Next Generation Sequencing and the impact on prenatal diagnosis

Abstract

Introduction: The advent of affordable and rapid next generation sequencing has been transformative for prenatal diagnosis. Sequencing of cell-free DNA in maternal plasma has enabled the development of not only a highly sensitive screening test for fetal aneuploidies, but now definitive non-invasive prenatal diagnosis for monogenic disorders at an early gestation. Sequencing of fetal exomes offers broad diagnostic capability for pregnancies with unexpected fetal anomalies, improving the yield and accuracy of diagnoses and allowing better counselling for parents. The challenge now is to translate these approaches into mainstream use in the clinic.

Areas covered: Here we review the current literature to describe the technologies available and how these have evolved. We discuss the opportunities and challenges at hand, including considerations for service delivery, counselling, and development of ethical guidelines.

Expert Commentary: As technology continues to advance, future developments may be towards non-invasive fetal whole exome or whole genome sequencing and a universal method for non-invasive prenatal diagnosis without the need to sequence both parents or an affected proband. Expansion of cell-free fetal DNA analysis to include the transcriptome and the methylome is likely to yield clinical benefits for monitoring other pregnancy-related pathologies such as pre-eclampsia and intrauterine growth restriction.

1. Introduction

Over recent years, advances in next generation sequencing (NGS) technology along with the discovery of cell-free fetal DNA (cffDNA) in maternal plasma have transformed prenatal diagnosis. As well as the development of a highly sensitive screening test for fetal aneuploidies, these advances have yielded progress in two main areas which we shall discuss here. The first is non-invasive prenatal diagnosis for monogenic disorders, which offers safe, accurate, definitive diagnosis of single gene disorders at an early gestation. The second is fetal exome sequencing, which can provide much broader prenatal diagnostic capability, offering better diagnoses and prognostic information for fetuses with abnormalities detected on prenatal ultrasound.

Here we will review recent advances in these areas, and discuss the new opportunities and challenges they bring, including ethical considerations and practical implications for service delivery.

2.0 NIPD for monogenic disorders

2.1 Background

Prior to the discovery of fetal cell-free DNA (cfDNA) circulating in maternal plasma, prenatal diagnosis of genetic disorders required direct sampling of fetal material by chorionic villous sampling or amniocentesis. These procedures are invasive and carry a small risk of miscarriage [1], creating a barrier to testing for some expectant parents.

The detection of cffDNA in maternal plasma [2] opened the door for non-invasive prenatal diagnosis, providing a means to perform definitive genetic analysis of fetal DNA without risking iatrogenic miscarriage of a wanted pregnancy.

Cell-free DNA describes short fragments of extra-cellular DNA found circulating in blood plasma. In pregnancy, the majority of cfDNA is maternal but from about four weeks' gestation a small amount of fetal DNA is released from the placenta, making up 5-20% of the total circulating cfDNA in maternal plasma [3]. The fetal fraction of cfDNA tends to increase with increasing gestation of the pregnancy and is also influenced by factors such as maternal weight, smoking and pre-eclampsia [4]. In the 20 years since its discovery, increasingly powerful methods have been developed to analyse cffDNA in maternal plasma, as previously reviewed by Chitty and Lo [5]. This has enabled the clinical implementation of non-invasive fetal aneuploidy screening, and now NIPD of monogenic disorders.

2.2 Development of NIPD approaches

Due to the co-existence of both maternal and fetal cfDNA in the maternal plasma, early efforts in NIPD focused on identifying paternally inherited or *de novo* alleles, which are easier to detect due to their absence in the background maternal cfDNA. Using this principle, non-invasive tests were developed to accurately determine fetal sex and Rhesus D status in RhD negative mothers and are now in widespread clinical use across Europe [6,7]. Non-invasive fetal sex-determination is commonly used in pregnancies at risk of severe X-linked disorders such as Duchenne Muscular dystrophy or haemophilia [8] where it reduces the need for invasive testing by 50% because only male-bearing pregnancies need further testing [6,9]. In congenital adrenal hyperplasia, it allows early identification of female fetuses, enabling *in utero* administration of dexamethasone to mitigate the *in utero* virilisation that otherwise occurs in affected females in this condition, and avoiding unnecessary treatment of male fetuses [10]. This application however remains somewhat controversial due to the possible effects of steroids on the developing fetal brain [11].

Next came the development of NIPD for autosomal dominant disorders caused by paternally inherited or *de novo* mutations, where the detection of the mutation in maternal plasma provided definitive diagnosis of an affected fetus [12]. The same principle was applied to some autosomal recessive conditions where the parents are heterozygous for different mutations. Here, the risk of an affected fetus was increased or excluded based on the presence or absence of the paternal mutant allele in the cfDNA [13]. A crucial element in any NIPD for paternal allele exclusion or *de novo* mutation detection is to verify the presence of fetal cfDNA in the sample, to avoid false negative results due to undetectably low fetal fractions.

