CHAPTER ONE

Cell-Free Fetal DNA Testing for Prenatal Diagnosis

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Abstract

Prenatal diagnosis and screening have undergone rapid development in recent years, with advances in molecular technology driving the change. Noninvasive prenatal testing (NIPT) for Down syndrome as a highly *sensitive screening test* is now available worldwide through the commercial sector with many countries moving toward implementation into their publically funded maternity systems. Noninvasive prenatal diagnosis (NIPD) can now be performed for definitive diagnosis of some recessive and X-linked conditions, rather than just paternally inherited dominant and de novo conditions. NIPD/T offers pregnant couples greater choice during their pregnancy as these safer methods avoid the risk of miscarriage associated with invasive testing. As the cost of sequencing falls and technology develops further, there may well be potential for whole exome and whole genome sequencing of the unborn fetus using cell-free DNA in the maternal plasma. How such assays can or should be implemented into the clinical setting remain an area of significant debate, but it is clear that the progress made to date for safer prenatal testing has been welcomed by expectant couples and their healthcare professionals.

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1. CELL-FREE FETAL DNA

The presence of cell-free fetal DNA (cffDNA) circulating in maternal plasma was first described in 1997 [1]. Prior to this, it was known that fetal cells circulated in the maternal blood stream; however, their clinical application is limited due to the paucity of fetal cells in the maternal circulation [2]. Conventionally genetic prenatal diagnosis requires an invasive procedure, namely chorionic villus sampling (CVS) or amniocentesis, which carries a small risk of miscarriage (approximately 0.5–1%) [3]. The discovery of cffDNA has enabled development of safer, earlier testing based on a simple maternal blood sample. A number of clinical diagnostic tests have now been implemented, including fetal sex determination [4,5], *RHD* blood group determination [6], and the detection or exclusion of de novo or paternally inherited monogenic disorders [7]. The advent of massively parallel next-generation sequencing (NGS) broadened opportunities to include widescreen detection of fetal aneuploidy [8,9]. The discovery that the fetal genome was present in its entirety [10] has further driven advances in this field.

The origin of cffDNA was initially thought to be fetal hematopoietic cells as it was known that a number of different cell types circulate in maternal blood [11,12]. However, it was considered unlikely that this was the sole source due to the relatively low volume of cells. A study by Zhong *et al.* [13] confirmed this in a group of patients where significantly increased cffDNA levels were observed without any incremental increase in fetal nucleated red blood cells. Fetal hematopoietic cells as a source of cffDNA were also disproved by Angert *et al.* [14] who originally hypothesized that after phlebotomy, apoptotic fetal cells would release their DNA, increasing cffDNA concentration.

Evidence now suggests that cffDNA is placental in origin. Differential methylation patterns between maternal blood cells and placental tissue have revealed the placental origin of cffDNA. The *mapsin* gene promoter is methylated in maternal blood cells and hypomethylated in placental tissue [15]. By genotyping a single-nucleotide polymorphism in the unmethylated *mapsin* sequence in maternal plasma, it was shown that this sequence was derived from the fetus. Furthermore, while detected throughout pregnancy, the signature was cleared 24 h after delivery. *RASSF1* has also been shown to be a fetal epigenetic marker, hypermethylated in placenta, and hypomethylated

in maternal blood [16] and this has been developed as a marker for fetal DNA in maternal blood [17]. Further evidence came from the study reported by Alberry and colleagues [18] who demonstrated that levels of cffDNA were the same in normal and anembryonic pregnancies, supporting the hypothesis that the trophoblast is the main source of cffDNA. Multiple studies have shown an association of cffDNA levels with gestational age [19], again indicating a trophoblastic origin for cffDNA with cffDNA levels coinciding with fetoplacental circulation establishment [20]. A recent case report where cultured mesenchymal cells and umbilical cord fibroblasts showed nonmosaic trisomy 18, while direct karyotyping of the cytotrophoblast and cfDNA analysis indicated that the fetus had a normal male karyotype, adds to the body of evidence as it seems that the normal cell line was confined to the placenta [21]. Finally, cffDNA levels are undetectable by 2 h after delivery [22], although fetal cells may persist [23]. This feature makes cfDNA in maternal plasma an ideal source of fetal genetic material for prenatal diagnosis as it is specific to an individual pregnancy.

1.1 Fetal Fraction of Cell-Free DNA

Initial quantification using real-time polymerase chain reaction (PCR) showed that 3.4–6.2% of cfDNA was of fetal origin in early (11–17 weeks) and late pregnancy (37–43 weeks gestation), respectively [24]. However, recent studies using digital PCR indicates that this is probably an underestimation with the cffDNA fraction being higher with medians of 9.7%, 9%, and 20.4% in the first (12–14 weeks), second (17–22 weeks), and third trimesters (38–30 weeks), respectively [25]. It is now clear that several factors influence the fetal fraction, including gestational age, maternal weight, aneuploidy, maternal disease, and the number of fetuses present. Fetal fraction is reported to increase by approximately 0.1% per week between 10 and 21 weeks of gestation and more rapidly after 21 weeks, approximately 1% per week [19,26]. Only 1–3% of pregnancies have a fetal fraction of less than 4% [27,28]. The fact that cffDNA is in the minority, the majority of cfDNA being maternal in origin, presents a challenge for sensitivity of assays and data analysis as low fetal fraction can cause false negative results. Negative correlation of fetal fraction with maternal weight is well documented [19,26,29,30]. Total cfDNA is directly related to maternal body mass index and the relatively lower fetal fraction with increasing maternal weight may reflect increased release of maternal cfDNA from adipose cells [31]. The

aneuploid status of the fetus has a varying effect on fetal fraction. Trisomy 21 is associated with increased fetal fraction [32]. However, trisomies 18 and 13 are associated with lower levels of cffDNA, thought to be due to the reduction in placental volume observed in these trisomies [19,29,33,34]. In contrast, in multiple pregnancies where there is an increased placental mass, for example dichorionic twins, fetal fraction tends to be higher than in singleton pregnancies [35].

Other clinical indications which have been studied in association with fetal fraction are preexisting hypertension, which is associated with significantly lower fetal fraction and preexisting diabetes, hyperthyroidism, or being a carrier of HbsAg, which do not affect fetal fraction [19]. Several studies have shown that there is an increase in fetal DNA in women with symptomatic preeclampsia [36–39] and those who will develop preeclampsia [40]. A sharp increase in cffDNA levels have been observed at 32 weeks gestation in normal pregnancies which may indicate impending delivery [24]. This was supported by the observation of increased cffDNA in women who had spontaneous preterm deliveries between 26 and 34 weeks gestation [41]. CffDNA is also higher in placenta previa [42] and hyperemesis gravidarum [43], although the underlying pathology here is unclear.

