Advancements in genetic testing in infancy

Recent advances in genetic medicine are changing the landscape of testing available in the neonatal period. A move away from karyotype to microarray-based comparative genomic hybridisation (aCGH) testing as first line chromosomal testing, has markedly improved diagnostic yield. This enhanced chromosomal testing, combined with the increased use of high-throughput sequencing of multiple genes, or of all protein coding genes, shows promise of a new era of diagnosis for genetic conditions that present in infancy. This article provides an overview of the current genetic tests available in the neonatal setting, looks to the future of testing in the field and highlights important points to consider when undertaking genetic testing.

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Key points

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1. Microarray-based CGH is now the first line chromosome test in the majority of cases in the neonatal setting.
2. While single gene testing is still applicable, the use of targeted gene panels and whole exome sequencing is changing the capabilities of molecular genetic testing in neonatal and paediatric medicine.
3. The new tests provide increased information over conventional chromosome testing, which may have implications for the patient and their family. As potential results may include clinically relevant incidental findings and/or variants of uncertain significance, informed consent prior to testing and communication with the clinical genetics department is important.

Since Lejeune et al[1] discovered in 1959 that Down Syndrome was caused by trisomy 21, our appreciation that neonatal syndromes may be caused by chromosomal aberrations or single gene mutations has become well established. The current rate of advancement of medical genetics, in terms of the technology available and the increased identification of the aetiology of genetic conditions, raises new possibilities for diagnosis in neonatal medicine.

With new technologies, however, also comes the increased possibility of deriving genetic data of uncertain clinical significance, ie data that is difficult to interpret based on current knowledge. In addition, tests may uncover genetic results which are only of significance to the health of the patient when they grow older, or which are of relevance to the wider family. This article will describe the new genetic tests and the pertinent points relating to the type of test to request and the interpretation/feeding back of results. A glossary of terms used is provided in **TABLE 1**.

Chromosome testing

Every cell within the body contains 46 chromosomes comprising 22 matching pairs of ordinary chromosomes (autosomes) and a 23rd pair, the sex chromosomes, either XX or XY. Recognisable human genetic disorders may result from a chromosomal abnormality in which there is a loss or gain of chromosomal material. Since the 1970s, such imbalances have traditionally been detected through the analysis of chromosomes by karyotype assessment.

Chromosome G-banding patterns may be visualised under a microscope leading to the identification of differences in:

- chromosome number (aneuploidies), such as trisomy 13, 18 or 21
- large balanced and unbalanced structural rearrangements, ie chromosome translocations
- mosaic structural and numerical abnormalities, ie where not all cells express the same chromosomal complement.

Karyotyping has thus long been an integral tool in the genetic evaluation of infants with congenital anomalies.

Routine karyotyping requires the culture of cells and microscopy and it is therefore labour intensive and time consuming. To expedite investigation if trisomy 13, 18 or 21 are suspected clinically, quantitative fluorescence polymerase chain reaction (QF-PCR) can be undertaken. QF-PCR uses probes that specifically assess the number of copies of chromosomes 13, 18 and 21. A result can be obtained within three working days, which may be important if a rapid diagnosis is needed to guide management decisions. The sex chromosomes can also be assessed by QF-PCR in cases of ambiguous genitalia, for example.

To assess chromosomal imbalance beyond aneuploidy, karyotype testing may be used but this has limitations in that chromosomal aberrations below approximately five megabases (Mb) in size

cannot be visualised. Therefore a loss or gain of genetic material below this size, termed a microdeletion or microduplication, which may be causative of the clinical phenotype, cannot be visualised.

The diagnosis of chromosome abnormalities below 5Mb can be enhanced through the use of fluorescently-labelled DNA probes, which bind to specific DNA sequences. This technique, called fluorescence *in situ* hybridisation (FISH), facilitates the identification of chromosomal aberrations at pre-determined locations. For example, the absence of a fluorescent probe signal on one copy of chromosome 22 at the q11.2 locus indicates 22q11.2 deletion syndrome, which may present in the neonatal period due to congenital cardiac defect or cleft palate.[2] While FISH is very useful, it is a targeted test that requires a specific diagnosis and chromosomal location to be considered.

A more hypothesis-free and all-encompassing approach is microarray-based comparative genomic hybridisation (aCGH). This technique uses thousands of probes spread across all chromosomes to interrogate chromosome copy number at each site (**FIGURE 1**). The data is computationally analysed to determine whether the expected number of copies of each probe are present (ie two copies of each probe for the autosomes). If there is a deviation from the expected copy number, such that a probe or group of probes are missing, this may indicate a deletion of material, while extra copies of probe(s), may be consistent with a gain of material. These variants are termed copy number variants (CNVs) and may represent normal human variation or may be associated with disease. CNVs associated with genetic syndromes are increasingly recognised,[3] although it should be noted that the expression of the clinical manifestations may be variable both within and between different families. Indeed CNVs that are considered to be at neurosusceptibility loci are recognised and they may be associated with developmental delay or neuropsychiatric disorders, but have also been reported in unaffected individuals.

