Prenatal genomic testing for ultrasound detected fetal structural anomalies

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Review

Prenatal genomic testing for ultrasound-detected fetal structural anomalies

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Key content

- In the presence of a fetal structural anomaly, fetal DNA can be obtained through invasive testing (e.g. amniocentesis and chorionic villus sampling) in order to undertake genomic testing to attempt to uncover a unifying genetic diagnosis.
- There are number of traditional and more novel genomic tests available, which can identify aneuploidy, chromosomal structural variation and/or sequence variants within genes.
- The cumulative diagnostic yield of such technologies is approximately 25%, 6% and up to 80% in some cohorts for QF-PCR/G-banding karyotype, chromosome microarray and exome sequencing, respectively.

Learning objectives

- To understand the technical basis and clinical indications for QF-PCR, G-banding karyotype, chromosome microarray and exome sequencing.
- To appreciate the potential benefits and challenges associated with exome sequencing.
- To gain awareness of modern technologies that may be utilised to address recurrence risk, e.g. preimplantation genetic diagnosis and non-invasive prenatal diagnosis.

Ethical issues

- Not all technologies are currently available across all four nations of the UK, hence challenges are raised regarding healthcare equity.
- There can be uncertainty around the interpretation of prenatal genomic test results, which can have implications in counselling, particularly regarding termination of pregnancy.
- Incidental findings may be revealed, which can have implications for counselling and the future health of the fetus and the parents.

Keywords: chromosome microarray / exome sequencing / fetal structural anomaly / fetus / quantitative fluorescence polymerase chain reaction

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Introduction

Fetal structural anomalies (FSAs) affect between 3% and 5% of pregnancies. Detectable on prenatal ultrasound, they can range from a single minor defect to multisystem anomalies. Almost half of all FSAs are proposed to have a genetic or chromosomal aetiology with associated increased risks of neonatal morbidity and mortality.[1,2] Such underlying genomic differences include abnormal chromosomal number (aneuploidy), differences in chromosomal structure (e.g. translocations, inversions, micro-duplications, micro-deletions), a single nucleotide variant and epigenetic variations affecting gene expression (e.g. methylation abnormalities).[3] Various laboratory-based genomic strategies can be used to establish a genetic diagnosis that explains the sequence of FSAs identified and therefore aid in counselling regarding the prognosis and clinical pathway for such fetuses/future children.[1] Up to 40% of the underlying chromosomal or genetic problems associated with FSAs can be identified from quantitative fluorescence-polymerase chain reaction (QF-PCR), G-banding karyotype and chromosomal microarray (CMA).[4] With the evolution of

genomic technologies since the 1960's (Figure 1) and the advent of next generation sequencing (NGS), it is now possible to interrogate the genome to the level of a single nucleotide. While this offers benefits in prenatal diagnosis, it brings challenges in terms of counselling and ethical considerations for families. This review will discuss on the different methods of prenatal genomic testing and propose a potential pathway for such families while discussing the ethical and future implications for prenatal testing.

Sources of fetal DNA

Prenatal determination of the fetal phenotype is limited because it relies on high resolution 2D-ultrasound, 3D/4D ultrasound and magnetic resonance imaging (MRI), and it is not always possible to obtain a deep phenotype (a detailed assessment of fetal structural anatomy and, where possible, dysmorphology).[2,4] If there are abnormal ultrasound findings, invasive testing may be offered in an effort to retrieve and test fetal DNA to establish a more definitive diagnosis and therefore accurately inform and counsel parents.[5] The principal procedures to retrieve samples for analysis are amniocentesis and chorionic villous sampling (CVS). These involve culture and assessment of amniocytes from amniotic fluid or chorionic villi from the placenta and have an associated risk of miscarriage of 0.5% (1 in 200).[6] Maternal cell contamination has a prevalence of 1–2% in invasive testing strategies[6-8] and must be excluded prior to reporting results of tests.[7]

Sampling of fetal blood via cordocentesis, which may be performed during fetocide prior to termination of pregnancy, or obtaining a pleural fluid sample via thoracocentesis may also yield fetal DNA.[9] Once DNA is isolated, the initial test routinely carried out is QF-PCR (replacing fluorescence in situ hybridization [FISH]), moving on to karyotype or CMA typically following cell culture. If QF-PCR reveals aneuploidy of autosomes 13, 18, or 21 or the sex chromosomes, G-banding karyotype should then be performed to rule out an inherited structural chromosomal rearrangement as the primary cause. Otherwise, where the QF-PCR is negative, the second-tier test in the presence of a FSA is CMA. NGS, typically via trio exome sequencing (ES), may be then be used as the third test of choice dependent on whether criteria are met.[10]

Fetuses with a structural anomaly are more likely to die in the perinatal period. When prior invasive testing has not been performed in the case of a neonatal death, umbilical cord blood can be retrieved at delivery or a fresh frozen skin, liver, spleen (most commonly) or placental biopsy (fetal side) at post-mortem can be retrieved following parental consent. At earlier gestations this may be challenging and it is important for clinicians to be aware that should they require DNA extraction, specimens should not be fixed in formalin as this impacts on the performance of genomic testing.[10] When proband DNA has not been obtained following perinatal demise, and fetal phenotypic information has been retrieved, an alternative testing method of 'molecular autopsy by proxy' may be considered. This is where genomic testing (predominantly via NGS) can be performed on biparental blood samples without the need for proband DNA.[11]

