Clinical utility of exome sequencing in the prenatal diagnosis of congenital anomalies:

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Abstract: Advances in prenatal genomics have enabled the assessment of not only the sub-microscopic structure of chromosomes using chromosomal microarray analysis, but also the detection of "pathogenic variants" to the resolution of a single base pair with the use of next generation sequencing. Research is emerging on the additional prenatal diagnostic yield that exome sequencing offers when structural fetal anomalies are detected on ultrasound examination, in particular with defining prognosis and recurrence of anomalies. Primarily assessed using fetal DNA obtained by invasive techniques (amniocytes or chorionic villi), this technology is progressing into a non-invasive approach using maternal plasma. There are several challenges, to be addressed before this technology can be introduced into routine clinical practice. These are primarily technical and interpretational but also relate to service provision; cost-effectiveness; turn-around time; patient acceptability and ethical dilemmas. With adequate pre- and post-test counselling many of these challenges may be overcome and such counselling will be multi-disciplinary, involving clinical geneticists, genetic scientists, paediatricians, perinatal pathologists and fetal medicine specialists. There is therefore a need for obstetricians to have an understanding of the application, advantages and challenges of such technologies before introduction into clinical practice.

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7th August 2018.

EJOGRB-18-18135

Submitted 19th July 2018.

Dear Janesh.

Thank you for discussing our submission last week of our review articles that discusses the role of *Prenatal Exome sequencing in fetuses with structural anomalies on ultrasound*.

We understand that this is a 'specialised area' but most of Europe and indeed the USA have been awaiting the results of our prospective study on 1000 cases to be published in the Lancet soon and it is likely this testing will find its way into clinical practice. It is therefore that obstetricians as well as healthcare professionals in related fields understand the general principles of this prenatal testing and the need for pre-test and post-test counselling.

As discussed, we have rationalised the original submission to shorten the text by ~50%. We have though re-submitted the abridged and the original article (which fulfils the word counts for EJOG).
I hope that you feel that this maybe an educational topic for your readers.
Yours sincerely.

Mark D Kilby DSc MD FRCOG FRCPI
Professor of Fetal Medicine & Therapy.

TITLE: CLINICAL UTILITY OF EXOME SEQUENCING IN THE PRENATAL DIAGNOSIS OF CONGENITAL ANOMALIES : A REVIEW

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Fionnuala Mone, Elizabeth Quinlan-Jones, Mark D Kilby

ABSTRACT

Advances in prenatal genomics have enabled the assessment of not only the sub-microscopic structure of chromosomes using chromosomal microarray analysis, but also the detection of "pathogenic variants" to the resolution of a single base pair with the use of next generation sequencing. Research is emerging on the additional prenatal diagnostic yield that exome sequencing offers when structural fetal anomalies are detected on ultrasound examination, in particular with defining prognosis and recurrence of anomalies. Primarily assessed using fetal DNA obtained by invasive techniques (amniocytes or chorionic villi), this technology is progressing into a non-invasive approach using maternal plasma. There are several challenges, to be addressed before this technology can be introduced into routine clinical practice. These are primarily technical and interpretational but also relate to service provision; cost-effectiveness; turn-around time; patient acceptability and ethical dilemmas. With adequate pre- and post-test counselling many of these challenges may be overcome and such counselling will be multi-disciplinary, involving clinical geneticists, genetic scientists, paediatricians, perinatal pathologists and fetal medicine specialists. There is therefore a need for obstetricians to have an understanding of the application, advantages and challenges of such technologies before introduction into clinical practice.

KEY WORDS: NEXT GENERATION SEQUENCING; EXOME SEQUENCING; PRENATAL; MONOGENTIC DISORDERS; FETAL ANOMALY; PAGE STUDY

INTRODUCTION

Fetal structural anomalies anomalies (FSA) complicate up to 5% of pregnancies.[1] Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR), conventional karyotype and chromosomal microarray (CMA) will detect up to 40% of the underlying chromosomal aetiology associated with FSAs. With advances in genomic technology, theoretically, we have the ability to increase the diagnostic yield of testing through the use of Next Generation Sequencing (NGS). These techniques allow examination of the genome down to one base pair, facilitating the diagnosis of monogenic disorders (Figure 1 and Box 1). The introduction of such technology into prenatal diagnosis will require complex clinical pathway development with multi-disciplinary team (MDT) working and communication.

<Insert Figure 1 and Box 1>

DISCUSSION

Approaches in Next Generation Sequencing

NGS is typically applied in proband cases where single major or multiple FSAs are identified on prenatal ultrasound scanning and standard prenatal testing strategies identify no chromosomal anomalies.[1] Common techniques include:

(i) **Clinical or targeted exome sequencing** – specific exonic regions of interest are sequenced and a panel of genes are tested which are known to be associated with specific phenotypes;[2]

(ii) **Whole Exome Sequencing (now referred to as 'exome sequencing (ES)')** - coding exons of all known disease coding genes are interrogated (Figure 2) and;

(iii) **Whole Genome Sequencing (WGS)**, where the whole genome inclusive of non-coding intronic regions is assessed. This technique is beyond the scope of this review and has yet to be assessed in prenatal diagnosis.[3]

NGS (primarily ES in the prenatal setting) may be performed to analyse fetal tissue/cells obtained by invasive prenatal testing or in certain specific cases, as a non-invasive prenatal diagnostic (NIPD) test.[4] Testing should ideally performed as a "trio" analysis (fetus [proband] and both parents) to aid in the assignment of variant pathogenicity .[5] CMA will delineate copy number variants and mosaicism in the case of an array-comparative genomic hybridisation technique, with the addition of genotyping (uniparental disomy, loss of heterozygosity and consanguinity) in the case of a single nucleotide polymorphism array technique. NGS has the ability to combine assessment of all of these measures in addition to the determination of single nucleotide variation.

