

Approaches to Finding the Molecular Basis of Mitochondrial Oxidative Phosphorylation Disorders

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Inherited disorders of mitochondrial oxidative phosphorylation are the most common group of inborn errors of metabolism and cause a wide range of clinical presentations. Mitochondrial DNA encodes 13 protein subunits required for oxidative phosphorylation plus 22 transfer RNAs and two ribosomal RNAs, and mutations in most of these genes cause human disease. Nuclear genes encode most of the protein subunits and all other proteins required for mitochondrial biogenesis and mitochondrial DNA replication and expression. Mutations in 64 nuclear genes and 34 mitochondrial genes are now known to cause mitochondrial disease and many novel mitochondrial disease genes await discovery. The genetic complexity of oxidative phosphorylation means that maternal, autosomal recessive, autosomal dominant and X-linked modes of inheritance can occur, along with de novo mutations. This complexity presents a challenge in planning efficient molecular genetic diagnosis of patients with suspected mitochondrial disease. In some situations, clinical phenotype can be strongly predictive of the underlying genotype. However, more often this is not the case and it is usually helpful, particularly with pediatric patients, to determine whether the activity of one or more of the individual oxidative phosphorylation enzymes is deficient before proceeding with mutation analysis. In this review we will summarize the genetic bases of mitochondrial disease and discuss some approaches to integrate information from clinical presentation, laboratory findings, family history, and imaging to guide molecular investigation.

Keywords: mitochondrial disease, genes, respiratory chain, OXPHOS

All cells require energy to function and the major site of cellular energy generation is the mitochondrion, where ATP is generated by the action of the oxidative phosphorylation (OXPHOS) system. OXPHOS consists of five multisubunit enzyme complexes embedded in the inner mitochondrial membrane. The respiratory chain (OXPHOS complexes I–IV) generates a proton gradient that drives ATP synthesis by the ATP synthase, complex V.

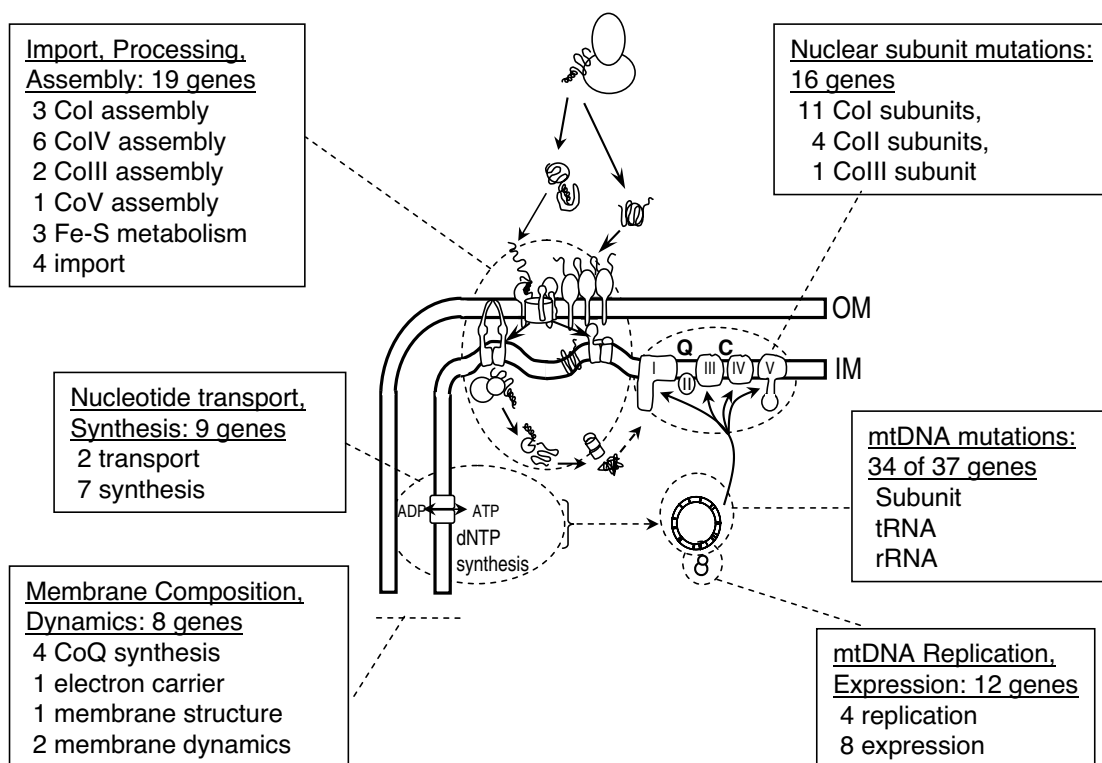
Mitochondria have other functions, including intermediary metabolism such as fatty acid β -oxidation, the urea cycle, some other amino acid metabolism pathways, heme biosynthesis, calcium homeostasis, detoxification of reactive oxygen species and regulation of apoptosis. However, in this review we focus on the restricted definition of mitochondrial diseases referring to OXPHOS dysfunction (Zeviani & Di Donato, 2004).

Mitochondria are unique in mammalian cells, being the only subcellular organelles other than the nucleus that contain functional DNA. Mitochondrial DNA (mtDNA) is a double-stranded circular genome of 16,569 base pairs in humans. It encodes 13 polypeptide subunits of the OXPHOS enzyme complexes, plus 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA). mtDNA is maternally inherited. Another ~80 OXPHOS subunits plus all other proteins and RNAs needed for mtDNA expression and mitochondrial function are encoded by the nucleus and require import into the mitochondria.

As with any biochemical process, OXPHOS can function abnormally. Since every cell requires energy to function, mitochondrial dysfunction can potentially affect any or all tissues or organs, potentially causing almost any symptom, with onset at any age. Due to the bigenomic origin of the OXPHOS enzyme complexes, inheritance of OXPHOS diseases can be autosomal recessive, autosomal dominant, X-linked or maternal, or be due to sporadic (de novo) mutations (Munnich & Rustin, 2001). Brain, muscle and heart are particularly affected, and while symptoms may be mild, in children they are more usually severe and progressive, leading to disability and often death. Mitochondrial dysfunction is important not only in primary OXPHOS disorders, but is also implicated in the pathogenesis of neurodegenerative conditions such

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**Figure 1**

The categories of genes that can cause OXPHOS disorders, including both mitochondrial and nuclear genes. Genes encoding OXPHOS subunits, those involved in import, processing and assembly, mtDNA replication and expression, nucleotide transport and synthesis, and mitochondrial membrane integrity can all cause OXPHOS disease.

Note: CoI, complex I; CoII, complex II; CoIII, complex III; CoIV, complex IV; CoV, complex V; mtDNA, mitochondrial DNA; tRNA, mitochondrial transfer RNA; rRNA mitochondrial ribosomal RNA; CoQ and Q, coenzyme Q; C, cytochrome c; ATP, adenosine triphosphate; ADP, adenosine diphosphate; dNTP, deoxynucleotide; Fe-S, iron sulfur; OM, outer membrane; IM, inner membrane.

as Parkinson disease and Alzheimer disease, and in ageing. Somatic mtDNA deletions accumulate in neurons in both ageing and Parkinson disease and are associated with OXPHOS dysfunction (Bender et al., 2006).

