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Non-invasive prenatal testing for Down syndrome

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SUMMARY

Prenatal screening and diagnosis of Down syndrome and other major aneuploidies may be transformed following the identification of cell-free fetal DNA in maternal plasma at the end of the last millennium. Next generation sequencing has enabled the development of tests that accurately predict the presence of fetal trisomies by analysis of cell-free DNA in maternal blood from as early as 10 weeks of gestation. These tests are now widely available in the commercial sector but are yet to be implemented in publicly led health services. In this article we discuss the technical, social, and ethical challenges that these new tests bring.

Introduction

Down syndrome (DS) or trisomy 21 is the most frequently observed aneuploidy associated with long-term survival, with an incidence of 1 in 800 live births. Antenatal screening for DS is routinely offered to all pregnant women in the UK and many parts of the developed world. This is usually performed using a combination of maternal age, ultrasound, and maternal serum biomarkers to estimate a pregnancy-specific individual risk of carrying a DS fetus [1]. Traditionally, definitive diagnosis in pregnancies identified as being at high risk is then offered by amniocentesis or chorionic villus sampling (CVS), both of which are invasive procedures and have miscarriage risk rates of around 0.5–1% [2]. Over the last few decades research has focused on identifying a less invasive approach to prenatal diagnosis. This was initially based on the isolation of fetal cells in the maternal circulation [3] but, following the identification of cell-free fetal DNA (cffDNA) in maternal plasma [4], efforts to develop non-invasive prenatal testing (NIPT) turned towards the analysis of cell-free DNA (cfDNA) [5].

Cell-free fetal DNA is a useful potential source of fetal genetic material to use for prenatal diagnosis as it is present in the maternal circulation from early in pregnancy and is rapidly cleared from maternal plasma shortly after delivery [6], making it pregnancy specific. However, the majority of cell-free DNA in a mother's blood is maternal in origin [7], which makes analysis of cffDNA challenging. Early clinical applications for cffDNA included the polymerase chain reaction (PCR)-based detection or exclusion of paternally inherited alleles for fetal sex determination in pregnancies at high risk of sex-linked genetic disorders [8], fetal *RHD* typing in RhD-negative mothers [9] and, more recently, for the diagnosis of some single gene disorders, which have arisen de novo or are paternally inherited [10,11].

NIPT for DS poses different challenges because of the high background levels of maternal chromosome 21 cfDNA. It is not feasible to use the PCR methods described above, as these are not sufficiently sensitive to detect the relatively small changes in level of chromosome 21 when the fetus has DS as most of the cfDNA is maternal in origin. Detection and quantification of this small difference requires either the analysis of targets that are free from maternal background interference, i.e. are fetal specific, or the use of technologies that enable extremely accurate copy number 'counting'. Initial attempts at NIPT for DS targeted a fetal-specific marker in mRNA in maternal plasma rather than cffDNA. This approach was based on testing cell-free mRNA from *PLAC4*, a gene located on chromosome 21, which is expressed in the placenta but not in maternal blood (i.e. it is fetal specific) [12]. By extracting cfRNA (rather than cfDNA) from maternal plasma and testing a single nucleotide polymorphism (SNP, a common sequence variation found in the normal population) located in the *PLAC4* fetal mRNA sequence, the chromosome 21 allelic ratio was determined to infer chromosome 21 dosage [12]. Named the 'RNA-SNP' method, this represented the first demonstration of NIPT for DS, achieving a sensitivity and specificity of 90% and 96.5%, respectively. However, a major drawback to SNP-based approaches is the reliance on polymorphisms within the DNA carrying the placenta-specific expression, thus limiting their use to families where parents carry

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different alleles and whose fetuses are therefore heterozygous. For the *PLAC4* RNA-SNP method this was estimated to apply to around 40% of pregnancies [12], rendering this approach impractical for routine clinical use.

Alternative approaches to NIPT for DS have been based on epigenetic differences between the maternal and fetal DNA, fetal DNA from the placenta being hypermethylated compared to maternal DNA, which is hypomethylated. The basis of this approach is that by using methylation-sensitive restriction enzymes, hypomethylated maternal sequences can be digested leaving only hypermethylated fetal sequences available for analysis by real-time PCR. Papageorgiou and colleagues published a set of fetal-specific epigenetic markers for all common aneuploidy chromosomes [13], and subsequently reported accurate NIPT for DS using methylated DNA immunoprecipitation (MeDIP) real-time PCR [14]. However, to date no large-scale validation study has been reported using this method. Hypermethylation of the *HLCS* gene promoter on chromosome 21 has also been successfully reported for NIPT of T21 by comparing to dosage of the *ZFY* gene. However, this test is restricted to male-bearing pregnancies [15]. NIPT based on differential methylation has yet to find a place in clinical practice, not least because the use of epigenetic markers is limited by relatively labour-intensive and time-consuming bisulphite conversion or restriction enzyme digestion, making them less practical for use in a routine service laboratory.

