

# BEST PRACTICE GUIDELINES FOR THE MOLECULAR DIAGNOSIS OF MITOCHONDRIAL DISEASE

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## 1 Introduction

Mitochondria are double-membrane bound organelles found in all nucleated human cells. Their primary function is the generation of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS)<sup>[1]</sup>. Located in the inner mitochondrial membrane, the OXPHOS system comprises five multi-subunit complexes and two electron carriers and involves electron transfer via complexes I-IV in a series of redox reactions to release energy, which is used for ATP synthesis via complex V. However, mitochondria are also involved in many other essential cellular processes including the synthesis of phospholipids and heme, calcium homeostasis and apoptosis in addition to signalling pathways and responses to stressors<sup>[2],[3]</sup>. Mitochondria are dynamic organelles and have the ability to remodel through mitochondrial fission and fusion in response to changes in respiratory states<sup>[4]</sup>.

Mitochondria are under the dual control of their own mitochondrial genome and the nuclear genome<sup>[5]</sup>. Human mitochondrial DNA (mtDNA) is a double-stranded circular molecule consisting of 16,569bp and contains 37 genes: 13 encoding subunits of the OXPHOS components, 22 tRNA and 2 rRNA required for the transcription and translation of the mtDNA-encoded proteins<sup>[6]</sup>. The vast majority of mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm, and then imported into the organelle. These include proteins required for OXPHOS processes, as well as those responsible for the maintenance, replication and transcription of the mitochondrial genome. Currently, the mitochondrial proteome is estimated to consist of 1158 mitochondrial proteins<sup>[7]</sup>. Hence, the genetic defect in patients with mitochondrial disorders may occur in either the mitochondrial<sup>[1]</sup> or nuclear genome<sup>[8]</sup> (refer to **Section 1.2: Mitochondrial Disease**). Over 300 mitochondrial disease-associated genes have been described to date, affecting a wide range of mitochondrial functions including OXPHOS, protein import and processing, mtDNA replication and maintenance, tRNA modification and maturation, mitochondrial protein translation, mitochondrial

membrane dynamics and composition, and other metabolic pathway defects (reviewed in [9]), with this number likely to continue to increase.

### 1.1 Heteroplasmy, threshold and mtDNA segregation

Mitochondria exist in varying numbers in different cells and generally, cells with higher energy demands contain more mitochondria. mtDNA is present in multiple copies within the cell (e.g. >100,000 copies in mature oocytes). These mtDNA copies can be identical (referred to as *homoplasmy*) or there may be a coexistence of more than one type of mtDNA within the same cell (referred to as *heteroplasmy* – literally “heterogeneity of the cytoplasm”). For any heteroplasmic, pathogenic variant, the proportion of mutant mtDNA in a cell typically exceeds a critical threshold before a clinical manifestation is apparent. This is termed the *threshold effect*, and the threshold can vary for different mtDNA pathogenic variants as well as between different tissues, organs and patients. Whilst the level of many heteroplasmic, pathogenic mtDNA variants is stable in post-mitotic tissues such as skeletal muscle (which is often the clinically-affected tissue), the levels of some variants - such as the common heteroplasmic m.3243A>G *MT-TL1* variant - have been shown to progressively decline in blood with age<sup>[10]</sup>. Moreover, some pathogenic variants can be restricted to skeletal muscle (for instance m.13513G>A p.(Asp393Asn) in *MT-ND5*<sup>[11]</sup> and large scale mtDNA deletions<sup>[12]</sup>), which highlights the importance of testing an appropriate tissue for accurate genetic diagnosis. Nevertheless, some mtDNA variants can reliably be detected in blood (refer to **Section 4: Testing methodologies and approaches**).

mtDNA exhibits strict maternal inheritance<sup>[13]</sup> which is complicated by the mitochondrial genetic bottleneck that occurs during embryonic development of the female germline and can result in mature oocytes containing different levels of heteroplasmy<sup>[14]</sup>.

Primary mtDNA pathogenic variants include single nucleotide variants (SNVs), genomic rearrangements such as insertions, duplications, triplications and single large-scale deletions. Multiple mtDNA deletions and decreased mtDNA copy number leading to mtDNA depletion can both occur (secondary to a primary nuclear gene defect).

### 1.2 Mitochondrial Disease

Mitochondrial disease is a collective term used to describe a diverse group of neuro-metabolic disorders characterised by impaired OXPHOS. These disorders are clinically heterogeneous and may present at any age spanning from prenatal and neonatal onset to adulthood onset, affecting isolated or multiple organ systems<sup>[15]</sup>. The dual involvement of nuclear and mitochondrial genomes, further adds to the complexity of mitochondrial disease which is therefore associated with all possible inheritance patterns: maternal, autosomal dominant, autosomal recessive, X-linked and *de novo*<sup>[16]</sup>.

Mitochondrial disease prevalence is estimated to be approximately 12.5 per 100000 in adults<sup>[17]</sup> and approximately 4.7 per 100000 in children<sup>[18]</sup>. The frequency of pathogenic mtDNA variants in the general population is estimated to be higher, with approximately 1 in 200 healthy individuals carrying a pathogenic mtDNA variant at low levels of heteroplasmy<sup>[19]</sup>.

Recognition of mitochondrial disease can be challenging due to the presenting clinical heterogeneity and limited genotype-phenotype correlations, with the same clinical presentation being due to pathogenic variants in different genes and/or the same variant giving rise to several distinct presentations.

### 1.3 Diagnosis of mitochondrial disorders

The advent of next generation sequencing technologies (NGS) has greatly improved the diagnosis of mitochondrial disorders<sup>[20]</sup>. The systematic analysis of the entire mtDNA by NGS is quicker and facilitates accurate heteroplasmy assessment thus improving sensitivity and increasing diagnostic yield. Moreover, the use of whole exome sequencing (WES) and/or custom panels has expedited the diagnosis of many cases associated with nuclear gene defects and has been associated with improved diagnostic yield especially in highly selected patient cohorts<sup>[21], [22]</sup>. The application of NGS and other emerging “omics” tools including RNA-seq has also greatly assisted the identification of novel candidate disease genes involved in mitochondrial function. Recent examples include genes associated with mitochondrial protein synthesis and mitochondrial tRNA synthetases (reviewed in [9]).

Diagnostic practices are rapidly evolving with a shift from the “biopsy first” approach to high yield genome-wide strategies. Yet even in the genomic era, the investigation and diagnosis of patients with mitochondrial disease often remains a challenge, necessitating a combination of techniques including muscle histochemistry and biochemical assessment of respiratory chain function alongside molecular genetic studies and clinical assessment<sup>[23]</sup>. Furthermore, muscle and other tissue biopsies may still be required to guide functional evaluation of variants or for biochemical diagnosis when no variants have been detected<sup>[24]</sup>.

Current therapies for mitochondrial diseases are limited and therefore early diagnosis is crucial<sup>[25]</sup>. A number of therapeutic approaches and therapies are emerging for mitochondrial diseases, including the molecular shifting of mtDNA heteroplasmy by antigenomic therapies as well as genomic engineering techniques using nucleases such as mitochondrially targeted zinc-finger nucleases (mtZFNs) and mitochondrially targeted transcription activator-like effector nucleases (mitoTALENs) and CRISPR/Cas9 genome editing technologies<sup>[26]</sup>.

Other promising interventions of recent years include the options for prevention of transmission of mitochondrial disease through preimplantation genetic diagnosis (PGD) and mitochondrial donation<sup>[16]</sup>. Approval of mitochondrial donation in the UK followed a long process through scientific and clinical studies, public consultation and parliamentary amendments in legislation. In 2017, the first licence for mitochondrial donation was issued to

Newcastle Fertility Clinic at Life, which is currently the only clinic in the UK that has a licence to treat suitable families.

