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Non-invasive prenatal testing for aneuploidy, copy number variants and single gene disorders

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Abstract

The discovery of cell-free fetal DNA (cffDNA) in maternal plasma has enabled a paradigm shift in prenatal testing, allowing for safer, earlier detection of genetic conditions of the fetus. Non-invasive prenatal testing (NIPT) for fetal aneuploidies has provided an alternative, highly efficient approach to first-trimester aneuploidy screening, and since its inception has been rapidly adopted worldwide. Due to the genome-wide nature of some NIPT protocols, the commercial sector has widened the scope of cell-free DNA (cfDNA) screening to include sex chromosome aneuploidies, rare autosomal trisomies and sub-microscopic copy number variants. These developments may be marketed as “expanded NIPT” or “NIPT Plus”, and bring with them a plethora of ethical and practical considerations. Concurrently, cfDNA tests for single gene disorders, termed non-invasive prenatal diagnosis (NIPD), have been developed for an increasing array of conditions but are less widely available. Despite the fact that all these tests utilise the same biomarker, cfDNA, there is considerable variation in key parameters such as sensitivity, specificity and positive predictive value depending on what the test is for. The distinction between diagnostics and screening has become blurred, and there is a clear need for the education of physicians and patients regarding the technical capabilities and limitations of these different forms of testing. Furthermore, there is a requirement for consistent guidelines that apply across health sectors, both public and commercial, to ensure that tests are validated and robust, and that careful and appropriate pre-test and post-test counselling is provided by professionals who understand the tests offered.

Introduction

Cell-free fetal DNA

In pregnancy, DNA from the developing fetus can be detected in maternal plasma, and is referred to as cell-free fetal DNA (cffDNA). The presence of cffDNA was proven during studies which detected Y chromosomal DNA within the plasma of women carrying male fetuses (Lo *et al.*, 1997). cffDNA originates from the syncitiotrophoblast layer of the placenta (Flori *et al.*, 2004, Alberry *et al.*, 2007), and is released into the maternal bloodstream following endonuclease degradation as short double-stranded DNA fragments with a median length of 143bp (Lo *et al.*, 2010). cffDNA is therefore shorter on average than maternal cell-free DNA (cfDNA), which has a median length of 166bp and derives from the natural lysis of cells from multiple bodily tissues, with the majority originating from haematopoietic cells. Intriguingly, cffDNA shows different fragment end sites to maternal cfDNA, with maternal cfDNA ends more commonly located within the linker regions between nucleosomes (Sun *et al.*, 2018). The plasma of a pregnant woman therefore comprises a mixture of cfDNA from placental and maternal tissue, which can be used to test for genetic conditions in the fetus. Traditional invasive methods of collecting fetal genetic material for prenatal diagnosis via amniocentesis or chorionic villous sampling are associated with a small risk of miscarriage (Alfirevic *et al.*,

2017), which can present a barrier to some parents, whilst no such risk is associated with the "non-invasive" sampling of maternal peripheral blood. Although the perceived miscarriage risk is an important factor shaping the views of patients, pregnant women also prefer non-invasive alternatives due to the pain and discomfort of invasive procedures, and the ability to test earlier in the pregnancy (Hill *et al.*, 2014). In addition to cffDNA, fetal cells isolated from the maternal bloodstream and cervix represent another source of fetal genetic material for non-invasive testing. The development of non-invasive testing methods using fetal cells has been reported for aneuploidy (Beaudet, 2016) and microdeletions (Vossaert *et al.*, 2018) but is not yet in clinical practice and hence is not discussed further in this review.

cffDNA is usually first detectable from 6-7 weeks gestation, with the earliest reported detection at 4.5 weeks (D'Aversa *et al.*, 2018), often allowing non-invasive tests to be performed earlier in pregnancy than standard biochemical screens or invasive testing procedures (Figure 1). The proportion of cffDNA to total cfDNA is referred to as the fetal fraction, which increases throughout pregnancy and can be as high as 30% in the third trimester. Following birth and the removal of the placenta, cffDNA is cleared from the maternal circulation within hours (Lo *et al.*, 1999). Consequently, cffDNA is a suitable source of fetal genetic material as it is specific to the ongoing pregnancy at the time of sampling. Since its discovery, cffDNA has revolutionised prenatal genetic testing, allowing the development of non-invasive screening methods for common aneuploidies, referred to as Non Invasive Prenatal Testing (NIPT) or Non Invasive Prenatal Screening (NIPS), and diagnostic testing for single gene disorders, referred to as Non-Invasive Prenatal Diagnosis (NIPD). NIPT is a screening test, with positive results requiring confirmation via invasive testing. This is because there are several factors, such as confined placental mosaicism (CPM), which may lead to a false positive NIPT result. On the other hand, CPM for single gene disorders has not been reported. Consequently, cfDNA test results for single gene disorders do not require confirmation via invasive methods, and are therefore considered diagnostic. The characteristics of cffDNA that allow NIPT for aneuploidy and diagnosis of monogenic disorders are summarised in Table 1. This review will summarise the technical parameters, clinical utility and limitations of NIPT and NIPD.