Genomic testing strategies

Quantitative fluorescent polymerase chain reaction

QF-PCR is a molecular genetic technique whereby markers specific to certain regions of the chromosomes are amplified using DNA polymerase, labelled with fluorescent tags and

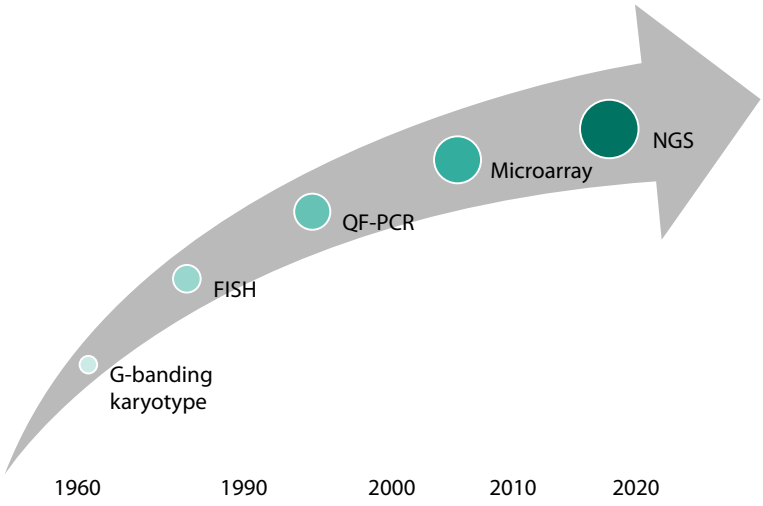

Figure 1. Evolution of prenatal genomic screening and diagnostics. FISH = fluorescence in situ hybridization; NGS = next-generation sequencing; QF-PCR = quantitative fluorescence-polymerase chain reaction.

then separated and quantified using capillary electrophoresis (a technique used to separate DNA fragments dependent upon size and electrical charge). Similar to FISH, QF-PCR is typically used to identify the most common aneuploidies, involving autosomes 13, 18 and 21 and the sex chromosomes. QF-PCR is less labour intensive, has higher throughput and is considered to be more cost-effective than FISH. However, it requires a skilled workforce and a well-established infrastructure, consisting of all necessary equipment and computer systems to acquire valid results.[12,13] The average turnaround time (TAT) is 48 hours. If an abnormality is detected on QF-PCR this is followed up with a karyotype to check whether the result is due to 'free trisomy' (a sporadic as opposed to an inherited event) or an unbalanced translocation. This establishes the origin of the trisomy abnormality and can have bearing on counselling regarding recurrence risk. QF-PCR results are depicted as peaks on an electropherogram, as demonstrated in Figure 2.[14]

G-banding karyotype

Karyotyping is a cytogenetic technique that has been in practice since the 1960s. It utilises Giemsa staining to visualise the number of chromosomes and their macro-structure.[15] Karyotype remains the gold standard for detecting mosaicism, polyploidy and rearrangements (e.g., translocations and inversions), which may be missed through other genetic testing modalities such as comparative genomic hybridisation with CMA. However, karyotyping is labour intensive and requires significant skill, and the TAT is typically ten days for urgent samples. Furthermore, the resolution to detect microdeletions and duplications of the chromosomes is relatively poor, at around 5 million base pairs, meaning that some relatively large microdeletions and microduplications may be missed. An example of a G-banding karyotype is shown in Figure 3.[1]

Chromosome microarray

A CMA, also referred to as array CGH (comparative genomic hybridisation), has been used in clinical genetics for three decades, however it has only been widely employed in the prenatal setting since 2010.[16] The underlying principle of DNA microarray is that oligonucleotide probes (short strands of synthetic DNA), which are complementary to sequences across the human genome, are annealed to a solid surface chip. The proband (fetal) DNA is fragmented and labelled with a fluorescent dye. When complementary sequences match up (hybridise) this releases a signal that can be captured through fluorescent microscopy. Oligonucleotide arrays have largely been superseded by single nucleotide polymorphism (SNP) arrays. In an SNP array, each probe is located at the site of a known polymorphism (a single base that commonly varies between individuals). Comparatively, SNP arrays can detect microdeletions and duplications at a higher resolution (typically 50–250 kb, depending on the genomic region), and they may also detect 'loss of heterozygosity'. Loss of heterozygosity suggests that the two copies of this locus are more similar than would be expected, for example in cases of consanguinity or uniparental isodisomy. While CMA can detect aneuploidies, microdeletions and duplications, it cannot detect balanced translocations and may miss small intragenic deletions or duplications. The average TAT of an urgent CMA is 10 to 14 days. If a copy number variant (CNV) is detected, parental DNA samples may be required to assist interpretation. A CNV may be interpreted as benign (normal variation), of uncertain significance or as