Wherever possible following diagnosis, it is recommended that the patient and their family are referred to the NHS Highly Specialised Services for Rare Mitochondrial Disorders, or their local Clinical Genetics department. Contact details of the three specialised centres which provide a comprehensive multi-disciplinary service for patients across the UK are given in the Appendix (Section 8). In addition, clinical care guidelines are available and are aimed to provide expert guidance to any health professionals involved in the management of patients with mitochondrial disease (<https://mitochondrialdisease.nhs.uk/professional-area/care-guidelines/>).

Given the complexities of molecular diagnosis of mitochondrial disease, the guidelines presented here aim to provide guidance for the genetic testing strategies for diagnostic and familial testing, variant interpretation and reporting. Guidance is also provided on prenatal diagnosis and reproductive options.

## 2 Reference sequence and Nomenclature

Current international nomenclature should be adopted, i.e. Human Genome Variation Society (HGVS) sequence variant nomenclature (<http://varnomen.hgvs.org/>).

For the mitochondrial genome, nucleotides are conventionally numbered sequentially with reference to a whole mitochondrial genome reference sequence prefixed by “m.”, and this nomenclature system should be followed when reporting results. An appropriate reference sequence should also be quoted on reports, currently **GenBank NC\_012920.1**. This sequence is a revised version of the Cambridge Reference Sequence<sup>[6]</sup>. Historical nucleotide numbering (from the original Cambridge Reference Sequence) is maintained by indicating the absence of a nucleotide at m.3107 as 'N'. In addition to whole mitochondrial genome numbering, for any reported variant, the affected gene(s) should be stated; for protein coding genes, the predicted protein change should also be described (using HGVS variant nomenclature).

For nuclear genes, HGVS nomenclature should also be followed using an appropriate reference sequence.

Further guidelines on use of variant nomenclature in laboratory reports are provided by the Association for Clinical Genomic Science (ACGS) General Genetic Laboratory Reporting Recommendations<sup>[27]</sup>.

## 3 Reasons for referral and appropriate molecular testing strategies

### 3.1 Diagnostic testing

As mitochondrial disorders are clinically and genetically heterogeneous, effective diagnosis requires a multi-disciplinary approach. A poor genotype-phenotype correlation, the type of variant (nuclear DNA vs mtDNA), the effect of heteroplasmy, sample type and the age of patient can all complicate the diagnosis of mitochondrial disease.

Traditional approaches whereby biochemical and histological investigation precede and inform molecular analysis, have been replaced by a more pragmatic approach where the laboratories undertake the most appropriate analysis (based on the clinical information and sample type provided) and subsequently provide further advice based on the outcome of these investigations. In patients where no variant is detected, additional clinical information, an additional sample (e.g. urine) or a muscle biopsy with associated biochemical and histopathological investigations may be suggested. Refer also to **Section 4: Testing methodologies and approaches**.

As a consequence of the shift from “biopsy first” to first line genomic testing, analysis of extensive nuclear gene panels is increasingly appropriate early in the diagnostic pathway. The testing information outlined here is aimed to provide a reference framework for mitochondrial genetic testing in line with this approach and is based on the NHS England’s reconfigured Genomic Medicine Service National Genomic Test Directory for ‘Mitochondrial Disease’ specialism (<https://www.england.nhs.uk/publication/national-genomic-test-directories/>) and the Genomics England PanelApp (<https://panelapp.genomicsengland.co.uk/panels/>). The NHS Highly Specialised Services for Rare Mitochondrial Disorders are involved in the development of the test directory and curation of the nuclear gene panels. The gene content of all these panels is regularly reviewed and updated based on current literature and data from diagnostic and research laboratories. The latest version that has been signed off by the Genomic Medicine Service (GMS) can be viewed on the PanelApp website by searching for the relevant “R” codes as listed below.

### 3.2 Mitochondrial DNA testing

Recommendations for the minimum level of mitochondrial DNA testing for the most common diagnostic referral reasons is provided below (**Table 1**). Testing methodologies are covered in **Section 4: Testing methodologies and approaches**.

**Table 1: Recommendations for the minimum level of mitochondrial DNA testing for the most common diagnostic referral reasons.**

Note '>' used to indicate order of tissue type preference

Phenotype /Syndrome	Phenotype Details	Minimum Level of Testing	NHSE Test directory code(s)	Tissue Type	Possible Further Testing
Ataxia	Possible diagnosis of: Late-childhood or adult-onset peripheral neuropathy, ataxia, pigmentary retinopathy (NARP) <i>OR</i> Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Ataxia may be present in up to half of MELAS patients.	m.8993T>G p.(Leu156Arg) m.8993T>C p.(Leu156Pro) ( <i>MT-ATP6</i> )  + m.3243A>G ( <i>MT-TL1</i> )	R351.2 NARP syndrome or maternally inherited Leigh syndrome  + R64.1 MELAS or MIDD	Any (e.g. blood)  Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Cardiomyopathy	Familial hypertrophic cardiomyopathy with maternal inheritance	m.4300A>G ( <i>MT-TI</i> )  + m.3243A>G ( <i>MT-TL1</i> )	R397.1 Maternally inherited cardiomyopathy  + R64.1 MELAS or MIDD	Any (e.g. blood)  Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Diabetes and hearing loss	Diabetes mellitus and sensorineural hearing loss	m.3243A>G ( <i>MT-TL1</i> )	R64.1 MELAS or MIDD	Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing. Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination).

Phenotype /Syndrome	Phenotype Details	Minimum Level of Testing	NHSE Test directory code(s)	Tissue Type	Possible Further Testing
Encephalopathy/ seizures with lactic acidosis	Possible diagnosis of: Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) <i>OR</i> Infantile onset subacute relapsing encephalopathy, cerebellar and brain stem signs (MILS)	m.3243A>G ( <i>MT-TL1</i> )  + m.8993T>G p.(Leu156Arg) m.8993T>C p.(Leu156Pro) ( <i>MT-ATP6</i> )	R64.1 MELAS or MIDD  + R351.2 NARP syndrome or maternally inherited Leigh syndrome	Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)  Any (e.g. blood)	Nuclear DNA testing may also be appropriate. R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Hearing loss	Non-syndromic sensorineural hearing loss, particularly if onset following aminoglycoside exposure	m.1555A>G ( <i>MT-RNR1</i> )	R65.1 Aminoglycoside exposure posing risk to hearing	Any (e.g. blood)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) may be appropriate if there is a maternal family history.
Kearns-Sayre syndrome	Onset below the age of 20 years: PEO and pigmentary retinopathy with one of either cardiac conduction block, cerebrospinal fluid protein concentration greater than 100 mg/dL, or cerebellar ataxia	Large-scale mtDNA rearrangements (single and multiple deletions)  + m.3243A>G ( <i>MT-TL1</i> )	R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing  + R64.1 MELAS or MIDD	Muscle > Urine > Blood (may be detectable in blood but a normal result does not exclude presence in another tissue)  Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Leber hereditary optic neuropathy, ?LHON, optic atrophy	Childhood or Midlife (adult onset) acute or subacute painless bilateral central vision loss	m.3460G>A p.(Ala52Thr) ( <i>MT-ND1</i> ) m.11778G>A p.(Arg340His) ( <i>MT-ND4</i> ) m.14484T>C p.(Met64Val) ( <i>MT-ND6</i> )	R42.1 Leber hereditary optic neuropathy	Blood	Nuclear optic atrophy genes (R42.2). Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination).
mtDNA depletion syndrome	Neonatal or infantile hepatocerebral, myopathic, encephalomyopathic or neurogastrointestinal presentations; may also include growth failure, lactic acidosis, and hypoglycemia.	mtDNA copy number analysis	R301.1 Possible mitochondrial disorder - mitochondrial DNA depletion testing (mtDNA copy number analysis)	Muscle or liver (other post-mitotic tissues may be analysed if the testing laboratory has appropriate control data).	Nuclear DNA testing may also be appropriate - Disorder of Mitochondrial DNA Maintenance (R352.1) or Suspected diagnosis of nuclear encoded mitochondrial disease (R63.1).