Non-Invasive Prenatal Testing

Trisomies 13, 18 and 21

The first reports of NIPT for trisomies 13, 18 and 21 were published over a decade ago (Tsui *et al.*, 2005, Lo *et al.*, 2007, Fan *et al.*, 2008, Chiu *et al.*, 2008), and since then aneuploidy screening by NIPT has become firmly established within antenatal care pathways in many countries (Minear *et al.*, 2015). The principle of NIPT lies in detecting a statistically significant increase in the relative dosage of chromosomal material in maternal cfDNA, which is attributed to the presence of a trisomic cell line in the fetus (Figure 2). This is achieved using next generation sequencing (NGS) or microarray hybridisation of cfDNA in maternal plasma. The massively parallel functionalities of NGS and array technologies have enabled high-throughput testing on a scale amenable to population screening, and the reliable determination of the fetal fraction via detection of paternally-inherited single nucleotide polymorphisms (SNPs). Different methodologies may apply a targeted approach, in which only sequencing reads for defined chromosomes are generated. Alternatively, a genome-wide approach may be used in which sequencing reads are generated for all chromosomes, whilst analysis is restricted to the dosage chromosomes 13, 18 and 21. Targeted approaches are economically advantageous, whilst genome-wide approaches offer the potential to expand the scope of testing to include sex chromosome aneuploidies, rare autosomal trisomies and copy

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number variants (covered in later sections of this review) without altering the underlying method.

Whilst not diagnostic, NIPT has been shown to have a much higher specificity and sensitivity than first trimester biochemical screening and nuchal translucency measurement (Norton *et al.*, 2015). Several studies have examined the testing parameters of NIPT, with a meta-analysis reporting the specificity for all three trisomies to be 99.87%, and the sensitivity for trisomy 21 to be 99.7%, compared to 97.9% for trisomy 18 and 99.0% for trisomy 13 (Gil *et al.*, 2017) . The lower sensitivity of NIPT for trisomies 13 and 18 is a result of the low average content of guanine and cytosine bases of these chromosomes compared to chromosome 21, which introduces non-uniform bias into sequencing reactions. NIPT therefore has a considerable advantage over first and second trimester biochemical screening as it has a lower rate of false positives, meaning fewer unnecessary invasive tests are offered in healthy pregnancies (Norton *et al.*, 2015).

One key parameter to note is that whilst sensitivity and specificity are high, the positive predictive value varies both with prior risk factors, such as maternal age, and the individual trisomies (Petersen *et al.*, 2017). NIPT has been implemented into healthcare systems worldwide, either as a contingent test for women at a defined risk level following first trimester or serum screening, such as in Australia (Hui *et al.*, 2017a), or replacing first trimester biochemical screening entirely, such as in the Netherlands (van der Meij *et al.*, 2019).

Limitations and Quality Control

NIPT is a highly accurate test when used for screening purposes, however there are multiple limitations which mean that it cannot be considered diagnostic. Consequently, robust quality assessment is required to ensure that minimum standards of testing and reporting are upheld between laboratories (Deans *et al.*, 2019). For example, fetal fraction measurement is a key analytical and quality-control metric. Low fetal fraction has been linked to very early gestations, high maternal body mass index, maternal medications, smoking and factors which lead to a smaller placenta, such as trisomies 13 and 18 (Kuhlmann-Capek *et al.*, 2019). A fetal fraction quality threshold of 4% is commonly applied, below which results are often reported as inconclusive. Most NIPT platforms screen for the common trisomies with or without sex chromosome anomalies, and, whilst their use in routine screening for these trisomies seems clear, most other chromosomal rearrangements will not be detected. Thus, in the presence of fetal structural abnormalities on ultrasound, NIPT for the common aneuploidies should not be the test of choice as there is a higher incidence of chromosomal rearrangements in this pregnancy cohort (Al Toukhi *et al.*, 2019).

There are several potential causes of a discordant NIPT result. These include a ‘vanishing twin’: an aneuploid twin pregnancy that spontaneously miscarries early in pregnancy but still releases cffDNA into the maternal blood (Alberry *et al.*, 2007). In this scenario, the cffDNA released by the placenta after the demise of the aneuploid fetus may be detected by early NIPT and falsely attributed to a euploid twin. As NIPT analyses all cfDNA, both fetal and maternal, in maternal plasma, detection of abnormal maternal cell lines is another potential aetiology of discordant results. These include maternal cytogenetic anomalies, either in constitutional or mosaic form, but also malignancies where ‘chaotic’ results may indicate circulating cell free tumour DNA (Bianchi *et al.*, 2015). Indeed, women with known malignancies should not have NIPT as the results cannot be accurately interpreted (Lenaerts *et al.*, 2019). Finally, as mentioned previously, cell free “fetal” DNA may not represent the

genotype of the fetus: there is potential for NIPT to produce inaccurate results either due to CPM for aneuploidy (Pan *et al.*, 2013) or due to complete discordance between fetal and placental genotypes (Verweij *et al.*, 2014). This can result in both discordant positive and negative results depending on the predominant cell line in the placenta (Hartwig *et al.*, 2017).

