The Clinical Manifestations and Molecular Mechanisms of Mitochondrial Neuro-Ophthalmological Disorders

Patrick Yu Wai Man

BMedSci (Hons), MBBS (Hons), MRCOphth

Published work submitted to Newcastle University for the degree of Doctor of Philosophy in the Faculty of Medical Sciences

2010

To

Mum, Dad and Cynthia

Table of Contents

List of Tables

List of Figures

Abstract

Autosomal dominant optic atrophy (DOA) classically presents with bilateral, symmetric visual failure in early childhood, with the pathological hallmark being the selective loss of retinal ganglion cells (RGCs). In the first population-based epidemiological study of DOA, we were able to estimate its minimum prevalence at 1 in 35,000 in the North of England. In independent case series from Northern Europe and North America, the majority of families with DOA harboured pathogenic *OPA1* mutations (50.0-57.6%), and large-scale *OPA1* rearrangements were present in only a small subgroup (11.1-12.9%). We also confirmed that *OPA3* mutations were very rare in non-syndromal DOA cases. Visual deterioration was observed in over half (54.2-67.4%) of all patients during long term follow-up, and the rate of visual decline varied markedly both between and within families.

In a large multi-centre study of 104 *OPA1*-positive patients from 45 independent families, we established that additional neuromuscular complications are common in *OPA1* disease, affecting up to 20% of all mutational carriers. Bilateral sensorineural deafness beginning in late childhood and early adulthood was a prominent manifestation, followed by a combination of ataxia, myopathy, peripheral neuropathy and chronic progressive external ophthalmoplegia (CPEO) from the third decade of life onwards. We also identified novel clinical presentations with spastic paraparesis mimicking hereditary spastic paraplegia, and a multiple sclerosis-like illness. Patients with these syndromal disease variants (DOA+) had a worse visual prognosis, and this was associated with a more pronounced reduction in retinal nerve fibre layer thickness compared to patients with pure DOA. Interestingly, there was a two- to three-fold

increased risk of developing DOA+ features with missense *OPA1* mutations and those located within the GTPase domain.

The molecular investigations of our DOA families have confirmed that *OPA1* mutations result in mitochondrial DNA (mtDNA) instability, with the accumulation of multiple mtDNA deletions causing cytochrome *c* oxidase (COX)-deficiency. The frequency of COX-negative fibres in skeletal muscle biopsies was over four times higher in the DOA+ group compared to the pure optic atrophy group. Importantly, COX-negative muscle fibres were also more frequent among patients with pure DOA compared to age-matched healthy controls. These observations clearly implicate a contributory role for these secondary mtDNA defects in triggering multi-system cellular dysfunction among affected *OPA1* carriers. Using a real-time, quantitative polymerase chain reaction assay, we subsequently found that patients with DOA+ features had significantly greater mtDNA proliferation in their COX-negative skeletal muscle fibres compared to patients with isolated optic neuropathy. Low levels of wild-type mtDNA molecules were present in COX-negative muscle fibres from both pure DOA and DOA+ patients, implicating haploinsufficiency as the mechanism responsible for the biochemical COX defect.

Although the accumulation of somatic, clonally-expanded mtDNA deletions is likely to be a key pathological player in DOA, we found no evidence of COX-deficiency or multiple mtDNA deletions in post-mitotic tissues from two *Opa1* mouse models. These results are not surprising as *OPA1* mutations are likely to exert other deleterious consequences at the cellular level. To explore these disease mechanisms further, we used *in vivo* phosphorus magnetic resonance spectroscopy (^{31}P-MRS) to specifically measure mitochondrial oxidative function in 17 *OPA1*-positive patients manifesting

both pure DOA (N = 9) and DOA+ phenotypes (N = 8). *OPA1* mutations impaired mitochondrial oxidative phosphorylation, but there was no significant difference in ^{31}P-MRS parameters between these two *OPA1* disease subgroups. Our observations clearly suggest that additional factors must be contributing to RGC loss and cellular dysfunction in other organ systems.