Mitochondrial OXPHOS disorders are the most common group of inborn errors of metabolism and collectively affect ~1/5,000 births (Skladal et al., 2003). Some are caused by mutations in mtDNA genes, but most are due to nuclear gene defects, many of which are yet to be identified. Most patients do not have defects in OXPHOS subunits but in the processes of protein import, subunit processing and assembly, mtDNA replication, mtRNA expression and synthesis or transport of nucleotides (Thorburn, 2004). Mutations in more than 90 genes have been identified, in both mtDNA and nuclear DNA (Figure 1). Deficiencies of multiple OXPHOS complexes are often seen. Complex I deficiency is the most frequently reported single enzyme defect (Kirby et al., 1999), followed by complex IV deficiency.

The molecular era of OXPHOS disease began in 1988, when the first mtDNA mutations were reported (Holt et al., 1988; Wallace et al., 1988) (Figure 2). Mutations in nuclear-encoded OXPHOS genes were

first reported in 1995 (Bourgeron et al., 1995) and their number has been steadily increasing. Over 60 nuclear OXPHOS disease genes have now been reported, including 11 in 2007 plus a further five in the 3 three months of 2008 (Figure 2). Given the complexity of OXPHOS biogenesis, it seems likely that the final number of nuclear OXPHOS disease genes will exceed 100.

How Do We Decide Which Gene Might Be Causing the Problem?

With so many genes potentially causing OXPHOS disease, we need to integrate information from clinical presentation, family history, imaging and laboratory findings to guide molecular investigation.

Mitochondrial or Nuclear DNA?

mtDNA Mutations

Thousands of copies of mtDNA are present in each cell, and normally these are identical, a condition known as homoplasmy. However, mtDNA has a high mutation fixation rate, and mutant and wildtype mtDNA can coexist in the same cell, a condition called heteroplasmy (DiMauro & Schon, 2003;

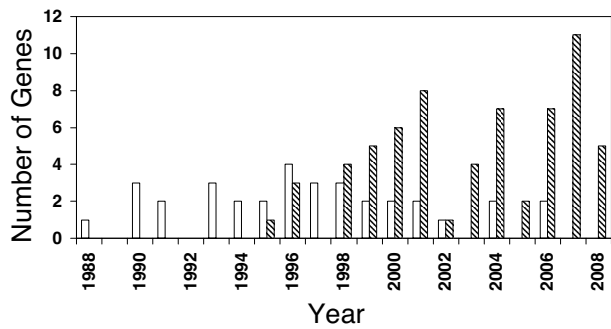


Figure 2

Timeline for discovery of OXPHOS 'disease' genes. The first gene, an mtDNA gene, was found in 1988, and now mutations have been described in 34 of the 37 mtDNA genes. The first nuclear gene was described in 1995, and until the end of March 2008, there have been 64 nuclear OXPHOS 'disease' genes discovered. Open columns, mtDNA genes; hatched columns, nuclear genes.

Schapira, 2006). Pathogenic mtDNA mutations can be tolerated at low levels without causing cellular dysfunction, but above a certain threshold level, they will cause OXPHOS dysfunction and disease. This threshold level varies for different mutations and between tissues (Taylor & Turnbull, 2005).

Pathogenic mutations have been reported in most of the 37 mtDNA genes encoding proteins, tRNAs and rRNAs. There remains some debate about how stringent the criteria should be for determining whether a mtDNA mutation is definitely pathogenic and a number of mutations initially reported as pathogenic now appear to be benign variants (McFarland et al., 2004; Mitchell et al., 2006). The Mitomap database lists only 21 of the 37 mtDNA genes as having confirmed pathogenic variants described but their very stringent criteria require that each mutation must be identified in two unrelated individuals (MITOMAP, 2008). One review listed 34 of the 37 mtDNA genes as having pathogenic mutations identified (DiMauro & Schon, 2003), and we regard this estimate as reasonable.

Many mtDNA mutations are associated with typical 'mitochondrial' syndromes, such as Leber Hereditary Optic Neuropathy (LHON), Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS), Myoclonic Epilepsy with Ragged Red Fibres (MERRF) and Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP) (DiMauro & Schon, 2003; Taylor & Turnbull, 2005). For example, in most (~80%) MELAS patients, the mt.3243A>G mutation in *MTTL1* is present, but other mutations in *MTTL1*, in other transfer RNAs and in subunit genes also cause MELAS (MITOMAP, 2008). Mutations in the same gene do not necessarily result in the same phenotype (e.g., mutations in *MTND1* can be associated with LHON and MELAS). The same mutation can cause different phenotypes, for example, the *MTND5* gene appears to be a hot spot for mutation, in particular for the mt.13513G>A mutation, which is not only associated with MELAS, but also with Leigh Syndrome (LS)

and overlap syndromes (Shanske et al., 2008). The mt.14459G>A mutation in *MTND6* was first reported in LHON, but is now associated with dystonia and with LS (Kirby et al., 2000). Rearrangements (deletions and duplications) of mtDNA are also seen, in such conditions as Kearns Sayre Syndrome (KSS), Pearson Syndrome and Chronic External Progressive Ophthalmoplegia (CPEO; DiMauro & Schon, 2003; Schapira, 2006; Taylor & Turnbull, 2005).

Nuclear DNA Mutations

OXPHOS disorders due to mutations in nuclear encoded genes can be divided into several categories (Figure 1). As of March 2008, pathogenic mutations have been reported in 64 nuclear genes causing OXPHOS defects: 16 OXPHOS subunit genes, 19 genes involved in import, processing and assembly (OXPHOS biogenesis), 12 genes involved in mtDNA replication and expression, nine genes involved in nucleotide transport and synthesis, and eight genes involved in membrane composition and dynamics. Mutations in most of these genes (49/64) are inherited in an autosomal recessive manner, with ten showing autosomal dominant inheritance and five X-linked. *POLG* and *PEO1* were included in the 10 genes with autosomal dominant mutations, but some mutations in these genes cause autosomal dominant multiple mtDNA deletions, while others cause autosomal recessive mtDNA depletion (Hakonen et al., 2007; Naviaux & Nguyen, 2004). Seventeen of the nuclear OXPHOS disease genes have had only a single pathogenic mutation reported, often in just one family, and most genes (35/64) have fewer than five mutations reported (Figure 3). Five genes (*POLG*, *SURF1*, *SDHB*, *SDHD* and *OPA1*) have had more than 50 mutations reported, and a further six genes (*PEO1*, *DGUOK*, *TYMP*, *FXN*, *TAZ* and *MFN2*) have more than 20 reported mutations. Of the nuclear DNA OXPHOS subunit genes, mutations have been reported in 11

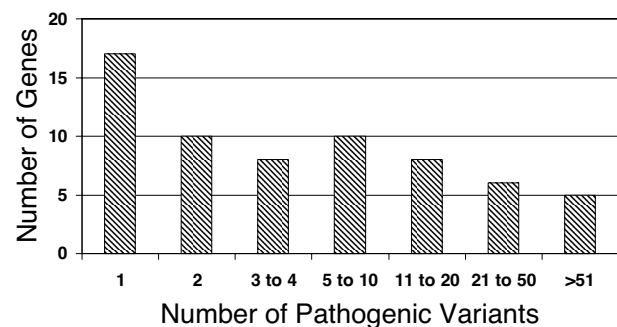


Figure 3

Number of pathogenic mutations reported in each of the nuclear OXPHOS 'disease' genes, based on the Human Gene Mutation Database Professional 8.1 version (<http://www.hgmd.cf.ac.uk/ac/index.php>) accessed on April 8, 2008 (Stenson et al., 2003).