<Insert Figure 2>

Processing and interpretation of variants

This is a complex process requiring experienced and specialist bioinformatic teams. It is one of the practical 'barriers' to introducing ES into clinical medical practice. Variant processing and interpretation involves a stepwise workflow, an example of which is demonstrated in Figure 3. Paramount to this workflow, is input of the MDT.[5] Following pre-test counselling and informed consent, DNA is extracted and library preparation using target enrichments are used to optimise coverage. ES can then be performed using a selected platform (e.g. Ilumina GAIIx®) by a sequencing by synthesis approach to determine the sequence of the exonic regions.[6] Following the sequencing process, masses of data regarding base alignment is produced and this is subsequently re-aligned and annotated by means of a bioinformatics pipeline, comparing the test sequence to an updated version of the reference genome.[2] Variants are then filtered and interpreted by means of an *in silico* tool to determine variants predicted to affect the protein sequence or exon splicing through use of prediction programs. Variants are graded using criteria including characteristics such as allele frequency of <5%; functional impact i.e. if they are synonymous and non-protein altering; nucleotide conservation between species and; the finding of a variant within clinical databases that are of benign consequence and/or are not related to the phenotype. Remaining variants are compared to the parental genome to assess if they are *de novo* (i.e. a variant which has developed for the first time during meiosis as opposed to one which is inherited from one/both parent(s)), which would imply pathogenicity.[6,7]

<Insert Figure 3>

Variants are graded using the Association of Clinical Genetic Science criteria by means of a five-class system ranging from benign or non-pathogenic, to variants of uncertain significance (VUS) to clearly pathogenic. Once or if a variant is identified the next step is to validate the variant, often using Sanger sequencing.[3]

Reporting of variants

ES may identify the presence of VUS. As knowledge of variant interpretation evolves, it is likely that the incidence of true VUS will reduce, as has been the demonstrated with CMA reporting.[8] ES may also unveil secondary findings (SF); variants considered to be medically actionable but unrelated to the primary indication for testing and often relating to late onset conditions, such as cancer. In the UK current practice gives patients the option of 'opting out' which is determined during pre-test counselling. In the prenatal setting, such secondary findings are not typically revealed in the research setting.[9] Incidental findings (ICF) are variants, which are discovered unexpectantly and are unrelated to phenotype referred. Currently these are also not routinely reported.[10] Presently, in the UK, NHS England is exploring the introduction of prenatal ES before the end of 2019 and with expected clinical pathway 'turnaround' duration of approximately 21 days. This is achievable but has health economic implications, not least because of laboratory and bioinformatic workforce implications.

Advantages

The primary advantage of the prenatal application of NGS is that it allows, in a proportion of cases, the revelation of a causative variant allowing an improved prognostic definition for the fetus. Parents will be able make a prospective, autonomous decision about their pregnancy and an early-prenatal identification may allow the future development of fetal therapies.[11] A defined single gene aetiology aids in counselling relating to recurrence risk, opening opportunities for pre-implantation genetic diagnosis and future prenatal testing.[12] Following trio analysis it is often discovered that most pathogenic variants are *de novo* in origin, however the potential of gonadal mosaicism cannot be out-ruled hence prenatal testing in subsequent pregnancies for both inherited pathogenic variants and those felt to be de novo may be offered once the pathogenic variant of the initial affected pregnancy is known. In paediatric practice, the diagnostic yield of ES (of approximately 25%) has been well established in various cohort series with congenital malformations and developmental morbidity[3].

Diagnostic yield

There are limited prenatal cohort studies which have assessed the diagnostic utility of ES and more research is needed before it can be applied in the routine clinical setting.[13,14] Two of the largest prenatal studies in structurally abnormal fetuses thus far suggest an ES diagnostic yield of 7.5-8.5%.[15,16] The largest of these, the *Prenatal Assessment of Genomes and Exomes* (PAGE) study has presently prospectively assessed 610 fetuses with structural anomalies on ultrasound through ES trio analysis and found it to have an additional diagnostic yield of 8.5%.[14] As noted by the smaller cohort study from the US by Wapner, *et*

al. the diagnostic yield was greater where there were multiple FSAs present on ultrasound, where the yield was a high as 15%.[15,16] It also varied between different phenotypic sub-groups with pathogenic variants more common in fetuses with hydrops, cardiac or skeletal abnormalities but a low diagnostic yield in those with only an isolated elevated nuchal translucency and no pathogenic variants identified in the co-existence of isolated renal or thoracic abnormalities. The PAGE study unveiled several novel diagnoses and had a rate of VUS of 3.9%.[16] Accurate and detailed phenotyping (including dysmorphological examination) has been shown to optimise the accuracy of ES, as evident from its' efficacy when testing is based upon post-mortem findings with a reported diagnostic yield of 37% in a subset of the PAGE study cohort where fetuses underwent autopsy (n=27).[17] Additionally, where prenatal cases are selected specifically by a geneticist (highlighting a high suspicion of an underlying pathogenic variant) the yield is significantly higher, at 47% in one study (n=15).[18] Where cases are pre-selected based upon the severity of the anomaly, as opposed to the unselected population assessed in PAGE, diagnostic yields, particularly in the presence of skeletal and brain anomalies are greater.[4,18] The utilisation of ES is dependent upon the accurate recognition of phenotypic patterns to target fetuses with the greatest risk of having an underlying pathogenic variant. This requires input from a clinical geneticist with expertise in dysmorphology to recognise phenotypical patterns and classify features appropriately.