Phenotype /Syndrome	Phenotype Details	Minimum Level of Testing	NHSE Test directory code(s)	Tissue Type	Possible Further Testing
Myoclonic epilepsy	Myoclonus, Seizures; Cerebellar ataxia; Myopathy	m.8344A>G ( <i>MT-TK</i> )  + m.3243A>G ( <i>MT-TL1</i> )	R350.1 MERRF syndrome  + R64.1 MELAS or MIDD	Any (e.g. blood)  Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Pearson syndrome	Sideroblastic anaemia of childhood; Pancytopenia; Exocrine pancreatic failure	Large-scale mtDNA rearrangements	R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing	Any (e.g. blood)	N/A
Progressive external ophthalmoplegia (PEO), ptosis	Typically adult onset ptosis, paralysis of the extraocular muscles (ophthalmoplegia), oropharyngeal weakness, and variably severe proximal limb weakness.	Large-scale mtDNA rearrangements (single and multiple deletions)  + m.3243A>G ( <i>MT-TL1</i> )	R299.1 Possible mitochondrial disorder - mitochondrial DNA rearrangement testing  + R64.1 MELAS or MIDD	Muscle > Urine > Blood (blood is not suitable from patients aged > 20 years)  Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).
Stroke-like episodes	Stroke-like episodes, typically before age 40 years.	m.3243A>G ( <i>MT-TL1</i> )	R64.1 MELAS or MIDD	Muscle > Urine > Blood (a normal result in blood from individuals aged ≥18 years does not exclude presence in another tissue)	Nuclear DNA testing may also be appropriate. Whole mtDNA sequencing (R300.1) (e.g. after further clinical examination and/or muscle biopsy).

### 3.3 Nuclear gene testing

Pathogenic variants in >300 nuclear encoded genes are known to cause mitochondrial respiratory chain disease and these can broadly be grouped into the categories below. It is important to remember that, depending on the gene, the inheritance may be autosomal dominant, autosomal recessive, X-linked or *de novo*<sup>[16]</sup>.

#### 3.3.1 Single gene testing

There are a number of indications where screening for a single gene or indeed common pathogenic variants within a single gene may be appropriate. These include:

##### 3.3.1.1 *POLG*-related disorders (R351.1 and R351.2):

*POLG* encodes one of the two subunits of DNA polymerase gamma, the only polymerase in human mitochondria which is required for replication and repair of mtDNA. Pathogenic *POLG* variants are the most common cause of inherited mitochondrial disorders (reviewed in [28]) and can lead to the secondary accumulation of mtDNA variants, including single nucleotide variants but more commonly, large-scale mtDNA rearrangements (so called “multiple mtDNA deletions”)<sup>[29]</sup> and/or mtDNA depletion<sup>[30]</sup>.

Autosomal recessive pathogenic variants in *POLG* are most commonly associated with Alpers syndrome or autosomal recessive PEO (with or without additional symptoms)<sup>[28]</sup>. Alpers syndrome is a severe infantile/childhood disorder characterised by myoclonic epilepsy (related to the gradual involvement of the grey matter) and hepatic involvement frequently triggered by valproate intake<sup>[31]</sup>. Some pathogenic variants in *POLG* are associated with autosomal dominant PEO<sup>[29], [32]</sup>.

Analysis by sequencing as a single gene may be appropriate in the following circumstances:

- Suspected mitochondrial DNA maintenance disorder
- Evidence of sodium valproate liver toxicity
- A first line test in epilepsy, neuropathy, ataxia etc. phenotypes

Alternatively, there are 4 autosomal recessive pathogenic variants/alleles in the *POLG* gene common in the European population, which may be prioritised in the first instance: c.1399G>A p.(Ala467Thr), c.1760C>T p.(Pro587Leu) in *cis* with c.752C>T p.(Thr251Ile), c.2243G>C p.(Trp748Ser), and c.2542G>A p.(Gly848Ser)<sup>[33],[34],[35]</sup>.

### **3.3.1.2 Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE, OMIM #603041) (R394.1):**

MNGIE is an autosomal recessive disorder leading to mtDNA deletions and depletion, caused by bi-allelic pathogenic variants in the thymidine phosphorylase (TP) gene, *TYMP*. Diagnosis is made by analysis of the *TYMP* gene, possibly following direct measurement of TP enzyme activity or measuring elevated plasma levels of thymidine and deoxyuridine. Pathogenic variants in other genes can also cause MNGIE-like disorders (e.g. *MT-TL1* (m.3243A>G), *POLG* and *RRM2B*) however, these would not be associated with reduced TP activity.

### **3.3.1.3 Thiamine metabolism dysfunction syndrome-2 (THMD2, OMIM #607483) (R395.1):**

The *SLC19A3* gene encodes a thiamine transporter and homozygous or compound heterozygous pathogenic variants in *SLC19A3* cause thiamine metabolism dysfunction syndrome-2 (THMD2), also known as biotin-responsive basal ganglia disease (BBGD) or thiamine-responsive encephalopathy. This is an autosomal recessive disorder with onset usually in childhood that presents as a subacute encephalopathy and can progress to severe cogwheel rigidity, dystonia, quadriplegia, and if left untreated can lead to coma or even death<sup>[36]</sup>.

### **3.3.1.4 Mitochondrial complex V (ATP synthase) deficiency nuclear type 2 (MC5DN2, OMIM #614052) (R396.1):**

This severe neonatal mitochondrial encephalocardiomyopathy can be caused by homozygous or compound heterozygous pathogenic variants in the *TMEM70* gene. Clinical features include psychomotor retardation, microcephaly, hypotonia, growth retardation, hypertrophic cardiomyopathy, dysmorphism, hypospadias, lactic acidosis, and 3-methylglutaconic aciduria and early death<sup>[37]</sup>.

## **3.3.2 Nuclear gene panels**

As stated in section 3.1, extensive nuclear gene testing is increasingly appropriate early in the mitochondrial diagnostic pathway. Comprehensive and more selective gene panels are outlined below.

### **3.3.2.1 Suspected diagnosis of nuclear encoded mitochondrial disease (R63.1):**

This extensive gene panel aims to include all nuclear genes definitively associated with Mendelian mitochondrial disease and includes all the genes within the smaller panels described below. Use of this panel is particularly appropriate when there is a strong clinical suspicion of mitochondrial

disease in the absence of a muscle biopsy or if muscle respiratory chain enzyme analysis demonstrates a combined deficiency, and where mtDNA variants have been excluded.

### 3.3.2.2 OXPHOS complex subunits and assembly factors:

The mitochondrial OXPHOS system comprises five multi-subunit complexes, the four complexes of the respiratory chain and ATP synthase (complex V). The correct assembly of many individual proteins into the fully functioning complexes is assisted by a family of genes known as mitochondrial respiratory chain complex assembly factors. The following NHSE gene panels are relevant:

#### **Mitochondrial disorder with complex I deficiency (R353.1):**

Isolated Complex I deficiency is the most frequently observed biochemical defect in respiratory chain disorders<sup>[38]</sup>. Complex I (NADH dehydrogenase) is comprised of 44 different subunits and hence there are a considerable number of nuclear genes encoding structural subunits and assembly factors, which may be considered as candidates. Approximately a third of paediatric patients have a pathogenic mtDNA variant in a mtDNA gene encoding one of the seven Complex I subunits or in a mt-tRNA gene<sup>[39]</sup> and therefore, sequencing of the mitochondrial genome is a worthwhile line of investigation to either establish the presence of a causative mtDNA variant or exclude maternally-transmitted mtDNA disease before embarking on nuclear gene analysis.