These initial applications of NIPD for monogenic disorders used PCR followed by restriction enzyme digest (PCR-RED) in order to detect the paternally inherited mutant allele and were employed successfully by several groups for the prenatal diagnosis of cystic fibrosis [14] and β-thalassaemia [15]. However, subsequent invasive testing was required for definitive diagnosis when the paternal allele was detected in maternal plasma, because the high background level of maternal cfDNA precluded use of this approach to determine whether the fetus had also inherited the maternal mutation. Another limitation was that PCR-based restriction enzyme digest approaches could only test for one mutation at a time, which is not practical for diagnosis of conditions with multiple possible causative variants, such as thanatophoric dysplasia [16].

More recently, advances in the speed and affordability of NGS, and the advent of bench-top sequencers have allowed NGS techniques to move from research into clinical diagnostic laboratories

and provided superior approaches to NIPD that can assess panels of mutations in a single test. For example, Chitty *et al* developed a panel for NIPD of skeletal dysplasias caused by numerous different mutations in the *FGFR3* gene and demonstrated superior accuracy of panel testing using NGS compared to the previously employed PCR-RED method [17] as well as the ability to screen for 29 potentially causative mutations in a single test. This NGS panel-based approach was brought into clinical practice in the UK National Health Service (NHS) in 2014 and is now in use for NIPD for paternal exclusion in autosomal recessive disorders such as cystic fibrosis [13] and B-thalassaemia [18] and for developing tests on a bespoke basis for a wide range of monogenic disorders [19].

Non-invasive prenatal diagnosis of X-linked disorders and autosomal recessive conditions where the parents carry the same mutation always posed a greater challenge, as any diagnosis that depends upon detection of a maternally inherited allele in the fetus is made difficult by the high background amount of maternal mutant allele already present in cfDNA. However, sequencing advances have expanded the scope of NIPD to include testing for such conditions, using relative mutation dosage (RMD). This approach has been applied using both droplet digital PCR (ddPCR) and NGS methods to precisely quantify alleles in maternal plasma and determine the relative proportions of mutant versus wild type alleles [20,21]. This reveals an allelic ratio which correlates to the fetal genotype (Figure 1). If the fetus is heterozygous for the mutation (the same as the mother) there will be allelic balance in the maternal plasma. Allelic imbalance occurs when the fetus is homozygous (or hemizygous in X-linked conditions), with over-representation of the mutant allele relative to the wild-type indicating an affected fetus and under-representation of the mutant allele indicating an unaffected fetus [21]. Quantification of the fetal fraction of cfDNA is inherent to determining allelic ratio, therefore a separate step to verify the presence of fetal DNA is not required for the RMD approach. Since RMD depends on fetal fraction to interpret the result, it is vital that this is determined accurately.

Figure 1 here

A further improvement on the RMD approach is relative haplotype dosage (RHDO), which goes beyond comparing allelic ratios at a specific mutation or single nucleotide polymorphism (SNP), to measure the allelic balance between two haplotypes [22]. These haplotypes are derived from informative heterozygous SNPs within or in close proximity to the gene of interest, which gives the test increased statistical power compared to RMD.

2.3 Benefits of NIPD for monogenic disorders

A major advantage of NIPD for fetal monogenic disorders is its ability to provide a definitive diagnosis from only a maternal blood sample, eliminating the need for an invasive procedure and the small risk of iatrogenic miscarriage that accompanies it. This is in contrast to NIPT for aneuploidy, where the possibility of confined placental mosaicism or detection of aneuploid maternal cell lines necessitates invasive confirmation of abnormal screening results [23]. Studies confirm that service users and clinicians alike value the safety element of NIPD highly [24]. Furthermore, since it can be performed as soon as the fetal fraction of cfDNA is sufficient, usually from 7-9 weeks of gestation [6], it offers a definitive diagnosis at an earlier stage in the pregnancy than an invasive test, thus relieving parental anxiety sooner and allowing couples more time to make decisions about the pregnancy.

The application of NGS approaches has been transformative for NIPD, enabling more accurate detection of mutations than the previously employed PCR-RED based methods [17]. The high number of sequence reads obtained from NGS allows results to be interpreted with greater confidence. Furthermore, NGS enables a more flexible approach to testing, with panels able to detect multiple mutations at once rather that multiple restriction enzyme digest reactions being required. For this reason, NGS approaches reduce the cost of assay design per family compared to PCR-RED based approaches. Samples from different patients can be multiplexed within one sequencing run, thereby further reducing the cost per sample [12,17,25].

The development of more complex genetic analysis by RHDO means NIPD can now be offered to families at high risk of a wider range of monogenic disorders, including autosomal recessive disorders where both parents carry the same variant [19], and X-linked disorders [26]. The use of RHDO also offers the advantage of enabling diagnostic testing in genes that would not be amenable to direct sequencing due to the presence of a pseudogene, such as CAH and spinal muscular atrophy [27], because RHDO depends on linked markers that define the haplotype, rather than the specific gene sequence itself.

2.4 Challenges for NIPD of monogenic disorders

Whilst advances in NGS technology have enabled immense progress, there remain certain limitations to consider when implementing NIPD in widespread clinical practice.