Challenges

The challenges of prenatal ES are focused within four primary areas; (i) technical – there are sample considerations such as DNA quality and quantity and the presence of maternal cell contamination. Tissue culturing and the use of appropriate library preparation can optimise processing but there is a risk of clonal selection.[2] ES does not analyse intronic regions or assess for epigenetic modifications and depending upon the depth of base pair coverage, all exonic regions may not be fully assessed with the risk of false negative results and a further 65% of FSAs which go unexplained. As the prenatal phenotype is based upon detailed ultrasonography this may be associated with poor true diagnostic potential.[2] (ii) service provision – genetic services are limited and training of the MDT in the prenatal setting will be required before NGS can be introduced routinely.; (iii) cost – the cost effectiveness of prenatal ES is not yet known although this is currently being evaluated as part of the PAGE Study collaboration.[19] In line with Moore's Law, the costs associated with NGS technologies have reduced significantly since inception and are optimised by the multiplex nature and as further understanding of the technology evolves over time.[5]; (iv) turnaround time - studies have suggested that an 11-41 day turnaround time is possible, and this should improve as more sophisticated bioinformatic analysis pipelines are developed and understanding improves.[5] The prenatal setting is unique as timely results are required to facilitate decision making with regards continuation of pregnancy. It is anticipated that the 100,000 genome project (not primarily focused upon prenatal anomalies) and subsequent PAGE study findings will address these pitfalls and offer solutions to optimise the process. [9,19]

Ethical considerations

Primary ethical concerns with regards NGS focus around several topics:

(i) ES may not identify the primary cause for investigation and may uncover ICFs or SFs, which can have implications for both the open future of the child and for the wider family[20];

(ii) non-paternity or consanguinity may be revealed;

(iii) ownership of genetic results and the rights of the wider family to access this information[21];

(iv) NGS is an evolving technology and with time new pathogenic variants will be identified. There are challenges in relation to who is responsible for such re-analysis and reporting to the child[11];

(v) limiting diversity in society

There are consensus recommendations to aid with managing such scenarios.[2,22]

Recommendations

The American College of Medical Genetics and American College of Obstetricians and Gynecologists agree that ES may be considered when targeted testing for a specific phenotype fails to identify a cause for multiple fetal anomalies, where a genetic condition is likely and following consultation with a clinical geneticist.[23,24] A joint position statement from the *International Society of Prenatal Diagnosis, Society of Maternal Fetal Medicine and Perinatal Quality Foundation* states that the routine use of NGS as a prenatal diagnostic test cannot currently be supported until adequate validation studies are performed. Outside a

research setting, this it must only be considered on a case-by-case setting with the input of appropriate expertise [Box 2]. The statement also highlights the need for; (i) trio analysis (testing of biological parents); (ii) consideration of "panels" vs. genome wide applications; (iii) adequate pre and post-test counselling with separate consent from both parents and; (iv) appropriate laboratory quality standards.[13]

<Insert Box 2>

Non-invasive application

NGS technologies can be performed on cffDNA.[25] Thus far, this approach has been assessed for two main applications. Firstly, prenatal design of a bespoke NIPD test for a typically *de novo* (where gonadal mosaicism cannot be out-ruled) or a paternally inherited autosomal dominant variant in a previous pregnancy to assess for recurrence in an index pregnancy.[26,27] Proband (in this context, fetal) and parental DNA is required. The second application for NIPD is with use of a gene panel where a genetic syndrome is suspected, such as the case of an FGFR3 panel assessing for achondroplasia, hypochondroplasia and thanatophoric dysplasia.[28] Limitations of this approach relate to cost and the need for a fast turn-around time.[29] Technological challenges include having an appropriate quantity of fetal DNA, in addition to challenges of assessing highly polymorphic regions the presence of pseudogenes and homologous regions or maternal mosaicism.

CONCLUSION

In the presence of FSAs identified using prenatal ultrasound, the application of ES can obtain a genetic diagnosis in over 8% of cases compared to standard techniques. The diagnostic yield is optimised by the presence of multiple anomalies, involvement of genetic expertise and accurate phenotyping. This approach can be applied to both fetal tissue and more recently maternal plasma with assessment of the fetal DNA fraction. The information provided from NGS serves valuable in autonomous parental decision making with regards continuation of pregnancy, perinatal planning and counselling as to recurrence risk. Although currently primarily a research tool, NGS will likely come into routine practice in the near future. While there are challenges, which need to be addressed, with time and on-going research it is hoped than many of these may be overcome, notably in relation to turnaround time, interpretation, cost and clinical provision as part of an ethical framework.