Sex Chromosome Aneuploidies

Sex chromosome aneuploidies, such as Turner syndrome (45,X) and Klinefelter syndrome (47,XXY), are variably reported using NIPT, for example they are not reported in the Netherlands (van der Meij *et al.*, 2019). The sensitivity of NIPT for sex chromosome aneuploidies is lower than for the common trisomies, with much lower positive predictive values (PPVs) than NIPT for Down syndrome, particularly for Turner syndrome. False positive rates of up to 90% have been reported in low-risk cohorts, which raises questions about the clinical utility of this information (Reiss *et al.*, 2017), although lower false positive rates are reported for cohorts with ultrasound anomalies such as cystic hygroma. The reasons for such high false positive rates for sex chromosome aneuploidies include CPM, but also constitutional or mosaic sex chromosome aneuploidies in the mother, such as X chromosome segmental duplications, triple X syndrome and mosaic Turner syndrome. In a recent study confirming the poor PPV, 20% of false-positive sex chromosome aneuploidy results were due to a maternal aneuploidy and a further 23% of fetal X chromosome copy number variants were maternally inherited (Zhang *et al.*, 2019a). Such results highlight the variability in clinical presentation of these conditions, as they may be detected incidentally in pregnant women with no apparent clinical features. The rationale for including sex chromosome aneuploidies in screening programmes is therefore debated, as “affected” individuals may not have significant adverse health outcomes. However, a potential advantage of NIPT for sex chromosome aneuploidies is that detection during pregnancy may allow early initiation of postnatal interventions that may improve neurodevelopmental outcomes.

Rare Autosomal Trisomies

The term “rare autosomal trisomy” (RAT) refers to a trisomy for any autosome other than 13, 18 and 21. Constitutional forms of these aneuploidies are almost invariably lethal, and hence the overwhelming majority of cases represent mosaicism which may be confined to placental tissue (Grati *et al.*, 2019). Originally, NIPT methods were designed solely to detect trisomies 13, 18 and 21. However, NGS-based NIPT methods generate low-depth sequencing coverage for all autosomes, which has allowed retrospective re-analysis of these datasets to detect trisomies for any chromosome (Pertile *et al.*, 2017). Several studies applying this analytical methodology have revealed that the prevalence of RATs is approximately 0.1-0.3% in general obstetric population cohorts (Table 2). Trisomy 7 is the most commonly detected RAT, whilst trisomies 15, 16 and 22 are more frequently detected via NIPT than previous studies using chorionic villous sampling data (Benn *et al.*, 2019) (Figure 3). Constitutional RATs are usually associated with spontaneous miscarriage, but mosaic RATs may be associated with a range of adverse outcomes such as placental insufficiency, low birth weight, miscarriage and structural anomalies due to fetal mosaicism (Scott *et al.*, 2018). In addition, CPM for a chromosome containing imprinted regions can lead to a clinical phenotype via generation of uniparental disomy in the developing fetus following trisomy rescue. A key example is uniparental disomy for chromosome 15, which causes Prader Willi syndrome or Angelman syndrome, dependent on a maternal or paternal origin, respectively. However, mosaic RATs are also associated with normal births: a recent meta-analysis reported that 41% of RATs detected via NIPT resulted in a normal postnatal outcome (Benn *et al.*, 2019). Thus, whilst

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extending the diagnostic scope of NGS-based NIPT to include detection of RATs is possible without significant amendment of most technical laboratory protocols, the utility of this approach is controversial and there is as yet no consensus on value from the clinical community.

Copy Number Variants

NIPT has also been extended to the detection of chromosomal deletions and duplications within the fetal genome, by applying the same principles of dosage as for aneuploidy analysis (Advani *et al.*, 2017). Microdeletions and microduplications are copy-number variants (CNVs) which lie below the resolution of traditional karyotyping methods, and are associated with a broad range of genetic syndromes. Whilst individually rare, these conditions are collectively common and do not exhibit a maternal age affect, unlike the common trisomies. Pathogenic CNVs can occur across the genome but around 25% are recurrent, the most common being the 22q11.2 deletion, which is causative of Di-George syndrome and has been demonstrated to have a prevalence of 1 in 992 in a low-risk obstetric population (Grati *et al.*, 2015). Whilst NIPT can be extended to include CNV screening, the majority of commercial platforms only report the detection of several recurrent microdeletion syndromes (Table 3), with only one claiming to detect all CNVs that are 7 Mb or greater. However, as most microdeletion syndromes and non-recurrent pathogenic CNVs are smaller than 5 Mb, such strategies will only detect the minority of relevant CNVs. Those platforms targeting specific recurrent microdeletions are also limited as non-recurrent CNVs occur across the genome. In a review of prenatal cases analysed in our Regional Cytogenetic Laboratory from 1997 to 2013, 173 pathogenic CNVs were detected in 23,000 cases, 77% were non-recurrent and would not be detected by the currently available commercial platforms (Chitty *et al.*, 2018). Not only is the sensitivity poor for most of these conditions, but the PPVs are considerably lower than for the common trisomies, and can vary significantly depending on the patient's clinical details. Using the 22q11.2 deletion as an example, the PPV of NIPT can range from 21% in low-risk pregnancies (Petersen *et al.*, 2017) to 50-97% in pregnancies with ultrasound anomalies (Helgeson *et al.*, 2015, Gross *et al.*, 2016). It is clear that practitioners offering extended NIPT which includes CNVs should provide comprehensive counselling before and after testing, including the possibility of no findings and the need for confirmation of positive results with invasive testing (Grati and Gross, 2019). There is also the consideration that many CNV syndromes present with variable expression, and accurate prediction of phenotypic severity in the absence of ultrasound findings is not possible. For the reasons discussed here, NIPT for CNVs and RATs is not currently endorsed by any professional society, and some national bodies do not endorse its use for fetal sex determination in the absence of a family history of sex-linked disorders.