#### **Mitochondrial disorder with complex II deficiency (R354.1):**

All four subunits of Complex II (succinate dehydrogenase) are encoded by the nuclear genome and therefore mtDNA analysis for isolated Complex II deficiency is not indicated. Several assembly factors encoded by the nuclear genome have also been identified<sup>[40]</sup>. Primary mitochondrial complex II deficiency is rare, accounting for ~2% of mitochondrial chain deficiency diagnoses; most cases are due to bi-allelic pathogenic variants in *SDHA*<sup>[41]</sup> but recessive variants in *SDHB*<sup>[42]</sup>, *SDHD*<sup>[43]</sup> and *SDHAF1* and dominant *SDHA* cases have also been reported<sup>[44]</sup>. Certain mono-allelic SDHx variants are associated with predisposition to cancer (e.g. *SDHB* and pheochromocytoma, reviewed elsewhere [45]) and this should be considered in genetic counselling and reporting of carrier status.

#### **Mitochondrial disorder with complex III deficiency (R355.1):**

Complex III (ubiquinol–cytochrome c oxidoreductase) is composed of 11 structural subunits and only one of these (MT-CYB) is encoded by the mitochondrial genome. *BCS1L* encodes one of several assembly factors for Complex III and bi-allelic pathogenic variants in this gene are associated with a range of phenotypes involving isolated Complex III deficiency including GRACILE (growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis, and early death) syndrome<sup>[46]</sup>.

**Mitochondrial disorder with complex IV deficiency (R356.1):**

Complex IV (also known as cytochrome c oxidase or COX) is composed of 13 subunits and all except 3 (MT-CO1, MT-CO2 and MT-CO3) are encoded by the nuclear genome. Also, many additional nuclear encoded genes are required for its assembly and maintenance, such as *SURF1*. Loss of function variants in the *SURF1* gene are associated with autosomal recessive *SURF1*-deficiency, which is the most common cause of Leigh syndrome in the UK<sup>[47]</sup>.

**Mitochondrial disorder with complex V deficiency (R357.1):**

Complex V (ATP synthase) is made up of 13 subunits and two of these are encoded by the mitochondrial genome (*MT-ATP6* and *MT-ATP8*). The most common cause of isolated Complex V deficiency is bi-allelic pathogenic variants in the *TMEM70* gene encoding an assembly factor. A *TMEM70* founder variant prevalent in the Roma population accounts for a proportion of cases<sup>[48]</sup>.

**3.3.2.3 Disorder of Mitochondrial DNA Maintenance (R352.1):**

There are a number of nuclear DNA encoded genes/proteins that function in part to maintain mitochondrial DNA (mtDNA). Pathogenic variants in these genes have been associated with a group of disorders characterised by multiple mtDNA deletions and/or mtDNA depletion<sup>[49]</sup>. Analysis of muscle or liver DNA may have revealed evidence of a mitochondrial DNA maintenance disorder (e.g. multiple mitochondrial DNA deletions or mitochondrial DNA depletion), or there may be a clinical suspicion of a mitochondrial DNA maintenance disorder (e.g. adult PEO).

Multiple mtDNA deletions are typically associated with adult-onset disease involving chronic progressive external ophthalmoplegia (CPEO or PEO). PEO is a slowly progressing, bilateral myopathy of the extraocular muscles. The levator muscles of the upper lids are usually affected first, with ptosis resulting, followed by progressive, total ocular paresis (also known as ocular myopathy)<sup>[50]</sup>. These disorders can be autosomal recessive or autosomal dominant.

MtDNA depletion syndromes (MDS) are typically infantile or childhood onset with severe multisystem disease and can be broadly characterised into the following types, hepatocerebral, myopathic, encephalomyopathic, cardiomyopathic, or mitochondrial neurogastrointestinal encephalopathy (MNGIE). MNGIE generally presents in late childhood or adulthood. MDS are typically autosomal recessive; however, autosomal dominant *de novo* pathogenic variants have been reported in *SLC25A4* related MDS<sup>[51]</sup>.

Mitochondrial dynamics are the processes of mitochondrial fusion and fission, mitochondrial transport, and mitophagy. Fusion and fission work in concert to maintain morphology, size and

number of mitochondria within cells. These processes are increasingly recognised as critically important to mitochondrial function, and defects in fusion or fission have been reported in a small number of Mendelian disorders<sup>[52]</sup>. These include *OPA1* and *MFN2* associated disorders, which are incorporated when testing for disorders of mitochondrial DNA maintenance. The reason for the overlap between impaired mitochondrial dynamics and impaired mtDNA maintenance is not entirely clear, but it appears that altered mitochondrial dynamics can affect mtDNA stability<sup>[53]</sup>.

#### **3.3.2.4 Mitochondrial liver disease (R317.1):**

In patients presenting with infantile acute liver failure or autosomal recessive transient infantile liver failure, sequencing of genes included in the mitochondrial liver disease panel may be appropriate. Urgent analysis may be required to inform whether a liver transplant is appropriate.

#### **3.3.2.5 Pyruvate dehydrogenase (PDH) deficiency (R316.1):**

The pyruvate dehydrogenase (PDH) complex catalyses the conversion of pyruvate to acetyl-CoA in the mitochondrial matrix, and is comprised of multiple copies of five protein subunits, encoded by the *PDHA1*, *PDHB*, *DLAT*, *DLD* and *PDHX* genes<sup>[54]</sup>. A number of genes encoding proteins, which function in PDH regulation or co-factor biosynthesis are also required for normal PDH function<sup>[55]</sup>. Pathogenic variants in *PDHA1* are most common and are associated with X-linked PDH deficiency, which can affect hemizygous (or mosaic) males or heterozygous females; *PDHA1* variants often arise *de novo* in the proband<sup>[56]</sup>. Pathogenic variants in the other genes are associated with autosomal recessive PDH deficiency. Defects in co-factor biosynthesis often lead to deficiency of multiple mitochondrial enzymes. PDH deficiency typically presents as Leigh syndrome; however, there is a broad clinical spectrum and pathogenic variants in some of these genes are associated with different phenotypes. A PDH enzyme activity assay can be carried out using cultured fibroblasts from patients<sup>[57]</sup>.

### **3.4 Family testing (presymptomatic/carrier)**

Following the genetic diagnosis of mitochondrial disease (mtDNA or nuclear DNA variants) in a patient or family, clinical referral to one of the three centres providing Highly Specialised Services for mitochondrial disorders or their local Clinical Genetics department is recommended. This is also important in the context of subsequent family testing as genetic counselling is complex and requires specialist input.

### 3.4.1 Nuclear DNA variants

In families in which a nuclear defect(s) is responsible for disease, counselling/testing should be undertaken following the same established principles as for other autosomal dominant, autosomal recessive or X-linked disorders.

### 3.4.2 mtDNA variants

Testing of maternally related family members is indicated when a mtDNA variant is found. Levels of heteroplasmy as well as the tissue tested (and sex of individual in the case of LHON) should be taken into account when reporting results and patients counselled appropriately.

Testing of asymptomatic children below the age of 16 with a family history of mtDNA disease provides a particular challenge. Routine practice is NOT to test these children. An echocardiogram may be performed (in children over 5 years of age) to ensure there is no evidence of cardiomyopathy and continued cardiac monitoring may be offered (on a case-by-case basis). Clinicians can also discuss genetic testing with clinical colleagues at the nearest specialised centre (for contact details refer to **Appendix (Section 8): Contact details of NHSE Highly Specialised Mitochondrial Service Centres**).

#### 3.4.2.1 Risk to Family Members – Mitochondrial Inheritance (mtDNA)

##### **Parents of a proband:**

Mitochondrial DNA single-nucleotide variants and duplications: Mitochondrial DNA single-nucleotide variants and large-scale duplications may be transmitted through the maternal line. The father of a proband is not at risk of having the mtDNA pathogenic variant. The mother of a proband usually has the mtDNA pathogenic variant and may or may not have symptoms, unless the variant is *de novo*.