Technical challenges include the need to analyse low concentrations of fetal cfDNA against a background of mostly maternal DNA. As mentioned above, quantification of the fetal fraction is crucial to confirm that sufficient fetal cfDNA is present in the sample to enable detection of a mutation if present and avoid false negative results. Whilst in male-bearing pregnancies the fetal fraction can be determined relatively easily using Y-chromosome sequences as a definitive marker of fetal DNA, female fetal DNA must be identified using other markers, such as *HLA-C*. Methylation of *RASSF1A* has been suggested as a universal fetal marker which is hypermethylated in fetal DNA and hypomethylated in maternal DNA [28] but this method is not commonly employed in clinical diagnostic laboratories. A superior approach is to assess fetal fraction using panels of informative heterozygous SNPs around a gene; RHDO provides an advantage here since deducing fetal fraction from heterozygous SNPs forms an inherent part of the technique [22], but it is a more complex and hence more costly test. Another technical challenge is the short length of fetal cfDNA fragments, which are typically shorter than their maternally-derived counterparts [29]. This limits the design of assays to detect a given target, particularly in certain types of mutations, such as triplet repeats, large deletions/duplications or mutations in loci that are homologous to other regions of the genome.

In addition to false negatives due to low concentrations of fetal cfDNA, false positives can result due to the presence of a ‘vanishing twin’. Where there has been early embryonic demise of an undetected twin the cfDNA from that twin can persist in the maternal circulation because it emanates from the trophoblast [30]. This can be avoided by careful ultrasound scanning to rule out the presence of an additional empty sac in an apparently singleton pregnancy. False positive NIPD results can also be due to maternal mosaicism, which can be detected by concurrent analysis of maternal genomic DNA.

Finally, NIPD is not ideal for use in multiple pregnancies, unless there are clearly discordant sonographic findings between the two fetuses, because when a mutation is detected it is not

possible to ascertain if only one or both fetuses are affected without invasive confirmation [19]. Nonetheless, NIPD can still be offered for sexing and bespoke testing in multiple pregnancies, where it removes the need for an invasive test in cases where the mutation is *not* detected (or where both fetuses are female, in the case of fetal sexing for X-linked disorders).

Most of the technical challenges are likely to be overcome in due course, however a significant barrier to widespread implementation of NIPD currently is the high cost associated with bespoke design of tests on an individual family basis. Bespoke test development can also be labour intensive and difficult to scale up. A particular limitation for autosomal recessive disorders is the requirement for an affected proband to enable haplotype phasing in RHDO. Newer methods in development hold promise for providing approaches that do not require a proband but at present remain prohibitively expensive [19,31].

2.5 Acceptability and ethics of NIPD for monogenic disorders

Studies to date show overwhelmingly positive views from women undergoing NIPD for single gene disorders [13,24,32]. Women interviewed particularly appreciated the safety of the test and its ability to provide them with a definitive diagnosis earlier in the pregnancy. This allowed them more time to make decisions about management of the pregnancy and prepare for the birth of an affected child or, in the case of a negative result, alleviated anxiety earlier and allowed them to 'normalise' the pregnancy sooner [24].

However, technological advances also bring new ethical and social considerations. Many of the ethical issues in prenatal diagnosis are not novel as they apply equally to invasive tests, but the advent of a non-invasive diagnostic test raises specific concerns around the ease of performing the test. As a straightforward blood test some fear that NIPD could be viewed as a routine part of pregnancy care and that women might not give it the careful consideration that they would an invasive test [24,33]. Another concern is that since the test does not pose any additional risk to the fetus, women may perceive an increased pressure to test a pregnancy they would not have tested invasively. Some argue that a societal pressure to test could by extension create a pressure to terminate pregnancies affected by genetic disorders. However, early evidence from studying NIPT for aneuploidy screening may allay these fears to some extent as it shows no increase in termination rates following NIPT for Down Syndrome, with some families using this safer approach to testing to gain information about the health of their baby [34].

These important concerns are largely addressed by thorough, non-directive pre- and post-test genetic counselling to safeguard informed consent and promote autonomous choice.European consensus guidelines provide a framework for counselling for prenatal diagnosis of monogenic disorders and after careful consideration they conclude that this guidance applies equally to both invasive and non-invasive tests [35]. In accordance with these guidelines, counselling should be individualised for each woman or couple's educational and cultural background, their values and beliefs. The content covered should include information about the condition being tested for and about the test itself. This includes practical information, what the test can or cannot detect, and the possible results (including the possibility of an inconclusive result, or need for a repeat test). Possible implications for family members must also be explained. Counselling should address psychosocial elements and encourage parents to take time to decide about testing, to consider the possible outcomes of the test and what the impact would be on them of each possible outcome.

One point to emphasise in pre-test counselling for NIPD is to ensure that parents understand that testing is optional, rather than part of routine maternity care. Separating appointments for NIPD from any 'routine' pregnancy blood tests could help to maintain the distinction between the two and ensure appropriate counselling from trained individuals. Studies of stakeholder views suggest this approach would be well-received; women and healthcare professionals interviewed preferred to see the test delivered through existing specialist genetics and fetal medicine services, separate from usual maternity care [24,33].