DISCLOSURE: Authors report no conflict of interest

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FIGURE AND TABLE LEGENDS

Figure 1 and Box 1 Diagnostic precision of prenatal chromosomal/genetic testing

Figure 2 Exome sequencing output

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Figure 3 Sample workflow pattern for variant interpretation in prenatal exome sequencing.[2,6]

Box 2 International Society of Prenatal Diagnosis, Society of Maternal Fetal Medicine and Perinatal Quality Foundation recommendations of when prenatal ES may be considered[18]

TITLE: WHOLE GENOME AND EXOME SEQUENCING IN PRENATAL DIAGNOSIS

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WHOLE GENOME AND EXOME SEQUENCING IN PRENATAL DIAGNOSIS

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ABSTRACT

Advances in prenatal genomics have enabled the assessment of not only the sub-microscopic structure of chromosomes through chromosomal microarray analysis, but also the detection of pathogenic variants to the resolution of one base pair with the use of next generation sequencing. Research is emerging on the additional prenatal diagnostic yield which whole exome sequencing offers when structural fetal anomalies are detected on ultrasound examination. Primarily assessed using fetal DNA obtained by invasive techniques (amniocytes or chorionic villi), this technology is progressing into a non-invasive approach using maternal plasma. There are several challenges, to be addressed before this technology could be introduced into routine clinical practice. These are primarily technical and interpretational but also relate to service provision; cost-effectiveness; turn-around time; patient acceptability and ethical dilemmas. With adequate pre- and post-test counselling many of these challenges may be overcome. However, it is likely that this counselling will be multi-disciplinary and will involve geneticists, genetic scientists, paediatricians, perinatal pathologists and fetal medicine specialists. There is a need for obstetricians to be educated about the application, advantages and challenges of such technologies before introduction into clinical practice.

KEY WORDS: NEXT GENERATION SEQUENCING; WHOLE EXOME SEQUENCING; PRENATAL; MONOGENTIC DISORDERS; FETAL ANOMALY; PAGE STUDY

INTRODUCTION

Fetal structural anomalies anomalies (FSA) complicate up to 5% of pregnancies (depending upon the population studied). Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR), conventional karyotype and chromosomal microarray (CMA) will detect up to 40% of the underlying chromosomal aetiology associated with structural anomalies. With advances in genomic technology, theoretically, we have the ability to increase the diagnostic yield of testing through the use of Next Generation Sequencing (NGS) technologies. This technique has a resolution down to one base pair, facilitating the diagnosis of monogenic disorders (Figure 1 and Box 1). Current advances in the use of NGS in the research field means that we will soon see a shift in the use of this technology into clinical practice. Already, there is a move towards using these tests in paediatrics to assess developmental disorders with interesting data sets being reported. The potential introduction of such technology into prenatal diagnosis will require complex clinical pathway development with multi-disciplinary team working and communication.

<Insert Figure 1 and Box 1>

DISCUSSION

Approaches in Next Generation Sequencing

Next generation sequencing is typically applied in proband cases where single major or multiple FSAs are identified on prenatal ultrasound scanning and standard prenatal testing strategies (i.e. QF-PCR or CMA) identify no chromosomal anomalies.[1] Common techniques used in NGS include:

(i) Clinical or targeted exome sequencing – where specific exonic regions of interest are sequenced and a panel of genes are tested which are known to be associated with specific phenotypes;[2]

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Such techniques (primarily exome sequencing in the prenatal setting) may be performed to analyse fetal tissue or in certain specific cases, through analysis of placental cell-free fetal DNA (cffDNA) as a non-invasive prenatal diagnostic (NIPD) test using maternal plasma.[4] Testing should ideally performed as a "trio" analysis (fetus and both parents) to aid in the assignment of variant pathogenicity.[5] CMA can delineate copy number variants and mosaicism in the case of an array-comparative genomic hybridisation technique, with the addition of genotyping (uniparental disomy, loss of heterozygosity and consanguinity) in the case of a single nucleotide polymorphism array technique. NGS has the ability to combine

assessment of all of these measures in addition to the determination of single nucleotide variation.

<Insert Figure 2>

Processing and interpretation of variants

This is a complex process requiring experienced and specialist bioinformatic teams. It is one of the practical 'barriers' to introducing exome sequencing into clinical medical practice. Variant processing and interpretation involves a stepwise workflow, an example of which is demonstrated in Figure 3. Paramount to this workflow, is the input of the multidisciplinary team inclusive of clinical scientists, geneticists, bioinformaicians, genetic counsellors and fetal medicine specialists.[5] Following adequate pre-test counselling, informed consent and provision of a trio sample, DNA is extracted and library preparation using target enrichments such as Amplicon® are used to optimise coverage. WES can then be performed using a selected platform (e.g. Ilumina GAIIx®) by a massive parallel sequencing and bridge amplification technique whereby sequencing by synthesis is performed utilising fluorescently labelled bases to determine the sequence of the exonic regions.[6] Following the sequencing process, masses of data regarding base alignment is produced and this is subsequently re-aligned and annotated by means of a bioinformatics pipeline, comparing the test sequence to an updated version of the reference genome (currently GRCh37 human reference version).[2] Variants are typically then filtered and interpreted by means of an *in silico* tool or a similar in house version to determine variants predicted to affect the protein sequence or exon splicing through use of prediction programs. As yet there are no currently recognised programmes or clinical databases validated for use in the prenatal setting, hence those used in rare Mendelian disorders such as DECIPHER [https://decipher.sanger.ac.uk]

are used. Variants are evaluated or graded using criteria including characteristics such as allele frequency of <5%; functional impact i.e. if they are synonymous and non-protein altering; nucleotide conservation between species and; the finding of a variant within clinical databases that are of benign consequence and/or are not related to the phenotype. Such variants can then be excluded from the analysis and the remainder are subsequently compared to the parental genome to assess if they are *de novo* (i.e. a variant which has developed for the first time during meiosis as opposed to one which is inherited from one/both parent(s)), which would imply pathogenicity.[6,7]