Patient and Health Professional Perspectives and Ethical Issues

Uptake of NIPT has been high with both women and health professionals welcoming the potential for an earlier and more accurate screening test, which can result in increased detection of Down syndrome with a significantly reduced rate of invasive testing (Chitty *et al.*, 2016). However, the simplicity of sample collection and the number of routine blood tests performed during pregnancy could mean that women do not fully consider the consequences of a "high chance" result without appropriate counselling. This has led to calls for better patient and health professional education to ensure that women have the opportunity to make

informed choices regarding testing (Lewis *et al.*, 2017). Despite fears that NIPT would increase termination rates of fetuses with Down syndrome, data do not support this conclusion. Instead, findings from international studies suggest that many women take this more accurate screening test to gain information about their baby rather than to terminate a pregnancy (Hill *et al.*, 2017). Another consideration is the potential for NIPT to facilitate sex-selective termination of pregnancy, as it is available very early in pregnancy and can be used to determine genetic sex. At present, ultrasound remains the primary method for prenatal determination of fetal sex in this context, and evidence that NIPT can facilitate sex selection is anecdotal (Bowman-Smart *et al.*, 2019).

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NIPD for Single Gene Disorders

The expansion of diagnostic testing for single gene disorders using cfDNA has been comparatively slower than NIPT for aneuploidy screening. This is probably due to multiple factors, including the relative rarity of some conditions, the technical complexity of testing, and the need for development on an individual family basis in many instances, meaning that, unlike NIPT for Down syndrome, there has been less potential for commercialisation (Jenkins *et al.*, 2018).

Methodologies for NIPD can be broadly divided into two categories. Firstly, there is the detection of a genetic variant in the fetus that is not present in the mother. This approach is appropriate for the detection of paternally-inherited variants for dominant and recessive conditions and for variants that have arisen *de novo* at conception. Secondly, there are dosage-based techniques targeting genetic variants which are carried by the mother, and are therefore present in maternal cfDNA. Detection of dosage imbalances of these variants in the total circulating cfDNA of pregnant women can be used to predict the fetal genotype (Figure 4).

De Novo and Paternally Inherited Conditions

The first clinical use of NIPD for the detection of paternally inherited markers was for fetal sex determination using quantitative PCR for Y chromosome sequences (Devaney *et al.*, 2011) and for the detection of Rhesus D positive (*RHD+)* fetuses in Rhesus D negative (*RHD-)* mothers (Finning and Chitty, 2008). Non-invasive fetal sex determination is now widely used across Europe to direct invasive testing in pregnancies at risk of sex-linked conditions where it has been shown to be highly accurate, cost effective and reduces invasive testing by around 50% (Hill *et al.*, 2011). NIPD for fetal sex determination can also clarify the genetic sex of the fetus when ambiguous genitalia are detected via ultrasound, which informs parental counselling. Fetal *RHD* typing was initially used to direct fetal monitoring and treatment in pregnancies at high risk of haemolytic disease of the new-born (Finning and Chitty, 2008). However, in many parts of Europe this is now used to direct routine immunoglobulin therapy in *RHD*- mothers (Clausen *et al.*, 2019), but the clinical and cost effectiveness in some parts of the world has been challenged (Moise *et al.*, 2019).

Subsequently NIPD methods for detecting *de novo* and paternally-inherited pathogenic variants were developed, since these can easily be distinguished against the high background of maternal cfDNA. This has been successfully developed for autosomal dominant disorders such as the *FGFR3*-related skeletal dysplasias: firstly via restriction enzyme digest to target individual mutations (Chitty *et al.*, 2011), and then extended to targeted NGS panels allowing multiple variants to be assessed in a single and more accurate test (Chitty *et al.*, 2015). Application of NIPD to this patient cohort is strengthened by well-characterised fetal phenotypes on ultrasound scanning. For autosomal recessive conditions such as cystic fibrosis (Hill *et al.*, 2015) and β-thalassaemia (Xiong *et al.*, 2015), where the father and mother are heterozygous for different pathogenic variants, targeted testing for the paternal variant in the cfDNA can be performed to offer paternal exclusion testing. Invasive testing will then only be required if the paternal mutant allele is detected.

Bespoke amplicon-based NGS assays can also be developed for a range of rare monogenic diseases caused by known mutations specific to a particular family. As each assay is developed and validated on an individual family basis, bespoke testing is considerably more expensive than invasive testing and other forms of NIPD (Verhoef *et al.*, 2016). Some have

argued that as the recurrence risk in these situations is extremely low, it may not be appropriate to offer this testing within a publicly funded healthcare system (Wilkie and Goriely, 2017).

X-linked and Recessive Conditions

Development of NIPD is more technically challenging for X-linked conditions, and for autosomal recessive conditions when both parents are carriers of the same mutation. This is due to the high background of the relevant mutation from maternal tissue in the circulating cfDNA.

Relative Mutation Dosage

NIPD using relative mutation dosage (RMD) requires the precise quantification of mutant and wild type alleles in cfDNA, and the application of statistical methods to clarify that measured imbalances reflect the signal of the fetal genotype rather than technical noise. Unfortunately, standard protocols of NGS are insufficiently sensitive for these applications, as amplification bias between mutant and wild type alleles can lead to inaccurate allelic fractions. Potential solutions to these challenges include the use of nested PCR (Xiong *et al.*, 2018, Cutts *et al.*, 2019), unique molecular indexes and synthetic reference amplicons that have known amplification dynamics (Tsao *et al.*, 2019).