Note: For recently described genes, the data were supplemented from references listed in Tables 2 to 6.

Table 1
Some OXPHOS Disorders In Which The Clinical Phenotype Predicts The Causative Gene

Disorder	Clinical Phenotype	Gene(s)	Role of Gene Product	Reference
LHON	Leber hereditary optic neuropathy	<i>MTND4</i> or <i>MTND1</i> , <i>MTND6</i>	Complex I subunits	(Taylor & Turnbull, 2005)
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes	<i>MTTL1</i> or <i>MTND1</i> , <i>MTND6</i>	tRNA leucine or Complex I subunits	(Taylor & Turnbull, 2005)
MERRF	Myoclonic epilepsy, ragged red fibres	<i>MTTK</i>	tRNA lysine	(Taylor & Turnbull, 2005)
KSS	Ophthalmoplegia, retinal degeneration, cardiomyopathy	mtDNA deletion	Both subunit and tRNA genes deleted	(Taylor & Turnbull, 2005)
Pearson Syndrome	Sideroblastic anemia, exocrine pancreatic dysfunction	mtDNA deletion	Both subunit and tRNA genes deleted	(Taylor & Turnbull, 2005)
Alpers Syndrome	Refractory epilepsy, liver failure	<i>POLG</i>	mtDNA gamma polymerase	(Naviaux & Nguyen, 2004)
Barth Syndrome	X-linked cardioskeletal myopathy	<i>TAZ</i>	cardiolipin remodeling	(Bione et al., 1996)
MNGIE	Encephalomyopathy plus chronic intestinal pseudo-obstruction	<i>TYMP</i>	thymidine phosphorylase	(Nishino et al., 1999)
LBSL	Characteristic magnetic resonance imaging and spectroscopy	<i>DARS2</i>	mitochondrial aspartyl-tRNA synthetase	(Scheper et al., 2007)

Note: LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; KSS, Kearns Sayre syndrome; MNGIE, mitochondrial neuro-gastro-intestinal encephalomyopathy; LBSL, leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation; mtDNA, mitochondrial DNA; tRNA, transfer RNA.

complex I genes, all four complex II genes and one of the complex III genes.

So, given the genetic complexity of OXPHOS disorders, how can we prioritize investigation of the most likely mtDNA or nuclear DNA genes to obtain a molecular diagnosis? Clues come from the pedigree, the phenotype, imaging studies and laboratory findings.

Pedigree

Sometimes there is an obvious family history implying maternal, X-linked, autosomal dominant or autosomal recessive inheritance. It is always worth taking a detailed family history to assess whether or not there may be oligosymptomatic relatives, as can occur with mtDNA mutations. However, in our experience, many pediatric patients with mtDNA mutations appear to have de novo mutations. For most children in whom we have identified a pathogenic mtDNA mutation, there was no obvious maternal family history (Thorburn, 2004). mtDNA mutations were traditionally thought to be a minor cause of OXPHOS disease in children (Shoffner, 1996), but we and others now believe that 20% to 30% of mitochondrial disease presenting in childhood is due to mtDNA mutations (Lebon et al., 2003; Thorburn, 2004). An X-linked pedigree might suggest involvement of one of the five genes on the X-chromosome known to be linked to OXPHOS disease, namely *NDUFA1*, *TAZ*, *TIMM8A*, *ABCB7*, or *HCCS*. These disorders present quite differently, and integration of clinical presentation with the pedigree may guide mutation analysis. The presence of consanguinity suggests an autosomal recessive cause is most likely, and consanguinity is more common in families with OXPHOS disease than in the general community (Skladal et al., 2003). One

needs to be wary that when investigating large numbers of patients, the role of chance will occasionally result in family histories that can be misleading. Consanguinity does not protect a family from having a mtDNA mutation but merely changes the odds. Similarly, we initially suspected maternal or autosomal dominant inheritance in a family with four of five children having a mitochondrial encephalopathy but subsequently identified homozygous autosomal recessive *POLG* mutations in the affected children (personal observation).

Phenotype

In some cases, clinical phenotype is strongly predictive of genotype (Table 1). This is the case with MELAS, where the majority of patients have the mt.3243A>G mutation in *MTTL1*, as already noted. Most patients with LHON have one of three common mutations, mt.3460G>A in *MTND1*, mt.11778G>A in *MTND4* or mt.14484T>C in *MTND6* (Taylor and Turnbull, 2005). Patients with Kearns Sayre syndrome (ophthalmoplegia with retinal degeneration and cardiomyopathy) and Pearson syndrome (sideroblastic anemia and exocrine pancreatic dysfunction) usually have single mtDNA deletions.

For nuclear DNA genes, there are a number of conditions where the clinical phenotype is strongly indicative of mutations in a specific gene (Table 1). For example, mutations in *POLG* are a frequent cause of Alpers Syndrome. There are three common *POLG* mutations (p.A467T, p.W748S, p.G848S) in people with European ancestry, representing about two-thirds of mutated alleles (Hakonen, 2006). We should note here that most children with OXPHOS disorders lack specific clinical presentations that are highly predictive

Table 2
Genes Causing Isolated Complex I Deficiency

Role	Gene	Presentation	Inheritance	References
Nuclear subunit	<i>NDUFV1</i>	LS, Leukodystrophy	AR	(Benit et al., 2001)
	<i>NDUFV2</i>	Cardioencephalomyopathy	AR	(Benit et al., 2003)
	<i>NDUFA1</i>	LS, myoclonic epilepsy	X-linked	(Fernandez-Moreira et al., 2007)
	<i>NDUFA11</i>	LS, Leukodystrophy	AR	(Berger et al., 2008)
	<i>NDUFS1</i>	LS	AR	(Benit et al., 2001)
	<i>NDUFS2</i>	Cardioencephalomyopathy	AR	(Loeffen et al., 2001)
	<i>NDUFS3</i>	LS	AR	(Benit et al., 2004)
	<i>NDUFS4</i>	LS	AR	(van den Heuvel et al., 1998)
	<i>NDUFS6</i>	LIMD	AR	(Kirby et al., 2004)
	<i>NDUFS7</i>	LS	AR	(Triepels et al., 1999)
<i>NDUFS8</i>	LS	AR	(Loeffen et al., 1998)	
mtDNA subunit	<i>MTND1</i>	MELAS, LHON	Mat, Sp	(MITOMAP, 2008)
	<i>MTND2</i>	LHON	Mat, Sp	(MITOMAP, 2008)
	<i>MTND3</i>	LIMD, LS	Mat, Sp	(MITOMAP, 2008)
	<i>MTND4</i>	MELAS, LS	Mat, Sp	(MITOMAP, 2008)
	<i>MTND4L</i>	LHON	Mat, Sp	(MITOMAP, 2008)
	<i>MTND5</i>	MELAS, LS, LHON	Mat, Sp	(MITOMAP, 2008)
<i>MTND6</i>	LS, LHON, dystonia	Mat, Sp	(MITOMAP, 2008)	
Assembly factors	<i>NDUFAF2 (B17.2L)</i>	Leukodystrophy	AR	(Ogilvie et al., 2005)
	<i>NDUFAF1 (CIA30)</i>	Cardioencephalomyopathy	AR	(Dunning et al., 2007)
	<i>C6ORF66</i>	LIMD	AR	(Saada et al., 2008)