A review by the International Standard Cytogenomic Array (ISCA) Consortium[4] found that in children with unexplained developmental delay or multiple congenital anomalies, a causative abnormality was detected in 15-20% of patients by aCGH, compared to 3% by karyotype analysis (excluding Down syndrome and other recognisable chromosomal syndromes). The improved diagnostic yield afforded by aCGH has a marked positive impact on both patient care and healthcare economics. It is for these reasons that in 2010 ISCA recommended that aCGH should become the first-tier test for individuals with developmental disabilities or congenital anomalies.[4] UK genetic services have moved to follow this recommendation.

Interestingly, with tens of thousands of data points on the microarray (eg 60,000 on the platform used in the Manchester Genomics Laboratory), in some regions resolution is down to the single gene level. This means that this test can sometimes identify single gene causes of disease.

Term	Definition
Karyotype	A test to identify structural and numerical chromosomal anomalies, which can be visualised by microscopy
Chromosome G-banding	Chromosomes in metaphase are stained with Giemsa stain to produce a banding pattern that allows each chromosome to be identified and described precisely. This allows accurate karyotype analysis
Quantitative fluorescence PCR (QF-PCR)	Fluorescent probes, which bind to specific chromosomal regions, allow assessment of chromosome number to be performed quickly (typically three working days)
Fluorescence *in situ* hybridisation (FISH)	Fluorescent probes chosen for pre-determined locations facilitate the identification of chromosomal aberrations – typically chromosome deletions or duplications
Microarray-based comparative genomic hybridisation (aCGH)	Thousands of probes spread across all chromosomes are applied to interrogate chromosome copy number in much greater resolution than possible by karyotype
Copy number variants (CNVs)	A variation from the expected number of copies of a chromosomal region, which for all chromosomes except the sex chromosomes would be two. This may be a benign variant or may be associated with disease
Gene sequencing	Determining the precise order of nucleotides within a DNA molecule
Next generation sequencing (NGS)	A technique in which 'massively parallel' genetic sequencing allows large amounts of genetic material to be sequenced
Exome	All protein coding (expressed) aspects of the genome
Genome	All of the genetic material in a cell or organism
Trinucleotide repeat disorders	Disorders caused by a trinucleotide repeat expansion. Trinucleotide repeats (three repeating nucleotides) in certain genes exceed the normal, stable number of repeats (precise number differs between genes). When the expansion threshold is exceeded disease occurs
Imprinting	For some genes, only one copy of the gene is expressed. Expression of these genes is determined by which parent the gene was inherited from, the process of silencing expression of one gene is known as imprinting
100,000 Genomes Project	A project which aims to sequence 100,000 genomes in around 70,000 NHS patients and their families with rare inherited diseases, cancers or infectious diseases

TABLE 1 A glossary of terms.

Single gene testing

Distributed throughout the chromosomes are an estimated 20,000 to 25,000 protein coding genes, and mutations in many of these genes have been associated with human disease. A number of single gene disorders manifest biochemical features that can be measured by blood spot and are considered of sufficient severity that they are part of the newborn screening programme, such as cystic fibrosis and certain inherited metabolic diseases. Abnormalities on the screening test can be

followed up with testing of the relevant single gene as required.

For the majority of diseases caused by a variant in a single gene, however, testing is arranged when the clinical presentation is considered fitting of a specific diagnosis. In cases where the constellation of symptoms is such that the condition is relatively easily recognisable and caused by a single gene, Sanger sequencing of that gene can be arranged to confirm the clinical suspicion. Typically a genetics laboratory takes around 40 days to sequence and report the result from a single gene test and such testing has been the mainstay of gene testing for several decades.

Single gene testing is less effective when a number of differential diagnoses may be under consideration for a given child, potentially caused by a number of different genes; or when a single diagnosis may be suspected that is known to be caused by more than one gene (ie genetic heterogeneity). In such cases sequential single gene testing is both time consuming and expensive and the development of 'next generation' or 'massively parallel' sequencing has revolutionised testing in this setting.

Next generation sequencing (NGS) technologies utilise a number of different methods that are reviewed by Metzker.[5] In brief, the essence of NGS involves the preparation of a nucleic acid template, typically broken down into small fragments and immobilised spatially, thus allowing billions of sequencing reactions to be performed simultaneously. A DNA template may involve the capture of a targeted region of interest, or all of an individual's genetic material, ie their whole genome. The fragments are sequenced multiple times and the derived sequenced fragments, known as NGS reads, are then aligned against a known reference sequence. The data is then analysed, looking for deviation from the norm, which is considered clinically relevant. The speed with which sequencing technology has evolved in recent years means that producing the sequencing is not difficult, but processing, storing and managing the data creates a challenge. Indeed, the data analysis or bioinformatics stage is typically the major bottleneck in the NGS pipeline.