The method of sample collection is important for maintaining fetal fraction. More cffDNA is found in the plasma of maternal blood, as opposed to the cellular fraction [44]. It is important to maintain maternal cell integrity when collecting samples for cfDNA extraction. Inappropriate sample collection leads to reduced detection of fetal components as lysis of maternal cells reduces the fetal fraction. Use of K_3EDTA tubes if the sample can be processed within 8 h is appropriate, otherwise cell-stabilizing tubes should be used [45].

Due to the relatively low amount of cffDNA, a number of approaches have been tried to enrich this component, including use of formaldehyde [46]. Cell-free DNA is fragmented, with maternal cfDNA approximately 166 bp, while the fetal component is 143 bp [47,48]. Gel size selection to enrich for the short fragments of cffDNA has been shown to improve the sensitivity of paternal allele detection for β-thalassemia mutations [49]. An alternative is to count short DNA molecules only, using PCR which amplifies short and long amplicons [50]. Using primers targeted to *ZFX/ZFY*, the authors found the greatest discrimination between maternal and fetal molecules was using 179 and 64 bp amplicons. However, enrichment based on size has not yet found a place in routine clinical practice.

2. CLINICAL APPLICATIONS FOR cffDNA

2.1 Noninvasive Prenatal Diagnosis

Traditional methods of prenatal testing for monogenic disorders use invasive sampling, subjecting the pregnancy to a small risk of miscarriage [3]. This risk can deter some couples from undergoing prenatal diagnosis. The purpose of noninvasive prenatal diagnosis (NIPD) is to confirm the presence or absence of a specific genomic region in a fetus and is therefore diagnostic, hence the terminology NIPD. This approach can be used in:

(1) RhD-negative mothers to identify fetuses at risk of hemolytic disease of the newborn by detecting the presence of *RHD* in the maternal plasma.

(2) Pregnancies at risk of sex-linked conditions to triage for invasive testing or treatment by determining fetal sex using markers on the Y chromosome.

(3) Pregnancies at risk of de novo, dominant, or recessive conditions because of a known family history or ultrasound findings.

2.1.1 Antenatal Determination of Fetal RHD Status

Rhesus disease, or hemolytic disease of the fetus and newborn (HDFN) occurs when maternal IgG antibodies to red cell surface antigens cross the placenta and cause the immune destruction of fetal red cells or erythroid progenitors. The RhD antigen is most commonly involved and to prevent immunization, it is now routine practice to give pregnant RhD-negative mothers anti-D immunoglobulin antenatal and after delivery. However, only around 60% of these women carry an RhD positive baby, the remaining 40% are not at risk as they carry an RhD-negative baby. This means many women are exposed to anti-D immunoglobulin, a human blood product, unnecessarily. The *RHD* status of the fetus in RhD-negative mothers can be determined by using qPCR to detect presence or absence of the *RHD* gene in cfDNA in maternal plasma. Initially, this technology was applied to women at high risk of HDFN when early identification of the fetal RHD type allowed those women found to be carrying an RhD-negative baby to avoid unnecessary intervention of anti-D immunoglobulin, a human blood product, anxiety, and the inconvenience of intensive and expensive surveillance of the pregnancy for signs of fetal anemia. However, the result can also be used to stratify routine anti-D administration and this approach has been implemented in a number of countries where testing is done at around 28 weeks gestation and

anti-D only administered when the fetus is found to be *RHD*− [6,51]. A UK study has shown testing is highly accurate if done earlier in pregnancy with the sensitivity of determining RhD positivity at different weeks of gestation being 96.9% (<11 weeks), 99.8% (11–13 weeks), 99.7% (14–17 weeks), 99.8% (18–23 weeks), and 100% (>23 weeks) [52]. Introduction of routine testing prior to 28 weeks gestation would be beneficial as anti-D immunoglobulin treatment is given from 12 weeks after any potential sensitizing event.

2.1.2 NIPD for Fetal Sex Determination

Although much prenatal genetic testing continues to rely on invasive methods, the discovery of cffDNA combined with advances in technology mean increased availability of NIPD assays. The first application was prenatal sex determination, clinically indicated in pregnancies at risk of a serious genetic disorder affecting a particular sex. Knowledge of fetal sex can be used to avoid invasive testing in up to 50% of cases at risk of X-linked disorders where a female fetus would not be affected [53]. It can also be used to inform pregnancy management and guide treatment in pregnancies at risk of congenital adrenal hyperplasia (CAH), where dexamethasone treatment can be administered to mothers bearing female fetuses to reduce virilization of the external genitalia in affected female [54,55] fetuses. However, lack of long-term follow-up data and potential neurodevelopmental side effects has led to cautionary use. Noninvasive fetal sex determination can be performed using a variety of laboratory approaches [56] but most commonly uses qPCR to detect the presence of *SRY* on the Y chromosome in maternal plasma [4,57,58]. Traditionally ultrasound has been used for sex determination, which is accurate in >99% of cases with normal genitalia [59]. Ultrasound at early (12–14 weeks) gestation can also be performed but is restricted to specialist centers [60–62]. The benefit of noninvasive fetal sexing compared to traditional ultrasound means of sex determination are earlier detection from 7 weeks [53,56,63] and it is more than 99% accurate if performed after 7 weeks gestation [4]. In addition, it is useful for cases such as CAH where external genitalia can be abnormal and so ultrasound is not appropriate for sex determination. NIPD for fetal sex determination has been reported to be cost neutral in the UK for conditions such as Duchenne muscular dystrophy where most carriers choose to have invasive testing [64]. However, comparative costs may vary in other societies [64].

2.1.3 Definitive NIPD for Monogenic Diseases

While fetal sex determination obviates the need for invasive testing for some pregnancies in certain conditions, NIPD for definitive diagnosis of monogenic disorders has been widely reported in the literature, usually on a case-by-case basis or small series [65] and is gradually being introduced into clinical practice. The technical approaches for diagnosis vary by inheritance mode, gene structure, and in the case of recessive conditions, whether the parents carry the same mutation. For diseases that arise de novo or are paternally inherited in an autosomal dominant manner, NIPD is relatively straightforward. In this instance, determination of the presence or absence of a mutation in the maternal plasma is required. NIPD for achondroplasia was the first example brought into routine clinical practice. This condition most commonly arises de novo and presents in the third trimester, when levels of cffDNA are higher, and the femur length falls below the normal range [66]. It is cause by de novo mutation in *FGFR3* (c.1138G > A, p.Gly380Arg) in 98% of cases [67]. Early tests utilized polymerase chain reaction-restriction enzyme digest (PCR-RED). However, this approach was hindered by low sensitivity and the subjective nature of the interpretation of the PCR image. The introduction of NGS to the diagnostic repertoire enables a more sensitive and quantitative approach, which is also better suited to greater sample numbers as well as having greater flexibility in terms of mutations which can be tested for. This approach has been shown to diagnose cases which were ambiguous by PCR-RED [7].