As this technology enters the mainstream and it becomes feasible to test for a greater range of disorders, we must also consider which conditions should be tested for and to whom the test should be offered. Equity of access must be ensured, whilst guarding against testing for inappropriate or 'trivial' things – a concern legitimately expressed by some healthcare professionals [33] and service users [24].

2.6 Service delivery of NIPD for monogenic disorders

Initially NIPD was largely provided on a research basis, but since 2011, when NIPD for fetal sex determination was approved for use in the UK NHS, there has been a gradual increase in its use in the clinic for diagnosis of a growing range of inherited conditions, albeit largely confined to the UK [12] .

Specific issues to address regarding the practicalities of service delivery include the need for quality assurance and standardisation of assays across regional labs. One challenge here is that, owing to the rarity of individual monogenic disorders, it can take a long time to gather sufficient plasma samples to develop and validate each assay. Once validated, tests need to have stringent quality control measures in place, both internally and externally. A key element is measuring the fetal fraction of cfDNA, to avoid false negatives where the fetal fraction is too low to be detected by the assay.

Appropriate handling of samples is also important to ensure high quality testing. Plasma must be separated from the cellular components of blood as soon as possible, because maternal white blood cells continue to lyse and release their DNA into the plasma after the sample is taken. This increases the amount of maternal cfDNA in the sample, and thus decreases the relative fetal fraction, making analysis more difficult and less reliable [36]. A necessary consideration in service delivery is to ensure that referring centres follow clear guidelines for sample handling. If it is not practical to transport samples immediately then cell-stabilising sample tubes should be used.

The other key factor for planning service delivery is the turnaround time of the test. Time is limited for making clinical decisions within a pregnancy, especially if fetal abnormalities are detected at a relatively late gestation, so turnaround times for NIPD tests need to be rapid. This requires the development of efficient pipelines that allow collection, transportation and analysis of samples and return of results to clinicians and patients all in the shortest time possible. Higher throughput of tests will maximise efficiency and reduce costs [25]. This may be best achieved by centralisation of services, which would be relatively straightforward for NIPD compared with invasive tests because blood samples can be easily sent by post.

2.7 Cost evaluation of NIPD for monogenic disorders

When implementing NIPD in a public healthcare system, cost is necessarily an important consideration. The PCR-RED technique used in some of the earliest clinical NIPD tests for monogenic disorders is relatively less expensive but, as discussed above, it is outperformed by more expensive NGS techniques due to the subjective nature of PCR-RED test interpretation leading to high inconclusive rates. The falling cost of NGS technology helps to mitigate this, as does the use of panels to detect multiple mutations in a single sequencing reaction, and the ability to multiplex several samples simultaneously. Analysis shows that for diagnosis of paternally inherited or *de novo* autosomal dominant conditions NIPD using NGS is cheaper than invasive testing, but costs rise for the more complicated analyses required for definitive diagnosis of X-linked and autosomal recessive disorders, and when a bespoke test needs to be developed for a family [12,25].

It is anticipated that if made widely available uptake of NIPD will increase as many women who would have declined invasive testing will opt for NIPD, including those who would not consider termination of pregnancy [13]. Whilst increased throughput could help to bring costs down, the higher volume of tests may increase costs overall, and brings into question whether testing for 'information only' or for reassurance in cases of very low recurrence risk is cost-effective [37]. In a financially constrained public health service resources must be allocated wisely, yet the value of alleviating psychological burden in a pregnancy should not be underestimated. Further, parental decision making may be difficult in the absence of a definitive diagnosis.

3.0 Fetal exome sequencing

3.1 Background

Pregnancies at risk of single gene disorders fall into two broad categories: those in which a mutation is known within the family (either via an affected family member or through carrier testing of the parents prior to pregnancy), and those in which fetal abnormalities suggestive of a monogenic disorder have been detected on ultrasound. In cases where the causative mutation in the family is known, diagnostic testing is relatively straightforward, however those with sonographic abnormalities in the absence of a family history have always posed a much greater diagnostic challenge as in many cases there are multiple mutations in different genes that could cause similar appearances on ultrasound, and testing for each one individually would not be feasible within the timescale of the pregnancy. Next generation sequencing is now providing a means to address this challenge.

Cytogenetic testing (karyotyping and microarray) on chorionic villus or amniotic fluid samples yields a diagnosis in up to 40% of these cases, where abnormalities are due to aneuploidy or medium/large regions of copy number variation, but still leaves the majority undiagnosed [38]. The advent of NGS has facilitated fast, accurate, targeted sequencing of relevant genes or panels where a defined monogenic disorder is suspected but such cases are rare, limited only to those conditions where a clear-cut fetal phenotype is recognised [39]. However, more often fetal phenotypes are non-specific, so it is desirable to cast the diagnostic net wider. As the cost of NGS continues to fall, interest has therefore turned to sequencing fetal exomes, or even whole genomes. Whole exome sequencing (WES) allows assessment of all the protein coding regions of the genome (harbouring around 80% of known disease-causing variants) in a single test. Fetal WES is now possible owing to advances in NGS and has been used to good effect in multiple published case series, recently reviewed by Best *et al* [38], to successfully reach genetic diagnoses where conventional cytogenetic tests were uninformative.

