Exome sequencing in the assessment of congenital malformations in the fetus and neonate

Mone, Fionnuala; Quinlan-Jones, Elizabeth; Ewer, Andrew; Kilby, Mark

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AUTHORS: Fionnuala MONE,[1,2] Elizabeth QUINLAN JONES,[1,2] Andrew K EWER[3,4], Mark D KILBY[2,4*]

AFFILIATIONS:

1. Department of Clinical Genetics, Birmingham Women's & Children's NHS Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham, B15 2TG, UK
2. West Midlands Fetal Medicine Centre, Birmingham Women's & Children's NHS Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham, UK.
3. Neonatal Unit, Birmingham Women's & Children's NHS Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham, UK.
4. Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK

***CORRESPONDING AUTHOR:** Professor Mark D. Kilby, West Midlands Fetal Medicine Centre, Birmingham Women's & Children's NHS Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham, B15 2TG, UK. E: m.d.kilby@bham.ac.uk T: +44 (0)121 627 2778

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What is known about this topic?

- Exome sequencing increases the diagnostic yield over and above standard investigations in the assessment of congenital anomalies but is not yet routinely performed in clinical practice.
- There is a paucity of guidance for clinicians and an urgent need to identify the clinical application of exome sequencing and the appropriate pre- and post-test counselling in the perinatal setting.

What this study adds:

- Provides information about the potential clinical utility of exome sequencing in the perinatal setting
- The prenatal and early postnatal application of exome sequencing are explored

ABSTRACT

Major congenital anomalies are often associated with perinatal mortality, long-term morbidity and prolonged hospitalisation. Prenatal ultrasound remains the principle diagnostic test for many anomalies but despite this up to one third are only identified in the neonatal period. The primary step in determining underlying aetiology is to define accurately the phenotype by recognition of dysmorphology (both prenatally and postnatally). The potential introduction of Next Generation Sequencing, primarily through exome sequencing into perinatal practice may improve the pathologic diagnostic yield. However, clinicians must understand both the benefit and potential harms of this technology in facilitating the discovery of relevant pathogenic variants in the diagnosis and management of congenital malformations.

INTRODUCTION

Congenital structural anomalies complicate ~2% of pregnancies (20 per 1000 live births) but are responsible for 13% of neonatal intensive care unit (NICU) admissions and up to a third of neonatal deaths.[1] Although overall neonatal mortality has halved in the last decade, mortality rates due to congenital anomalies remain unchanged.[1] Aneuploidy and copy number variation (detected using G-banding karyotype and chromosome microarray analysis (CMA)) are detected in up to 40% of pregnancies with malformations.[2] In approximately 60% of malformations the underlying aetiology is unresolved with a proportion of cases being the result of monogenic disorders.[3] Careful prenatal imaging is vital in the detection and classification of fetal structural anomalies. It is important to establish whether an anomaly is isolated or if there are multiple abnormalities, as is the subsequent classification into malformations, deformations and disruptions. This is helpful in formulating a clinical risk of a monogenic aetiology and aids the selection of further investigations. Traditionally, genomic testing (either pre- or postnatally) has been based upon the use of 'targeted' gene tests and has been limited by incomplete phenotypic information and false negative diagnosis if a variant gene is not represented in the selected panel of tests. Through the application of genomic databases cataloguing prenatal findings with confirmatory postnatal diagnosis to complement the results of next generation sequencing (NGS), perinatal prognostic information for the purpose of counselling will improve.

CURRENT PERINATAL ANOMALY INVESTIGATION

A primary malformation is a structural defect in an organ that can be traced back to its embryological development, whilst a secondary malformation is interruption of the normal development of an organ following external influences.[2] This review focuses upon those malformations which result from a genetic aetiology. The presence of single or multiple anomalies, identified by systematic prenatal imaging and postnatal examination,[2,4] is associated with a genetic or chromosomal aetiology in up to 40% of cases, which, if diagnosed prenatally, can aid in counselling with regard to long-term prognosis by a multi-disciplinary team.[2]

Review by a clinical geneticist either prenatally or postnatally, who after family pedigree analysis and clinical examination may instigate targeted gene testing, which typically involves serial sequencing of single genes or gene panels to explore a potential molecular genetic diagnosis. This is time consuming, relying on a narrow differential diagnosis, and choosing a specific test to identify a pathologic variant. The examination of the whole exome (ES) and genome (WGS) by NGS may be a potentially valuable tool in both prenatal and postnatal investigation of a unifying molecular diagnosis.