Thanatophoric dysplasia (TD) is also caused by mutations in *FGFR3*. This is a lethal skeletal dysplasia that can be caused by at least 13 different mutations and as such molecular diagnosis requires a genetic screen, rather than a mutation-specific approach [66]. PCR-RED and dPCR are of limited use for NIPD of this situation; PCR-RED is limited by the availability of enzymes which recognize the restriction site created or are abolished by the mutation and both approaches require individual assays for each mutation. Thus for conditions such as TD multiple assays would have to be set up to provide a comprehensive diagnosis. Chitty *et al.* [7] have described the introduction of a NGS assay covering 29 known mutations in *FGFR3* which cause achondroplasia and TD. Using this approach, indexed primers targeting the *FGFR3* gene are used to amplify the regions of interest. The high depth to which the samples can be sequenced means the number of reads for wild type and mutant allele can be counted. Use of indexed primers mean different samples can be sequenced on the same run, making the

approach scalable. In addition, as new mutations are identified, new primer sets can be designed and incorporated into the workflow. The same approach can be used for the ultrasound-detected Apert syndrome, caused by mutations in *FGFR2*. By using the same indexed primer approach, cases of suspected Apert can be tested alongside achondroplasia and TD, making this a flexible and economic approach for the diagnostic service laboratory. This is especially important when considering the short turnaround time required for prenatal diagnosis. Such an assay means that suspected diagnoses can be confirmed without putting a potentially unaffected fetus at risk. This makes the test suitable for determining recurrence in cases where there is a low risk, such as for TD and for twin pregnancies, where one fetus appears to be affected and the other unaffected. However, this approach cannot be used when the mother is affected and therefore a carrier of the mutation herself, as may be the case in some instances of achondroplasia, for example. This is because the contribution from the fetal DNA needs to be distinguished from that of the mother.

NIPD for dominant diseases caused by expansion repeats were some of the earliest assays described. Myotonic dystrophy is caused by expansion of a CTG repeat (50–4000 repeats) in the 3′ untranslated region of *DMPK* on chromosome 19 [68]. In 2000, Amicucci *et al.* [69] demonstrated detection of the expansion of this region noninvasively in a male fetus which was inherited from the father. The paternal sample had 70 CTG repeats and after confirming the presence of fetal DNA using Y chromosome markers, 150 repeats were detected in the maternal plasma, demonstrating the utility of this approach. Huntington's disease (HD) is caused by expansion of a CAG polymorphic trinucleotide repeat in exon 1 of the *HTT* gene. Alleles <27 CAG repeats are normal, 27–35 intermediate, and >35 cause HD. Repeats in the intermediate range are unstable and may expand over generations. This is primarily from paternal transmission. NIPD for HD has been described using (semi-)quantitative fluorescent PCR in seven cases, with a correct diagnosis in five [71–73]. A more recent study recruited couples with fathers with expansions in the intermediate range who were undergoing invasive testing for prenatal diagnosis. A blood sample was taken prior to the invasive test and tested using PCR and automated fragment analysis. The group demonstrated that normal, HD, and intermediate length expansions could be detected [74]. However, this was restricted to couples where the difference in size between maternal and paternal expansion is sufficient. In addition, it was helpful to have the repeat profiles of both mother and father as a reference and this may not always be possible. In addition, the paternal expansion may be too large to be detected in the short fragments of cfDNA.

A targeted sequencing approach can be applied to pregnancies at risk of an autosomal recessive condition, where the parents are known carriers of recessive disease but carry different mutations. In this way, the same principles as de novo or paternally inherited dominant disease can be applied for exclusion of the paternal mutant allele. In this instance, the fetus would be at most a carrier of the maternal mutant allele and therefore unaffected. Hill *et al.* [75] recently described the introduction to clinical practice of a cystic fibrosis assay for noninvasive exclusion of 10 *CFTR* mutations. NIPD for *CFTR* is welcomed by stakeholders, with the majority of those asked saying they would choose NIPD for CF, while more said they would decline invasive testing for the same disease [75]. Bespoke testing can also be offered for parents who are carriers of different mutations for a range of conditions, for example autosomal polycystic kidney disease [65]. While this particular example reported employed PCR-RED, in our molecular genetics service laboratory we now use NGS and a targeted amplicon approach. Indeed, NGS has enabled us to increase the range and number of NIPD tests done (Table 1) to the extent that in 2014, 32% of all prenatal diagnostic tests for monogenic disorders were done using NIPD.

Table 1 NIPD Service Delivered by the North East Thames Regional Genetics Laboratory at Great Ormond Street Hospital to September 2015

Condition	Gene	Cases
Achondroplasia	*FGFR3*	115
Thanataphoric dysplasia	*FGFR3*	71
Apert syndrome	*FGFR2*	13
Crouzon	*TRPV4*	6
Torsion dystonia	*DYT1*	4
Osteogenesis imperfecta	*COL1A1*	3
	COL1A2	2
Fraser syndrome	*FGFR3*	2
Cystic fibrosis	*CFTR*	7
Tuberous sclerosis	*TSC1*	1
	TSC2	1
Neurofibromatosis	*NF1*	2
Rhabdoid tumor predisposition syndrome	*SMARCB1*	1

For recessive conditions where parents carry the same mutation, X-linked conditions or for maternally inherited dominant diseases, diagnosis is complicated by the need to detect inheritance of the maternal allele against a high background of the maternal cfDNA. A useful clinical example to highlight different approaches to NIPD, both technical and analytical, are mutations in the *HBB* gene, which cause sickle cell anemia and β-thalassemia. These are recessive conditions, ie, two mutant copies are required to cause disease, one from each parent. This may be two different mutations, or the same mutation, which restricts the application of the paternal mutation exclusion approach described earlier. When the parents carry the same mutation, or when paternal exclusion confirms the fetus is a carrier, a different approach is needed to determine whether or not the fetus carries the maternal mutation. An approach termed relative mutation dosage (RMD) can be used [50]. Here the mutation of interest is targeted and a counting method applied, typically dPCR, which uses probes of different florescence to distinguish cfDNA molecules bearing the wild type or mutation. These are counted and Fig. 1 summarizes the method used to establish the fetal genetic state. The ability to detect the overrepresentation of the mutant