<Insert Figure 3>

Variants are classed or graded using the Association of Clinical Genetic Science criteria by means of a five-class system ranging from benign or non-pathogenic, to variants of uncertain significance (VUS) to clearly pathogenic. Joint American College of Medical Genetics and Genomic and Association for Molecular Pathology standards for the interpretation of sequence variants have been formally adopted for use in United Kingdom (UK) laboratories to assist clinical scientists with classification of sequence variants identified prenatally.[7-9] Once or if a variant is identified the next step is to validate the variant, often using Sanger sequencing or an alternative technique such as multiplex ligand-dependent probe amplification in a local / regional genetics laboratory.[3]

Reporting of variants

WES may identify the presence of VUS. One of the options for parents is to receive VUS at a later time, or not at all, depending on the judgement of a multidisciplinary team. As knowledge of variant interpretation evolves, it is likely that the incidence of true VUS will reduce, as has been the demonstrated with CMA reporting.[10] WES may also unveil secondary findings (SF); variants considered to be medically actionable but unrelated to the primary indication for testing and often relating to late onset conditions such as cancer and cardiac disease. The American College of Medical Genetics and Genomics recommend that diagnostic laboratories actively look for and report on such variants in the case of 59 preselected disease-associated genes.[11] In the UK, there is no such equivalent guidance and current practice gives patients the option of 'opting out' which is determined during pre-test counselling. In the prenatal setting, such secondary findings are not typically revealed in the research setting.[12] Incidental findings (ICF) are variants, which are discovered unexpectantly and are unrelated to phenotype referred. Currently these are also not routinely reported, however again guidance is lacking.[13] Presently, in the UK, NHS England is exploring the introduction of prenatal WES before the end of 2019 and with expected pathway 'turnaround' duration of approximately 21 days.

Advantages

The primary advantage of the prenatal application of NGS is that it allows, in a proportion of cases, the revelation of a causative variant allowing an improved prognostic definition for the fetus. Parents will be able make a prospective, autonomous decision about their pregnancy and an early-prenatal identification may allow the future development of fetal

therapies.[14] A defined single gene aetiology aids in counselling relating to recurrence risk in subsequent pregnancies, opening opportunities for pre-implantation genetic diagnosis and future prenatal testing.[15] Following trio analysis it is often discovered that most pathogenic variants are *de novo* in origin, however the potential of gonadal mosaicism can not be out-ruled hence prenatal testing in subsequent pregnancies for both inherited pathogenic variants and those felt to be de novo may be offered once the pathogenic variant of the initial affected pregnancy is known. In paediatric practice, the diagnostic yield of WES (of approximately 25%) has been well established in various cohort series with congenital malformations and developmental morbidity[3]. The diagnostic yield has been demonstrated to be greatest for children with an underlying neurological phenotype or intellectual disability, with a high prevalence of pathogenic autosomal dominant *de novo* mutations.[16,17] NGS is not currently part of routine clinical practice and it is the aim of the current 'proof of principle' 100,000 genomes project in the UK to act as a bridge for NGS into mainstream clinical practice for the diagnosis of rare Mendelian disorders.[12]

Diagnostic yield

There are limited prenatal cohort studies which have assessed the diagnostic utility of WES and more research is needed before it can be applied in the routine clinical setting.[18,19] A recent review of the literature suggested a diagnostic yield over conventional chromosomal assessment, ranging from 6.2 to 80%[5].

The variation appears dependent on:

(i) the type of analysis (trio or solo proband);

(ii) clinical or whole exome sequencing;

(iii) the number, certainty of diagnosis and type of anomalies present and;

(iv) if the test was performed on a selected or unselected population.[5]

The application of 'targeted' prenatal exome sequencing has yielded promising results, notably in the area of suspected skeletal dysplasia where use of a panel of 240 known skeletal dysplasia gene mutations in appropriately phenotyped fetuses, yielded a diagnostic rate of 81% in selected cases.[4] Two of the largest prenatal studies in structurally abnormal fetuses thus far suggest a WES diagnostic yield of 7.5-8.5%.[20,21] The largest of these, the Prenatal Assessment of Genomes and Exomes (PAGE) study assessed 610 fetuses with structural anomalies on ultrasound through WES trio analysis and found it to have an additional diagnostic yield of 8.5%.[19] As noted by the smaller cohort study from the US by Wapner, *et al*. the diagnostic yield was greater where there were multiple FSAs present on ultrasound, where the yield was a high as 15%.[21] It also varied between different phenotypic sub-groups with pathogenic variants more common in fetuses with hydrops, cardiac or skeletal abnormalities but a low diagnostic yield in those with only an isolated elevated nuchal translucency and no pathogenic variants identified in the co-existence of isolated renal or thoracic abnormalities. The PAGE study unveiled several novel diagnoses and had a rate of VUS of 3.9%.[21]

Accurate and detailed phenotyping (including dysmorphological examination) has been shown to optimise the accuracy of WES, as evident from its' efficacy when testing is based upon post-mortem findings with a reported diagnostic yield of 37% in a subset of the PAGE study cohort where fetuses underwent autopsy (n=27).[22] Additionally, where prenatal cases

are selected specifically by a geneticist (highlighting a high suspicion of an underlying pathogenic variant) the yield is significantly higher, at 47% in one study (n=15).[23] Where cases are pre-selected based upon the severity of the anomaly, as opposed to the unselected population assessed in PAGE, diagnostic yields, particularly in the presence of skeletal and brain anomalies are greater.[4,23] In addition to the clinical benefit of obtaining a diagnosis, WES has the benefit of being an automated multiplex technology which can reduce bias in interpretation and in time reduce the cost of testing, although this is yet to be determined. Table 1 outlines the advantages and challenges posed by the application of prenatal WES.