Note: LS, Leigh syndrome; LIMD, lethal infantile mitochondrial disease; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; LHON, Leber hereditary optic neuropathy; AR, autosomal recessive; Mat, maternal; Sp, sporadic.

of the underlying genotype. An interesting example is Leigh syndrome, which is a distinct phenotype of developmental regression with specific magnetic resonance imaging (MRI) changes (see below). This well defined phenotype can be caused by mutations in at least 26 different genes located on mtDNA, autosomes and the X chromosome (Thorburn & Rahman, 2003; Tables 2–5).

Brain Imaging

Brain imaging can also provide clues. The ‘metabolic’ strokes in MELAS typically cross vascular territories, which may prompt investigation of the mtDNA mutations associated with MELAS. In LS there are typical lesions in the basal ganglia, brainstem or thalamus, which would prompt investigations of genes associated with LS, both nuclear and mtDNA. Leukoencephalopathy with brain stem and spinal cord involvement and lactic acidosis (LBSL), is a more recently described condition with a characteristic MRI pattern (Scheper et al., 2007; Table 1).

Laboratory Investigations

Histochemical staining of muscle biopsies is frequently undertaken in patients being investigated for mitochondrial dysfunction. Staining with Gomori trichrome can reveal accumulations of mitochondria around the periphery of the muscle fibres, so-called ragged red fibres (RRF). Staining for cytochrome c oxidase (COX,

complex IV) activity can show a mosaic pattern, with some fibres staining positively, and others negatively, for COX. The mosaic pattern is typically due to heteroplasmy, a characteristic of mtDNA (i.e., some muscle fibres have reached the threshold level of mtDNA mutation necessary for expression of an OXPHOS defect, while others have not). Both RRF and mosaic COX staining are often seen in patients with mtDNA deletions and tRNA mutations, and prompt investigation of a mtDNA aetiology. However, these findings can also be caused by nuclear gene defects, particularly those causing depletion or multiple deletions of mtDNA. Globally reduced COX staining is more likely to be due to a nuclear DNA mutation, where all cells are affected equally.

Results of OXPHOS enzyme analysis are usually the primary laboratory findings that guide investigation of the molecular basis of an OXPHOS defect. However, we should emphasize that normal results of OXPHOS enzymology do not exclude an OXPHOS defect. There are a number of reasons for this, including tissue specificity (due to mtDNA heteroplasmy or nuclear gene defects), which occurs in approximately half of all OXPHOS enzyme defects, and limitations of enzyme analysis. OXPHOS enzyme assays do not always reflect in vivo enzyme function and may not detect certain defects such as kinetic variants or mutations that affect proton pumping but not electron transfer. Additionally, many centers including our own laboratory, which acts as the Australasian referral

Table 3
Genes Causing Isolated Deficiencies Of Complexes II, III, IV and V

Role	Gene	Presentation	Inheritance	References
COMPLEX II				
Nuclear Subunit	<i>SDHA</i>	LS	AR	(Bourgeron et al., 1995)
	<i>SDHB</i>	paranglioma, phaeochromocytoma, gastric stromal sarcoma	AD	(DiMauro & Schon, 2003)
	<i>SDHC</i>	paranglioma, gastric stromal sarcoma	AD	(DiMauro & Schon, 2003)
	<i>SDHD</i>	paranglioma, phaeochromocytoma, gastric stromal sarcoma	AD	(DiMauro & Schon, 2003)
COMPLEX III				
Nuclear subunit	<i>UQCRCB</i>	Hypoglycaemia, lactic acidosis	AR	(Haut et al., 2003)
mtDNA subunit	<i>MTCYB</i>	Exercise intolerance, hypertrophic cardiomyopathy	Sp, Mat	(MITOMAP, 2008)
Assembly factor	<i>BCS1L</i>	Encephalo-tubulo-hepatopathy	AR	(de Lonlay et al., 2001),
Prosthetic group synthesis	<i>HCCS</i>	Microphthalmia, skin defects	X-linked	(Wimplinger et al., 2006)
COMPLEX IV				
mtDNA subunit	<i>MTCO1</i>	Encephalomyopathy	Mat, Sp	(MITOMAP, 2008)
	<i>MTCO2</i>	Encephalomyopathy	Mat, Sp	(MITOMAP, 2008)
	<i>MTCO3</i>	Encephalomyopathy	Mat, Sp	(MITOMAP, 2008)
Assembly	<i>SURF1</i>	LS	AR	(Tiranti et al., 1998; Zhu et al., 1998)
	<i>SCO1</i>	Encephalopathy, hepatopathy	AR	(Valnot et al., 2000a)
	<i>SCO2</i>	Cardioencephalomyopathy	AR	(Papadopoulou et al., 1999)
	<i>COX10</i>	Encephalopathy, tubulopathy	AR	(Valnot et al., 2000b)
	<i>COX15</i>	Cardioencephalomyopathy	AR	(Antonicka et al., 2003)
	<i>ETHE1</i>	Ethylmalonic encephalopathy	AR	(Tiranti et al., 2004)
mtDNA expression	<i>LRPPRC</i>	SLSJ-LS	AR	(Mootha et al., 2003)
COMPLEX V				
mtDNA subunit	<i>MTATP6</i>	NARP, LS	Mat, Sp	(MITOMAP, 2008)
Assembly	<i>ATPAF2</i>	Encephalopathy	AR	(De Meirleir et al., 2004)

Note: LS, Leigh syndrome; LIMD, lethal infantile mitochondrial disease; SLSJ-LS, Saguenay Lac St Jean Leigh syndrome; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; AR, autosomal recessive; Mat, maternal; Sp, sporadic.

center for diagnosis of pediatric OXPHOS disorders, analyze frozen tissues rather than fresh. This precludes functional studies, so a proportion of functional OXPHOS defects will not be detected. Examples of this include the phosphate and adenine nucleotide translocator defects discussed later and limitations of enzyme analysis are described in more detail elsewhere (Thorburn et al., 2004a). OXPHOS enzymology is important in planning molecular investigations, but such studies should still be considered even in patients with normal enzyme results. Here we discuss the approaches that may be taken in the various enzyme deficiencies, both isolated and combined, to elucidate the molecular defect.