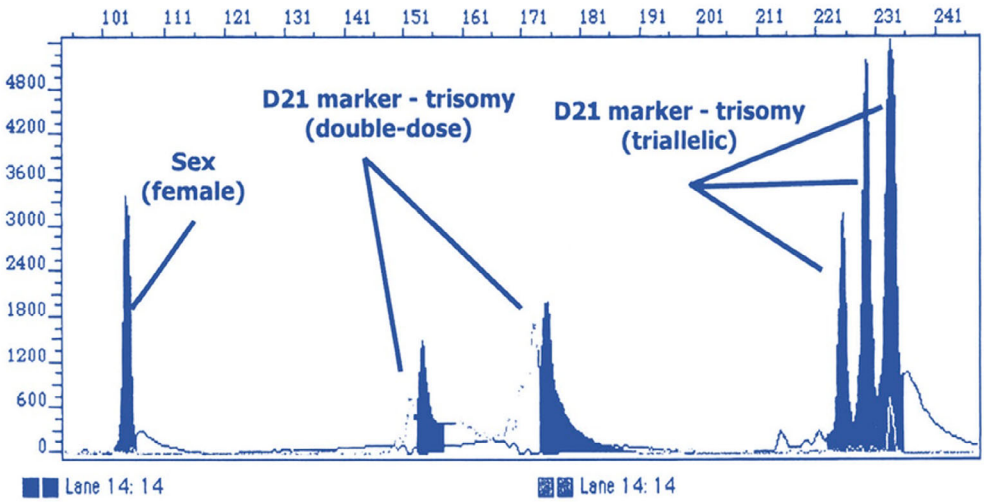

Figure 2. Electropherogram depicting Trisomy 21 in a female fetus with a 2:1 signal peak and triallelic 1:1:1 signal peak. Image reproduced from Findlay et al.[15] with permission from Springer Nature.

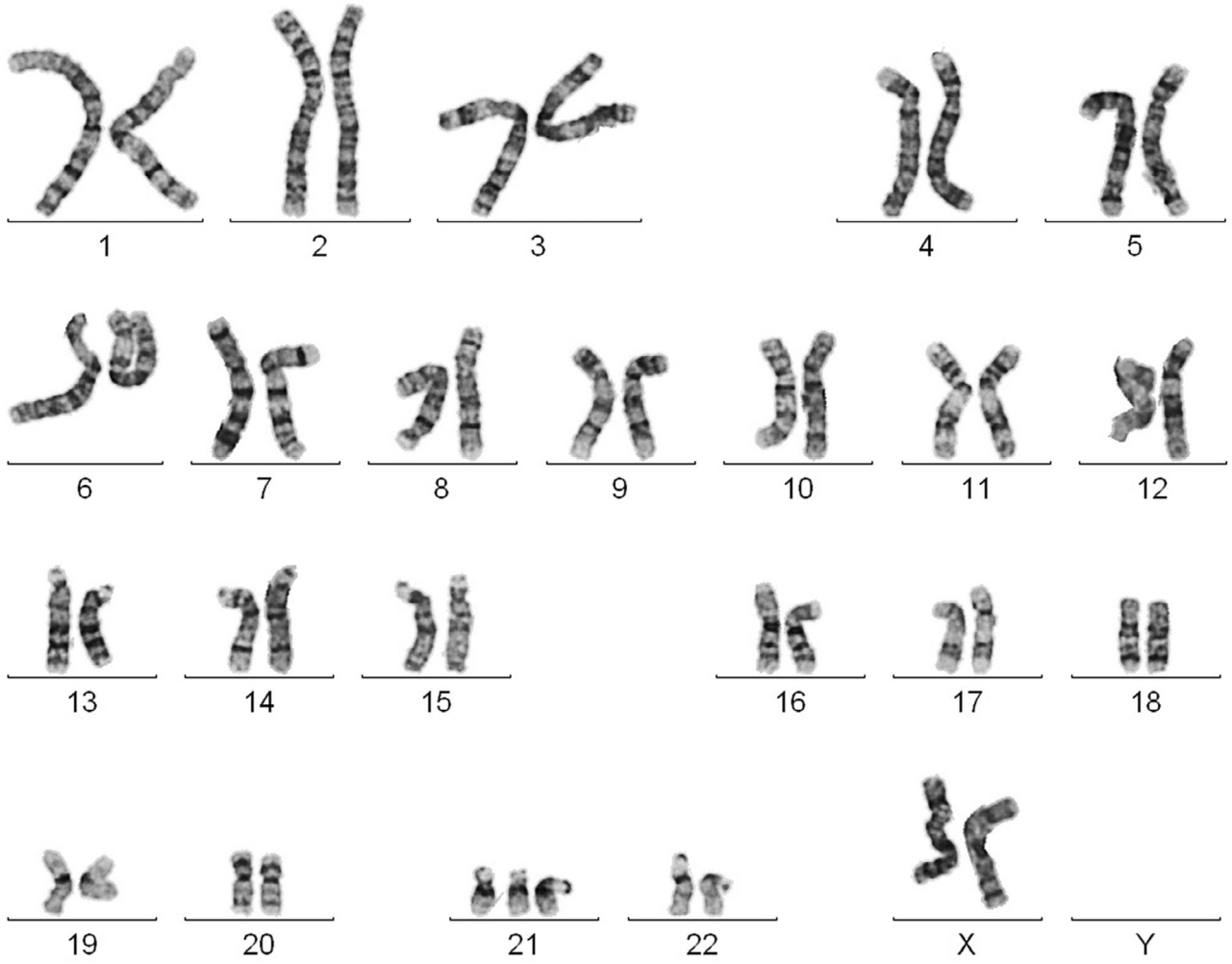

Figure 3. G-banding karyotype depicting Trisomy 21 in a female fetus. Reproduced with permission from Cambridge University Press.[1]

pathogenic, i.e., disease causing.[17] This may be inherited from either parent or not (i.e., *de novo*). Figure 4 depicts the steps involved in a CMA.