NEXT GENERATION SEQUENCING

NGS can interrogate the human genome down to the level of one base pair through either; (i) ES assessing all 20,000 gene coding regions (responsible for 85% of disease-causing variants), or by; (ii) WGS assessing the entire genome including introns, non-coding RNA and mitochondrial DNA in addition to assessment of copy number variation and structural rearrangements.[5] In perinatology, there is growing evidence from several 'proof of concept' studies (e.g. Prenatal Assessment of Genomes and Exomes (PAGE) study and the UK Nationwide 100,000 Genomes Project) suggesting that there is a significant additional molecular diagnostic rate through introducing NGS into mainstream clinical practice while simultaneously serving as translational studies with a proposed up and running framework for ES.[6,7] It is anticipated that through NGS, the rate of diagnosis of monogenic disorders presenting with congenital anomalies will increase, as will our understanding as to why such anomalies arise during development, bringing us a step further to possible prevention.[8]

Prenatal next generation sequencing

The PAGE study is the largest prospective, prenatal study to date (*Lancet 2019, In press*), assessing the clinical utility of ES in investigation of the malformed fetus. So far, 610 trios (fetus and both parents) have been analysed in cases where fetal structural anomalies have been identified using ultrasound and where autosomal/sex aneuploidy and large copy number variants have been excluded.[6] This prospective study demonstrated that prenatal ES provides up to a 8.5% additional diagnostic yield of pathological variants when compared to conventional genetic testing.[6] Another smaller prospective prenatal series conducted by Columbia University *(Lancet 2019, In press)* demonstrated similar findings with a diagnostic yield of 10.3% (n=234 trios).[9] The differences in detection rates between studies may have been secondary to variation in interpretational approaches; with the PAGE study utilising a

virtual panel of 1,628 genes (from the Deciphering Developmental Disorders study) [10] and the Columbia study including all genes. This resulted in 0.42 variants requiring manual interpretation per case in PAGE versus 4.8 variants per case in the Columbia Study, demonstrating the challenge of balancing increased interpretational burden with increased sensitivity. The PAGE study also noted that the pathologic variant rate varied according to the anatomical anomaly identified and whether these were isolated or multiple [Figure 1]. A relatively low rate (4%) of variants of uncertain significance (VUS) was described.[6] Only if the 'variant' was considered causative was it fed back to parents after the end of the pregnancy. The PAGE study is unique as it assessed the application of ES in a relatively unselected population (as opposed to ES being performed following clinical genetic consultation) and the cohort included a heterogeneous mix of congenital anomalies (from increased nuchal translucency (>4mm) to multiple structural anomalies). The relatively low diagnostic yield in the PAGE study is at variance with paediatric series where complex investigation and phenotyping is feasible as opposed to reliance upon relatively subjective prenatal ultrasound findings.[11] The use of trio (parental) analysis as opposed to proband (fetus) only, enriched the variant interpretation process and the study unmasked the challenges posed by ethical issues such as identification and uncertainty of VUS, and the importance of informed consent and parental counselling (both pre- and post-test). In addition, this study will elucidate the contribution of different forms of genetic variation in prenatal structural anomalies and determine the cost-effectiveness of prenatal WES potentially catalysing the clinical adoption of this technology by in the UK.[12]