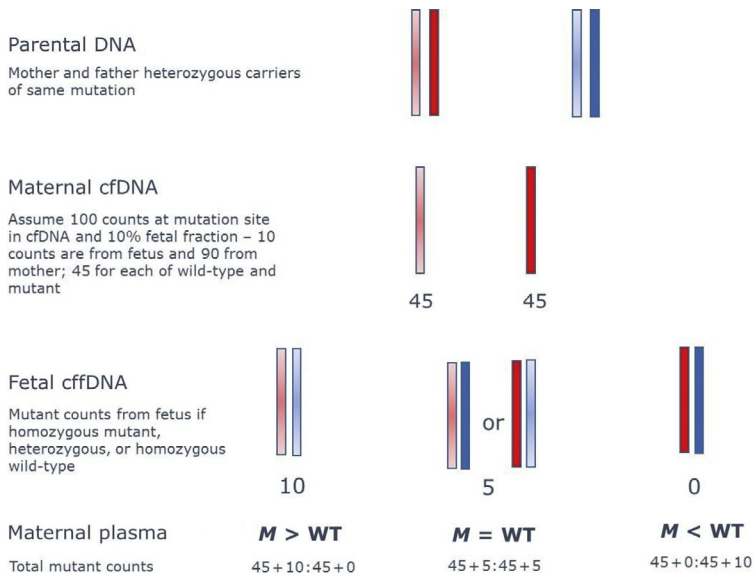

Fig. 1 Principles of relative mutation dosage. *Adapted from F.M.F. Lun, N.B.Y. Tsui, K.C.A. Chan, T.Y. Leung, T.K. Lau, P. Charoenkwan, et al., Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma, Proc. Natl. Acad. Sci. 105 (2008) 19920–19925. doi: 10.1073/pnas.0810373105.*

or wild-type allele by the fetus is affected by the fetal fraction. Assuming a fetal fraction of 10% in a cfDNA sample of 100 genomic equivalents at the mutation, we can assume that 10 copies are fetal specific and 90 maternal specific. If the fetus is homozygous for the mutation, 55 copies would be expected in cfDNA; 45 from the mother, and 10 from the fetus. Thus the mutant allele is overrepresented with a total 55 counts and 45 wild-type maternal counts.

Sequential probability ratio testing (SPRT) is used to calculate the statistical significance of the imbalance [50]. This approach has been applied for diagnosis of a number of conditions, summarized in Table 2. In order to apply this method, the fraction of cfDNA contributed by the fetus must be known. This can be measured by using a marker specific to the fetus, for example in male fetuses the chromosome Y marker *ZFY* [45,50]. The fraction of counts from this marker will be half the fetal fraction. For female fetuses a different marker must be used, for example polymorphic indel markers [45]. Here, maternal and paternal samples are genotyped using qPCR for a panel of indels and alleles present in the father and absent in the mother selected for each family; informative markers can then be used to determine fetal fraction in cfDNA. A drawback of such a method is the requirement of multiple separate assays: (1) to genotype the parents, (2) to determine fetal fraction of cfDNA, and (3) to measure dosage at the mutation site, which has implications in clinical practice in terms of time to obtain diagnosis and cost of multiple assays. In addition, SPRT relies on sufficient accumulation of data for statistical significance; this may not be obtained by a single dPCR assay and more runs may be needed until statistical confidence reached [45]. This again has further implications of repeated testing and, in the context of a diagnostic laboratory; the ability to meet turnaround times could be affected [50].

As part of the discovery that the whole fetal genome was present in maternal plasma, Lo *et al.* [10] described NIPD for a couple where the father carried the *HBB* mutation c.126_129del p.(Phe42Leufs*19) and the mother carried $-28A>G$. Using DNA from parental bloods, whole genome genotyping was performed and resulting single nuclear polymorphisms (SNPs) were categorized and a new approach called relative haplotype dosage (RHDO) analysis applied to determine the genetic status of the fetus. Following WGS sequencing of cfDNA, SNPs unique to the father were used for calculating fetal fraction as they represented fetal-specific markers. cfDNA analysis showed that the paternal mutant allele had been inherited. To establish whether the fetus also carried the maternal mutation, the

Table 2 Summary of Published NIPD Applications and Methodologies

Inheritance	Methodology	Gene	Disease	References
Autosomal dominant/ De novo	PCR-RED, dPCR, Amplicon NGS	*FGFR3*	Achondroplasia; Thanatraphoric dysplasia	[7]
	PCR	*DMPK*	Myotonic dystrophy	[69]
	Semi-qPCR; PCR and automated fragment analysis	*HTT*	Huntington	[71–74]
Autosomal recessive—paternal exclusion	Allele-specific PCR Amplicon NGS	*CFTR*	Cystic fibrosis	[70,75–77]
	dPCR	*PKHD1*	Autosomal recessive polycystic kidney disease	[65]
	Polymorphic markers-fluorescence PCR and fragment size analysis	*CYP21A2*	Congenital adrenal hyperplasia	[78]
	qPCR	*HBB*	β-thalassemia	[79]
Autosomal recessive—definitive diagnosis/ X-linked	dPCR	*F8, F9*	Hemophilia	[80]
	RMD–NASS	*HBB*	β-thalassemia	[50]
	dPCR	*HBB*	Sickle cell anemia	[45]
	ddPCR	*MUT*	Methylmalonic acidemia	[81]
	cSMART	*ATP7B*	Wilsons disease	[82]
	NGS—RHDO	*CYP21A2*	Congenital adrenal hyperplasia	[83–85]
	dPCR + NGS-RHDO	*HBB*	β-thalassemia	[86]

Table 2 Summary of Published NIPD Applications and Methodologies—cont'd

Inheritance	Methodology	Gene	Disease	References
	NGS-RHDO	*DMD*	Duchene muscular dystrophy/ Becker muscular dystrophy	[87]
	NGS-RHDO	*BCKDHA*	Maple syrup urine disease	[88]
	NGS-RHDO	*GJB2*	Hearing loss	[89]

Key: *PCR-RED*, polymerase chain reaction-restriction enzyme digest; *dPCR*, digital PCR; *NGS*, next-generation sequencing; *RMD*, relative mutation dosage; *cSMART*, counting single-molecule amplification and resequencing technology; *NASS*, nucleic acid size selection; *RHDO*: relative haplotype dosage.

authors could not look for allelic imbalance at the maternal mutation site as depth of sequencing required to reach SPRT significance could not be achieved cost effectively. Instead, SNPs which were heterozygous in the mother and homozygous in the father were analyzed on the same chromosome as haplotype blocks and RHDO analysis applied. This uses an accumulation of data from different SNPs to reach statistical significance, rather than many counts at a single mutant locus. By linking the SNPs to the mutation in the matched CVS sample, this showed that the fetus had not inherited the mutant allele and was therefore predicted to be a carrier of β-thalassemia.

Clearly this whole genome sequencing (WGS) remains relatively costly for diagnosis, but when target enrichment by hybridization was shown to capture maternal and fetal DNA in an unbiased manner [90] this opened the way for application of RHDO using targeted panels. In these cases, an affected proband is used to construct parental haplotypes and link informative SNPs to the mutant allele as described in Fig. 2.