In prenatal cohorts with FSAs, CMA has a diagnostic yield of 3.5–6% over and above QF-PCR/karyotype.[18] The most common pathogenic CNV detected on prenatal CMA is 22q11.2 microdeletion (DiGeorge syndrome). At present, UK guidelines from the Royal College of Obstetricians and Gynaecologists (RCOG) and the Joint Committee on Genomics in Medicine suggest that CMA is indicated for any fetus with a normal QF-PCR, and one or more of the following: (i) isolated nuchal translucency ≥3.5 mm when the crown-rump length measures from 45 mm to 84 mm (at approximately 11^{+0} weeks to 13^{+6} weeks); (ii) one or more FSAs identified on ultrasound scan; or (iii) fetuses with a sex chromosome aneuploidy that is unlikely to explain the ultrasound anomaly (e.g. XXX, XXY and XYY).[20] As well as recognised, highly penetrant CNVs, CMA may also detect CNVs that are less well understood or entirely unrelated to the phenotype of the fetus ('incidental findings'). The UK joint guidelines on CMA in pregnancy provide recommendations both on which variants should always be reported (e.g. "high penetrance neuro-susceptibility loci that are associated with a risk of a severe phenotype"), and those which should not. In general, reporting is not recommended for CNVs that are "not linked to potential phenotypes for the pregnancy (future child) in question or [has] no clinically actionable consequence for that child or family in the future. Reporting is also not recommended for low penetrance neuro-susceptibility loci and unsolicited pathogenic variants for which there is no available intervention".[19] Examples of these include 15q11 microdeletion and 16p13 duplication, which are low penetrance CNVs primarily associated with susceptibility to neurodevelopmental delay.

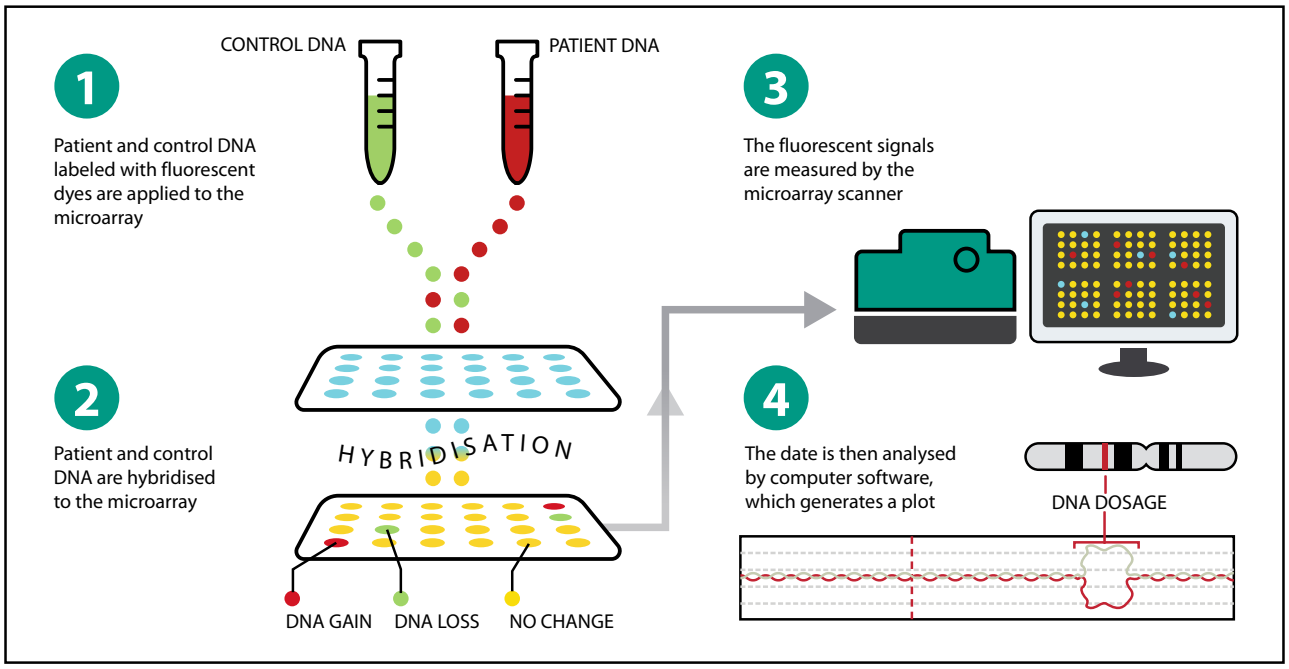

Figure 4. Steps involved in chromosome microarray. Reproduced with permission from Cambridge University Press.[1]