Primary open angle glaucoma is a progressive optic neuropathy characterised by the selective loss of RGCs, pathological optic disc cupping, and visual field defects. It has a major genetic basis and it shares striking clinical and pathological overlap with DOA. Since the OPA1 protein is closely linked with normal RGC function, we investigated whether *OPA1* single nucleotide polymorphisms (SNPs) influenced the risk of developing glaucoma. We found a strong association between two specific *OPA1* SNPs (IVS8+4c>t and IVS8+32t>c) and the risk of developing normal tension glaucoma, but not high tension glaucoma. These findings support a possible role for the OPA1 protein in the pathophysiology of normal tension glaucoma.

Leber hereditary optic neuropathy (LHON), with DOA, are the two most common inherited optic neuropathies seen in clinical practice. Over 90% of LHON cases are due to one of three primary mtDNA mutations: m.3460G>A (*MTND1*), m.11778G>A (*MTND4*), and m.14484T>C (*MTND6*). Visual loss in LHON is usually severe with little functional improvement. Using the Visual Function Index (VF-14) questionnaire, qualify of life among LHON carriers was quantified for the first time. LHON had the worst VF-14 score when compared to other acquired and inherited ophthalmological disorders, highlighting the significant visual impairment caused by this disorder. Patients with the m.14484T>C mutation had higher VF-14 scores compared with those harboring the m.3460G>A and m.11778T>C mutations, which is consistent with

previous observations that this primary mutation carries a better prognosis for visual recovery. LHON is a complex disease, and we systematically explored the role of potential environmental triggers in a large cohort of 196 affected and 206 unaffected carriers (N = 402) from 125 Northern European LHON pedigrees. We found a strong and consistent association between visual loss and smoking, with heavy smokers being more likely to be affected than light smokers. There was also a trend towards an increased risk of visual failure with alcohol, but only among heavy drinkers. Based on our results, asymptomatic LHON carriers should be strongly advised not to smoke and to moderate their alcohol intake.

Extraocular muscles (EOMs) are frequently involved in patients with mitochondrial genetic disorders and it is often the major cause of morbidity. In order to explore this selective vulnerability, we studied the mitochondrial changes seen in EOMs as part of the normal ageing process. COX-negative fibres were seen in EOMs from the third decade of life, and in the majority of these fibres (72.5%), mtDNA deletions were detected at levels over 70%. An exponential, age-related increase in COX-deficiency was observed in EOMs, and this occurred at a faster rate than skeletal muscle. In a parallel study, we investigated EOM and skeletal muscle biopsies from 13 patients with confirmed CPEO. We found significantly higher levels of COX-negative fibres in EOMs (41.6%) compared to skeletal muscle (13.7%), and this difference was linked to a lower mutational threshold for COX deficiency in EOMs. Because of their intrinsic biological properties, EOM fibres accumulate mtDNA deletions and COX deficiency at an accelerated rate as part of normal ageing. In the presence of an underlying mtDNA defect, this greater susceptibility is accentuated further, resulting in the prominent and often restricted EOM involvement seen in patients with CPEO.

Author's Declaration

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy. The research detailed within was performed between the years 2007-2010 and it was supervised by Professor Patrick F. Chinnery and Mr Philip G. Griffiths. I certify that none of the material offered in this thesis has been previously submitted by me for a degree or any other qualification at this or any other university.