Complex I Deficiency

Isolated complex I deficiency is the most common OXPHOS enzyme diagnosis and suggests mutations in one of the 45 subunit genes, both mtDNA and nuclear DNA encoded, or in nuclear DNA encoded assembly factors. In our experience, mtDNA mutations are responsible for approximately 25% of pediatric complex I deficiency and mutations have been reported in all seven mtDNA encoded complex I subunit genes.

There are several recurrent mutations, in *MTND3* (mt.10158T>C, mt.10191T>C, mt.10197G>A), *MTND4* (mt.11777C>A), *MTND5* (mt.12706T>C, mt.13513G>A, mt.13514A>G), and *MTND6* (mt.14459G>A, mt.14487T>C) (Sarzi et al., 2007a; Thorburn et al., 2004b). Analysis for these specific mtDNA mutations or sequencing of all mtDNA complex I subunits (and flanking tRNA genes) is probably the most fruitful first approach to investigation of complex I deficiency. Each of the nuclear genes associated with complex I deficiency is responsible for only a small proportion of complex I defects, with most accounting for < 5% of patients.

Eleven nuclear DNA subunit genes have had mutations identified that are causative of complex I deficiency (Table 2). Mutations in *NDUFS1*, *NDUFS3*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1*, *NDUFA1* and *NDUFA11* all cause LS. *NDUFV2* and *NDUFS2* mutations cause cardioencephalopathy. *NDUFS6* mutations cause lethal infantile mitochondrial disease (LIMD). *NDUFV1* and *NDUFA11* mutations are also associated with leukodystrophy, and *NDUFA1* with myoclonic epilepsy.

Mutations in three complex I assembly factor genes have been shown to cause complex I deficiency.

Table 4

Correlation Of Clinical Phenotype And Expression Of Enzyme Defect In The More Common Genes Causing Complex IV Deficiency

Gene	Complex IV deficiency	Phenotype	Common mutation
<i>SURF1</i>	Systemic	LS, motor symptoms more than cognitive, hypertrichosis	c.326insATdelTCTGCCAGCC c.855delCT c.604G>C
<i>SCO2</i>	H & M deficient, L & F near-normal	Cardioencephalopathy	p.E140K
<i>ETHE1</i>	M deficient, F near-normal	Encephalopathy, EMA	
<i>LRPPRC</i>	B & L deficient, F & M near-normal	SLSJ-LS	p.A354V

Note: LS, Leigh syndrome; SLSJ-LS, Saguenay Lac St Jean Leigh syndrome; H, heart; M, skeletal muscle; L, liver; F, fibroblasts; B, brain; EMA, ethylmalonic aciduria.

NDUFAF2 (*B17.2L*) mutations are associated with leukodystrophy, *NDUFAF1* (*CIA30*) mutations with cardioencephalopathy and *C6ORF66* mutations with LIMD.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) immunoblotting is a useful technique for guiding molecular investigations in isolated complex I deficiency (Thorburn et al., 2004b). Mutations in some nuclear subunit genes result in incorrect assembly of the mature complex. This can result in a very much reduced amount of the mature complex (e.g., when *NDUFS8*, *NDUFS2* or *NDUFAF1* are mutated), or the presence of immature intermediates (e.g., when *NDUFS4*, *NDUFS6* or *NDUFV1* are mutated). In contrast, mutations in some subunits (e.g., *MTND3*) result in virtually normal amounts of fully assembled, but non-functional complex I.

We should note that mutations in *TAZ*, *POLG* and *MTTL1* can also cause an isolated complex I defect. These genes can be associated with particular phenotypes (Barth Syndrome with *TAZ*, Alpers Syndrome or CPEO with *POLG*, and MELAS with *MTTL1*), so consideration of the clinical picture is important when deciding on the direction of mutation investigation. In some Alpers Syndrome patients the enzyme defect can evolve over time, progressing from predominantly complex I deficiency to a more profound defect affecting all complexes containing mtDNA encoded subunits, often with a secondary increase in the wholly nuclear-encoded complex II, reflecting mitochondrial proliferation (personal observation).

Complex II Deficiency

Complex II (Succinate Dehydrogenase, SDH) has no mtDNA encoded subunits. Of the four subunit genes, only *SDHA* mutations cause a typical 'mitochondrial encephalopathy' (LS), while those in *SDHB*, *SDHC* and *SDHD* are associated with autosomal dominant tumor syndromes (DiMauro & Schon, 2003; Table 3). Recently, a splice-site mutation in an iron-sulfur scaffold protein (ISCU) has been shown to cause SDH deficiency and myopathy with exercise intolerance (Mochel et al., 2008).

Complex III Deficiency

Mutations in an assembly factor gene, *BCS1L*, are the most frequent cause of complex III deficiency due to nuclear genes (Table 3). A third of complex III-deficient patients in one study had GRACILE Syndrome (renal tubular acidosis, encephalopathy and liver failure), (de Lonlay et al., 2001). Not all patients with GRACILE Syndrome have detectable complex III deficiency; Finnish patients with GRACILE Syndrome typically do not have complex III deficiency, but do have *BCS1L* mutations (Visapaa et al., 2002). *BCS1L* mutations are also seen in Bjornstad Syndrome (sensorineural deafness plus pili torti), but complex III is not reduced (Hinson et al., 2007). Of the nuclear DNA encoded complex III subunits only one, *UQCRCB*, has had a mutation reported (Haut et al., 2003). *MTCYB* mutations are more common in adults, are often sporadic and limited to skeletal muscle and associated with exercise intolerance (Taylor & Turnbull, 2005). As well as cytochrome b, complex III contains cytochrome *c*₁. The X-chromosome located *HCCS* gene encodes holocytochrome c-type synthetase, which functions in the synthesis of both apocytochrome *c*₁ and cytochrome *c*. Mutations in *HCCS* cause an X-linked dominant syndrome of microphthalmia and linear skin defects (Wimplinger et al., 2006).