Next generation sequencing

The testing outlined above may detect aneuploidies, rearrangements and CNVs, but in order to look for smaller changes in the genetic code, sequencing must be undertaken. A technique called massive parallel sequencing is used to read the DNA code down to the level of the nucleotide and has made it possible to look at much more DNA at once – and much faster – than its technological predecessors could.[20] Analogous with Moore's law, the cost of sequencing has decreased significantly over time and continues to fall, making this an increasingly cost-effective test (the current cost for trio ES is approximately £2200).[21] There are different sub-types of NGS: whole genome sequencing (WGS) refers to sequencing of the entirety of the genetic code (3 billion base pairs), while ES focuses on the estimated 1% of the genome which codes for proteins (30 million base pairs). As the exome encodes proteins, it is believed to be the most clinically relevant portion of the DNA. However, modern research is shedding increasing light on the importance of the 'non-coding' genome.[22] Exome sequencing can incorporate a 'whole exome', where all of the exome is interrogated, or a clinical – or targeted – exome, where a panel or list of relevant disease causing genes is assessed. Advantages of the latter include greater in-depth coverage or accuracy but at the stake of novel gene discovery, which whole ES can offer (Figure 5).[4]

The 2018 PAGE (Prenatal Assessment of Exomes and Genomes) study[3] undertook ES in 610 unselected fetuses with FSAs, after exclusion of aneuploidy and large CNVs. A diagnostic genetic variant was identified in 8.5%, and a further 3.9% had a variant of uncertain significance (VUS), which still had potential clinical usefulness. Diagnostic yield tended to be higher in FSAs affecting multiple systems and skeletal dysplasias, while yield was relatively low in cases with isolated elevated nuchal translucency (≥4.0 mm). Variants in *KMT2D* (Kabuki Syndrome), *CHD7* (CHARGE syndrome) and *PTPN11* (Noonan syndrome) were among the most common diagnostic findings.[3] Since PAGE, in more selected populations where cases are selected by a prenatal geneticist with set inclusion criteria, the diagnostic yield of ES has been reported to be greater than 50% in some instances.[23-25]

The NHS England National Genomic Test directory provides recommendations for when a rapid prenatal exome (R21) may be appropriate. Inclusion and exclusion criteria for this pathway are shown in Box 1.[9] These criteria are revised iteratively as more evidence emerges. Before ES is undertaken, the fetus should have had a normal QF-PCR and CMA result. To optimise the TAT (currently 10 to 14 days), CMA may be performed in parallel, and interpretation of sequencing findings should be suspended should a pathogenic CNV be detected. Rapid prenatal ES is not recommended when imminent fetal loss or termination is expected; in these scenarios, postnatal genetic testing would be more appropriate. There is less of a need for a rapid TAT, which is required prenatally to facilitate decision making for

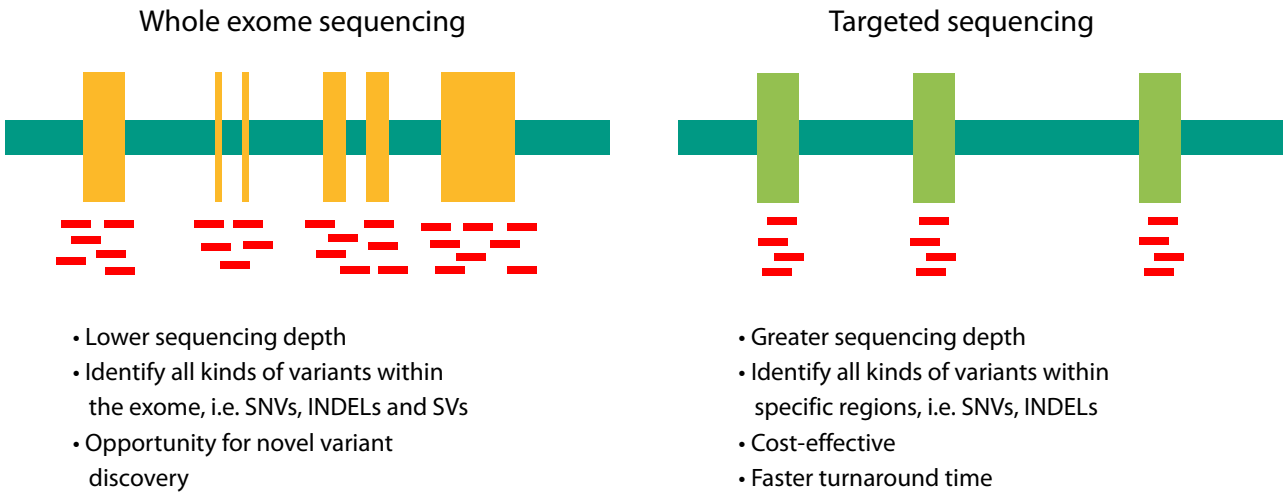

Figure 5. Types of exome sequencing. INDELs = insertions & deletions; SNV = single nucleotide variant; SV = structural variants.