Another key technique in this area is digital PCR (dPCR). dPCR is a highly sensitive technique in which a single PCR reaction is separated into many thousands of partitions. Detection of the presence or absence of an allele-specific fluorescent signal from each partition allows the concentration of the target sequence to be precisely quantified according to Poisson statistics. Proof-of-principle studies for NIPD using dPCR have been reported for several recessive and X-linked conditions, including β-thalassemia (Lun *et al.*, 2008, Camunas-Soler *et al.*, 2018), sickle cell disease (Barrett *et al.*, 2012), haemophilia (Hudecova *et al.*, 2017, Tsui *et al.*, 2011) and recessive forms of deafness (Chang *et al.*, 2016). Whilst dPCR is highly sensitive, it has limited capacity for multiplexing comparative to NGS, which restricts the number of mutations that can be assayed in one test.

Relative Haplotype Dosage

Rather than directly detecting pathogenic variants, NIPD using relative haplotype dosage (RHDO) determines which parental haplotypes have been inherited by the fetus based on the inheritance of SNPs at the locus of the relevant disease gene (Lo *et al.*, 2010). Using NGS, the inherited paternal haplotype can be determined by detection of low-level SNPs in the cfDNA which differ from the maternal haplotype, whilst the inherited maternal haplotype can be determined by changes in dosage of SNPs which differ from the paternal haplotype. The inherited haplotypes are then compared to those inherited by a previous pregnancy, usually an affected proband, to determine the genetic status of the fetus (Figure 5). In this manner, RHDO employs both low-level variant detection and dosage techniques to determine haplotype inheritance. As multiple SNPs are used for classifying haplotypes, RHDO is not affected by the technical noise of NGS to the same degree as RMD, and hence standard NGS protocols are sufficient without modifications for molecular counting.

Notably, RHDO is able to determine the inheritance of complex genomic variants which are beyond the resolution of cfDNA fragmentation, such as exonic deletions and the intron-22-related inversions within the *F8* gene which cause severe haemophilia A (Hudecova *et al.*, 2017). RHDO can also be applied to genes with homologous pseudogenes that complicate

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direct mutation detection, most notably *CYP21A2*-related congenital adrenal hyperplasia (New *et al.*, 2014).

In contrast to RMD approaches, RHDO has been implemented clinically, and services for Duchenne muscular dystrophy (Parks *et al.*, 2016), spinal muscular atrophy (Parks *et al.*, 2017) and cystic fibrosis (Chandler *et al.*, 2019) are now available in the United Kingdom National Health Service. Over 90 cases of proband-based RHDO have been reported for several monogenic disorders, with no false positive or false negative results (Table 4). The limitations of this approach include the high cost of testing, and the potential for inconclusive results due to recombination events within the target locus (Chandler *et al.*, 2019). Moreover, RHDO may not be applicable in pregnancies with consanguineous parents, as the technique relies on a large number of different SNPs to differentiate between maternal and paternal haplotypes.

Currently, clinical RHDO services require familial samples for haplotype phasing, most commonly genomic DNA from the father, mother and an affected proband. This unfortunately restricts the application of RHDO to families with children, or where DNA from a previous child or pregnancy has been stored. Proband-free RHDO, using methods to haplotype the parents directly, has so far been shown to be possible by two different approaches: targeted locus amplification (TLA) (Vermeulen *et al.*, 2017) and microfluidics-based linked-read sequencing (Hui *et al.*, 2017b, Jang *et al.*, 2018). In addition, long-read sequencing technologies offer the potential to directly haplotype parental genomic DNA, although proof-of-principle is yet to be reported for this approach.

Commercial NIPD for Screening Low-Risk Pregnancies

The development of NIPD thus far been concentrated on pregnancies at high-risk of single gene disorders, either due to a pre-existing family history or ultrasound findings consistent with a specific condition. There are now, however, increasing efforts by the commercial sector to develop NIPD to screen the general population for monogenic disorders. Two key areas are emerging: low-level variant NIPD for *de novo* mutations in dominant disease genes, such as for Noonan syndrome and achondroplasia (Zhang *et al.*, 2019b), and RMD approaches for mutations with high population carrier frequencies, such as sickle cell disease and spinal muscular atrophy, which are amongst the most common indications for invasive testing (Tsao *et al.*, 2019). Both of these tests are now commercially available and it is argued that these applications have considerable potential to impact prenatal care by providing definitive diagnosis of genetic conditions early in pregnancy, and facilitating the potential for postnatal or *in utero* treatment.

However, there remain many issues with the provision of these tests. In the rare disease area, the technical information on gene coverage and test sensitivity from commercial providers is limited, and compounded by a lack of follow-up data for reported cases. The provision of these tests to women with no family history or clinical indication such as ultrasound anomalies, may lead either to unnecessary stress and concern, or inappropriate reassurance that the fetus does not have a genetic condition. In particular, mutation agnostic approaches may detect variants of uncertain clinical significance, which pose major counselling issues. Furthermore, as there is currently limited follow-up or validation data available, these tests should be used with caution and positive results confirmed by invasive testing. Larger scale studies with comprehensive follow-up are required to determine the true sensitivity and specificity of these tests. A significant concern is that of false negative results, which can either be due to incomplete coverage of genes tested or other potential causes, such as variation in enzymatic

cut-sites in the fragmented cfDNA (Sun *et al.*, 2018). Conversely, false positives may occur if somatic mosaicism for a variant is misinterpreted as a fetal genotype, and consequently maternal genomic DNA should always be simultaneously tested in order to exclude this possibility. These concerns mean that rigorous standards of technical validation should be applied to all new tests whether developed in the commercial or public health sector, and that parental counselling should include all the potential technical limitations.