NGS can be used to assess predetermined groups of genes at one time, in a 'gene panel'. Gene panel testing has numerous advantageous in terms of enhancing diagnoses and thus, potentially,

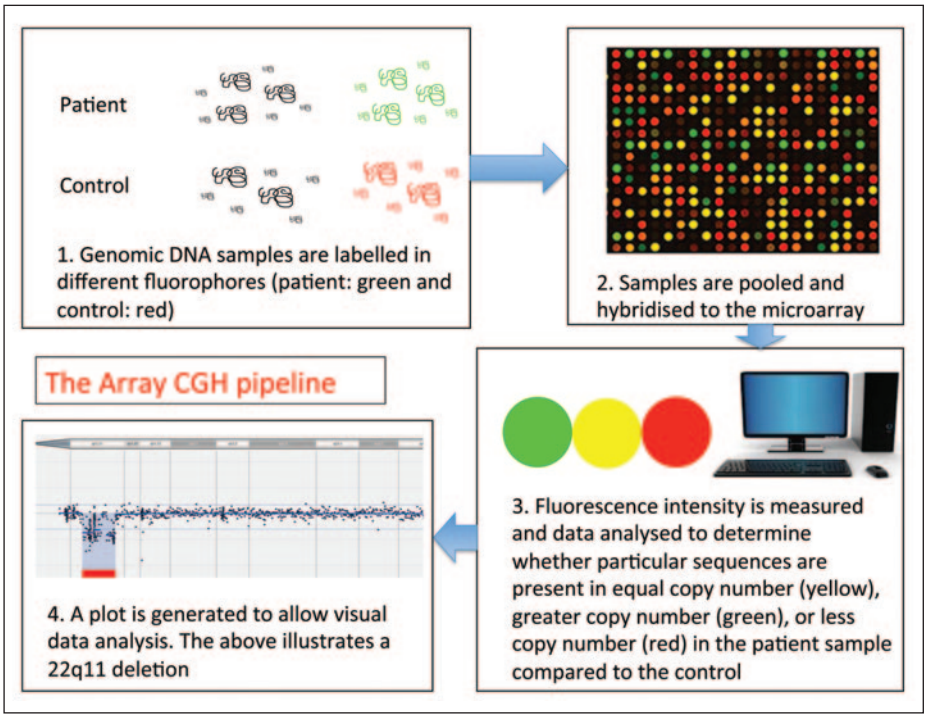

FIGURE 1 The array CGH pipeline. As depicted, patient and control samples are differentially fluorescently labelled, pooled and hybridised. Copy number is then determined based upon fluorescent intensity and computationally represented.

management, as well as time and cost efficiencies (with typically 80 days to test and report on all genes in the panel). Examples of areas where panel testing may be used in the infant setting include:

- ophthalmology (eg congenital cataract)
- cardiology (eg neonatal cardiomyopathy)
- neurology (eg epilepsy panels).

Gillespie et al[6] demonstrated how NGS panel testing in the case of congenital cataract affords: "Extended access to genetic testing, which can lead to improved diagnostic and management outcomes." Using an NGS panel consisting of 115 genes known to cause non-syndromic and syndromic forms of congenital cataract, they determined the underlying genetic aetiology in 75% of cases.[6]

While a gene panel allows simultaneous assessment of many genes, it must be acknowledged that the panel needs to be updated as new genes are discovered. Furthermore, clinical symptoms may fit into several different disease categories and possible causative genes may be spread across several different pre-determined panels. In such cases, an alternative to panel testing is the use of whole exome NGS where all the protein coding genes are studied. This method can be used to good effect to identify causative gene variants: an exome from the affected infant may be assessed alone, or a trio assessment may be undertaken where parental and infant exomes are compared looking for a novel (*de novo*) variant that is present in the infant's exome, but not in the parents'.

At present in the NHS, diagnostic service gene panels and, in selected cases, whole exome sequencing, are being used to facilitate genetic diagnosis. Increasingly, however, it is recognised that genetic disease is caused not only by variants in protein coding genes (and thus found in the exome), but also by variants within the non-coding regions, such as variations in sequences that regulate gene expression.[7] A whole genome approach is needed to test non-coding regions and at present such testing is not available in the NHS, but can be accessed via research or development projects in certain cases, eg as part of the 100,000 Genomes Project (www.genomicsengland.co.uk). This project, run by Genomics England and funded by UK government, aims to sequence 100,000 whole genomes in around 70,000 NHS patients and their families with rare inherited diseases, cancers or infectious diseases. The amount of data generated by whole genome sequencing is sizeable and interpretation time may be considerable. At present such testing is only considered when other established NHS tests have not yielded a result but in time the use of whole genome, and certainly whole exome sequ-

encing, will undoubtedly increase, which is the aim of the 100,000 Genomes Project.