Acknowledgements

The years spent working on this PhD were exciting, challenging, and packed full with new adventures around every corner!! I am greatly indebted to my two mentors, Professor Patrick Chinnery and Mr Philip Griffiths, who have shaped my professional and personal development over the past decade. I had the good fortune of meeting them as a medical student, and over these formative years, their sound advice, unwavering support, and pep talks have guided my steps through the sometimes daunting maze of academia and career choices. I am also very grateful to Dr Gavin Hudson, Dr Angela Pyle, Dr Lynsey Cree, and Dr Joanna Stewart for their help and patience, especially during my first few months on the bench. This list is certainly not exhaustive but the following people deserve special mention for their contribution to the work presented in this thesis: Professor Douglass Turnbull, Professor Robert Taylor, members of the mitochondrial diagnostic service, Dr Rita Horvath, Dr Grainne Gorman, Dr Matthew Kirkman, Mr Kamil Sitarz, Dr Michael Trenell, Dr Kieren Hollingsworth, Dr Marcela Votruba, Professor John Leigh, and all the clinical colleagues who have referred patients and provided research samples. Finally, I would like to express my gratitude to the Medical Research Council (UK) for providing me with a Clinical Research Training Fellowship and making this whole endeavour possible.

Abbreviations

AAV	Adenovirus-associated virus
ADP	Adenosine diphosphate
AGE	Agarose gel electrophoresis
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
ANT1	Adenine nucleotide translocator 1
ATP	Adenosine triphosphate
BCVA	Best corrected visual acuity
bp	Base pair
CF	Counting fingers
CGH	Comparative genomic hybridization
CMT	Charcot Marie Tooth
CoQ_{10}	Coenzyme Q_{10}
COX	Cytochrome *c* oxidase
CPEO	Chronic progressive external ophthalmoplegia
CSB	Conserved sequence block
Ct	Threshold cycle value
Da	Dalton
dCK	Deoxycytidine kinase
dGK	Deoxyguanosine kinase
DGUOK	Deoxyguanosine kinase

DIDMOAD	Diabetes insipidus and mellitus with optic atrophy and deafness
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dThd	Deoxythymidine
DOA	Dominant optic atrophy
dUrd	Deoxyuridine
EOM	Extraocular muscle
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide hydrogen
FMN	Flavin mononucleotide
FRDA	Friedreich ataxia
GED	GTPase effector domain
GTP	Guanosine triphophate
H&E	Haematoxylin and eosin
HSCT	Haematopoietic stem cell transplantation
HSMN	Hereditary sensory motor neuropathy
HSP	Hereditary spastic paraplegia
HTG	High tension glaucoma
HVR	Hypervariable region
IOP	Intraocular pressure
KDa	Kilodalton
LHON	Leber hereditary optic neuropathy
LogMAR	Logarithm of the minimum angle of resolution

M	Maternal
MDS	Mitochondrial depletion syndrome
MEF	Mouse embryonic fibroblast
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged red fibres
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MLPA	Multiplex ligation probe amplification assay
MMP	Matrix metalloproteinase
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
MtDNA	Mitochondrial DNA
MTERF	Mitochondrial transcription terminator factor
mtSSB	Mitochondrial single-stranded binding protein
NADH	Nicotinamide adenine dinucleotide hydrogen
NARP	Neurogenic weakness with ataxia and retinitis pigmentosa
nDNA	Nuclear DNA
NPDK	Nucleoside diphosphate kinase
NTG	Normal tension glaucoma
O_H	Heavy-strand origin of replication
O_L	Light-strand origin of replication

OCT	Optical coherence tomography
OD	Right eye
OMIM	Online mendelian inheritance in man
OPA1	Optic atrophy 1
OPA3	Optic atrophy 3
OPMD	Oculopharyngeal muscular dystrophy
OS	Left eye
OXPHOS	Oxidative phosphorylation
OU	Both eyes
PARL	Presenilin associated rhomboid-like protease
PCR	Polymerase chain reaction
PCr	Phosphocreatine
PEO1	Progressive external ophthalmoplegia 1
PGC	Primordial germ cell
Pi	Inorganic phosphate
PNA	Peptide nucleic acid
POAG	Primary open angle glaucoma
POLG	Polymerase gamma
POLRMT	Mitochondrial RNA polymerase
RFLP	Restriction fragment length polymorphism
RGC	Retinal ganglion cell
RI	Replication intermediate
RITOLS	Ribonucleotide incorporation throughout the lagging strand