The principles of this approach have since been applied for NIPD of a number of recessive and X-linked conditions, including profound hearing loss [89], β-thalassemia [86], maple syrup urine disease [88], Duchene Muscular Dystrophy [87], and CAH, caused by mutations in *CYP21A1*. NIPD for CAH is confounded by the presence of a pseudogene, *CYP21A1P*. The short fragments which constitute cfDNA mean that the long range PCR used for postnatal diagnosis cannot be used. Chiu *et al.* [78] first described the use of polymorphic markers for NIPD of CAH and this principle was implemented on a larger scale on a

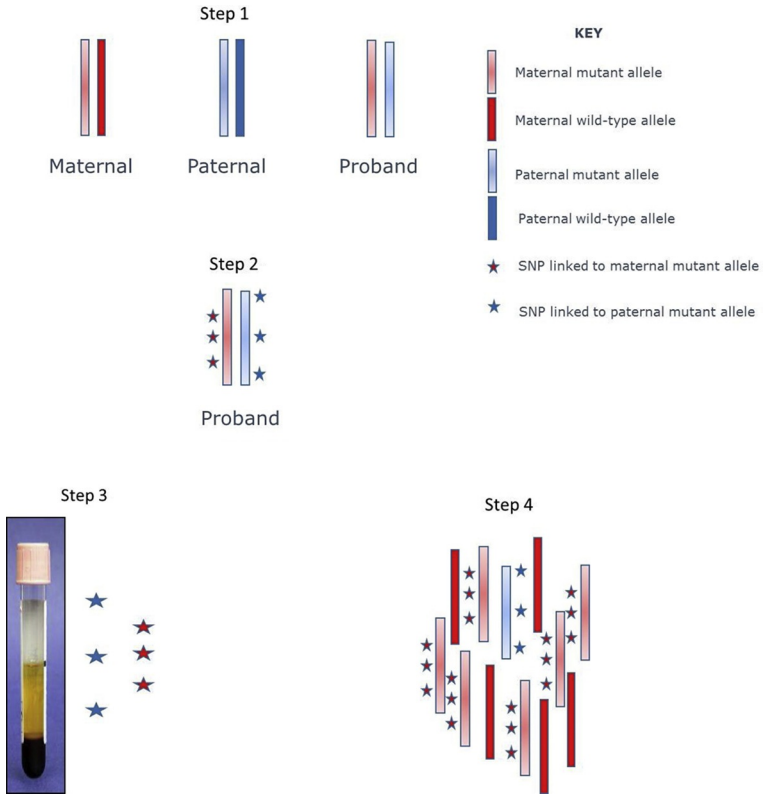

Fig. 2 Haplotyping approach for NIPD of recessive conditions. (1) DNA from couple at risk of affected pregnancy and their affected proband is required. (2) DNA enriched for heterozygous SNPs and SNPs linked to affected allele. (3) In subsequent at risk pregnancy, cell-free DNA is extracted from maternal plasma and cfDNA enriched for SNPs linked to affected allele. (4) To determine if paternal mutant allele inherited by fetus, for presence or absence of SNPs linked to paternal mutation (*blue* (*dark gray* in the print version) *stars*) is established. Inheritance of maternal mutant allele is established if there is an overrepresentation of SNPs linked to maternal mutation (*red* (*dark gray* in the print version) *stars*) using RHDO.

cohort of patients, using NGS [10,83,85] and is being introduced to the UK NHS [84].

RHDO has increased the potential for NIPD, however challenges remain, in particular the availability of proband DNA with which to construct the haplotypes and the bioinformatics skills required for data analysis and interpretation. There are additional costs associated, due to the need for genotyping parental and proband samples.

2.1.3.1 Identification of De Novo Mutations by WGS

While targeted analysis can be reliably used to identify mutations present in the fetus but not in either parent, whole genome approaches currently prove more challenging. Kitzman *et al.* [91] found approximately 25 million potential de novo mutations on sequencing cfDNA of a patient; sequencing of the matched cord blood found only 44 high quality de novo mutations. The discrepancy is in large part due to sequencing error rate and PCR error occurring at a higher rate than de novo mutations. By varying the stringency of analysis criteria, 39 of the 44 de novo mutations were filtered in the cfDNA sample, increasing the potential application of prenatal cfDNA testing.

2.2 Noninvasive Prenatal Testing

Noninvasive prenatal testing (NIPT) for aneuploidy using cell-free DNA has been available clinically though the commercial sector since 2011, following two proof-of-concept publications using massively parallel sequencing in 2008 [8,9] and a series of large-scale validation studies [27,92,93]. As in other situations, detection of fetal aneuploidy by analysis of cfDNA is challenging due to the preponderance of cell-free maternal DNA in the plasma. Thus detection of aneuploidy relies on the ability to detect a relatively small overrepresentation of the chromosome in question (Fig. 3). Since these early reports, the pace of development has been rapid and entirely commercially driven to the extent that NIPT for aneuploidy is now available in more than 60 countries across the globe [94]. At the end of 2014, over 800,000 women had been tested by the main commercial providers, with testing extended to trisomy 18, 13, and sex chromosome aneuploidies. Detection rates for Down syndrome have been reported in excess of 99%, and slightly lower for trisomies 18 and 13, 96% and 92%, respectively [95]. NIPT for aneuploidy has been shown to be very sensitive in both high [95] and low risk [96] pregnancies.

2.2.1 Technical Approaches to NIPT

There are three main approaches currently used in practice for the detection of fetal aneuploidy, WGS, targeted sequencing, and SNP analysis. WGS involves preparation of libraries of cfDNA by the end-repair, A-tail, adapter ligation and limited amplification of sample prior to sequencing. Millions of short sequences (most commonly single-end 50 bp reads) are obtained from across from the whole genome. These short sequences are aligned with a reference human genome sequence, mapped to unique sequences on each