Next generation sequencing in the critically ill neonate

Exome sequencing is more established in the postnatal setting. This is demonstrated by the significant increase in the number of monogenic disorders which are now identifiable in the

new-born period.[1] Many genetic conditions present within the first 28-days of life, often resulting in a critically ill neonate where the cause is not clearly identifiable using standard investigations.[3] Onset progression of such monogenic disorders tends to be rapid in neonates and there is insufficient time for serial screening of a selection of the thousands of known single gene disorders using standard methods. Additionally, variable phenotypes and neonates which may 'grow into' their diagnoses mean that assessment is challenging and phenotyping may not clearly identify the primary pattern of disease. Hence it is unsurprising that current research is focusing on the feasibility of introducing rapid NGS technologies into the NICU setting.[13] When applied to critically ill infants in the NICU and intensive care setting, NGS results can be obtained in 50 hours,[13] achieving a molecular diagnosis in up to 37% of subjects and subsequently affecting clinical decision making (i.e. redirecting care, considering new subspecialist care, and medication/dietary modifications) in over half of cases.[3] Future introduction of NGS has the potential to be highly cost-effective by reducing mortality and length of hospital stay as well as bypassing the prolonged course of investigation such neonates would likely face through childhood.[13]

In older infants and children, NGS has been shown to be beneficial in the understanding and management of congenital diarrhoeas and enteropathies[14] as well as those with unexplained seizures and neurodevelopmental delay, where a preponderance for autosomal dominant *de novo* pathogenic variants is commonly seen, which can be discovered using ES.[15] There have been several prospective large scale studies assessing the clinical utility of ES in children with suspected monogenic disorders, in whom the definitive diagnosis is often significantly delayed. The yield in this group is between 25-52% dependent on the group assessed, being greater in the dysmorphic child with a suspected heterogeneous disorder or overlapping disorders as opposed to one with an isolated intellectual disability.[3,16] Once

again, ES has been shown to lead to a significant alteration in management and improved long-term outcomes, in addition to offering prenatal genetic diagnosis in subsequent pregnancies once a mutation has been identified.[3,13,17] Such studies support referral by paediatricians to a genetic service offering NGS early in the diagnostic stage. Appropriate counselling is vital to inform parents of the potential to uncover a pathogenic variant through future re-analysis. This has been demonstrated by the Deciphering Developmental Disorders research consortium where, through contemporary re-analysis of 1,333 trios in children with an undiagnosed developmental disorder, by applying updated bioinformatics pipelines and variant calling systems, the diagnostic yield increased over time from from 27% up to to 40%.[10]

In relation to WGS in children with suspected disease of Mendelian inheritance, an additional diagnostic yield compared to ES alone of 8.7% has been proposed, although potential pitfalls include challenges with variant interpretation.[18] The main advantage of WGS is it's potential use as an 'all-in-one' genetic test combining copy number variation, structural rearrangements and single base pair changes, in addition to assessment of intronic and epigenetic regions.

Next generation sequencing in perinatal autopsy

NGS also has the potential to extend the clinical perinatal autopsy examination, for example through use of ES in infants with suspected sudden death due primarily to cardiac arrhythmias.[19] There are limited studies which have assessed use of NGS use in the perinatal post-mortem setting, although the scope for added genetic variant diagnostic yield is potentially huge.[20] In a sub-cohort of the PAGE study 27 trios involving fetuses with

significant congenital anomalies identified prenatally that had undergone autopsy were retrospectively assessed. In this cohort, WES provided an additional diagnostic yield of pathogenic variants in 37% of cases compared with standard investigation [Figure 1].[20] The possible reason for a higher diagnostic yield in fetuses undergoing post-mortem examination was secondary to improved identification of anomalies (including subtle dysmorphology) and a trend to a larger proportion of probands with multiple anomalies. As we become increasingly aware of the strong association of single gene disorders with congenital anomalies and perinatal death, it is vital that even where NGS is not currently offered, snap frozen samples of fetal tissue should be obtained so that DNA is available for future analysis.[21] However, extracting high quality DNA is less successful from post-mortem tissue than that from living tissue.[22] The added clinical utility of WGS has yet to be assessed in perinatal post-mortem.[12]

Clinical considerations with Next Generation Sequencing

1. Multidisciplinary team - The potential benefit of NGS in both the pre- and post-natal setting, highlights the need for comprehensive perinatal MDTs, incorporating clinical pathologists, genomic scientists, geneticists, neonatologists and fetal medicine sub-specialists to assist with variant interpretation and pre and post-test counselling.[23]