Challenges

The challenges of prenatal WES are focused within four primary areas; (i) technical – there are sample considerations such as DNA quality and quantity and the presence of maternal cell contamination which must be overcome when performing WES. Tissue culturing and the use of appropriate library preparation can optimise processing but there is a risk of clonal selection.[2] WES does not analyse intronic regions or assess for epigenetic modifications and depending upon the depth of base pair coverage, all exonic regions may not be fully assessed with the risk of false negative results and a further 65% of FSAs which go unexplained (Figure 4). As the prenatal phenotype is based upon detailed ultrasonography this may be associated with poor true diagnostic potential.[2] Currently, Sanger sequencing is used to validate WES findings, which has additional resource implications; (ii) service provision – genetic services are limited and training of the multi-disciplinary team in the prenatal setting will be required before NGS can be introduced into

routine clinical practice. Counselling is challenging in a prenatal setting due to ethical considerations and the difficulty in knowing how penetrant a pathogenic variant may be or as to how severe a child may be affected; (iii) cost – the cost effectiveness of prenatal WES is not yet known although this is currently being evaluated as part of the PAGE Study collaboration.[24] In line with Moore's Law, the costs associated with NGS technologies have reduced significantly since inception and are optimised by the multiplex nature and as further understanding of the technology evolves over time. There are also significant costs related to data storage and re-interpretation[5]; (iv) turnaround time - studies have suggested that an 11-41 day turnaround time is possible, and this should improve as more sophisticated bioinformatic analysis pipelines are developed and understanding improves.[5] The prenatal setting is unique as timely results are required to facilitate decision making with regards continuation of pregnancy. It is anticipated that the 100,000 genome project (no primarily focused upon prenatal anomalies) and subsequent PAGE study findings will address these pitfalls and offer solutions to optimise the process. In addition to elucidating the relative contribution of different forms of genetic variation in prenatal FSAs and determine the cost effectiveness of prenatal WES, the PAGE study also aims to catalyse the adoption, by the National Health Service in the UK, of prenatal diagnostic sequencing through translation of acquired knowledge, rigorous health economic assessment, and establishment of an ethical social science framework for clinical implementation.[24]

<Insert Figure 4>

Ethical considerations

Primary ethical concerns with regards NGS focus around several topics, which must be the focus of counselling and consent for patients prior to undergoing WES:

(i) WES may not identify the primary cause for investigation and may uncover ICFs or SFs – revealing the findings of pathogenic variants which may lead to adult-onset disease can have implications for both the open future of the child and for the wider family[25]; Certainly in the UK PAGE study these were *á priori* not reported, unless they were on a specific predefined gene list.

(ii) non-paternity or consanguinity may be revealed;

(iii) ownership of genetic results and the rights of the wider family to access this information in the so called 'joint-account' concept[26];

(iv) NGS is an evolving technology and with time new pathogenic variants will be identified. There are challenges in relation to who is responsible for such re-analysis and reporting to the child[14];

(v) limiting diversity in society – are we on the brink of creating a selected designer population? Adequate pre and post-test counselling may ease the management of some of these dilemmas and there are consensus recommendations to aid with managing such ethical scenarios.[2,27]

<Insert Table 1>

Recommendations

The American College of Medical Genetics and American College of Obstetricians and Gynecologists agree that WES may be considered when targeted testing for a specific phenotype fails to identify a cause for multiple fetal anomalies, where a genetic condition is likely and following consultation with a clinical geneticist.[28,29] A joint position statement from the *International Society of Prenatal Diagnosis, Society of Maternal Fetal Medicine and Perinatal Quality Foundation* acknowledges the challenges which the prenatal application and prospective use of NGS may pose. This document states that the routine use of NGS as a prenatal diagnostic test cannot currently be supported until adequate validation studies are performed. While recognising that NGS is currently being performed in a research setting, outside this it must only be considered on a case-by-case setting with the input of appropriate expertise. The statement also highlights the need for; (i) trio analysis (testing of biological parents); (ii) consideration of "panels" vs. genome wide applications; (iii) adequate pre and post-test counselling with separate consent from both parents and; (iv) appropriate laboratory quality standards. Situations where WES could be considered are demonstrated in Box 2.[18] There is currently no recognised international guideline for the use of prenatal NGS in routine clinical practice and the cost-effectiveness of its' application has yet to be assessed.

<Insert Box 2>

Non-invasive application

NGS technologies can be performed on cffDNA isolated from maternal plasma.[30] Once a plasma sample is obtained, the fetal DNA fraction is quantified and assessed. Sequencing may then be performed using Amplicon® technology. Thus far, this approach has been assessed for two main applications. Firstly, prenatal design of a bespoke NIPD test for a typically *de novo* (where gonadal mosaicism cannot be out-ruled) or a paternally inherited autosomal dominant variant in a previous pregnancy to assess for recurrence in an index pregnancy.[31,32] Assessment for an autosomal recessive disorder is also feasible using a haplotype analysis approach, however the parents must have different mutations. Proband (in this context, fetal) and parental DNA is required. The second application for NIPD is with use of a gene panel where a genetic syndrome is suspected, such as the case of an FGFR3 panel assessing for achondroplasia, hypochondroplasia and thanatophoric dysplasia.[33] A non-invasive approach is favourable as it is a safe option for patients, however the focus of testing is upon obtaining appropriate validation and accreditation, which will take time. Limitations of this approach relate to cost and the need for a fast turn-around time.[34]

Additionally there are technological challenges, which include having an appropriate quantity of fetal DNA, in addition to challenges of assessing highly polymorphic regions the presence of pseudogenes and homologous regions or maternal mosaicism.