Conclusions

Non-invasive prenatal tests based on analysis of cfDNA have transformed prenatal care. NIPT provides a cost-effective, high-sensitivity screening test for the common trisomies, and its global implementation has dramatically reduced the number of invasive prenatal procedures performed. Conversely, NIPD for single gene disorders is less widely available, and may be significantly more expensive dependent on the approach chosen. Given the high cost of particular NIPD methods, such as bespoke mutation exclusion and RHDO, a wider debate is required on who should be offered testing and for which conditions within publicly funded healthcare systems. The entire fetal genome is represented in cffDNA, and genome-wide sequencing methodologies have allowed commercial providers to report on a broader range of fetal genetic abnormalities, including sex chromosome abnormalities, RATs and CNV syndromes. However, these developments are controversial, and the low PPVs, debatable clinical utility and associated counselling challenges mean that screening for these conditions is not currently supported by any international society. In addition, there is now commercial interest in providing NIPD to screen for monogenic conditions in low-risk pregnancies. The continuing education of physicians and patients about the technical capabilities and limitations of different testing methods is crucial to ensure these tests are implemented appropriately to provide maximal benefit for families.

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Declaration of Interest

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Author Contributions

JS and LSC wrote the manuscript, with editing performed by ES and NC. JS designed the tables and figures, excluding Table 1 which was designed by LSC.

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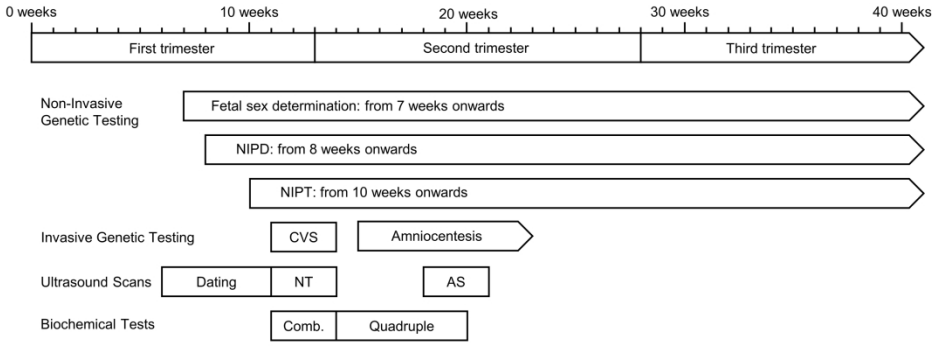

Figure 1: this figure shows the timing of non-invasive prenatal tests within pregnancy relative to routine ultrasound scanning, biochemical screening for common aneuploidies, and traditional methods of invasive testing. This diagram is intended to be illustrative rather than definitive, and is based on current practices within the United Kingdom National Health Service. The timings of non-invasive tests offered by commercial providers may differ from those quoted here. First and second trimester biochemical screening is indicated by the "comb" (combined screen: PAPP-A and free β-hCG) and "quadruple" (AFP, total hCG, uE3 and inhibin A) test boxes. Abbreviations: AS, anomaly scan; CVS, chorionic villous sample; NT, nuchal translucency; RDHO: relative haplotype dosage.

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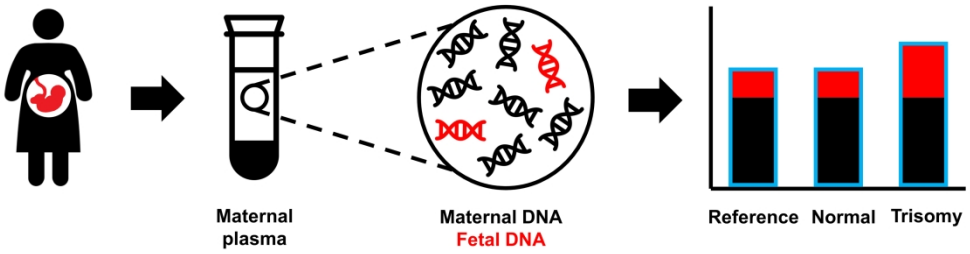

Figure 2: this figure illustrates the concept of NIPT for aneuploidy. The cfDNA in the plasma of a pregnant woman is a mix of maternal cfDNA (black) and fetal (cffDNA) released from the fetal placenta (red). Measurement of cfDNA by NGS or microarray analysis is used to calculate the dosage of each chromosome. The maternal cfDNA and cffDNA are not distinguishable from each other but are measured in aggregate. An over-representation of sequences mapped to a particular chromosome compared to a reference chromosome indicates a fetal trisomy for that chromosome. Figure images were sourced from https://www.flaticon.com/.