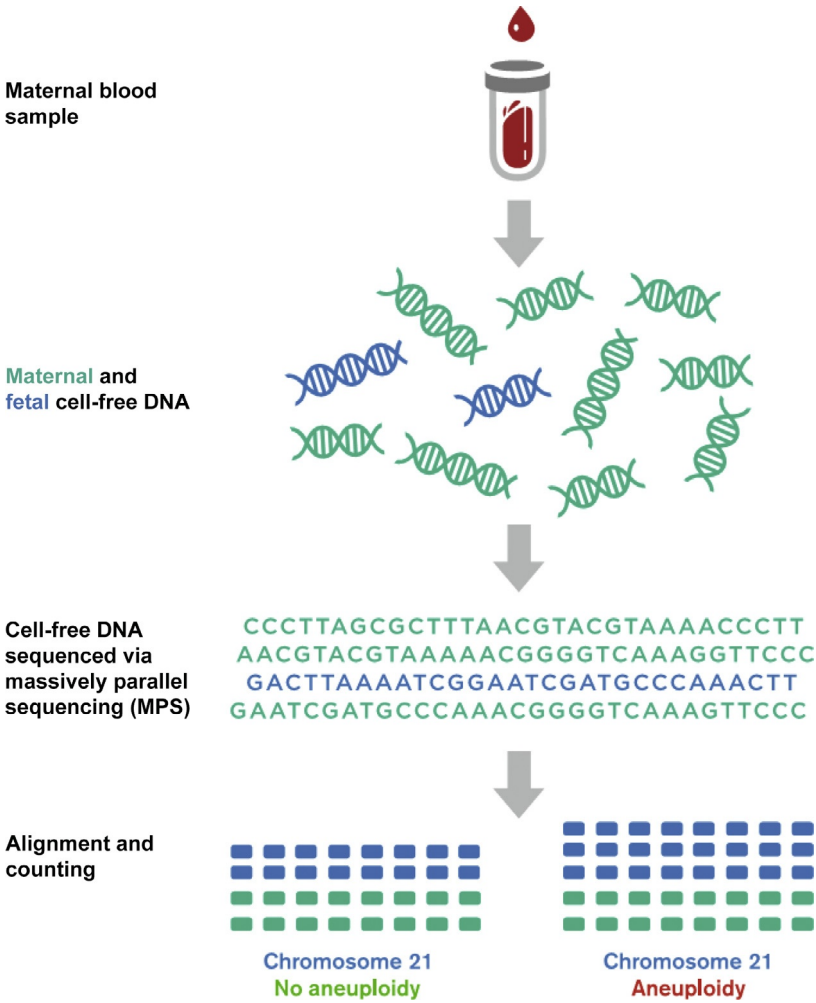

Fig. 3 Detection of fetal aneuploidy by overrepresentation of fetal DNA at chromosome of interest. *Reproduced from A. Swanson, A.J. Sehnert, S. Bhatt, Non-invasive prenatal testing: technologies, clinical assays and implementation strategies for women's healthcare practitioners, Curr. Genet. Med. Rep. 1 (2013) 113–121. doi: 10.1007/s40142-013-0010-x.*

chromosome and then counted. Bioinformatic analysis is performed to determine whether the chromosome of interest is overrepresented. If the fetus has trisomy 21, for example, more fragments from chromosome 21 will be present in maternal plasma than expected. Various bioinformatic algorithms are available to do this, some proprietary associated with commercial ventures and others publically available including RAPIDR [97] and

WISECONDOR [98,99]. The targeted approach involves selective amplification of specific genomic loci on the chromosome of interest followed by sequencing. This has the advantages of allowing estimation of fetal fraction and reduced sequencing costs, but it only delivers analysis of preselected regions of interest and development is more labor intensive [100]. The third approach is based on the amplification of large numbers of SNPs on the chromosome of interest [101]. The targeted SNP sequencing approach involves the amplification of large numbers of polymorphic loci on the chromosomes of interest that are then sequenced [101,102]. Billions of theoretical fetal genotypes are generated and the relative likelihood for each is determined by comparison with the maternal genotype. Use of microarrays for the detection of aneuploidy is also being explored, although there are comparatively fewer data available [103].

2.2.2 NIPT Performance for the Common Aneuploidies

Detection rates for NIPT are high, regardless of the approach used. Metaanalysis has demonstrated that for T21 sensitivity was 99.0% and specificity 99.9% (1051 affected and 21,608 unaffected pregnancies) [95]. For T18 sensitivity was 96.8% and specificity 99.9% (389 affected and 21,306 unaffected pregnancies). For T13 sensitivity was 92.1% and specificity was 99.8% (139 affected and 18,059 unaffected pregnancies).

Initial validation studies were undertaken in pregnancies at high risk because of high risk DSS result, abnormal ultrasound findings or advanced maternal age. More recently, it has become clear that NIPT is highly accurate in the general population [96,104] and twin pregnancies. A recent metaanalysis found that the detection rate for T21 was 93.7% with a false positive rate of 0.23% in 31 affected and 399 unaffected twin pregnancies [95]. Fetal fraction is a particularly important consideration for twin pregnancies. In twins who are discordant for Down syndrome, the effective fetal fraction is less than that of an affected singleton pregnancy. In addition, the fetal fraction of each fetus may be very different and this could lead to false negative results if the twins are discordant for the aneuploidy [105]. Amniocentesis of each fetal sac is required to definitively diagnose each pregnancy. Women with twin pregnancies at risk of Down syndrome can be offered NIPT to refine their risk and aid decision making. This should also apply to T13 and T18, but at this stage numbers are small. However, ACOG guidelines do not currently recommend NIPT for multiple pregnancies [106].

NIPT relies on analysis of cfDNA in maternal plasma, the majority of which comes from the mother herself with the fetal fraction being shed by the placenta. While concordance between NIPT and traditional invasive sampling is high, a number of false positive or discordant cases have been reported [93,107–111]. These cases are predominantly due to confined placental mosaicism (CPM). CPM is of particular relevance in T18 and T13 cases, which may have a substantial euploid cell line in the trophoblasts [112]. This can lead to false positive (abnormal cells in placenta but normal in fetus); or false negative results (abnormal cells in the fetus but not placenta) [113]. Other causes of discrepancy include false negatives due to low fetal fraction, with higher concentration of fetal DNA associated with more accurate aneuploidy results [32]. True fetal mosaicism can also lead to discordant NIPT results, which has been predicted to occur in 1/107 cases [113].

The fact that the majority of cfDNA analyzed is maternal in origin is the other main cause for discordant results. Another consideration for false positive results is maternal malignancy. The presence of maternal tumors, benign and malignant, has been identified as a rare etiology caused by the tumors shedding abnormal cell lines into the maternal circulation [114–118]. Osborne *et al*. [114] reported a case of double aneuploidy detected for chr13 and 18, with low chr18 counts when two additional samples were tested. The mother was found to have a malignancy and a recent review found at least 40 women had results suggesting maternal malignancy, with 26 of these confirmed as such on further investigation [119]. Reporting on a case series of 125,426 maternal samples, abnormal results (one or more aneuploidy of chromosome 13, 18, 21, X, or Y) in otherwise asymptomatic pregnant women were found in 3757 (3%). From these 3757, 10 cases of maternal cancer were identified [116]. Cancer was most common with a finding of more than one aneuploidy by NIPT (7 known cancers in 39 cases of multiple aneuploidy). This raises specific issues about informed consent [119]. How women are counseled for the potential of a return of such results in their pregnancy requires further discussion. A further maternal etiology for discordant results can arise if the mother is a mosaic herself, this is particularly the case when using NIPT to screen for sex chromosome aneuploidies [120] but can also arise the mother carries a chromosomal rearrangement [121,122].