RNA	Ribonucleic acid
RNFL	Retinal nerve fibre layer thickness
ROS	Reactive oxygen species
RRF	Ragged red fibre
RRM2B	Ribonucleotide reductase M2 B
rRNA	Ribosomal RNA
S	Sporadic
SCA	Spinocerebellar ataxia
SDH	Succinate dehydrogenase
SLC25A4	Solute carrier family 25, member 4
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SPG7	Spastic paraplegia 7
SUCLA2	Succinate-CoA ligase, ADP-forming, beta subunit
SUCLG1	Succinate-CoA ligase, alpha subunit
TAS	Termination associated sequence
TFAM	Transcription factor A
TK1	Thymidine kinase 1
TK2	Thymidine kinase 2
tRNA	Transfer RNA
TYMP	Thymidine phosphorylase
VDAC	Voltage dependent anion channel
VEP	Visual evoked potential

Chapter 1

Introduction

1.1 The Mitochondrion

1.1.1 Evolutionary Origin

Mitochondria are ubiquitous intracellular organelles and they fulfil a fundamental role by providing most of the adenosine triphosphate (ATP) requirements of eukaryotic cells (DiMauro and Schon, 2003). The prevailing endosymbiotic hypothesis suggests that mitochondria evolved from aerobic α-proteobacteria, which were then gradually assimilated by primitive glycolytic eubacteria (Margulis, 1971; Gray et al., 1999). During evolution, the α-proteobacteria gradually transferred the majority of their genetic material to the eubacteria's nuclear chromosomes, resulting in a symbiotic relationship (Gabaldon and Huynen, 2004). Phylogenetic comparison of mitochondrial DNA (mtDNA) between modern humans and other organisms, including *Rickettsia prowazekii*, an α-proteobacterium, support this common evolutionary origin for the mitochondrial genome (Martin and Muller, 1998; Gray et al., 1999).

1.1.2 Mitochondrial Structure

Mitochondria are tubular-shaped organelles bounded by an outer and an inner membrane, and these delineate two distinct compartments: an intermembrane space and an internal matrix space (**Figure 1**) (Alberts et al., 1994; Frey and Mannella, 2000). The outer membrane allows passive diffusion of low molecular weight molecules up to 10 kDa, and this permeability is conferred by a family of channel-forming proteins known as porins, or voltage dependent anion channels (VDAC). The inner membrane is highly convoluted and these multiple infoldings, known as cristae, greatly increase its effective surface area (Perkins et al., 1997). Compared to the outer membrane, the inner membrane is relatively impermeable except for specific active