Box 1. Clinical examples where rapid fetal exome (R21) may be appropriate and suggested exclusion criteria. National Genomic Test Directory April '22, NHS England.[25]

Inclusion criteria

- Fetuses with multiple anomalies
- Suspected skeletal dysplasias (fetal growth restriction should be excluded)
- Large echogenic kidneys with a normal bladder
- Major central nervous system (CNS) abnormalities (excluding neural tube defects)
- Multiple contractures (excluding isolated bilateral talipes)
- Nuchal translucency (NT) >6.5 mm plus another anomaly (that can include a minor finding) with a normal array comparative genomic hybridisation.
- Isolated nonimmune hydrops fetalis defined as fluid/oedema in at least two compartments (e.g. skin, pleural, pericardial or ascites) with a normal microarray

Persistently elevated nuchal translucency (>3.5 mm) can only be considered in the presence of other structural abnormalities in two or more systems. Mild ventriculomegaly should only be considered as an abnormality if the posterior horn is persistently >11 mm. Under these circumstances it is not considered a major CNS abnormality in isolation.

Exclusion criteria

- Confirmed aneuploidy or pathogenic copy number variant consistent with fetal anomalies detected by microarray
- Minor 'markers of aneuploidy' – choroid plexus cysts, echogenic foci, mild renal pelvis dilation, small nasal bone, long bones on 3rd centile, etc.
- Fetuses with confirmed thanatophoric dysplasia, achondroplasia or Apert syndrome on other relevant rapid tests are excluded
- Cases where familial causative variant(s) are known – targeted testing should be performed
- Where sonographic findings indicate a specific monogenic disorder, targeted testing should be applied where appropriate
- Where termination of pregnancy has already been decided or when fetal demise has occurred or is imminent. Appropriate testing should be implemented postnatally.

the couple and multidisciplinary team (MDT) regarding the course of the pregnancy and direction of neonatal care.[26]

Identifying cases that are most likely to benefit from a prenatal ES, counselling families and interpreting genomic results requires the collaboration and cooperation of multiple specialities and professions. The decision to undertake a prenatal ES should be made as part of an MDT review including clinical geneticists, tertiary fetal medicine specialists, genomic scientists, genetic counsellors, midwives and any other relevant specialities.[9] Undertaking prenatal ES is not without risk, including the potential to identify VUS and incidental findings. VUS can pose significant counselling challenges, particularly when parents are deciding about whether to proceed with a pregnancy.[1,27]

In current prenatal practice, ES is more commonly used than WGS. WGS is more expensive, requires higher concentrations of DNA and generates much larger volumes of data, which can be difficult to store and analyse rapidly because it includes not just the exome but also intronic regions, which we might not yet fully understand how to

interpret. However, with advancements in bioinformatics and knowledge about disease association of variants in the 'non-coding' DNA, it is likely that WGS will eventually supersede ES in prenatal genetic diagnosis, predominantly owing to its ability to serve as an 'all-in-one test' assessing for aneuploidy, structural variants, CNVs and single nucleotide variants and beyond.[1]

Proposed clinical pathway

Currently in the NHS, following fetal medicine referral, families are counselled regarding options for invasive testing and fetal DNA is sent for genomic testing using the pathway outlined in Figure 6. The earlier in pregnancy that a genetic aetiology is suspected from ultrasound findings, the earlier the recourse to MDT input for further testing including ES, which prenatally is typically via a clinical exome, based on a fetal anomaly 'panel'.[9] If the CMA is abnormal and specialist counselling is required, or if there are FSAs and the CMA is normal, the couple can be referred to a genetics clinic (ideally a joint fetal medicine–prenatal genetic clinic).[2] If the QF-PCR and CMA are normal, further investigations such as NGS may be considered, however this is currently only routinely available in NHS England via the R21 pathway.[9] This pathway directed by the Genomic Medicine Service is used in England for rapid prenatal ES and has been proven to be successful, providing a unifying genetic diagnosis diagnoses in over 50% of those with ultrasound-detected FSAs so could be modelled upon for extended prenatal ES pathways internationally.[9,25,28]

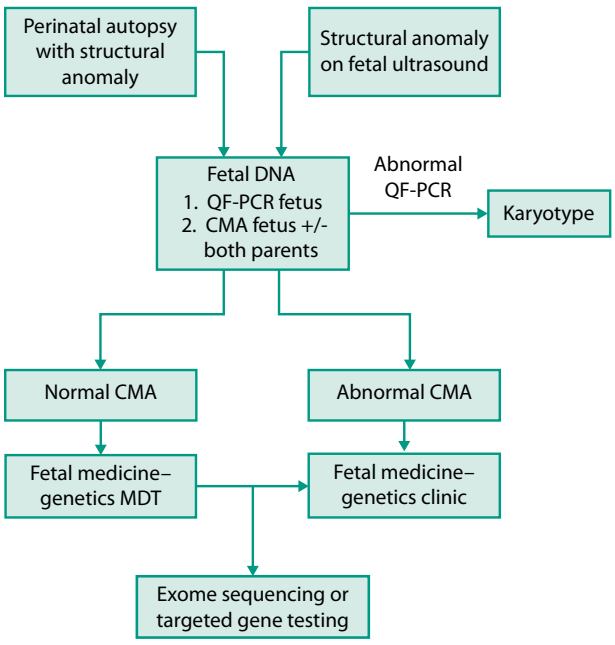

Figure 6. Proposed genomic testing pathway for fetal structural anomalies. Reproduced with permission from John Wiley & Sons.[2]