Whole genome sequencing

The primary focus of this review has been on exome sequencing, mainly because WGS has not been formally assessed in the prenatal setting. However, WGS facilitates sequencing of the entire genome inclusive of deep intronic regions, non-coding RNA and mitochondrial DNA, extending to the detection of copy number variants and structural rearrangements, hence may improve diagnostic yield and can serve as an 'all in' genetic test. Our understanding and ability to interpret intronic variants is limited, hence the current primary focus is on WES.[35] The efficacy of WGS has been assessed in the setting of rare Mendelian disease in children and suggests an additional diagnostic yield over WES of 8.7% although how this will translate into prenatal practice is as yet unknown and it is postulated that the rate of VUS may be more significant.[36]

CONCLUSION

In the presence of fetal structural anomalies identified using prenatal ultrasound, the application of WES can obtain a genetic diagnosis in over 8% of cases compared to standard techniques. The diagnostic yield is optimised by the presence of multiple anomalies, involvement of genetic expertise and accurate phenotyping. This approach can be applied to both fetal tissue and more recently maternal plasma with assessment of the fetal DNA fraction. The information provided from NGS serves valuable in autonomous parental decision making with regards continuation of pregnancy, perinatal planning and counselling as to recurrence risk. Although currently primarily a research tool, NGS will likely come into routine practice in the near future. While there are challenges, which need to be addressed, with time and on-going research it is hoped than many of these may be overcome, notably in relation to turnaround time, interpretation, cost and clinical provision as part of an ethical framework.

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FIGURE AND TABLE LEGENDS

Figure 1 and Box 1 Diagnostic precision of prenatal chromosomal/genetic testing

Figure 2 Same whole exome sequencing output Sequence output

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Figure 3 Sample workflow pattern for variant interpretation in prenatal exome sequencing.[2,6]

Figure 4 – The diagnostic yield of prenatal tests in the presence of a structural fetal anomaly

Table 1 Advantages and challenges of prenatal whole exome sequencing over standard genetic testing (MCC=maternal cell contamination; NIPD=non-invasive prenatal diagnosis)

Box 2 International Society of Prenatal Diagnosis, Society of Maternal Fetal Medicine and Perinatal Quality Foundation recommendations of when prenatal WES may be considered[18]

Box 2

Situations where WES may be considered prenatally include:

1. Fetal anomalies or a single major anomaly suggestive of a genetic disorder, but microarray is negative
2. No microarray result is available, but the fetus exhibits anomalies strongly suggestive of a single gene disorder (multidisciplinary review required)
3. Previous undiagnosed fetus (or child) from either parent, with single or multiple anomalies suspicious for a genetic syndrome that has now recurred in the current pregnancy
4. Karyotype and microarray in the current pregnancy have not yielded a diagnosis
5. If samples are unavailable from previously affected offspring or the current pregnancy, consider offering parents sequencing to determine if they may be carriers for an autosomal recessive disorder
6. History of recurrent stillbirths with negative karyotype and/or microarray, where the fetus is exhibiting a similar pattern of anomalies

Figure 1 and Box 1

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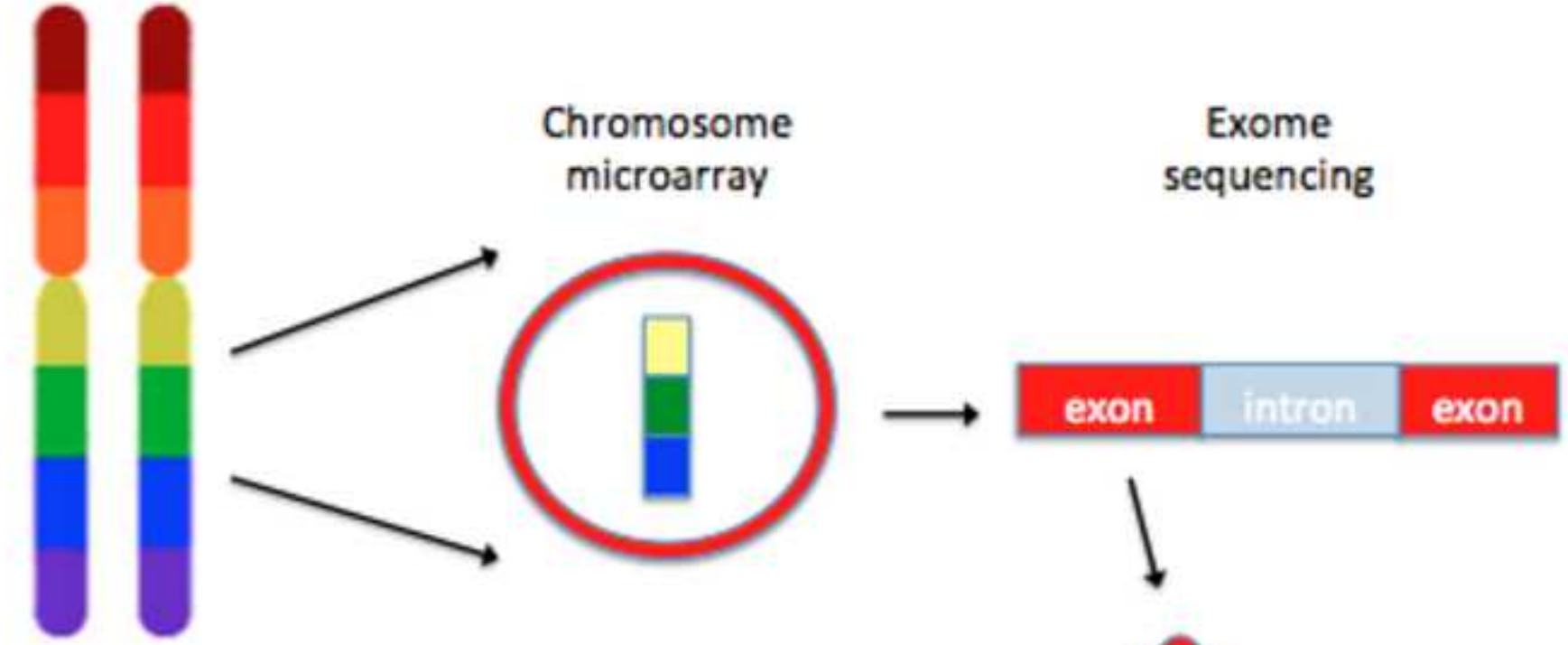