Single mtDNA deletions: Single large-scale mitochondrial DNA deletions generally occur *de novo* and thus affect only the proband. When single mtDNA deletions are transmitted, inheritance is from the mother.

##### **Siblings of a proband:**

Mitochondrial DNA single-nucleotide variants and duplications: The risk to the siblings of an affected proband is dependent on the genetic status of the mother. If the mother has the mtDNA pathogenic single nucleotide variant, all siblings are at risk of inheriting it. However, the heteroplasmy level both in the mother (and in particular in her ova) as well as the heteroplasmy level and tissue distribution of the mutant mtDNA in any siblings will influence the risk of these siblings developing symptoms.

If the variant is presumed to be *de novo* in the proband (after testing maternal blood and other tissues, e.g. urine) all siblings are at low risk although maternal gonadal mosaicism cannot be excluded <sup>[58]</sup>.

Single mtDNA deletions: As mtDNA deletions are usually sporadic, in the absence of any family history, the recurrence risk is considered low.

### **Offspring of a proband:**

Mitochondrial DNA single-nucleotide variants: Offspring of males with a mtDNA pathogenic variant will not inherit the variant.

All offspring of females with a mtDNA pathogenic single nucleotide variant are at risk of inheriting the pathogenic variant. A female harbouring a heteroplasmic mtDNA single nucleotide variant may transmit a variable amount of mtDNA with the variant to her offspring, resulting in considerable clinical variability among sibs within the same family<sup>[59]</sup> (See above for 'Siblings of a proband').

Single mtDNA deletions: A clinically affected mother with a mtDNA deletion is estimated to have a 1 in 24 risk of transmitting the deletion <sup>[60]</sup>.

### **Other family members of a proband:**

Other maternal relative may be at risk of inheriting the familial mtDNA pathogenic variant. Testing is available as appropriate. Ideally testing of the most closely related family members should be undertaken in the first instance to inform risks to more distant family members.

## **3.5 Reproductive options for mitochondrial disease prevention**

### **3.5.1 Reproductive options for nuclear variants**

Reproductive options for couples with a previously affected child or family history of mitochondrial disease caused by nuclear genetic defects are similar to other Mendelian conditions. For autosomal recessive conditions, confirmation of parental carrier status should be established prior to offering prenatal diagnosis by chorionic villous sampling or amniocentesis. Preimplantation genetic diagnosis is also available for some autosomal recessive mitochondrial diseases (see Human Fertilisation and Embryology Authority (HFEA) website for current approved lists of conditions including mitochondrial).

### 3.5.2 Reproductive options for mtDNA variants

The European Neuro Muscular Centre (ENMC) international workshops have been valuable in developing guidelines for the management of reproductive options in mitochondrial disease<sup>[59], [61], [62]</sup>. Due to the strict maternal inheritance<sup>[13]</sup> of mtDNA, men with pathogenic mtDNA variants can be reassured that they are not at risk of transmitting a pathogenic mtDNA variant to their offspring.

Within the UK, it is estimated that there are approximately 150 women per year at risk of transmitting pathogenic mtDNA variants<sup>[63]</sup>. Genetic counselling is challenging due to the complexities of mtDNA variant transmission and prediction of disease risk. Available reproductive options vary between women depending on the nature of the mtDNA variant and the level of heteroplasmy. It is therefore recommended that counselling is provided by specialised mitochondrial services, to offer appropriate advice and allow informed reproductive choices. It is advised that women are referred for discussion of their reproduction options as early in their reproductive lives as possible, as options will become more limited with advanced age and decline of ovarian reserve. A reproductive care pathway is available for all women of reproductive age within the UK with a personal or family history of mtDNA disease and involves two linked clinics in Newcastle consisting of a Mitochondrial Reproductive Advice Clinic (MRAC) and Mitochondrial Assisted Reproductive Technology (MART) clinic. MRAC reviews and confirms the genetic diagnosis, assesses fitness for pregnancy and offers psychological support and reproductive advice for all available options including voluntary childlessness, adoption, natural conception without medical intervention, prenatal diagnosis, oocyte donation, PGD and mitochondrial donation. In addition, there is an integrated IVF pathway which encompasses the MART clinic, a donor pathway and a paediatric care pathway for children born following mitochondrial donation<sup>[64]</sup>.

Enquiries regarding both MRAC and MART, should be directed to, Mitochondrial Reproductive Advice Clinic, Wellcome Centre for Mitochondrial Research, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH (Email: [nuth.mitochondrialreproductiveadvice@nhs.net](mailto:nuth.mitochondrialreproductiveadvice@nhs.net); Tel: 0191 282 4876).

To evaluate the risk of disease transmission in women with heteroplasmic mtDNA variants, heteroplasmy levels should be determined in at least two different tissues (e.g. blood, urine, buccal) and where possible in other maternal relatives and any previously affected offspring.

*De novo* occurrences of pathogenic mtDNA variants have been reported<sup>[58]</sup>. Where a pathogenic variant has been detected only in the proband, the possibility that it may be below the level of

detection in the tissues tested from the mother must be considered, in addition to germline mosaicism. Referral to a reproductive care pathway is appropriate for further discussion.

Consideration of all available data may assist in determining whether CVS, PGD or mitochondrial donation may be the most appropriate option.

### 3.5.2.1 Prenatal diagnosis by CVS or amniocentesis

Prenatal testing for pathogenic mtDNA variants should be offered only on direct CVS/amniocytes and not on cultured cells<sup>[59],[65]</sup> as heteroplasmy levels of mtDNA variants within cell lines can change during culture. It is advised that heteroplasmy levels in the mother and family members where appropriate are evaluated in advance of obtaining the chorionic or amniotic sample, where a rapid turnaround time will be expected in order to facilitate informative interpretation of the results.

#### **Heteroplasmic mtDNA variants:**

A UK wide retrospective review of 17 prenatal tests performed for different mtDNA variants concluded that prenatal diagnosis can be an informative and reliable option for certain mtDNA variants<sup>[66]</sup>. Prediction of risk from the mutant load can be challenging especially where correlation between level of heteroplasmy and disease severity is complex. For the m.3243A>G *MT-TL1* pathogenic variant, a prediction model has been developed using available datasets to enable predictions of likely outcomes below a specified threshold on the basis of maternal heteroplasmy levels<sup>[67]</sup>. For other variants with distinct segregation patterns (e.g. *MT-ATP6* m.8993T>G p.(Leu156Arg)) a reliable prediction may be possible for most mutant loads<sup>[68]</sup>. For variants at high level of heteroplasmy, prenatal diagnosis is generally not appropriate. There are a few exceptions where markedly skewed heteroplasmy levels have been observed in the oocytes from a female with high mutant loads<sup>[69]</sup>, and prenatal diagnosis would be offered in the clinic for similar couples. Mitochondrial donation may be considered (refer to **Section 3.5.2.3: Mitochondrial donation (Mitochondrial Replacement Therapy MRT)**).

This once more demonstrates the importance of seeking specialist advice regarding risk predictions for all mtDNA variants.

Prenatal diagnosis for the m.1555A>G *MT-RNR1* variant which is associated with susceptibility to aminoglycoside-induced ototoxicity and non-syndromic sensorineural deafness is not considered to be appropriate.

#### **Homoplasmic mtDNA variants:**

For any homoplasmic variants, prenatal diagnosis is generally not appropriate. Mitochondrial donation may be considered (refer to **Section 3.5.2.3: Mitochondrial donation (Mitochondrial Replacement Therapy MRT)**).

#### **Single, large-scale mtDNA deletions:**

Single, large-scale mtDNA deletions are usually sporadic with low recurrence risks. Risk estimates were described in a study of 226 families where a single mtDNA deletion had been detected in the proband<sup>[60]</sup>. Clinically unaffected women were highly unlikely to have more than one affected child and affected women had a 1 in 24 risk of having a clinically affected child. Discussion of risks should be offered during counselling and prenatal diagnosis may be available if requested for reassurance.

