal recessive disease	M W	W W	90	110	0.82:1
		M W	100	100	1:1
		M M	110	90	1.22:1
X-linked disease	M W (X)(X)	W - (X)(Y)	90	100	0.9:1
		M - (X)(Y)	100	90	1.11:1

Abbreviations: W: wild-type; M: mutant; GE: genome equivalent; (X): X chromosome; (Y): Y chromosome; fe% = fractional fetal DNA concentration.

In order to detect such subtle changes in the allelic ratio, a very precise molecular quantification method is needed, such as single molecule counting techniques. In 2008, Lo et al. developed a digital PCR-based method named "relative mutation dosage" (RMD) analysis for this kind of application [63]. In digital PCR analysis, the DNA templates were diluted to a single molecule which enabled the precise measurement of the wild-type and mutant alleles in maternal plasma. The detection rate depends on the fractional fetal DNA concentration and the number of molecules used for analysis. For example, with a concentration of 0.5 template per well, a 765-well reaction can achieve a detection accuracy of greater than 95% with a fractional fetal DNA concentration of 20% [63]. If the fractional fetal DNA concentration decreases by 2-fold (i.e., 10%), the number of wells would need to increase by 4-fold (i.e., 2^2) in order to maintain the detection accuracy (i.e., >95%). Pilot studies have demonstrated the feasibility of RMD analysis in NIPT of autosomal recessive (e.g., β-thalassemia [63] and sickle cell anemia [64]) and X-linked diseases (e.g., hemophilia [65]).

Another single molecule counting platform is MPS. In 2010, Lo et al. introduced the concept of genome-wide fetal profiling by MPS of maternal plasma DNA as described above [11]. In Lo's study, they also demonstrated the combination of deep sequencing and RHDO analysis for NIPT of monogenic diseases, using β-thalassemia as an example. For the family recruited in that study, both parents were heterozygous carriers of β-thalassemia. The father carried the CD41/42(−CTTT) mutation, while the mother carried the nt-28(A → G) mutation. Deep sequencing of maternal plasma DNA enabled the detection of CD41/42(−CTTT), indicating that the fetus had inherited the paternal mutation. This result prompted the further investigation of fetal inheritance of maternal mutation. RHDO analysis was performed across the disease-causing region (i.e., *HBB* gene), and revealed that the maternal haplotype carrying the wild-type allele was over-represented in maternal plasma. The fetus did not inherit the maternal mutation. Therefore, the fetus was a heterozygous carrier with mutation inherited from the father. In clinical practice, however, deep sequencing in a genome-wide manner would not be the most cost-effective approach. Recently, Gabriel et al. introduced a targeted approach for RHDO analysis, in which hybridization-based enrichment was performed to enrich the region of interest before sequencing [66]. By using this approach, the NIPT of monogenic diseases could be performed in a cost-effective and disease-tailored manner.

Compared with RMD analysis, RHDO analysis requires additional effort to work out the parental haplotypes. However, targeted RHDO analysis demonstrates advantages in detecting multiple mutations across multiple disease-causing regions, whereas RMD analysis typically targets one or a limited number of loci. In addition, as RHDO analysis targets the adjacent alleles linked to the mutations rather than the mutations per se,

it allows NIPT of certain monogenic diseases when direct interrogation of mutations is considered challenging. For example, in congenital adrenal hyperplasia, pseudogenes interfere with the mutation interrogation due to their homology [67].

6. Conclusion

The existence of cffDNA in maternal blood holds enormous promise for the development of NIPT. However, it is technically challenging to detect circulating fetal DNA which is only a minor portion in the large background of maternal DNA. In recent years, the technical barrier has been overcome through the development of molecular techniques, such as digital PCR and MPS. These highly sensitive and precise methods have enabled the successful introduction of NIPT into clinical practice, such as fetal sex assessment, RhD genotyping, and fetal chromosomal aneuploidy detection. In addition, the introduction of genome-wide profiling of cffDNA by MPS holds great promise for detecting many single-gene disorders in the near future.

Because of its noninvasive nature, broad applications, and availability at an earlier gestational age, cffDNA testing provides a tremendous opportunity to change prenatal genetic testing. However, such changes will not be realized without answering related ethical, practical, and social questions. How will clinicians be educated? How might prospective parents and physicians use the fetal genomic information? Can NIPT be applied to pregnancies with complications (e.g., diabetes)? These and many other questions have yet to be addressed.

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