Prior to undergoing genomic testing, families must receive extensive pre-test counselling and give informed consent.[1,12,26] As per the NHS guideline 'Rapid exome sequencing service guidance', there is a checklist that should be discussed with these patients prior to consent for NGS.[9] Owing to the adoption of the variant classification guidelines from the American College of Medical Genetics and Genomics and the Association for Clinical Genomic Science, the consistency for variant reporting has improved.[29,30] The current grading of variants is via a five-class system from benign to pathogenic. Pathogenic (class V) and likely pathogenic variants (class IV) viewed to be causative of the fetal phenotype are diagnostic and are validated by further sequencing (Sanger) or a similar technique.[31] It is important to communicate with parents that although VUS are not currently reported, there is potential in the future that with increasing knowledge of the human genome these variants may be upgraded and yield a diagnosis.[32]

Mitigating recurrence

Identification of a unifying genetic diagnosis has advantages in terms of not only planning pregnancy and neonatal course, but also identifying carriers for genetic conditions and risk of recurrence, which can have implications for future pregnancies. While most prenatally identified single gene disorders are not inherited but *de novo*, it is important to determine the inheritance pattern for both interpretation and recurrence risk, hence the value of trio analysis.[33,34] The risk of recurrence is dependent upon the inheritance pattern, e.g. one in four if autosomal recessive and parents are heterozygous, or one in two in the case of an autosomal dominant condition where one parent is a carrier. Even in apparently *de novo* abnormalities there remains a risk of gonadal mosaicism where parental gonads may harbour cells carrying the variant, conferring a recurrence risk of up to 4% for some conditions, such as some skeletal dysplasias, although for most conditions this is considerably less.[35]

Testing in future pregnancies can include non-invasive prenatal diagnosis (NIPD), which was initially developed to identify rhesus-positive fetuses in rhesus-negative mothers for tailored anti-D therapies and to identify Y chromosome sequences to determine fetal sex in sex-linked disorders.[36] Since then it has developed to detect *de novo* mutations that have occurred in a previous pregnancy and paternally derived mutations, as these can be easily detected by comparing against the cell free maternal DNA.[36] Using a relative haplotype dosage technique and bespoke design it has now evolved to test for most inherited conditons. In the current clinical climate NIPD is used in pregnancies with a pre-existing family history of a specific genetic disease and requires months of prior notice to develop the appropriate

probes for testing.[37] When a causative pathogenic variant has been identified in a previous pregnancy another option is an elective invasive test, however one must weigh up recurrence risk, which for some *de novo* conditions may be <1%, which is the same as the risk of miscarriage for an invasive procedure. Clinicians must debate whether it is more optimal to await the results of genomic testing (which in the post-natal setting may take some time) when a potentially recurring diagnosis is suspected versus delaying opportunities for child-bearing for the couple, also considering advancing parental age. More recently NIPD has been used to diagnose specific monogenic disorders when they are phenotypically suspected prenatally, e.g. Apert syndrome, achondroplasia and thanatophoric dysplasia.[38]

A further option is that of preimplantation genetic diagnosis, which can be utilised not only for single gene disorders but for structural chromosomal rearrangements carried by a parent. This involves in vitro fertilisation with selection of an unaffected embryo following a biopsy and genomic testing, followed by implantation (Figure 7).[39] Although unlikely, there remains a small risk of an affected embryo, hence invasive testing may still be offered and it is important again to ensure couples are aware that time is required to prepare appropriate set up and probe testing development in advance.[40]

Ethical challenges

Genomic testing provides us with a substantial amount of information about the human genome. Incidental findings present ethical challenges as they may detect adult-onset disorders, such as cancer predisposition genes, which may introduce unanticipated anxiety into the family, as well as potentially leading to stigmatisation and a loss of autonomy for the future child. A trio exome may also uncover non-paternity, which has an estimated median rate of around 4%, and consanguinity, where the rate is dependent upon regional demographics.[41,42] Consensus recommendations exist to support these challenging circumstances.[19,43] Another important consideration for counselling is who has ownership of the results, which can pose a concern for the wider family and raise the question of who owns the genetic material as part of the 'joint account'.[44] Other family members can be informed of clinically significant findings once consent has been obtained from the parents, but this should be discussed prior to invasive investigations, otherwise clinicians must consider avoidance of breach of confidentiality by disclosing results in a way that does not identify the patient.[1] This highlights the importance of informed consent and accurate counselling prior to invasive tests to establish how the parents would wish to proceed in