It is important to note that NGS cannot be used to test for all single gene defects, and gene dosage assessments may be required if a gene deletion or duplication is causative. Furthermore, trinucleotide repeat disorders such as congenital myotonic dystrophy[8] cannot be assessed by NGS, nor can imprinting errors such as those causing transient neonatal diabetes.[9]

Important considerations with genetic testing

The primary aim of diagnostic genetic testing is to establish the diagnosis in order to maximise patient care, advise the family and ultimately work towards personalised medicine, with therapy driven by the underlying molecular diagnosis. The advancement of genetic testing, in terms of aCGH and NGS, provides exciting new opportunities to facilitate the advancement of this aim.

However, caution must be exercised in using these tests and counselling of parents/guardians prior to testing, and early consultation with clinical genetics services as required, is important. Factors to discuss (summarised in the checklist, **FIGURE 2**) include that the result may take several months (or longer in the case of whole exome sequencing) to report and that there are several possible result outcomes. The two most obvious possible outcomes to consider with both aCGH and NGS are:

1. A diagnosis may be made and this may have implications for the wider family and future offspring of the couple, as well as the affected child.
2. No plausibly pathogenic variants may be identified at the time of testing, thus no diagnosis is made. It should be noted that the absence of a diagnosis today does not preclude a genetic diagnosis being made in the future when testing capabilities and knowledge advances.

There are other outcomes to consider and discuss prior to testing. One such outcome is the possibility that a variant of uncertain clinical significance may be identified, meaning that it cannot be determined whether an identified variant is likely to be causative of disease or not. In such cases discussion with colleagues in clinical genetics is pertinent as database and literature review, family studies and possibly repeated clinical review over time may be required.[3]

Another possible result is the identification of a clinically relevant incidental finding, the chance of which is increased as more data is derived. For example, a whole exome may be undertaken and a variant may be found in a gene, which is not related to the patient's current presenting complaint but has implications for their health either at present or in the future. The identification of a variant in an infant that infers a future health risk, such as an adult onset cancer predisposition or a neurological condition for which there is no treatment, raises a number of ethical concerns. Due to these concerns the use of targeted gene panels may be favoured, but the importance of obtaining informed consent to ensure that the patient's parents understand the possible nature of the results cannot be underestimated. Currently, consent for diagnostic exome sequencing often includes an 'opt-out' option for incidental findings.

Discussion papers and recommendations from both the American College of Medical Genetics and Genomics[10] and the British Society for Genetic Medicine[11] exist to guide the reporting of incidental findings, but this is a contentious subject as both disclosure and non-disclosure of clinically relevant incidental findings may undermine a patient's autonomy;[12] a factor which is exacerbated in paediatrics where the patient is unable to consent to testing.

Issues associated with genetic testing, particularly NGS, are the subject of great debate and opinions differ, even among experts in the field.[13] Therefore, while the new genetic tests provide invaluable diagnostic data, it is vital that users are aware of all potential outcomes.

- ✓ The test result can take time to become available and in some cases repeat or additional tests may be needed to provide clarity.
- ✓ Result outcomes are:
  1. A disease causing variant found – this may be a disease associated with additional features to those currently recognised in the infant.
  2. No plausible pathogenic variant identified. A negative result does not exclude a genetic diagnosis as each technique only looks for certain types of genetic disease.
  3. Variant of uncertain clinical significance found, ie data that is difficult to interpret based on current knowledge.
  4. Incidental finding identified, ie relevant to the health of the patient or the family but not related to the reason for requesting the test.
- ✓ Results may have implications for other family members in terms of health of parents, current and future children and the wider family.
- ✓ Following receipt of a result for the infant, parental samples may be requested to help clarify the result (particularly in the case of outcome 3), or for recurrence information. It is important to highlight this, as it may be problematic if biological parents are not present, or in cases of non-paternity.

FIGURE 2 A checklist of pertinent points for discussion prior to undertaking a genetic test. Specifics of the discussion will vary depending upon the test requested. Discussion with/referral to genetics colleagues is recommended, as required.

Conclusions

Genetic medicine is advancing at a rapid pace, new technologies are revolutionising testing capabilities and a new era of genetic medicine – with a goal to personalise medicine – has begun. A practitioner using these tests, particularly in the paediatric setting, should be aware of the capabilities of the tests, but also their limitations and possible additional findings that may impact upon the health of the family and the patient for years to come.

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