2. Pre- and post-test counselling – Parents must be given accurate information before deciding on proceeding with NGS testing.[23] They must be aware that testing is optional and that a clear, definitive diagnosis may not obtained and test turnaround time (TAT) can be of variable duration.[23] It is important that parents understand the challenges and complexity of variant interpretation and the levels of potential uncertainty, which may arise from this process. The possibility of secondary or incidental findings must also be discussed in addition to potential ethical issues that may arise. In the fetus and neonate this is most notable as there is the potential for unveiling a significant secondary results such as an

increased risk of subsequent adult-onset disease. In the PAGE study such 'variants' were not screened for but a more liberal bioinformatic screening process could identify such anomalies. A further challenge is to explain that the field of NGS is evolving, as are the pathologic variant lists (if utilised). As these variant lists are updated over time with the addition of new pathologic variants (and their associated phenotypes) identified, a proband (fetus/newborn) in whom no variant was found on initial testing may have a significant finding identified on future re-testing.[23] Therefore detailed informed, written consent should be obtained prior to testing.[24] For parents making decisions about pregnancy continuation, the level of potential uncertainty posed by ES is challenging as one is attempting to predict the phenotype of a child which has not yet been born and parents are expected to make autonomous decisions based upon this information. Despite the complex and sometimes non-definitive nature of the information to be relayed the health profession must not be tempted adopt a paternalistic approach and must fully inform patients to obtain consent prior to testing.[23] Studies assessing the views of health care professionals regarding ES have unveiled concerns from, notably Obstetricians who felt that "next generation testing could lead to increased levels of parental anxiety".[25] This again highlights the need for education of all health care professionals and the need for a multidisciplinary team approach to counselling.[25] As noted by the PAGE study,[6] before ES is rolled out into clinical care, there needs to be a consensus on the development of a good model of ethical practice exploring parental expectations, counselling and professional duties.[5]

3. Accurate phenotyping - Accurate variant interpretation requires detailed phenotyping with classification using systems such as Human Phenotype Ontology.[21] This allows targeted enrichment or application of a clinical exome panel to be selected to optimise coverage of areas of the genome known to be associated with system anomaly subtypes. The clinical exome panel provides a more efficient approach than exome sequencing, increasing the need for accurate phenotyping. A multi-disciplinary approach is required to obtain a so-

called *'deep phenotype'* prior to consideration of performing NGS. Additionally the application of clinical variant databases (although not specific to the fetus) such as *Clinvar* and DECIPHER may aid in phenotype matching and variant interpretation, and similarly any novel variants discovered should be reported to such database consortiums in addition to phenotype data. [6,23,26] Instances where phenotyping has been adequately obtained has been shown to optimise the diagnostic yield from ES, notably in cases of prenatally suspected skeletal dysplasias or in perinatal autopsy.[20,27]

4. Recommendations – Recent guidance from the American College of Obstetricians and Gynaecologists and a Joint Statement from International Society of Prenatal Diagnosis, Society of Maternal Fetal Medicine and Perinatal Quality Foundation does not recommend routine use of ES in the prenatal setting, but acknowledges that there are circumstances where it may be considered following liaison with an expert.[28,29] A recent publication from the UK Nuffield Council on Bioethics acknowledged the potential benefit of using NGS to improve the care and treatment of seriously ill babies but warned against the its use in widespread screening in otherwise healthy babies.[30]

Technical considerations with Next Generation Sequencing

A sample pathway for performing NGS is demonstrated in Figure 2. While a detailed technical description of the process of NGS is beyond the scope of this review it is based upon the principle of massive parallel sequencing with subsequent alignment to the human reference genome and identification of variants.[5] There are several challenges with regards clinical implementation of NGS which are considered below:

1. Trio analysis – In order to determine heritability and to aid in variant interpretation and reporting, It is recommended that DNA is obtained not only from the affected proband but

also from both biological parents, as has been the case in the majority of NGS studies assessing for single gene disorders.[6-8] The majority of mutations identified in congenital anomalies are of an autosomal dominant *de novo* nature, hence parental samples are important in defining this inheritance pattern.[23] There is emerging evidence that in the absence of sufficient proband DNA, where a lethal autosomal recessive disorder is suspected, sequencing of parental samples can still be performed to diagnose single gene disorders.[31]