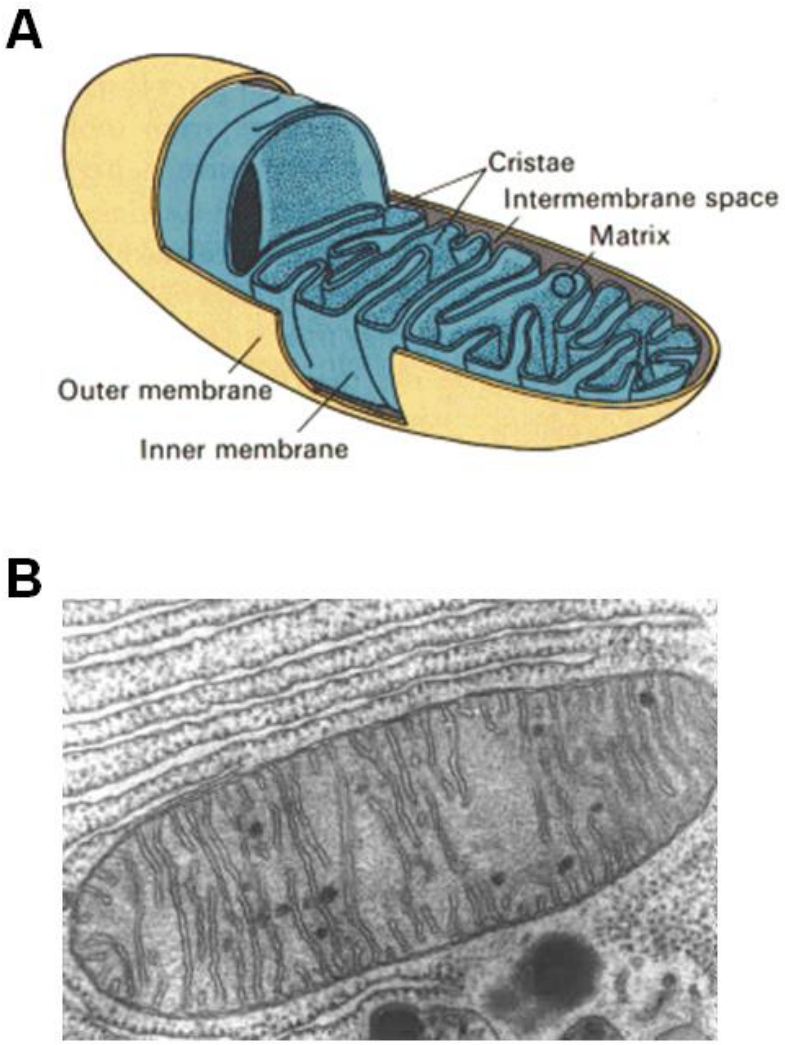

Figure 1. The major structural features of a mitochondrion: **(A)** Schematic representation, **(B)** Transmission electron micrograph. Reproduced from Alberts et al (1994).

transport channels, allowing an electrochemical gradient to be established across this barrier. The inner membrane also contains a number of highly specialised proteins, including the respiratory chain complexes and members of the mitochondrial

membrane protease family. The mitochondrial matrix compartment contains mtDNA molecules within nucleoid structures, and it is also the site of multiple metabolic pathways essential for normal cellular function: the citric acid cycle, β-oxidation of fatty acids, steroid, and amino acid biosynthesis (Raha and Robinson, 2000).

1.1.3 Oxidative Phosphorylation

The mitochondrial respiratory chain comprises five multi-subunit polypeptide complexes (I-V) which are embedded within the inner mitochondrial membrane (**Figure 2**) (Nijtmans et al., 2004). The production of ATP is tightly regulated and it is the end-product of a process known as oxidative phosphorylation (OXPHOS). Acetyl-CoA, an intermediate product of glycolysis and β-oxidation, is metabolised further by the citric acid cycle to the reducing equivalents nicotinamide adenine dinucleotide hydrogen (NADH) and flavin adenine dinucleotide hydrogen ($FADH_2$) (DiMauro and Schon, 2003). NADH and $FADH_2$ are then re-oxidised by donating electrons to complexes I and II respectively. The energy released by the shuttling of these high energy electrons along the respiratory chain complexes allows protons to be pumped from the matrix compartment into the intermembrane space. Two additional carriers, ubiquinone (Coenzyme Q_{10}) and cytochrome *c*, also play critical roles in the efficient transfer of electrons through these successive oxidation-reduction reactions. The electrochemical gradient generated across the inner mitochondrial membrane is then used by complex V (ATP synthase) to catalyze the conversion of adenosine diphosphate (ADP) and inorganic phosphate (Pi) to ATP.

1.1.3.1 Complex I

Complex I (NADH-ubiquinone oxidoreductase) is an L-shaped molecule with two arms, one in the same plane as the inner mitochondrial membrane, and the other projecting into the mitochondrial matrix (Grigorieff, 1999; Hirst et al., 2003). It is the largest respiratory chain complex, with a total molecular mass of 980 kDa, and it consists of ~ 46 subunits, seven of which are mitochondrially-encoded (**Table 1**). Complex I contains the co-enzyme flavin mononucleotide (FMN) and eight iron-sulphur complexes involved in the electron transfer process (Koopman et al., 2007).