3.2 Benefits of fetal exome sequencing

Exome sequencing of the fetus offers much broader diagnostic capability than current prenatal genetic tests for fetuses with unexpected abnormalities on US. It provides superior resolution, down to the single nucleotide level and does not rely upon designing assays for a single mutation at a time, but rather sequencing the whole exome in one reaction then targeting panels for analysis as needed. Studies of prenatal WES so far indicate that this method can provide diagnostic yields of anything between 6% and 80% in fetuses with undiagnosed sonographic abnormalities and normal karyotype/microarray [38]. The observed diagnostic yield seems to vary significantly depending on case selection and on whether proband only versus trio sequencing was employed. Studies selecting only fetuses with multiple structural abnormalities have yielded higher diagnostic rates than those testing unselected cases, particularly if cases of isolated raised nuchal translucency (NT) were included. The most successful diagnostic yields, and greatest speed of returning a diagnosis have been achieved by sequencing trios of fetus, mother and father in parallel, rather than sequencing the fetus first and subsequently testing the parents for any candidate variants identified [38,40]. Most published case series of fetal WES to date have been relatively small but much more information on diagnostic yields and the clinical utility of fetal WES is expected to emerge from two ongoing large unselected cohort studies in the UK and USA [40–42].

Despite the variable diagnostic yields observed so far, exome sequencing clearly improves upon existing prenatal genetic tests in its ability to provide a more accurate prenatal diagnosis. Accuracy of diagnosis is hugely important to enable more informative counselling during pregnancy about likely prognosis for the fetus and recurrence risk for future pregnancies, as highlighted in European guidelines for prenatal diagnosis [35]. Receiving an accurate prenatal diagnosis allows parents more informed choice about pregnancy and perinatal management, and can avoid a postnatal 'diagnostic odyssey' which may involve a prolonged stay in hospital undergoing multiple tests, some of which may be invasive [38].

In some cases, an accurate prenatal diagnosis has a direct effect on shaping plans for delivery, early postnatal treatment, and increasingly targeted *in utero* treatment. For example, early trials in osteogenesis imperfecta (OI) indicate that *in utero* transplantation of mesenchymal stem cells may result in a reduction in fractures [43]. Further work on this is now underway in the BOOSTB4 trial, which will use rapid fetal exome sequencing to reach an early definitive prenatal diagnosis of OI before administering *in utero* stem cell therapy [44]. With ongoing developments in stem cell research and gene therapies, it is reasonable to assume that *in utero* treatments for other genetic conditions will be developed in time and fetal exome sequencing could be instrumental for identifying candidates for these therapies. Of equal importance can be the exclusion of a genetic diagnosis before *in utero* therapy. For example, fetal surgery is now possible for certain cardiac lesions [45] but some of these anomalies have a poor prognosis when they are part of a genetic syndrome, which may be indistinguishable sonographically. The same applies for congenital diaphragmatic hernia, which may be ameliorated by *in utero* endotracheal occlusion [46,47]. Knowledge of the genetic diagnosis provided by fetal exome sequencing would contribute to weighing up risks and benefits and guide choices for performing these high-risk, invasive procedures.

Beyond improved diagnostic capabilities and access to appropriate treatment, the wider potential of fetal exome sequencing lies in deepening our understanding of how genetic disorders present in the prenatal period. Fetal phenotypes are notoriously difficult to characterise and interpret due to factors including the limitations of ultrasound scanning and interpretation and the existence of

phenotypes that simply cannot be detected prenatally, such as developmental delay, intellectual disability or metabolic derangements. Candidate gene discovery through exome sequencing can reveal new genotype-phenotype correlations that have not previously been characterised prenatally and thus help us to better interpret fetal phenotypes in future.

3.3 Challenges for fetal exome sequencing

The potential benefits of fetal exome sequencing are vast, but there are challenges to overcome before it can enter widespread clinical use. One difficulty, as mentioned above, is the fact that prenatal phenotypes are often incomplete and difficult to interpret. Data generated from fetal exomes will in itself help to address this problem but in the meantime it poses a challenge for interpretation of variants detected, and we must be wary of both over and under-reporting of new variants as a result. For example, Sotos syndrome – caused by haploinsufficiency of the *NSD1* gene – is an overgrowth syndrome characterised by macrocephaly and intellectual disability and is rarely diagnosed prenatally. However, a recent case was reported of a fetus with microcephaly and intrauterine growth restriction, where fetal genomic analysis revealed a *de novo* deletion of *NSD1* [48]. In this case the pregnancy was terminated but we have now encountered a similar case in a local study, where an *NSD1* variant was identified in a microcephalic growth-restricted fetus but could not be classified as pathogenic at the time due to insufficient evidence relevant to the fetal phenotype. Postnatally, the infant subsequently developed macrocephaly and is now reported to have clinical features of Sotos syndrome. This may indicate a previously unrecognised evolving phenotype and highlights the importance of sharing information about new genotype-phenotype associations as cases like these arise.