#### **3.5.2.2 Preimplantation genetic diagnosis (PGD)**

PGD is an IVF-based procedure that facilitates quantification of the level of a pathogenic mtDNA variant to allow the most appropriate embryo(s), based on mutant load and quality, to be selected for transfer. For PGD to be successful, embryos must be produced with heteroplasmy levels below the critical threshold for disease expression<sup>[16]</sup>. Predicting the percentage threshold below which clinical symptoms are unlikely to occur later in adult life is challenging. A threshold of  $\leq 18\%$  has been proposed<sup>[70]</sup>, however the exact threshold is different for each mtDNA variant, and can vary between cases. Comprehensive studies to investigate the threshold in each family should be performed prior to offering PGD for pathogenic mtDNA variants. It should be noted that PGD is a risk reduction procedure and may benefit women who are likely to produce oocytes with low mutant load and this should be considered at the pre-test counselling stage, thus illustrating the importance of specialist counselling. PGD is not indicated for women with high levels of heteroplasmy (except in cases of variants with distinct segregation patterns such as m.8993T>G/C or m.9176T>C/G *MT-ATP6* variants).

#### **3.5.2.3 Mitochondrial donation (Mitochondrial Replacement Therapy MRT)**

Two techniques are currently approved for use by UK parliament: Maternal spindle transfer (MST) and pronuclear transfer (PNT)<sup>[71]</sup>. For a description of these techniques, refer to [16]. Currently, only Newcastle Fertility at Life has been granted an HFEA licence to provide mitochondrial donation using PNT. This licence allows PNT to be offered for women whose offspring are at risk of serious mitochondrial disease for which PGD is not suitable. All individual applications must be approved by the HFEA. All enquiries should be directed to MRAC in Newcastle (for contact details refer to **Section 3.5.2: Reproductive options for mtDNA variants**).

Consent for participation in follow up of any offspring via the integrated paediatric pathway is also discussed, as the outcomes of this follow up are vital in informing for the long-term safety of mitochondrial donation.

## 4 Testing methodologies and approaches

### 4.1 Testing of Mitochondrial DNA

#### 4.1.1 Sample type

Sample type is important and affects the likelihood of detecting a variant in certain referral categories. Age of the patient must also be taken into consideration when advising on the most appropriate sample type. Preferred sample types for different variants are given in **Table 1: Recommendations for the minimum level of mitochondrial DNA testing for the most common diagnostic referral reasons.**

Important points to consider when performing mtDNA analysis:

- mtDNA rearrangements are unlikely to be detected in blood in adults >20 years<sup>[72]</sup>
- m.3243A>G *MT-TL1* levels decline in blood with age; therefore, in older patients, may be absent in blood or low levels may be missed<sup>[10], [73]</sup>
- Muscle should be snap frozen and delivered to laboratories on dry ice
- Liver (snap frozen and delivered to laboratories on dry ice) is an appropriate tissue for mtDNA depletion analysis in patients with liver disease
- Urine is a very good alternative for the analysis of variants, especially *MT-TL1* m.3243A>G, as levels in urine correlate more closely with levels in muscle: 20ml of an early morning sample is sufficient for most applications<sup>[74]</sup>

#### 4.1.2 Testing for common mtDNA single nucleotide variants

General considerations when choosing/validating/verifying a method include<sup>[75]</sup>:

- Sensitivity: absence of false negatives
- Limit of detection: ability to detect low levels of heteroplasmy; the ability to detect at least as low as 10% heteroplasmy is important for some variants and sample types (e.g. to test for *MT-TL1* m.3243A>G in blood DNA from adults; for family tests for heteroplasmic variants); however, a higher limit of detection may be adequate for certain referral types (e.g. diagnostic testing for common mtDNA variants associated with LHON)
- Specificity: absence of false positives

- Quantitative: a quantitative method is preferable in order to determine the level/percentage heteroplasmy (although this may not necessarily be the first line test employed by a laboratory)
- Robustness: the method should have a low failure rate

A variety of methods is available to test for common mtDNA variants; these include:

- Pyrosequencing
- Real-time PCR
- Digital PCR/Droplet digital PCR
- Fluorescent restriction digest PCR
- Restriction digest PCR and agarose gel electrophoresis: limit of detection is relatively high and percentage heteroplasmy cannot be determined accurately
- Sanger sequencing: limit of detection is relatively high and percentage heteroplasmy cannot be determined accurately
- Next generation sequencing

If the test employed is not sequencing based, then laboratories should consider whether any variants detected should be confirmed by a sequencing-based approach (to exclude a false positive result).

Laboratories should also consider whether polymorphisms (in particular, more common polymorphisms) could lead to false negative or false positive results. For example, if testing for m.8993T>G/C in *MT-ATP6* is undertaken by restriction digest PCR (RFLP) analysis, then the presence of an m.8994G>A polymorphism will lead to a false negative result; hence, if using this assay, testing for the polymorphism should be undertaken, and if detected alternative analysis for the *MT-ATP6* m.8993T>G/C should be performed.

#### **4.1.3 Testing for mtDNA rearrangements**

The same general considerations apply as for mtDNA single nucleotide variants. If testing muscle, the method employed should be able to detect multiple mtDNA deletions as well as single mtDNA rearrangements. The ability to detect at least as low as 10% heteroplasmy is important, particularly since levels of single rearrangements in blood may be low even in children and levels of multiple mtDNA deletions may be low in muscle. Quantitation of the level of heteroplasmy and determination of mtDNA rearrangement breakpoints are not possible by simple long-range PCR, which is currently the most widely used routine methodology and this is acceptable. However,

determination of mtDNA rearrangement breakpoints<sup>[76]</sup> and level of heteroplasmy<sup>[77]</sup> may be useful for prognosis. Also, if the clinical significance is uncertain, for example, if the clinical phenotype is atypical, quantitation may prove useful to aid interpretation.

Again, a variety of methods is available, and these include:

- Long range PCR: primers should be chosen such that the vast majority of mtDNA rearrangements will be detected (i.e. primers should amplify most of the major arc)<sup>[78]</sup>
- Southern blotting: can be useful to quantify and characterise mtDNA rearrangements, including distinguishing deletions, duplications and deletion dimers<sup>[79]</sup>
- Real-time PCR / Quantitative PCR: can be useful to quantify mtDNA rearrangements<sup>[80]</sup>
- Next generation sequencing/Whole genome sequencing: this approach can be used to determine breakpoints and potentially quantify mtDNA rearrangements (if a PCR-free library preparation method is used).

#### 4.1.4 Testing for mtDNA depletion

MtDNA depletion analysis is a quantitative test to assess mtDNA copy number. This is of diagnostic utility when testing a clinically affected post-mitotic tissue (such as muscle or liver) in patients with suspected mtDNA depletion syndrome. Data from normal control samples is necessary to determine/estimate the range of normal variation or reference range for each tissue type. In muscle from normal controls, there is evidence that mtDNA copy number changes with age<sup>[81], [82]</sup> and so an age-matched reference range may be necessary.

Real-time PCR to quantitate mtDNA relative to nuclear DNA is currently the most widely used routine methodology, although newer approaches such as droplet digital PCR are increasingly being adopted<sup>[83]</sup>. Possible approaches include:

- Real-time PCR / Quantitative PCR
- Digital PCR / Droplet digital PCR
- Southern blotting
- Next generation sequencing / Whole genome sequencing

Results may be reported in a number of different ways. Examples include: as a percentage compared to the mean normal level; as absolute mtDNA copy number relative to haploid/diploid nuclear genome; as an arbitrary number (an output from the method employed) with reference to the normal range.

#### 4.1.5 Sequence analysis of the whole mitochondrial genome

The same general considerations apply as for testing for common mtDNA single nucleotide variants.