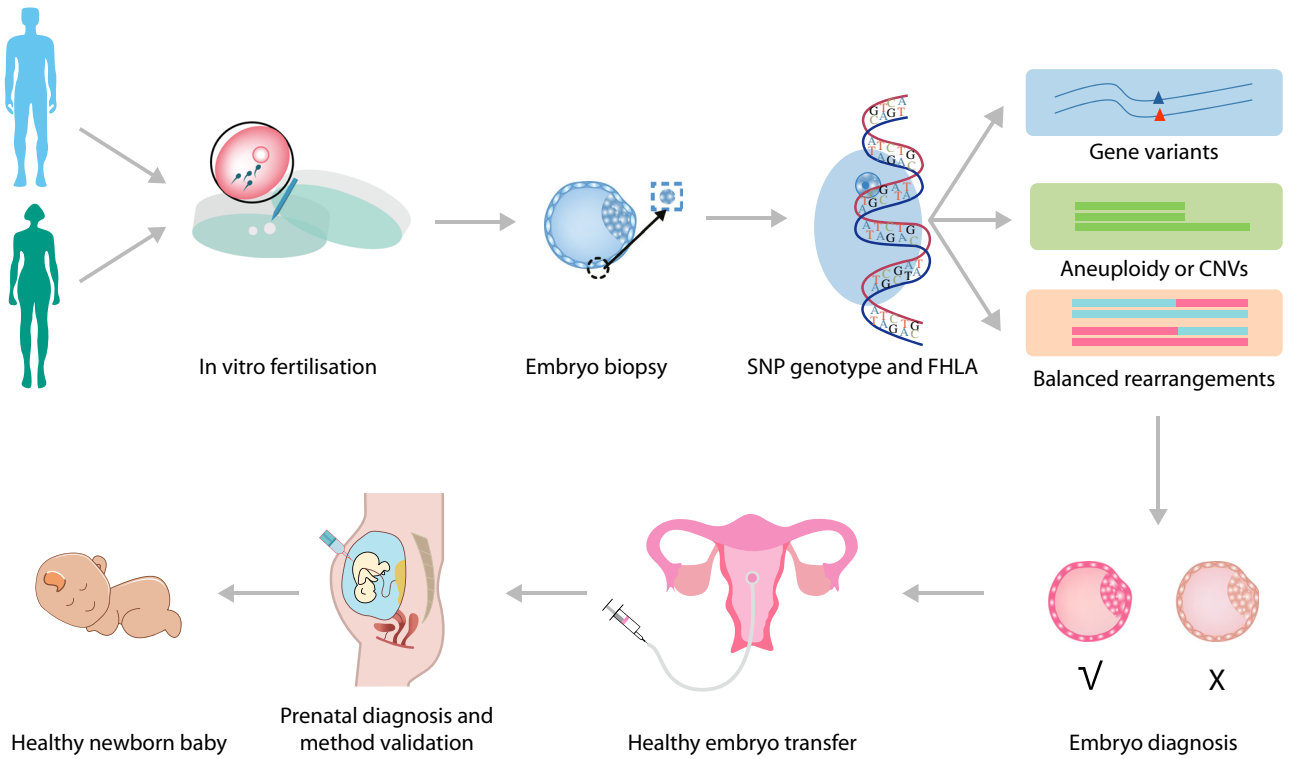

Figure 7. Steps involved in preimplantation genetic diagnosis. CNV = copy number variant; FHLA = family haplotype linkage analysis; SNP = single nucleotide polymorphism.[39]

these scenarios. We currently do not have adequate information to be able to interpret all variants in the human genome, and there is therefore significant potential for reclassification at a later stage.[26] Key considerations are the burden on healthcare staff to keep up to date with potential changes in re-categorising variants and keep the family informed, as well as the query as to who is directly responsible for re-analysis of bioinformatic data.[1,45] Currently, in the UK, not all technologies are available as standard for all patients. In order to ensure equal and ethical care for all patients in the UK, care should ideally be standardised throughout. Owing to the complex nature of consent and confidentiality pertaining to the ethics of these cases, consensus documents have been created to aid with management of these circumstances.[46]

The future

As prenatal genomic testing evolves, the situations in which it is best utilised, along with the criteria for use, will be better recognised.[26] On the horizon is the development of WGS to encompass 'non-coding intronic' sections of DNA and splice-site regions as well as epigenetic modification with assessment histone modifications, methylation and promoter and enhancer regions, which have the potential to uncover even more unifying genetic diagnoses.[3,22,23] All of these investigations that provide more accurate and detailed prenatal diagnoses have even more exciting implications for not only the investigation of fetuses but also potential interventions and therapies. Novel work has shown that with accurate prenatal diagnosis, in-utero gene therapies can be used for conditions such as neurodegenerative diseases and thalassaemias.[47-49]

Conclusion

In fetuses with prenatally identified structural anomalies, advanced genomic investigation can aid in determining a unifying diagnosis and thus limit the diagnostic odyssey that children with rare disease typically endure, as well as empowering couples to make informed decisions about their pregnancy, aid the MDT in planning care and utilise methods to mitigate recurrence. However, there are serious ethical challenges to be considered prior to roll out of exome sequencing, and robust guidance must be included.

Disclosure of interests

FM is a member of the Scientific Impact Committee for the RCOG and has written the e-learning module on prenatal genomics as well as the scientific impact paper on: Evidence to Support the Clinical Utility of Prenatal Exome Sequencing in Evaluation of the Fetus with Congenital Anomalies: Scientific Impact Paper No. 64.

Contribution to authorship

FM conceived and signed the manuscript. KR and CMK drafted the primary version. FM, SMC and SMK edited and approved the final draft, providing final approval and agreement regarding accountability. All authors approved the final version.

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