As with postnatal exome sequencing, the volume of data generated compared to targeted sequencing can create challenges for interpretation of variants. More variants will be detected from whole exome sequencing and all require expert scientist and clinician input to appropriately assign their pathogenicity. Counselling issues arising from variants of uncertain significance may be mitigated by use of a 'clinical' or targeted exome panel whereby only variants relevant to the phenotype are reported. This approach also decreases the identification of secondary and incidental findings; another very important point to consider in counselling. If fetal exome sequencing is to be rolled out in clinical use it will be vital to first have in place clear guidelines for reporting of secondary or incidental findings, and mechanisms to ensure fully informed consent prior to testing [49].

Technical challenges for fetal exome sequencing include incomplete coverage of some genes, which can lead to missed variants and significantly impair definitive diagnosis [50]. For example, Drury *et al* [40] reported two cases in which fetal exome sequencing detected a single copy of a likely pathogenic variant in a plausibly causative autosomal recessive gene but incomplete coverage of the gene rendered it impossible to detect or rule out a second variant. The first was a fetus with sonographic findings of echogenic lungs and a dilated trachea where exome sequencing revealed a heterozygous missense variant in *FREM2,* a gene in which biallelic mutations cause Fraser syndrome. Fraser syndrome is associated with congenital high airway obstruction syndrome (CHAOS) [51], which would have been consistent with the sonographic findings in the fetus. Post-mortem examination following neonatal death confirmed a diagnosis of CHAOS. A second case in the same series was a fetus with multiple sonographic abnormalities, including congenital diaphragmatic hernia, bilateral severe ventriculomegaly, atrioventricular septal defect (AVSD) and polydactyly. Here, fetal exome sequencing identified a novel splice site variant in *DYNC2H1,* a gene in which

previously reported biallelic variants have been associated with ciliopathy phenotypes [52]. Again, this was a plausible causative candidate for the fetal phenotype and post-mortem findings were suggestive of a ciliopathy but it was not possible to identify a second variant in *DYNC2H1* despite re-sequencing to attempt to improve coverage of the gene.

Next generation sequencing currently also has poor ability to detect certain types of variants, such as copy number variants, inversions and other structural rearrangements. For this reason, it will be important to continue to use existing cytogenetic techniques in conjunction with WES to ensure maximum variant detection. Pseudogenes and repetitive regions of the genome also continue to pose a challenge for all types of NGS, and exome sequencing, by definition, will not detect variants in non-coding regions. As NGS technologies advance further it may be the case that exome sequencing becomes obsolete in future, in favour of whole genome sequencing as standard.

Finally, the amount of sequencing and complex data analysis required for whole exome (or genome) sequencing means that, at present, turnaround times tend to be too long for the results to be returned within the pregnancy, particularly since most structural fetal abnormalities are not picked up until the 20 week anomaly scan, or even later. Most fetal exome sequencing performed in the research setting to date has not returned results to parents within the pregnancy, but in order to move this into clinical use it will be necessary to make analysis pipelines much faster. However, recent work using rapid exome sequencing with analysis targeted to skeletal genes for the diagnosis of fetuses with sonographic anomalies suggestive of a skeletal dysplasia have achieved turnaround times of less than two weeks, making analysis useful in the course of the pregnancy [39].

3.4 Acceptability and ethics of fetal exome sequencing

In a qualitative study of their experiences expectant parents undergoing prenatal exome sequencing after detection of a fetal anomaly on ultrasound perceived the test positively, as a way to gain more information about their baby's condition [53]. They also emphasised the importance of receiving clear and detailed information at the time of consent for the test, particularly regarding the scope of the findings that would be returned to them.

The main ethical consideration for whole exome sequencing, whether pre- or postnatal, is the question of which findings should be sought and reported within the sequence information. In all cases, variants directly relevant to the presenting phenotype will be reported but exome sequencing may also reveal incidental findings and provides the opportunity to intentionally look for secondary findings unrelated to the phenotype in question (for example carrier status for other conditions, or inherited cancer predisposition). The American College of Medical Genetics (ACMG) recommends a list of 59 genes in which pathogenic variants should be reported postnatally but it specifically excludes prenatal testing from the scope of its guidance [54]. Therefore, there is currently no published guideline on reporting secondary findings in prenatal WES, although one has recently been published recommending counselling and laboratory standards [49].

The prenatal setting poses unique ethical challenges around which variants to report for two main reasons. Firstly, predictive testing in the fetus removes the future autonomy of the unborn child to decide whether they would want to know that genetic information [55]. Guidelines are already established which caution against predictive genetic testing in minors [56,57] and their principles could be extended to create similar guidelines for prenatal testing. Secondly, reporting in prenatal WES differs from the postnatal setting because the information reported prenatally could influence parents' decisions on whether to continue or terminate a pregnancy. This difference in the potential

consequences of testing make it extremely important to evaluate which variants are appropriate to report prenatally, and to consider whether a tiered approach is necessary, allowing the unborn child to access further information from their exome sequence if they wish to when they are old enough to decide for themselves [58].