Two main approaches are currently routinely available:

- Sanger sequencing: typically PCR and sequencing of 30-40 overlapping amplicons to cover the whole mtDNA
- Next generation sequencing (NGS): typically by commercial panel, or proprietary long-range PCR (of 1 or 2 amplicons to cover the whole mtDNA), followed by library preparation and sequencing

In general, NGS is preferred over Sanger sequencing as the limit of detection is lower (as long as sequencing read depth is sufficiently high) and the percentage heteroplasmy can be determined<sup>[84]</sup>. For example, at a read depth of 500 there is expected to be 99% probability that a variant present at 7.5% heteroplasmy will be detected in  $\geq 5\%$  of sequence reads (based on binomial distribution calculations).

Laboratories should follow other relevant/appropriate best practice guidelines for NGS and Sanger sequencing (the latest ACGS guidelines can be found here: <https://www.acgs.uk.com/quality/best-practice-guidelines/>). Depending on local validation, variants identified by NGS that require reporting (i.e. pathogenic variants, likely pathogenic variants, and at least some variants of uncertain clinical significance) may need to be confirmed by an alternative method.

As sequencing technologies continue to develop, it is expected that alternative methodologies (such as whole genome sequencing using emerging NGS/third generation sequencing platforms) will increasingly be adopted for whole mitochondrial genome sequencing<sup>[85]</sup>.

#### 4.2 Screening nuclear-encoded genes

Screening for pathogenic variants in nuclear encoded mitochondrial disease genes is no different from analysis of other nuclear genes and all the same considerations apply. Therefore, any tissue sample is a suitable source of DNA. Blood is the usual sample received in a genetics laboratory; however, muscle or urine DNA are also suitable for analysis of nuclear encoded genes, although DNA from these tissues should be prioritised for mtDNA analysis as DNA from these samples is often limited. Variants in nuclear encoded genes may be heterozygous, homozygous, hemizygous, or rarely may be mosaic in the case of some *de novo* variants.

Sequencing analysis of genes is generally limited to coding exons plus intronic regions flanking exon/intron boundaries, typically plus and minus 10 nucleotides into the intron. However, it is

important to be aware that some genes have known recurrent pathogenic variants outside of these regions and regions analysed should include these known pathogenic variants.

#### **4.2.1 Screening for common pathogenic variants**

For *POLG*, screening 4 common autosomal recessive pathogenic variants/alleles may be the appropriate first line test (refer to **Section 3.3.1: Single gene testing**). This could be undertaken by a targeted method (e.g. pyrosequencing) or by Sanger sequencing of the relevant regions of the gene. The method used must be able to distinguish between homozygotes and heterozygotes. Identification of a single heterozygous common pathogenic variant should prompt screening the complete coding region for a second pathogenic allele.

#### **4.2.2 Screening of single genes**

When clinical features strongly suggest a specific diagnosis (e.g. *TYMP* or *TMEM70*), or a single heterozygous autosomal recessive pathogenic variant has been identified in *POLG* by testing for common variants, analysis of a single gene may be the most efficient test. This is usually by Sanger sequencing.

#### **4.2.3 Small gene panels**

Small panels of genes (<10) can be analysed to focus on a specific phenotype (e.g. infantile acute liver failure). This analysis could be by Sanger sequencing, as described above for screening of single genes, which may have the advantage of a fast turnaround in urgent cases. Alternatively, NGS approaches could be adopted, as discussed in the following section.

#### **4.2.4 Large gene panels**

When the clinical indication requires a large panel of nuclear encoded genes to be analysed, next generation sequencing (NGS) is the most practical approach. Library preparation may utilise a targeted capture approach; alternatively, whole exome sequencing (WES) or whole genome sequencing (WGS) followed by targeted analysis of the genes of interest can be applied. The latter approach has the advantage of being able to re-analyse the data for other gene panels or wider analysis if a molecular diagnosis is not found.

#### **4.2.5 Whole Exome Sequencing and Whole Genome Sequencing**

If targeted analysis of nuclear genes and mtDNA analysis has not identified pathogenic variants and there is still a strong clinical suspicion of mitochondrial disease, a gene agnostic approach via WES or WGS may be undertaken in order to reach a genetic diagnosis. In these cases, trio analysis is generally the most practical approach.

#### **4.2.6 Copy number analysis**

The above methods tend to focus on detection of single nucleotide variants (SNVs) or small deletions and/or insertions of several nucleotides; however, larger deletions or duplications involving one or more exons have been reported as pathogenic variants in some genes. Detection of these large-scale rearrangements requires copy number analysis, which is usually carried out by MLPA (multiplex ligation-dependent probe amplification) or a method based on analysis of NGS data.

## **5 Reporting guidelines**

### **5.1 Introduction / general comments**

Reporting is the crucial final step of a genetic test, and the report represents a lifelong document for the clinician, patient and his/her relatives. The report should be comprehensible, written in clear language, and provide a fully interpretative and authoritative answer to the clinical question. The analysed regions (genes, coding region and intron-exon boundaries etc.) and methods used (referring to commercial kit reference and version numbers if applicable) should be specified. General guidelines on reporting results of diagnostic genetic testing, including guidance on report format and essential information, have previously been published<sup>[27],[86]</sup>. Therefore, these guidelines should be adhered to and only limited additional general comments are provided here to add clarity in the context of mitochondrial disorders.

### **5.2 Variant Interpretation**

Variant pathogenicity should be determined in accordance with up-to-date guidelines, currently the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) guidelines<sup>[87]</sup> with modifications and clarifications provided by the ACGS; the latest ACGS guidelines can be found here: <https://www.acgs.uk.com/quality/best-practice-guidelines/>.

The evaluation of pathogenicity for mtDNA variants is more challenging than that of nuclear variants. Although the development of specific guidelines for mtDNA variant interpretation is beyond the scope of this document, a few brief key points are presented below to assist any laboratories querying the pathogenicity of mtDNA variants:

- Use of dedicated mtDNA databases: The MITOMAP database represents the most comprehensive resource for mtDNA variation (listing both benign and pathogenic variants) and is updated regularly (<https://www.mitomap.org/MITOMAP>). The information provided includes locus, disease phenotype, GenBank frequency and a link to relevant publications.

- Frequency: Variant frequency must be considered when assessing pathogenicity and this should be interrogated for the general population. Variants with a frequency of  $\geq 1/1000$  curated GenBank sequences in the MITOMAP database and without any disease association are unlikely to be pathogenic and do not require further review.
- Haplogroups: mtDNA haplogroups define geographic origins of mtDNA variation accumulated during human evolution. MITOMASTER provides a prediction of the variant distribution in different haplogroups that can be considered in the evaluation of variant frequency (<https://www.mitomap.org/foswiki/bin/view/MITOMASTER/WebHome>).
- Heteroplasmy: The majority of pathogenic mtDNA variants are heteroplasmic. The level of heteroplasmy must be accurately determined and it is essential that this be interpreted in the context of the tissue tested. The level of heteroplasmy in a clinically affected tissue should correlate with the clinical presentation and any additional histochemical and/or biochemical findings.
- Homoplasmy: Homoplasmic variants inherited from a homoplasmic clinically unaffected mother are unlikely to be pathogenic. However, there are known pathogenic homoplasmic variants which exhibit variable penetrance (e.g. *MT-RNR1* m.1555A>G, *MT-TI* m.4300A>G, *MT-ND4* m.11778G>A p.(Arg340His), other variants associated with LHON) and this must be considered.
- Evolutionary conservation and functionality: Amino acid and nucleotide conservation between different species may be evaluated for protein coding variation using *in silico* prediction tools. Special consideration should be given for mt-tRNA variants where the impact to the canonical structure and function should be assessed. Specialized databases for mt-tRNA variants may be consulted (<http://mttrna.bioinf.uni-leipzig.de/mtDataOutput/>). MitoTIP<sup>[88]</sup> provided through the MITOMAP database gives an *in silico* prediction score that may be used in addition to other evidence, for the assessment of mt-tRNA variants.
- Similar to the interpretation of nuclear variants, mtDNA variant evaluation should incorporate and consider all related family history and available clinical and histopathological findings. If required, specialised laboratories may be contacted to provide advice for interpretation of mtDNA variants and the following should be provided 1) variant

description 2) read depth and minor allele frequency (MAF) for the proband and mother (for NGS data if available) 3) clinical phenotype or HPO terms and the tissue tested.