False negatives also occur with NIPT and can be due to low fetal fraction which can be associated with maternal obesity or early gestation [31,123,124]. Inconclusive or failed samples occur in up to 5% of first samples [95] and in most cases this is the result of a low fetal fraction, however a

recent study has suggested that low fetal fraction is not always the cause of false negative results [93]. Other causes could include errors during sample collection, low library concentrations, and assay failures [113]. There is an association between NIPT failure, fetal fraction, and aneuploidy. In a cohort of 2157 pregnancies undergoing NIPT, Palomaki *et al.* [124] reported 13 with a fetal fraction less than 3%, all of which were uninformative. Of these 13, there were three confirmed trisomy 18 and three triploidy fetuses. They also showed that fetal fraction was significantly higher in T21 pregnancies. In addition, Pergament *et al.* [125] have reported a test failure rate of 8.1% in a series of 1064 maternal samples and found that the failed samples included a disproportionately high number of aneuploid samples. The current American College of Obstetricians and Gynecologists guidelines [126] suggest detailed ultrasound and invasive testing when an inconclusive or failed result occurs because there is an increased risk of aneuploidy.

2.2.3 Other Uses of NIPT

2.2.3.1 NIPT for Sex Chromosome Aneuploidy

The use of NIPT for the sex chromosome aneuploidies (Turner syndrome (monosomy X), Klinefelter syndrome (47,XXY or 48,XXYY), Triple X syndrome (47,XXX), and 47,XYY) has been considered in a number of studies, however, case numbers are small. Metaanalysis found a detection rate of 90.3% and a false positive rate of 0.23% for monosomy X from 177 affected and 9079 unaffected pregnancies [95]. All other sex chromosome aneuploidies were pooled and the detection rate was 93.0% with a false positive rate of 0.14% in 56 affected and 6699 unaffected pregnancies [95]. The accuracy of NIPT for the sex chromosome aneuploidies is clearly lower than for T21, T18, or T13. The reasons are postulated to be either mosaicism or an abnormal maternal karyotype due to X chromosome loss or mosaicism [112,121].

2.2.3.2 NIPT for Microdeletion and Microduplication Syndromes

There is the potential to use NIPT to analyze the fetal genome for conditions beyond the common aneuploidies. This is an area of intense debate regarding the accuracy and predictive value of such testing, along with how it might be possible to offer reasonable pretest counseling for a test that screens for an increasing number of rare conditions. Microdeletions and duplications of clinical significance occur in 1–1.7% of structurally normal pregnancies [127]. Several of the commercially available NIPT services now also

offer testing of common microdeletion syndromes, including di George and Cru-de-Chat. While a number of proof-of-concept studies have described microdeletion detection by NIPT [128,129], there is ongoing debate regarding the utility of this clinically [130–132]. This is in part due to the rarity of the conditions meaning there is an insufficient body of evidence to support an accurate diagnosis, due to low positive predictive value (PPV). Accordingly, recent professional guidelines state that NIPT screening for microdeletion syndromes is not recommended [126]. Some studies have shown good PPV's when using NIPT to detect subchromosomal abnormalities. In a review of 175,393 samples by Helgeson *et al.* [133], 55 subchromosomal microdeletions were reported. The PPV ranged from 60% to 100% with a false positive rate of 0.0017%, but outcomes of the majority of the pregnancies tested were unknown and so sensitivity could not be determined. Similarly, Wapner *et al.* [134] reported 358 cases which included maternal plasma from 352 normal pregnancies, 6 with a microduplication or microdeletion syndrome, and 111 artificial plasma mixes. Detection rates ranged from 97.8% to 100%, with false positive rates ranging from 0% to 0.76%. However, other studies suggest a need for caution. Lo *et al.* [135] report using NIPT to detect 15 out of 18 samples with fetal subchromosomal abnormality larger than 6 Mb. While, sensitivity (83%) and specificity (99.6%) were high, in cases with a low fetal fraction a negative result would not definitively rule out the possibility of a subchromosomal variant. In addition, Yatsenko *et al.* [136] reported one example of a phenotypically normal fetus and mother who had been shown to have a 22q11.2 microdeletion by NIPT. Diagnostic work-up using microarray and FISH did not confirm the NIPT result and the authors suggested that NIPT should be confirmed by invasive testing. In addition, Gross *et al.* [137] reported on results from SNP-based NIPT specifically for 22q11.2 deletion syndrome in 21,948 samples. Ninety-seven pregnancies were found to be at high risk for 22q11.2 deletion. In two cases, the mother was suspected to have the deletion. A diagnostic confirmation was available in 58/97 cases; only 11 were true positives and 47 were false positives; a PPV of 19%. Thus applying NIPT for microdeletion detection could increase the number of women undergoing unnecessary invasive testing and as a result there is the risk that the benefit of NIPT can be undone by screening for microdeletion syndromes. Furthermore, as NIPT cannot confidently exclude the presence of a microdeletion or duplication in fetuses at risk, an invasive test is required even in the presence of a negative NIPT result. Most authors and some national bodies recommend that NIPT should not be offered for this indication [126].

3. COUNSELING

The introduction of any new genetic test needs to be undertaken following the consideration of ethical, legal, social and economic issues and stakeholder views, to ensure new services are delivered in a way that best meets service user and service provider needs. The views of potential service users, the general public and the healthcare professionals involved in offering noninvasive testing have been assessed in a growing number of studies. Attitudes to noninvasive tests are generally very positive. The most frequently reported benefits of noninvasive tests are the safety to the fetus as the need for invasive testing is either removed or reduced, having information earlier in pregnancy, the relative ease with which the sample for testing could be given and as a result, the potential for improved access to prenatal tests (reviewed in Skirton and Patch [138]). Reasons for taking up testing include seeking reassurance and obtaining information to guide decisions about termination of pregnancy or for planning for the birth of an affected child.