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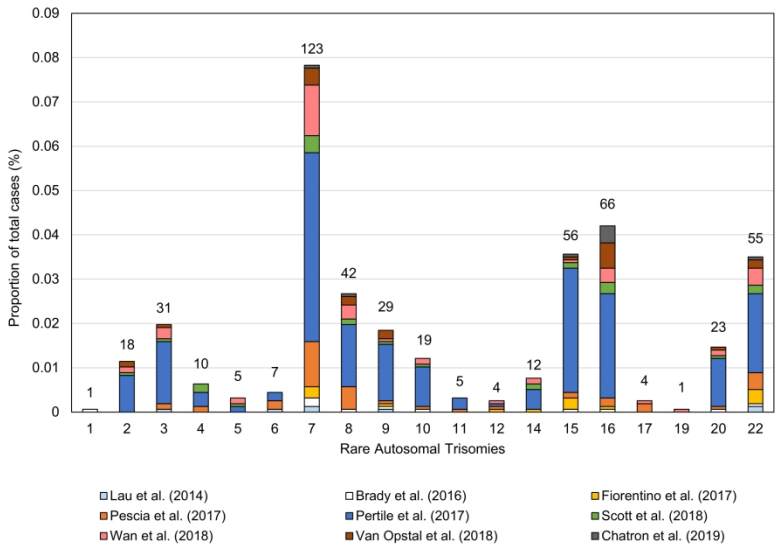

Figure 3: this figure shows the reported prevalence of each RAT from the studies shown in Table 2 as a proportion of the total cases tested (N=157,159). The absolute numbers of each RAT are indicated above each column.

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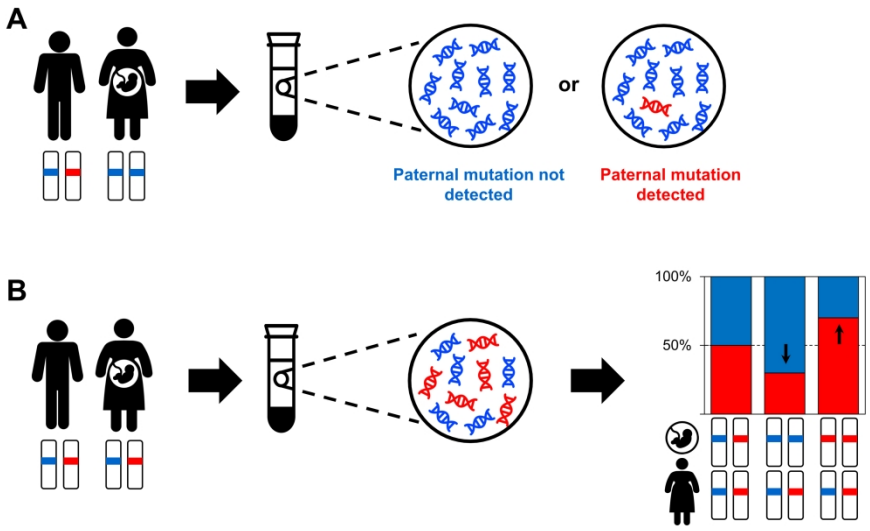

Figure 4: NIPD via low level variant detection (A) and relative mutation dosage (B). The chromosome ideograms show the mutation and wild type allele in red and blue, respectively. (A) In situations where the mother is not a carrier of the variant of interest, the presence or absence of the mutation at low levels with maternal plasma can be used for diagnosis in the fetus. This applies in cases of a dominant paternal condition (such as achondroplasia), chromosomal sex (using Y chromosome markers) or for recessive conditions in which the father and mother are heterozygous carriers of different mutations. (B) This illustrates relative mutation dosage in an autosomal recessive disease model. When both parents are carriers of the same mutation, the dosage of the mutant and wild type alleles in maternal plasma can be used to infer the fetal genotype.

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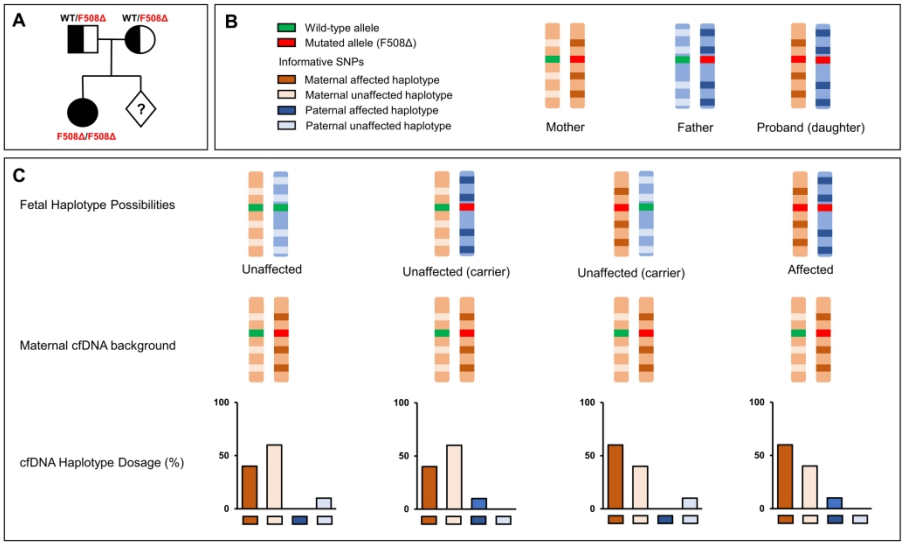