Box 1: Information to share with patients prior to prenatal whole exome sequencing

- WES will assess all exons encoding 20,000 genes and can detect a genetic fault to the precision of one base pair
- Genetic changes may be identified that are of uncertain significance and parental bloods are important to assess inheritance pattern
- If a mutation is detected in the prenatal setting the penetrance or severity to which the disease is expressed can not be predicted
- WES may not unveil a diagnosis

Figure 2

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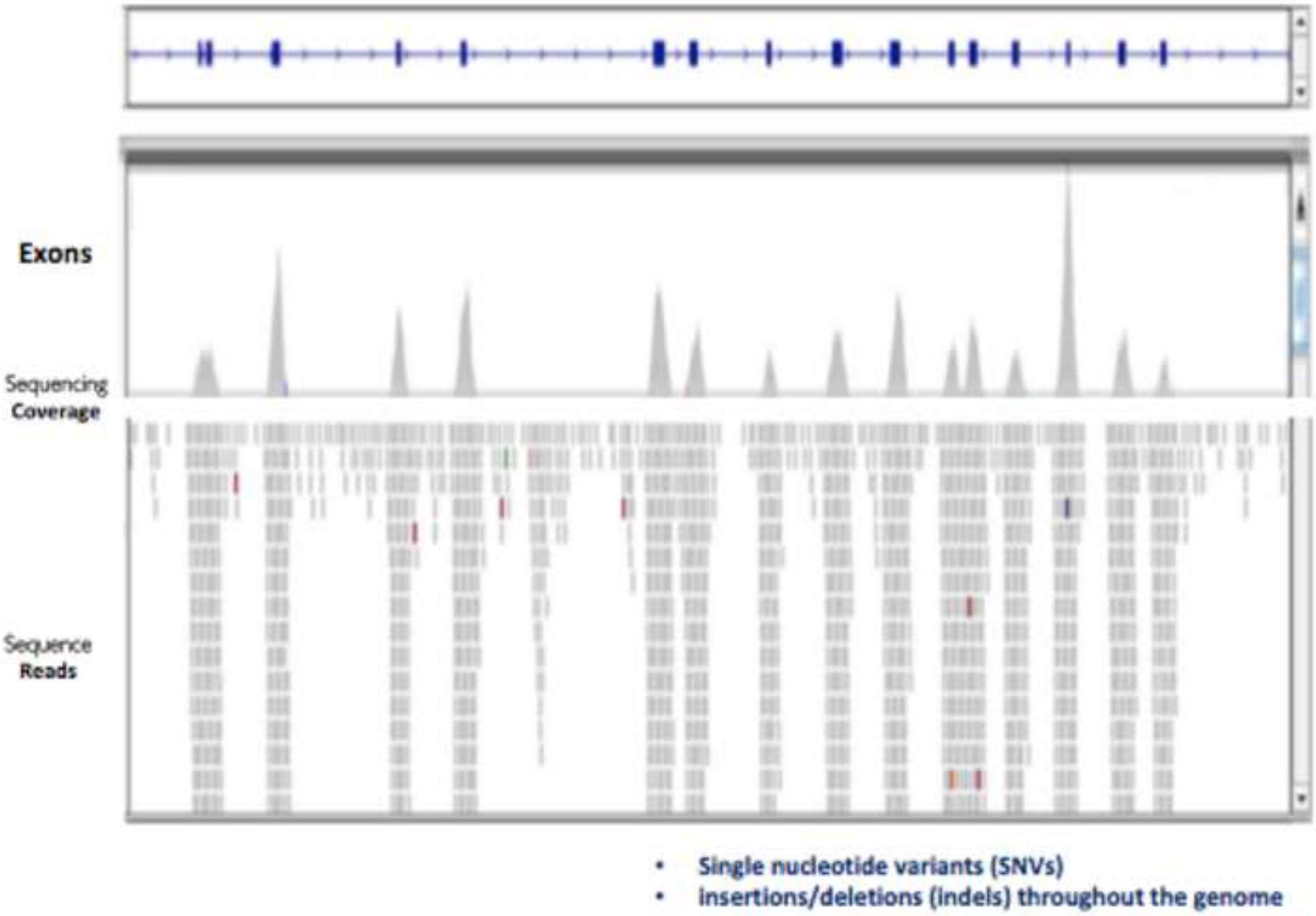

Figure 3

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