As well as incidental findings of known significance, whole exome sequencing also invariably reveals variants whose pathogenicity cannot currently be determined (variants of uncertain significance [VUS]) according to the ACMG guidelines for variant interpretation [59]. These pose a counselling challenge because it is not possible to give the family a definitive idea of whether such variants are relevant to the fetal phenotype or likely to pose any risk in the current or future pregnancies. As a result, they can generate much uncertainty and anxiety, so it is disputed as to whether they should be reported. In the research setting VUSs are not generally reported, and the pre-test consent process makes this clear. However, in clinical practice patients often express a desire to know 'everything' [60] and this can be challenging to manage. Popular discourse may portray genetic information as being more deterministic than it really is and it is difficult to counter this assumption during counselling. When the consequences of testing include the possibility of terminating a pregnancy it is crucial to ensure that women and their partners can base decisions on the most accurate information possible.

To add to the uncertainty, VUSs are liable to be re-classified as pathogenic or benign as more evidence emerges in the future. In this scenario, families must be re-contacted and re-counselled about the implications of the variant. If decisions have already been made based on the old information this may cause distress and must be handled very sensitively. A further dilemma relates to the logistics and practicalities of reanalysing sequencing data. In order to make sure something significant is not missed it would seem necessary to periodically routinely reanalyse all VUSs identified by fetal exome sequencing but it remains to be defined how exactly this could be achieved and whether the responsibility should lie primarily with clinicians or laboratories. It would certainly seem sensible, as recommended in the ISPD guideline [49] that negative data should either be re-analysed or resequencing undertaken if the family present planning another pregnancy. More than ever, these ethical issues necessitate careful, thorough and non-directive pre- and post-test genetic counselling. Particular counselling challenges include the complexity and scope of the information that may be revealed and the need to convey uncertainty [61]. This must be explained in a way that can be understood by patients from a range of educational, social and cultural backgrounds, and should include explicit discussion about incidental and secondary findings before testing [49,58].

Studies of patient and healthcare provider attitudes towards reporting of prenatal WES reveal some disparities between the two groups. Expectant parents and patient group representatives appear more inclined towards receiving the maximum information possible, including routine reanalysis of VUSs [60,62], whereas many clinicians surveyed expressed reservations about reporting incidental or uncertain findings [63]. In one study patients also tended to hold a more positive view of the promises of WES, despite having been counselled that diagnostic yield was only around 30% [64]. This underlines the importance of extensive education of both healthcare providers and the public before fetal WES enters mainstream medicine.

3.5 Service delivery of fetal exome sequencing

These technical practicalities, ethical and social issues all have implications for future service delivery of fetal exome sequencing as a mainstream clinical diagnostic service. Clear and unified guidelines

on interpretation, reporting and re-analysis of variants, as well as on counselling considerations will be paramount. The recent position statement from the International Society for Prenatal Diagnosis offers welcome and detailed consensus to this effect but does not currently support the routine use of prenatal exome sequencing outside of the research setting, until more validation data becomes available [49].

When considering the eventual implementation of fetal exome sequencing as a routine diagnostic test the complexity of the test will present an increased need for detailed pre- and post-test counselling as discussed. This should be delivered by qualified professionals, who are often in short supply, particularly if we are to depend on those who have a genetic qualification. The level of interest from patients suggests that demand for this test could be high, so hospitals and diagnostic laboratories must be equipped with both the resources and the workforce to handle high throughput for sequencing, analysis and counselling.

Analytical pipelines must be developed and validated to ensure rapid turnaround of results; one way to facilitate this is the application of phenotype-specific panels, which also helps to minimise unwanted incidental findings. However, this relies heavily on accurate phenotypic characterisation, which is variable and not always possible based on prenatal ultrasound alone.

3.6 Cost evaluation of fetal exome sequencing

The cost of any exome sequencing is currently high compared to single gene tests, but the cost of fetal WES has not yet been formally evaluated in comparison to other methods of prenatal diagnosis. The costs to consider are those of sequencing itself, analysis, counselling expertise, and the costs relating to storage of data for potential re-analysis. Despite falling costs of NGS technologies, the cost of sequencing of fetal whole exomes is increased by the requirement for trio analysis to facilitate rapid and accurate diagnosis meaning that three exomes must be sequenced for every proband. This, however, is increasingly not the biggest financial concern, as commercial competition between providers will continue to drive prices down. The cost of analysis is unlikely to fall so quickly, due to the manpower required and will require significant bioinformatic input. Adding to this the need to store sequence data and return to it for re-interpretation adds a further cost and infrastructure consideration.

It must be remembered however that, by definition, patients who would undergo testing using fetal exome sequencing are those whose diagnosis is not readily determined by more straightforward tests, so the avoidance of the postnatal diagnostic odyssey that they would otherwise have to reach a diagnosis may help to mitigate the cost of exome sequencing in some cases. A comparable study of the cost of exome sequencing in the postnatal setting indicated that for this reason, exome sequencing represented a saving in the longer term compared to conventional diagnostic tests [65].