The introduction of technologies such as digital PCR (dPCR) and next generation sequencing (NGS) have enabled accurate estimation of very small differences in chromosome-specific sequences in maternal blood, thus delivering an approach to NIPT that can be used in clinical practice.

Non-invasive prenatal testing for DS using NGS

Two seminal proof-of-principle experiments published in 2008 [16,17] demonstrated that massively parallel shotgun sequencing (MPSS) of cfDNA in maternal plasma had the potential to be an effective method for fetal T21 detection. In brief, whole genome cfDNA extracted from maternal plasma is sequenced to generate millions of short sequence reads or 'tags'. These tags are then aligned and uniquely mapped to the reference human genome sequence. Individual uniquely mapped reads to chromosome 21 are then counted, and compared to the number of counts obtained from a reference euploid sample. Fan et al. [16] successfully classified all nine trisomy 21 cases in a cohort of 18 samples, and Chui et al. [17] correctly detected all 14 T21 cases in a cohort of 28, with neither study producing false-positive results. Other groups have reproduced these findings in large validation studies (Table 1) with similarly high detection levels across a range of sequencing instruments and chemistries, using a variety of bioinformatics algorithms to open the door to the wider application of NGS for the NIPT of DS [18–28].

Two approaches to NIPT for DS using NGS are now in common use in the USA, Asia, and some parts of Europe [29]. The first uses the whole genome MPS approach described above (Table 1), which requires sequencing of many millions of DNA fragments in order to generate sufficient reads to detect differences in level of reads from chromosome 21, which constitutes around 1.5% of sequenced fragments. The alternative approach has been to target the sequencing to selected genomic loci on the chromosome of interest, e.g. chromosome 21 for NIPT for DS (Table 1) [30–36]. This significantly reduces the amount of sequencing required and is primarily aimed at reducing costs while increasing throughput and test performance. Regardless of the approach taken, the sensitivity and specificity of these methods are high, ranging from 98.6 to 100% and from 99.7 to 100%, respectively (Table 1). False-negative results may be related to low fetal fraction of cffDNA and have been shown to be more common in obese women [37,38], where it is thought that there is a higher than average level of circulating maternal cfDNA because of increased release of cfDNA from adipose tissue. The small, but regularly reported, false-positive rate results from a variety of factors and reflects the fact NIPT analyses both maternal and fetal cfDNA, and that the cffDNA emanates from the placenta. Thus, the aetiology of reported discordant results includes confined placental mosaicism [39,40], maternal chromosome abnormalities [41], and the presence of maternal malignancy [42].

NIPT for other common chromosomal abnormalities

NIPT for other common aneuploidies, trisomies 13 and 18, have been reported with lower detection rates, which some reports suggest is because of the larger chromosome size and higher GC content [20,28,31–33,43]. Combined data from five studies report a sensitivity of 97.4% (188/193) for trisomy 18 (Edwards syndrome) [20,28,31–33]. However, only three of these studies [20,28,43] include data for trisomy 13 (Patau syndrome) and report a lower sensitivity of 83.3% (30/38). However, more recent studies report improved detection rates [22,23], although the outcome data are not always reported in detail. False-positive rates are broadly similar, with trisomy 18 being consistent with those seen for trisomy 21, and slightly higher for NIPT of trisomy 13 (0.41%), but as the numbers reported are small, it is difficult to draw definitive conclusions at this time.

Table 1
Studies reporting the use of next generation sequencing for non-invasive prenatal testing for Down syndrome.

Type of approach	Test results					Sensitivity		Specificity	
	TP	FN	TN	FP	Total	%	95% CI	%	95% CI
MPS whole genome									
Enrich et al. [18]	39	0	409	1	449	100	89–100	99.7	98.5–99.9
Palomaki et al. [19]	209	3	1468	3	1683	98.6	95.9–99.5	99.8	99.4–99.9
Bianchi et al. [20]	89	0	404	0	493	100	95.9–100	100	99.1–100
Dan et al. [21]	139	0	2819	1	2959	100	97.3–100	99.96	99.8–99.99
Futch et al. [22]	154	2	5515	1	5672	98.7	95.5–99.7	99.98	99.9–100
Liang et al. [23]	40	0	372	0	412	100	91.2–100	100	98.98–100
Targeted MPS									
Sparks et al. [30]	39	0	252	0	291	100	91.0–100	100	98.5–100
Sparks et al. [31]	36	0	123	0	159	100	90.4–100	100	97.0–100
Ashoor et al. [32]	50	0	297	0	347	100	92.9–100	100	98.7–100
Norton et al. [33]	81	0	2887	1	2969	100	95.5–100	99.97	99.8–99.99
Nicolaides et al. [34]	8	0	1939	0	1947	100	67.6–100	100	99.8–100
Zimmerman et al. [35]	11	0	126	0	137	100	74.1–100	100	97.0–100
Nicolaides et al. [36]	25	0	197	0	222	100	86.7–100	100	98.1–100

TP, true positive; FN, false negative; TN, true negative; FP, false positive; CI, confidence interval; MPS, massively parallel sequencing.