Complex IV Deficiency

No mutations have been reported in nuclear DNA complex IV subunit genes, despite extensive searches. Mutations in each of the mtDNA subunit genes *MTCO1*, *MTCO2* and *MTCO3* have been reported but each is a rare cause of complex IV deficiency. Isolated complex IV deficiency is most often due to mutations in assembly factor genes (Table 3), particularly *SURF1*. More than 100 patients with *SURF1* mutations have been reported, and several common mutations have been reported in patients of British, Eastern European and Chinese origin (Bohm et al., 2006; Pequignot et al., 2001; Zhang et al., 2007). Patients with *SURF1* mutations frequently have relative sparing of cognition, with motor skills more affected. Unlike some other complex IV defects,

Table 5
Genes Causing Combined OXPHOS Enzyme Defects

Gene	Phenotype	Gene product role	Inheritance	References
NUCLEAR GENES				
mtDNA replication				
<i>POLG</i>	CPEO, multiple mtDNA deletions; hepatocerebral mtDNA depletion	Catalytic subunit mt polymerase gamma	AR, AD	(Hudson & Chinnery, 2006; Van Naviaux & Nguyen, 2004; Goethem et al., 2001)
<i>POLG2</i>	CPEO, multiple mtDNA deletions	Accessory subunit Mt polymerase gamma	AD	(Longley et al., 2006)
<i>PEO1 (TWINKLE)</i>	CPEO, multiple mtDNA deletions, mtDNA depletion	MtDNA helicase	AD, AR	(Hakonen et al., 2007; Sarzi et al., 2007b; Spelbrink et al., 2001)
<i>MPV17</i>	Hepatocerebral mtDNA depletion	Unknown function, inner mitochondrial membrane protein	AR	(Spinazzola et al., 2006)
Nucleotide synthesis and transport				
<i>SLC25A3</i>	LIMD	Mt phosphate transporter	AR	(Mayr et al., 2007)
<i>SLC25A4 (ANT1)</i>	AD CPEO, multiple mtDNA deletions	Adenine nucleotide translocator	AD	(Kaukonen et al., 2000)
<i>TYMP (ECGF1)</i>	MNGIE, multiple mtDNA deletions	Thymidine phosphorylase	AR	(Nishino et al., 1999)
<i>DGUOK</i>	Hepatocerebral mtDNA depletion	Deoxyguanine kinase	AR	(Mandel et al., 2001)
<i>TK2</i>	mtDNA depletion myopathy	Thymidine kinase	AR	(Saada et al., 2001)
<i>PUS1</i>	MLASA	Pseudouridylate synthase	AR	(Bykhovskaya et al., 2004)
<i>SUCLA2</i>	Encephalopathy, mtDNA depletion	β -subunit of ADP-forming succinyl Coenzyme A synthetase ligase	AR	(Carrozzo et al., 2007; Ostergaard et al., 2007b)
<i>SUCLG1</i>	LIMD, mtDNA depletion	β -subunit of succinyl-Coenzyme A ligase	AR	(Ostergaard et al., 2007a)
<i>RRM2B</i>	Encephalomyopathy, mtDNA depletion	Mt ribonucleotide reductase	AR	(Bourdon et al., 2007)
mtDNA expression				
<i>MRPS16</i>	LIMD	Mt ribosomal protein	AR	(Miller et al., 2004)
<i>MRPS22</i>	LIMD	Mt ribosomal protein	AR	(Saada et al., 2007)
<i>GFM1 (EFG1)</i>	LIMD/LS	Mt translation elongation factor G1 (EFG1)	AR	(Coenen et al., 2004; Valente, 2007)
<i>TSMF</i>	Encephalopathy or cardiomyopathy	Mt translation elongation factor EFTs	AR	(Smeitink et al., 2006)
<i>TUFM</i>	Leukoencephalopathy	Mt translation elongation factor EFTu	AR	(Valente, 2007)
<i>DARS2</i>	Leukoencephalopathy, LBSL	Mt aspartyl-tRNA synthetase	AR	(Scheper et al., 2007)
<i>RARS2</i>	Pontocerebellar hypoplasia	Mt arginyl-tRNA synthetase	AR	(Edvardson et al., 2007)
mtDNA Genes				
tRNA genes	MELAS, MERRF, LS, CPEO, myopathy, encephalopathy, deafness, diabetes, cardiomyopathy		Mat, Sp	(MITOMAP, 2008)
rRNA genes	Deafness, MELAS		Mat, Sp	(MITOMAP, 2008)

Note: CPEO, chronic external progressive ophthalmoplegia; LIMD, lethal infantile mitochondrial disease, MNGIE mitochondrial neuro-gastro-intestinal encephalomyopathy; MLASA, mitochondrial myopathy, lactic acidosis and sideroblastic anaemia; LS, Leigh syndrome; LBSL, leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; mtDNA, mitochondrial DNA; tRNA, transfer RNA; rRNA, ribosomal RNA; Mt, mitochondrial; AR, autosomal recessive; AD, autosomal dominant; Mat, maternal; Sp, sporadic.

patients with *SURF1* mutations have a systemic enzyme defect, with complex IV deficiency present in all tissues including skin fibroblasts (Table 4).

Mutations in five other complex IV biogenesis factors (Table 3) have been reported. Here the clinical and biochemical phenotype can give clues (Table 4). *SCO2* and *COX15* mutations are associated with cardioencephalopathy or LS, and all *SCO2* patients reported so far have the mutation p.E140K on at least one allele. *SCO1* mutations are associated with encephalopathy and liver dysfunction, while *COX10* mutations cause encephalopathy and renal dysfunction.

ETHE1 mutations cause encephalopathy with ethylmalonic aciduria.

Complex IV deficient LS in the Saguenay Lac St Jean region of Quebec is almost always caused by mutations in *LRPPRC*, a gene putatively involved in mtDNA expression, which seems to affect particularly *MTCO1* expression (Mootha et al., 2003; Xu et al., 2004). Almost all individuals harbour the same mutation (p.A354V), indicative of a founder effect (Table 4).

Patients with X-linked Menkes disease, a defect of copper metabolism caused by mutations in *ATP7A*, have a functional deficiency of complex IV because

Table 6

Genes Involved In Mitochondrial Protein Import and Biogenesis, or Membrane Composition and Dynamics

Gene	Symptoms	Gene product role	Inheritance	References
Import, Processing and Assembly				
<i>TIMM8A (DDP1)</i>	Deafness, dystonia	Protein import	X-linked	(Koehler et al., 1999)
<i>SPG7 (PARAPLEGIN)</i>	Hereditary spastic paraplegia	OXPHOS regulation?	AR	(Casari et al., 1998)
<i>HSPD1 (HSP60)</i>	Hereditary spastic paraplegia	Chaperone	AR	(Hansen et al., 2002)
<i>DNAJC19</i>	Cardioencephalomyopathy	Chaperone	AR	(Davey et al., 2006)
<i>ABCB7</i>	Sideroblastic anaemia/ataxia	Fe transport	X-linked	(Allikmets et al., 1999)
<i>FXN</i>	Friedreich ataxia	FeS metabolism	AR	(Schapira, 2006)
<i>ISCU</i>	Myopathy, exercise intolerance	FeS assembly	AR	(Mochel et al., 2008)
Membrane composition and dynamics				
<i>COQ2</i>	Nephroencephalopathy	CoQ synthesis	AR	(Mollet et al., 2007; Quinzii et al., 2006)
<i>PDSS1</i>	Deafness, obesity, optic atrophy, peripheral neuropathy, valvulopathy	CoQ synthesis	AR	(Mollet et al., 2007)
<i>PDSS2</i>	Nephroencephalopathy	CoQ synthesis	AR	(López et al., 2006)
<i>CABC1</i>	Cerebellar ataxia with seizures	CoQ synthesis	AR	(Mollet et al., 2008)
<i>CYCS</i>	Thrombocytopenia	Cytochrome c	AD	(Morison et al., 2008)
<i>TAZ</i>	Barth Syndrome	Cardiolipin remodeling	X-linked	(Bione et al., 1996)
<i>OPA1</i>	Optic atrophy	Dynammin-related GTPase (Membrane dynamics)	AD	(Alexander et al., 2000; Deleltre et al., 2000)
<i>MFN2</i>	Charcot-Marie Tooth Type 2A	Mitofusin 2 (Membrane dynamics)	AD	(Zuchner et al., 2004)

Note: OXPHOS, oxidative phosphorylation; Fe, iron; FeS, iron-sulfur; CoQ, coenzyme Q; AR, autosomal recessive; AD, autosomal dominant.

copper is not incorporated into the enzyme (Bertini & Rosato, 2008). We have not included *ATP7A* in this review, since the clinical phenotype reflects deficiency of several copper containing enzymes and would direct the clinician towards copper studies to make the diagnosis.