4. Conclusion

Next generation sequencing techniques have enabled the development of prenatal diagnostic tests that are safer, more accurate and have greater applicability than ever before. These tests are welcomed by expectant parents and healthcare professionals alike. They also render prenatal diagnosis perhaps more complex than ever before, bringing new technical, analytical and ethical challenges. Implementation into mainstream antenatal care will therefore require the development of clear guidelines and delivery of high quality genetic counselling before and after testing. It will be

particularly important to ensure consensus guidelines and counselling standards are in place before major interest in these tests emerges in the commercial sector, so that tests delivered commercially are held to the same high standards as those in the public healthcare service.

5.Expert Commentary

Next generation sequencing is revolutionising prenatal diagnosis through the use of NIPD for monogenic disorders and the advent of fetal exome sequencing but the future is likely to hold even further advances. As sequencing costs fall further and analytical capabilities continue to improve, it is likely that exome sequencing will be superseded by prenatal whole genome sequencing (WGS). This would offer even broader diagnostic capabilities by additionally detecting non-coding and copy number variants, but would bring with it an even greater number of variants of uncertain significance and their associated challenges.

The next goal will be to combine the concepts discussed here to achieve non-invasive prenatal WES/WGS on cfDNA. Proof of concept has already been established for WGS on cfDNA [66] but it remains too technically challenging at present to separate the fetal and maternal genomes. Other methods for non-invasive prenatal sequencing are also being studied, including single cell sequencing of circulating fetal cells isolated from maternal blood [67]. This has been successfully employed in some small series to detect fetal aneuploidy [68], copy number variants [69] and even to perform deep whole genome sequencing [70]. Whilst this approach is promising, and would circumvent the problem of high levels of background maternal DNA, it is not yet in widespread use due to ongoing difficulty isolating the fetal cells.

An alternative non-invasive source of fetal genetic material for sequencing is trophoblastic cells obtained from the mother's cervix; Pfeifer *et al* have demonstrated proof of principle for this technique, successfully ascertaining the fetal genotype in 6 cases at risk for cystic fibrosis or spinal muscular atrophy [71]. The pitfall of this approach, however, is that trophoblastic cells are thought to be present in the cervix only up until around 11-12 weeks' gestation, so this approach would not be appropriate for NIPD beyond the first trimester. This is impractical given that many fetal anomalies are not detected until the 20-week scan.

The need for parental haplotyping may also be eliminated in future, due to the development of microfluidics-based linked-read sequencing technology – a method to directly deduce the fetal haplotype without the need for sequencing both parents [72]. Prohibitively high costs currently preclude the use of this technology but these will undoubtedly fall in time [19]. Furthermore, we may now be approaching universal applicability of NIPD without the need for an affected proband thanks to the development of a haplotype-based protocol that obviates the need for designing mutation-specific assays [31].

As well as the diagnosis of monogenic disorders, the future holds promise for other applications of non-invasive cell-free nucleic acid sequencing. Recently, several groups have used cell-free nucleic acids to go beyond genomic analysis and examine the fetal transcriptome and epigenome. Some of these studies have demonstrated the potential of such techniques for use in assessment and monitoring of pregnancy-related pathologies such as pre-eclampsia, intra-uterine growth restriction and risk of preterm birth [73]. With the recent development by Sun *et al* [74]of an algorithm to non-invasively reconstruct the whole fetal/placental methylome from cffDNA in maternal plasma, a clinical application of this technique for pregnancy monitoring may be in sight.

Five-year view

We speculate that five years from now it will be possible to perform most prenatal diagnosis non-invasively. We envisage that NGS-based NIPD for an even wider range of monogenic disorders will be developed and that non-invasive sequencing of fetal exomes may become a reality for pregnancies complicated by fetal anomalies.

Key Issues

- Next generation sequencing (NGS) has advanced the field of prenatal diagnosis by enabling us to sequence cell-free DNA (cfDNA) in maternal plasma to offer safer, accurate diagnosis of monogenic disorders at an early gestation. Non-invasive prenatal diagnosis (NIPD) using a NGS panel-based approach can screen for multiple potentially causative mutations in the fetus simultaneously. The sophisticated technique of relative haplotype dosage (RHDO) can determine the fetal genotype even in autosomal recessive conditions where the mother is heterozygous.
- Next generation sequencing also enables sequencing of fetal genetic material obtained following invasive testing to allow screening of many genes in one test. This offers broad diagnostic capability for pregnancies with unexpected fetal anomalies and a normal microarray, improving the yield and accuracy of diagnoses and allowing better counselling for parents.
- Important considerations for service delivery of these technologies in mainstream clinical care include delivering tests with a rapid turnaround time at an affordable cost, and ensuring good quality genetic counselling from trained professionals.
- Ethical challenges including reporting of variants of uncertain significance and incidental findings require careful attention and the development of clear guidelines before these tests are implemented in mainstream clinical care.

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