Muscle and other tissue biopsies may still be required to guide further functional evaluation of prioritized variants and provide evidence for establishing pathogenicity and these can be carried out by specialised laboratories (e.g. single-fibre studies to investigate variant segregation with a histochemical defect <sup>[89]</sup>).

It is essential to report the pathogenicity of the variant, i.e. pathogenic, likely pathogenic or uncertain significance. Variants classified as benign or likely benign are not routinely included in patient reports. It may not be appropriate to report variants of uncertain significance. This is dependent on local practice and whether further investigations/information may aid interpretation; further UK guidance is available from ACGS (<https://www.acgs.uk.com/quality/best-practice-guidelines>). As stated in **Section 2: Reference Sequence and Nomenclature**, when reporting sequence variant(s), HGVS variant nomenclature should be followed and an appropriate reference sequence accession number (including version) must be included.

When a genetic diagnosis of mitochondrial disease is made or when an individual is identified as being at risk of developing mitochondrial disease, it is recommended to include a comment that the patient and their family can be referred to a specialised mitochondrial centre or their local Clinical Genetics department for assessment and follow up. In cases where no variant is detected, additional clinical information and/or further investigations, such as muscle biopsy with associated biochemical and histopathological investigations, may be suggested.

### 5.3 Reporting results of mitochondrial -molecular investigations

The original sample type (e.g. blood, urine or muscle), should be stated.

For nuclear gene testing, reporting should be undertaken as for other genetic tests for rare disease. For mtDNA testing, there are additional unique aspects to consider when reporting results and key points are summarised below.

Where an mtDNA variant is detected, homoplasmy or the level of heteroplasmy should be specified where possible. When a (likely) pathogenic mtDNA variant is detected in an affected individual at a level of heteroplasmy consistent with the clinical phenotype, the report should state that the result confirms, or is consistent with, a diagnosis of mitochondrial disease. The report should include a statement regarding any associated risk of developing (additional) symptoms, based on the available

evidence for the particular variant; this applies to testing of both affected individuals and asymptomatic relatives. It may also be appropriate to recommend testing of further tissues to refine this risk, particularly if only blood DNA has been tested. For females of reproductive age or who may wish to have a family in the future, the availability of prenatal diagnosis, preimplantation genetic diagnosis and/or mitochondrial donation should be mentioned where appropriate and referral to MRAC recommended. For males, it is important to include a comment that the individual is not at risk of transmitting the variant to his offspring (but maternal relatives remain at risk).

If no putative pathogenic mtDNA variant is identified, the report should state that the result neither confirms nor excludes a diagnosis of mitochondrial disease (or convey this meaning using alternative phrasing). For the mtDNA variants tested, the limit of mtDNA heteroplasmy detection must be specified. Appropriate interpretation is dependent on the limit of detection, as well as, referral reason, patient age, tissue tested and variant(s) tested. Depending on the tissue and variant(s) tested, it may be appropriate to recommend testing an additional sample such as urine or muscle. When testing for a known familial mtDNA variant and it is not detected, this reduces but does not completely exclude the possibility that the variant is present, as it may be present in other tissues and/or at low levels of heteroplasmy below the limit of detection. For familial variant testing in females where the familial variant is not detected, the risk of transmission to any offspring is also reduced but not excluded.

Interpretation of results of prenatal diagnosis can be difficult when intermediate levels of mtDNA heteroplasmy are detected (refer to **Section 3.5.2: Reproductive options for mtDNA variants**). The report interpretation and conclusions should be made on the basis of the available evidence for the specific mtDNA variant, and ideally these reports should be issued from a specialised centre. As for all prenatal genetic tests, maternal cell contamination must be excluded.

## 6 Summary

The diagnosis of mitochondrial disease is complex and challenging, confounded by dual genomic involvement and the vast degree of clinical, functional and genetic heterogeneity. Genetic testing strategies and methodologies are summarised, which are complicated by the unique characteristics of the mitochondrial genome. As with all genetic disorders, the interpretation of sequence variants is critical for accurate diagnosis. However, for Mendelian mitochondrial disorders, the sheer number of candidate genes alone demands the involvement of specialists in genomic analysis and genetic

diagnosis, whilst the clinical significance of mtDNA-defects is complicated by heteroplasmy level, variant-specific thresholds and the spectrum of clinical presentations. These guidelines have been drafted to highlight the fundamental aspects of the laboratory diagnosis of mitochondrial disease, including guidance on interpretation and reporting. Additional guidance is always available from the clinicians and scientists within the NHS Highly Specialised Services for Rare Mitochondrial Disorders.

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## 8 Appendix

Since 2007, the NHS Highly Specialised Services for Rare Mitochondrial Disorders have been delivered through three Highly Specialised centres based in Newcastle, Oxford and London. The three centres provide a comprehensive multi-disciplinary service for patients across the UK. Clinical advice and information on patient care pathways and management can be sought from any of these centres. In addition to a specialised clinical service, these centres deliver a multi-disciplinary diagnostic service, where biochemical, histochemical and immunohistochemical studies can provide functional support for genetic diagnoses. The table below provides contact details for the three centres.

Contact details of NHSE Highly Specialised Mitochondrial Service Centres		
Centre	Clinical Correspondence	Diagnostic Correspondence
Newcastle	Newcastle NHS Highly Specialised Service for Rare Mitochondrial Disorders The Medical School Newcastle University Framlington Place Newcastle Upon Tyne NE2 4HH Tel: 0191 2824375 Email: <a href="mailto:nuth.Mitochondrial.Clinical.Enquiries@nhs.net">nuth.Mitochondrial.Clinical.Enquiries@nhs.net</a>	Newcastle NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory The Medical School Newcastle University Framlington Place Newcastle Upon Tyne NE2 4HH Tel: 0191 2824375 Email: <a href="mailto:tnu-tr.newcastle-mitochondria@nhs.net">tnu-tr.newcastle-mitochondria@nhs.net</a>
Oxford	NHS Highly Specialised Services for Rare Mitochondrial Disorders – Oxford Centre ACE building Nuffield Orthopaedic Centre Oxford University Hospitals NHS Foundation Trust Oxford OX3 7HE Tel: 01865 221007 Email: <a href="mailto:mitohelp@ouh.nhs.uk">mitohelp@ouh.nhs.uk</a>	Oxford Genetics Laboratories The Churchill Hospital Oxford University Hospitals NHS Foundation Trust Oxford OX3 7LE Tel: 01865 226001 Email: <a href="mailto:oxford.mitogenetics@nhs.net">oxford.mitogenetics@nhs.net</a>

<p>London</p>	<p>NHS Highly Specialised Service for Rare Mitochondrial Disorders Centre for Neuromuscular Diseases The National Hospital of Neurology and Neurosurgery Box 102 London WC1N 3BG Tel: 020 3448 8014 Email: <a href="mailto:uclh.enquiries.londonmitonc@nhs.net">uclh.enquiries.londonmitonc@nhs.net</a></p>	<p>Rare &amp; Inherited Disease Genomic Laboratory North Thames GLH Level 5 Barclay House 37 Queen Square London WC1N 3BH Tel: 020 7829 8870 Email: <a href="mailto:ucl-tr.NHNNgenetics@nhs.net">ucl-tr.NHNNgenetics@nhs.net</a></p>
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