Complex V Deficiency

Complex V deficiency is usually due to mtDNA mutations, particularly the mt.8993T>G and mt.8993T>C mutations in *MTATP6*, which are associated with NARP and LS (Taylor & Turnbull, 2005). One assembly factor (*ATPAF2*) defect has been reported in a consanguineous patient with lactic acidosis, seizures, developmental delay and failure to thrive (De Meirleir et al., 2004; Table 3).

Multiple OXPHOS Complex Deficiencies

Deficiencies of multiple OXPHOS complexes (Complex I, III and IV or Complex I and IV) can be caused by mutations in mitochondrial tRNAs, rRNAs and by a multitude of genes in which mutations cause mtDNA depletion, multiple mtDNA deletions or abnormal expression of mtDNA-encoded genes (Table 5). Genes encoding proteins directly involved in mtDNA replication are *POLG*, *POLG2*, *PEO1* and perhaps *MPV17*, which encodes an inner mitochondrial membrane protein of unknown function. Genes encoding proteins involved in nucleotide transport and synthesis are *SLC25A4*, *TYMP*, *TK2*, *RRM2B*, *PUS1*, *DGUOK*, *SUCLA2*, *SUCLG1* and *SLC25A3*. Genes encoding proteins involved in expression of mtDNA-

encoded RNA include *MRPS16*, *MRPS22*, *GFM1*, *TSFM*, *TUFM*, *DARS2* and *RARS2*.

Mutations in some nuclear genes cause mtDNA depletion, while others cause multiple mtDNA deletions. Integration of enzymology with the clinical picture, tissue involvement and the level and/or integrity of mtDNA can predict candidate genes. So if there is hepatic mtDNA depletion, *POLG*, *DGUOK*, *MPV17* and *PEO1* are candidates, whereas muscle mtDNA depletion predicts mutations in *TK2* and *RRM2B*. Multiple mtDNA deletions in muscle implicate *POLG*, *PEO1* and *SLC25A4*. *POLG* and *PEO1* are notable in that some mutations cause autosomal dominant CPEO with multiple mtDNA deletions predominantly in muscle, while other mutations cause autosomal recessive hepatocerebral mtDNA depletion syndromes like Alpers syndrome. *POLG* mutations are now recognized as a major cause of mitochondrial disease, with diverse presentations (Hudson & Chinnery, 2006). *POLG* mutations are the single most common nuclear gene defects causing childhood mitochondrial disease and in our experience are responsible for approximately 10% of children diagnosed. We find that *POLG* mutations are more common than mtDNA mutations such as mt.3243G>A, mt.8993T>G or mt.8993T>C or mtDNA deletions. We consider it is worth testing for the three common European *POLG* mutations (p.A467T, p.W748S, p.G848S) in children or adults with seizures, ataxia, liver disease, CPEO, ptosis or peripheral neuropathy.

Deficiency of multiple OXPHOS enzymes with normal or increased mtDNA levels is likely with muta-

tions in genes involved in mtDNA expression. These include the genes encoding mitochondrial elongation factors (*GFM1*, *TSM* and *TUFM*), mitochondrial ribosomal proteins (*MRPS16* and *MRPS22*) and mitochondrial tRNA synthetases (*DARS2* and *RARS2*). *LRPPRC* mutations cause complex IV deficiency by interference with translation of the *MTCO1* gene (Xu et al., 2004).

Mitochondrial tRNA mutations are frequently associated with recognized mitochondrial syndromes such as MELAS and MERRF. It seems logical that all OXPHOS complexes with mtDNA encoded subunits (complexes I, III, IV and V) will be affected if there is a mutation in a tRNA gene. However, the reality is not quite so straightforward and complex I is typically most affected by *MTTL1* mutations, while there is a much lesser effect on complex IV activity. Mutations in *MTTK* are often associated with MERRF, and complex IV deficiency predominates in these patients.

Other Mitochondrial Diseases Closely Related to OXPHOS Defects

In addition to the above categories of genes with direct links to the composition or assembly of OXPHOS complexes, numerous other genes have been shown or are expected to affect OXPHOS function. These include genes encoding proteins involved in mitochondrial protein import, biogenesis or membrane composition and dynamics (Table 6). There is some debate about whether all of these disorders should be classified as mitochondrial diseases, that is, primarily affecting OXPHOS. Investigation of some of these genes is prompted primarily by their clinical phenotype and family history, although OXPHOS function is abnormal in many of them, if tested.

Two mobile electron carriers are involved in transfer of electrons between the OXPHOS complexes, namely coenzyme Q and cytochrome c. The latter is a protein located in the intermembrane space, which interacts with the outer face of the inner membrane. A pathogenic mutation in *CYCS*, the gene encoding cytochrome c has recently been reported to cause autosomal dominant thrombocytopenia (Morison et al., 2008). The phenotype seems remarkably mild and the mutation appears not to affect the protein's role in electron transport but instead enhances its ability to stimulate apoptosis when released into the cytosol.

Two categories of genes cause defects in composition of the mitochondrial inner membrane, the first of which are those involved in synthesis of coenzyme Q. Mutations in *PDSS1* cause cardiac valve dysfunction with deafness, obesity and optic atrophy (Mollet et al., 2007). Mutations in two other genes (*COQ2* and *PDSS2*) cause renal disease with or without neurological disease (López et al., 2006; Quinzii et al., 2006) while mutations in another gene, *CABC1*, are associated with cerebellar ataxia and seizures (Lagier-Tourenne et al., 2008; Mollet et al., 2008). Coenzyme Q deficiency can reveal itself by measurement of com-

bined OXPHOS enzyme activities that rely on intrinsic coenzyme Q (e.g., combined complex I+III and combined II+III activities). If these linked assays are low and the individual complexes I, II and III are normal, coenzyme Q deficiency is likely.

The second category of defects in membrane composition affect cardiolipin, a unique phospholipid with 4 fatty acyl sidechains that comprises ~20% of the inner membrane phospholipid composition and is required for maintaining the structure of OXPHOS complexes and supercomplexes (McKenzie et al., 2006). Barth syndrome (X-linked cardioskeletal myopathy, neutropenia and abnormal mitochondria) is caused by mutations in *TAZ*, a gene encoding a series of alternatively spliced mRNAs, whose protein products, the tafazzins, have a role in cardiolipin remodeling (Vreken et al., 2000).