Figure 5: (A) This figure illustrates the method of RHDO using an example pedigree for cystic fibrosis, in which the parents are both heterozygous for the common CFTR c.1521_1523del p.Phe508del mutation (F508Δ) and have a daughter affected with cystic fibrosis. (B) Sequencing of genomic DNA from the mother, father and daughter allows delineation of the parental haplotypes associated with each mutant and wild type allele. These haplotypes are defined by informative heterozygous SNPs, indicated by the dark and light blue (paternal) and orange (maternal) boxes, that lie within and surrounding the CFTR gene. A proband sample is required for this, as NGS cannot determine haplotypes using only parental samples: the short read length prevents phasing a particular SNP onto the same chromosome as the mutation.(C) Sequencing of cfDNA from maternal plasma then allows the haplotypes inherited by the fetus to be detected through dosage imbalance of the maternal haplotypes and low-level detection of the paternal haplotype. The four different fetal haplotype are shown, along with the maternal haplotype background, and the resulting relative dosage of each haplotype detected in the cfDNA. WT: wild type.

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Table 1: Characteristics of cffDNA and the impact on prenatal screening for aneuploidy and diagnosis of monogenic conditions

Characteristics of cffDNA	**Impact on NIPT for aneuploidy**	**Impact on NIPD for monogenic conditions**
cffDNA is present in maternal plasma from early gestation	Early screening	Early diagnosis without risk of miscarriage
cffDNA originates from the placenta	False negative and false positive results due to confined placental mosaicism (CPM)	No impact as CPM not reported for monogenic conditions
The majority of cfDNA in maternal plasma originates from maternal tissues	Incidental detection of maternal chromosomal rearrangements including microdeletion and duplication syndromes, chromosomal mosaicism, sex chromosome aneuploidy and malignancy	Maternal somatic mosaicism must be excluded to avoid false positives by analysing maternal genomic DNA in parallel with cfDNA
The relative proportion of cffDNA (fetal fraction) increases with gestational age	Ultrasound dating of pregnancy required Fetal fraction should be determined when testing to ensure sufficient cffDNA present	Ultrasound dating of pregnancy required Fetal fraction should be determined when testing to ensure sufficient cffDNA present
The placenta can shed fetal DNA into the maternal circulation for up to 6 weeks after demise of the fetal pole	To avoid discordant results from a vanishing twin, careful ultrasound is required	To avoid discordant results from a vanishing twin, careful ultrasound is required
cffDNA is cleared from maternal circulation within hours of birth	Testing is pregnancy specific	Testing is pregnancy specific

Table 1 summarises how the provision of NIPT and NIPD are impacted by different characteristics of cffDNA.

Table 2: Studies of RAT Detection using NIPT

Study	Population	Study type	Study size	Total RATs
Lau *et al.* (2014)	General population	Prospective	1,982	7 (0.35%)
Brady *et al.* (2016)	Increased risk	Prospective	4,000	11 (0.28%)
Fiorentino *et al.* (2017)	Increased risk	Prospective	12,078	17 (0.14%)
Pertile *et al.* (2017)	General population	Retrospective	89,817	306 (0.34%)
Pescia *et al.* (2017)	Not specified	Prospective	6,388	50 (0.78%)
Scott *et al.* (2018)	General population	Prospective	23,388	28 (0.12%)
Wan *et al.* (2018)	General population	Retrospective	15,362	53 (0.35%)
Van Opstal *et al.* (2018)	Increased risk	Prospective	2,527	29 (0.91%)
Chatron *et al.* (2019)	Increased risk	Prospective	1,617	10 (0.62%)

Table 2 summarises the results of published studies reporting rare autosomal trisomies (RATs) detected via NIPT. This table only includes studies publishing the prevalence of individual trisomies in each cohort.

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Table 3: Recurrent Chromosomal Deletions in expanded NIPT

Chromosomal locus	Condition
1p36	1p36 deletion syndrome
4p16	Wolf-Hirschorn syndrome
5p	Cri du Chat syndrome
8q24	8q24 deletion syndrome
11q23	Jacobsen syndrome
15q11-13	Angelman syndrome and Prader-Willi syndrome
22q11.2	Di-George syndrome

Table 3: recurrent chromosomal deletions causing genetic syndromes commonly offered in expanded NIPT by commercial providers.

Table 4: Studies of NIPD using Relative Haplotype Dosage

Publication	Condition	Cases	Methodology	Sensitivity
Lo *et al.* (2010)	BT	1	Proband-based	100%
New *et al.* (2014)	CAH	14	Proband-based	100%
Parks *et al.* (2016)	DMD, BMD	9	Proband-based	100%
Parks *et al.* (2017)	SMA	16	Proband-based	100%
Hui *et al.* (2017b)	CAH, BT, EVCS, F8-H, HS	13	Parental - linked-read	100%
Hudecova *et al.* (2017)	F8-H	3	Proband-based	100%
Vermeulen *et al.* (2017)	CF, CAH, BT	18	Parental - TLA	100%
Jang *et al.* (2018)	DMD	5	Parental - linked-read	100%
Chandler *et al.* (2019)	CF	51	Proband-based	100%

Table 4: studies reporting NIPD using relative haplotype dosage for a range of monogenic conditions. Acronyms: BMD, Becker muscular dystrophy; BT, β-thalassemia; CAH, congenital adrenal hyperplasia; CF, cystic fibrosis; DMD, Duchenne muscular dystrophy; EVCS, Ellis-van Creveld syndrome; F8-H, factor 8 haemophilia; HS, Hunter syndrome; SMA, spinal muscular atrophy; TLA, targeted locus amplification.