Although the risk of miscarriage has been removed, the fact that NIPD for monogenic disorders, fetal RHD typing and sex determination is diagnostic and NIPT for aneuploidy is highly sensitive means that pretest counseling should be equivalent to the pretest counseling undertaken when offering invasive diagnostic testing to expectant couples. This includes making parents aware of the full range of options for prenatal testing and providing information about the benefits and limitations of each option. Supporting informed decision making is essential and pretesting counseling should include a discussion of the implications of the test result and allow time for reflection. There has been concern that because noninvasive methods are safe and easy to perform that they may appear routine, and be viewed by parents as "just another blood test" [139–141]. This may undermine informed consent as parents may not think through the implications of the test result for them and their family [139,142]. Other concerns raised in the literature include societal pressure to have testing and then terminate an affected pregnancy as the test is safe and easy to perform and the potential for misuse of the technology for sex selection, less serious indications or testing for late onset conditions [141,143–145]. Concerns have also been raised that offering noninvasive tests would increase the number of prenatal tests and subsequent abortions [142]. Research with potential service users of both NIPD and NIPT suggest that interest in these tests is indeed high and the number of prenatal tests performed is likely to increase, but many will use the results to plan and prepare for the birth of an affected child

and not for termination of pregnancy [145,146]. With more women continuing their pregnancy knowing they have an affected child it is important that health services are prepared to provide support to these parents. Studies conducted in the United States [147] and the Netherlands [148] have also shown that in some countries there may be quite a large group of parents who would decline all prenatal testing. Accordingly, it is important that discussions in pretest counseling include a clear option to not have testing.

Research has also addressed how NIPD and NIPT should be delivered, including women's preference for which healthcare professionals are involved in the delivery of testing. For single gene disorders stakeholders have emphasized the need for care pathways for prenatal testing to remain relatively unchanged with NIPD offered through specialist genetic or fetal medicine services. This would ensure appropriate pre- and posttest counseling offered by health professionals with specialist knowledge of the condition and experience and training in counseling for prenatal testing [145,149,150]. The implementation of NIPT for Down syndrome and other aneuploidies will be more complicated, particularly in the public sector as changes will need to be made to current Down syndrome screening care pathways and approaches to counseling need revision to include discussion of the benefits and limitations of NIPT, the possibility of an unintended maternal diagnosis, and the recommendation of an invasive test for confirmation. Parents also need to be clear on what results they will receive and the accuracy of each test target as NIPT from commercial providers can now also include sex chromosome anomalies, rare aneuploidies with a high risk for early pregnancy loss (trisomies 9, 16, and 22), and microdeletion syndromes [151]. Several professional bodies have issued guidance on offering NIPT for Down syndrome [126,152–154]. At present contingent testing is seen by many as the most appropriate use of NIPT as costs are minimized by only offering NIPD to a subset of women and the additional information gathered from traditional Down syndrome screening is maintained [155–157]. This may change in the future, especially as NIPT has been shown to work as well in the general population as it does in the high risk population [96].

Recent research looking at offering NIPT in state funded healthcare systems [158] has highlighted some of the issues when offering NIPT as a contingent screening test. When NIPT was offered to women with a traditional screening risk of 1 in 1000 parents were overwhelmingly positive about a test that was safe, accurate, simple to perform and reduced the need for invasive testing and identified cases of Down syndrome that might otherwise have

been missed. The increased accuracy was found to be highly preferable to current Down syndrome screening. Reassurance was identified as the main motivator for accepting NIPT, particularly among women with an intermediate Down syndrome screening risk (1 in 151 to 1 in 1000), with higher risk women (>1 in 150) reporting that they had accepted NIPT to inform decisions around invasive testing. The major criticism of NIPT raised by parents was the length of time it currently takes to get a test result (7–10 working days) [159]. Qualitative findings have suggested that waiting for an NIPT result had brought about prolonged or additional anxiety for women with an intermediate risk result who traditionally would not have been offered further testing. Nevertheless, most parents thought that the benefits of the extra reassurance from NIPT outweighed any potential additional anxiety that occurred while waiting for test results. Rates of informed choice were high in the study (89%), but assessing informed choice in a clinical practice setting is needed in order to determine whether this was the result of NIPT being offered in a research setting with additional time available for pretest counseling [159].

Education and information gathering about noninvasive tests can take many forms and the media plays an important role in shaping public opinion, informing of medical advances, highlighting certain attributes, and suppressing others [160]. Lewis *et al.* [161] reviewed reports of 79 articles about noninvasive tests in the British press. Notably, the majority (85%) of articles was related to NIPT for Down syndrome and reflected a positive attitude toward NIPT, with most reporting benefits over and above any concerns or limitations of the technology. Similarly, review of 40 websites advertising NIPT for aneuploidy found that while some had balanced and accurate information the majority of websites did not provide information that matched recommendations from professional guidelines with a key omission being a lack of evidence to support the information provided [162]. Moving forward, guidelines that specifically cover information provision from test providers are essential.

4. FUTURE

In the postnatal setting, there has been a move toward whole exome (WES) and increasingly WGS, increasing scope of genetic diagnosis. There are a number of scenarios in which noninvasive diagnosis by WES or WGS could be useful. For pregnancies where ultrasound suggests a genetic abnormality (typically at the 12- or 20-week gestation scan in the UK), there are

currently limited options for genetic testing beyond suspected *FGFR3* mutations. Unless a specific disorder is suggested by the ultrasound, there are currently no prenatal genetic tests that are performed routinely and those which are available reply on an invasive procedure. NGS offers the potential to use NIPD for screening a number of genes which are associated with prenatal abnormalities and even whole exome sequencing. There are however a number of challenges which remain with this, including assay sensitivity, sequencing cost, and ability to accurately detect de novo variants in cfDNA. Only 500–1000 genome copies of cfDNA are present in each 1 mL of blood, meaning obtaining enough DNA for sensitive analysis is challenging. The benefit of sequencing parental samples in the prenatal setting has been described to assist variant analysis, [163] but these are not always available.

Despite the technical limitations that make analysis of whole genome data from noninvasive samples challenging, there has been demonstration of the principle in two recent publications [91,164]. Another challenge which has started to be addressed but requires further refinement to offer in a cost effective manner is accurate haplotyping of parental DNA in the absence of proband.

To overcome the challenge in NIPD of defining the fetal component of the cfDNA, there has been an interest in whether fetal cells rather than DNA can be used for diagnosis [165]. If sufficient DNA can be extracted and analyzed with enough sensitivity, there is the potential to use this material for direct diagnosis. However, this is currently technically challenging due to the low number of fetal cells circulating in maternal blood and the need to amplify this material to use for genetic analysis. Whole genome amplification is not 100% accurate, therefore this can pose challenges to analysis, in particular for de novo mutation detection and allele biases are also seen.

5. SUMMARY

The discovery of cffDNA circulating in maternal plasma [1], in combination with significant advances in technology have led to the rapid advancement and translation of NIPT and diagnosis into clinical practice. This will continue to reduce the number of couples needing to make difficult decisions about whether to subject their pregnancy to the risk of invasive sampling, in either the setting of screening for Down syndrome, or in the diagnostic setting of definitive diagnosis for monogenic conditions. Families and healthcare professionals have welcomed the advances made

but it is important to ensure adequate counseling, including limitations of the new technologies and potential incidental findings.

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