2. Variant interpretation – Following massive parallel sequencing of the DNA, bioinformatic pipelines are required to align and annotate reads and interpret variants graded using a five class system raging from benign to pathogenic. Most notable in the prenatal setting, variant interpretation is the most time-consuming and costly stage of the ES process as there are currently no variant databases specific to the fetus to aid interpretation, nor are there recognised guidelines. Interpretation relies upon the skill mix and experience of the multidisciplinary clinical review panel which are vital, to form a consensus in relation to variant pathogenicity and reporting. Investigation and decision-making with regards variant grading can be cumbersome, requiring an adjunctive literature review and research for each potentially pathogenic variant. [25,28,32,33]

3. Validation – Current practice and the majority of NGS research studies validate pathogenic variants using Sanger sequencing or an alternative technique.[34]

4. Turn-around-time – In the prenatal setting particularly, fast TAT is vital so that couples can make autonomous decisions about pregnancy management. Since the advent of NGS, the TAT has traditionally been protracted and in the PAGE study pathogenic variants were only fed back post-natally.[6] Recent studies utilising ES in the prenatal setting have reported much faster TAT of two to three weeks.[23,35] With the application of more accurate

phenotyping and targeted bioinformatics pipelines it is likely that as experience with prenatal NGS increases, TAT will reduce significantly. [23,25]

Benefits and Risks of Next Generation Sequencing

The risks benefits of the perinatal application of NGS are summarised in Table 1.

Future considerations

There is a significant amount of the human genome, that we do not yet fully understand, and much of the pathology underpinning congenital anomalies are suspected to lie within the intronic regions of the genome, where regulators such as the transcriptome and methylome control gene expression.[36] While WGS may go some way in assessing genetic variation in these regions, clinical studies thus far demonstrate a limited additional benefit over ES.[18] Figure 3 demonstrates the proposed the pathological weight of causes of congenital anomalies and what is yet to be discovered. The future of perinatology will likely see a move toward a WGS approach with the use of non-invasive analysis of free-fetal DNA in the maternal circulation or NGS performed on parental samples only, without the need for proband DNA.[36]

CONCLUSION

Next generation sequencing is proving to be a valuable diagnostic tool in the setting of perinatal congenital anomaly and suspected genetic disease. The data produced from prospective studies assessing its utility are reproducible and are a major step in relation to translation of NGS into routine perinatal clinical practice in order to obtain a unifying molecular diagnosis. Clinical, technical and ethical concerns must be thoroughly addressed through clinical guidelines before NGS is introduced into the perinatal setting. One must acknowledge the blind spots, which ES alone can leave in relation to detection of copy number variants and balanced chromosomal anomalies and consider combination testing with existing methods or eventual transition into WGS. Accurate phenotyping of congenital anomalies from the outset with appropriate target enrichment or clinical exome selection is the most vital stepping-stone to establishing a diagnosis. Through the application of NGS we can not only diagnose but also underpin the underlying aetiology of congenital anomalies, which will facilitate the development of future treatments and preventative therapies.

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Advantages	Challenges
Additional diagnostic yield Ends 'diagnostic odyssey'	Technical • Coverage – Incomplete exon capture, GC rich regions • Sample processing (MCC and DNA quality/quantity) • Does not assess whole genome • Validation with Sanger sequencing • Accurate phenotyping
Provides information on prognosis	Service provision • Pre and post-test counselling • Education of workforce • Need for increased resources
Gene discovery	Interpretational • Variant interpretation • Bioinformatic pipelines • Variants of unknown significance • Secondary or incidental findings • False negatives
Facilitates counselling for recurrence risk	Ethical • Non paternity or consanguinity • Parental expectations • Limiting child's *'open future'* • Data ownership • Implications for wider family • Reanalysis and reporting • Diversity in society
Automated	High cost
Multiplexed	Turnaround time
Can be extended to NIPD	Lack of international guidelines

Table 1 – Advantages and challenges of next generation sequencing

FIGURE LEGENDS

1. Percentage diagnostic yield of exome sequencing pre- and postnatally per system
2. Pathway for next generation sequencing
3. Proportion of anomalies diagnosed using existing genomic technologies (CMA = Chromosome microarray; ES = Exome sequencing; WGS = Whole genome sequencing)