OXPHOS dysfunction can be caused by mutations in genes affecting mitochondrial iron or iron-sulfur metabolism. Three such defects have been identified. Mutations in the *ABCB7* gene are associated with X-linked sideroblastic anaemia and ataxia (XLSA/A) (Allikmets et al., 1999). A trinucleotide (GAA) expansion in the *FXN* gene causes Friedreich's ataxia (FRDA) in approximately 95% of patients (Schapira, 2006). FRDA is characterized by degenerative atrophy of the dorsal root ganglia, progressive ataxia, sensory loss, muscle weakness and cardiomyopathy. *FXN* encodes frataxin, which plays a role in Fe-S biogenesis (DiMauro & Schon, 2003). Deficiencies of Fe-S containing enzymes (Complexes I, II, III and aconitase) are seen in heart tissue from FRDA patients (Rotig et al., 1997). ISCU, an iron-sulfur scaffold protein associated with complex II deficiency and myopathy with exercise intolerance, interacts with frataxin in Fe-S cluster biogenesis (Mochel et al., 2008).

Mohr-Tranebjaerg Syndrome is caused by mutations in *TIMM8A*, thought to encode a translocase of the mitochondrial inner membrane, which mediates the import of proteins to the inner membrane (Koehler et al., 1999). The syndrome is X-linked, and characterized by deafness, dystonia, muscle weakness, dementia and blindness. Mutations in *HSPD1*, encoding the mitochondrial chaperonin HSP60, are associated with autosomal dominant hereditary spastic paraplegia (Hansen et al., 2002). Mutations in another chaperonin gene, *DNAJC19*, cause cardioencephalomyopathy (Davey et al., 2006). An autosomal recessive form of hereditary spastic paraplegia is caused by mutations in *SPG7*, encoding paraplegin, a mitochondrial protein similar to yeast metalloproteases (Casari et al., 1998).

Integrity of the mitochondrial membrane is necessary for normal mitochondrial function. The mitochondria are not static and in many tissues are constantly dividing and fusing, forming dynamic networks. Mutations in *OPA1*, which encodes a dynamin-related GTPase, cause autosomal dominant optic atrophy (Alexander et al., 2000; Deletre et al., 2000), apparently by interfer-

ing with mitochondrial fission (Dimauro & Schon, 2008). *OPA1* mutations also show abnormalities of mtDNA maintenance and OXPHOS function (Hudson et al., 2008). The gene encoding mitofusin 2 (*MFN2*) is mutated in Charcot-Marie-Tooth neuropathy type 2A (CMT2A) (Zuchner et al., 2004). *MFN2* is an outer membrane protein involved in mitochondrial fusion and so plays a role in regulation of the mitochondrial network architecture (Dimauro & Schon, 2008).

What If We Still Have Not Found the Causative Gene?

Mutations in known genes account for only about half the cases of OXPHOS disease. The search for new causes and their mechanisms of action continues. Further investigations fall in to the realm of the research laboratory.

Cell biology studies can give an indication of mtDNA or nuclear DNA origin of the disease, and even which gene might be responsible. An example of this is *transmitochondrial* cybrid technology. If patient cells (platelets or enucleated fibroblasts) with a defined enzyme defect are fused with a cell line that contains no mtDNA (a rho zero cell line), the enzyme defect will be corrected if the gene responsible is located in the nucleus. If there is no correction, mtDNA is implicated. If, say, complex I is deficient, analysis of the *MTND* genes and the adjacent tRNA genes might reveal a mutation. If there is no other clue as to which mtDNA gene is implicated, whole mitochondrial genome sequencing can be undertaken. However, this is not without problems. The highly polymorphic nature of mtDNA means that many non-deleterious mtDNA sequence variants may be detected and detailed analysis of all these changes, and usually more cell biology and family studies are required to prove pathogenicity of a 'new' mtDNA mutation.

Complementation studies can identify patients with defects in the same gene. This can be done by creating hybrids, where patient cells are fused with a cell line with a known genetic defect. Lack of phenotypic rescue implies a defect in the same gene, be it mtDNA or nuclear DNA encoded. Dividing patients into 'complementation groups' can strengthen molecular studies such as SNP analysis and homozygosity mapping.

Families with multiple affected individuals and consanguineous families can be helpful for finding 'new' genes. In a consanguineous family with several affected children and obligate carrier parents, a genome-wide scan using a SNP array can narrow down a chromosomal region of interest by detecting regions of homozygosity shared by all affected individuals (i.e., identity by descent). Analysis of the same region in other affected families or individuals with a defect in the same gene (perhaps identified by complementation analysis) can further refine the region of interest, and candidate gene analysis can follow. Microcell-mediated chromosome transfer (MMCT), where a single human chromosome is transferred from a hybrid cell line on a

rodent background to deficient cells, can confirm on which chromosome the causative gene is located. Since the chromosome tends to fragment during the MMCT process, the chromosomal region of interest can often be refined by deletion mapping.

Expression arrays can also provide an indication of which genes are differentially expressed in patients as compared with controls, and direct subsequent investigation. Proving pathogenicity may involve correction of the defect in patient cells by transfecting with a normal copy of the gene and observing phenotypic correction, or by silencing the 'normal' gene by siRNA studies in unaffected cell lines and observing the effect.

Often no one technique will provide the answers. Integration of patient clinical presentation, OXPHOS enzymology, complementation analysis, homozygosity mapping, MMCT and RNA expression microarrays was crucial in identifying *NDUFS6* mutations as a cause of complex I deficiency (Kirby et al., 2004).

The large number of genes causing OXPHOS defects and the paucity of common mutations in most such genes have delayed the development of OXPHOS mutation chips, and if these were introduced they would likely need constant updating. Recent advances in sequencing technology make it likely that in coming years high throughput sequencing will become a feasible option for mutation detection. In complex I deficiency, for example, it may become economically feasible to sequence the ~60 genes encoding subunits and assembly factors, so that pathogenic mutations could be identified in most patients. Eventually such technology may make it feasible to sequence all genes associated with OXPHOS defects in any patient with suspected OXPHOS dysfunction. This could perhaps bypass the need for tissue biopsy and enzyme analysis, although confirming pathogenicity of novel sequence variants will often still require functional analyses.

In summary, integration of all available information about the patient and family are necessary for guiding us towards the most appropriate investigations for discovering the molecular basis of mitochondrial disease. Pedigree, ethnicity, clinical presentation, results of laboratory and imaging investigations, OXPHOS enzymology and other functional studies, all contribute. Since it is impractical for most groups to test all genes, there is a great need to collaborate and share resources and expertise.

Note

Emphasising the rapid rate of progress in identifying nuclear genes causing mitochondrial disease, four additional genes were described in the period May to June 2008, following submission of this manuscript. These include a new subunit gene associated with Complex III deficiency (UQCRQ), the first nuclear subunit gene associated with Complex IV deficiency (COX6B1) and new subunit (NDUFA2) and assembly factor (C8orf38) genes associated with Complex I deficiency, namely:

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