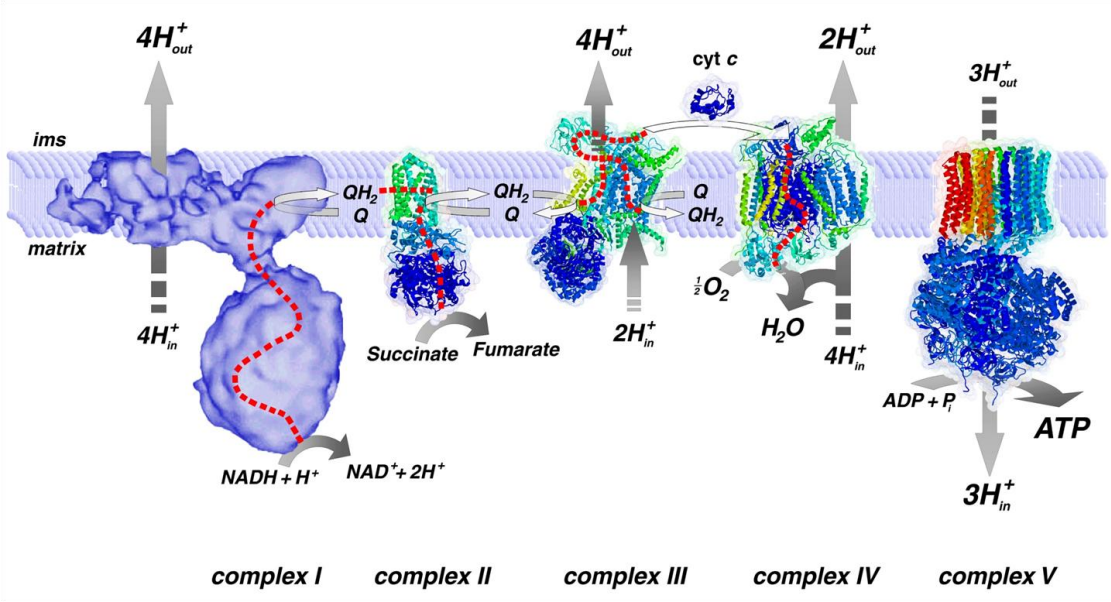

Figure 2. The mitochondrial respiratory chain and oxidative phosphorylation. Reproduced from Nijtmans et al (2004).

1.1.3.2 Complex II

Complex II (Succinate-ubiquinone oxidoreductase) has unique features which distinguish it from the other respiratory chain complexes. It is not a proton pump and it consists of four subunits (A-D), which are all nuclear-encoded (Hatefi and Galante, 1980; Yue et al., 1991). Complex II is the smallest respiratory chain complex, with subunits A (73kDa) and B (27kDa) located on the matrix side, and two smaller hydrophobic subunits, C and D, acting as anchoring membrane proteins (Lancaster and Kroger, 2000). Complex II contains various prosthetic groups: a covalently-bound flavin adenine dinucleotide (FAD) coenzyme (Subunit A), three iron-sulphur centres (Subunit B), and haem groups (Subunits C and D).

1.1.3.3 Complex III

Complex III (Ubiquinol-cytochrome *c* oxidoreductase) has a dimeric structure with a molecular weight of 480 kDa (Acin-Perez et al., 2004). Each monomer has 11 different polypeptide subunits, of which only one is mitochondrially-encoded (Cytochrome *b*). It contains various active redox prosthetic groups: cytochrome *b* which contains two haem groups, cytochrome c_1, and iron-sulphur clusters (Rieske protein), which assemble into a functional structure known as the cytochrome bc_1 complex (Yu et al., 1999).

1.1.3.4 Complex IV

Complex IV (Cytochrome *c* oxidase) has 13 subunits, and three of these subunits are encoded by the mitochondrial genome (Kadenbach et al., 1983; Tsukihara et al., 1996). It is a water soluble haemoprotein complex, 204 kDa in size, containing two non-covalently bound haem groups (Cytochromes *a* and a_3), and two copper centres (Cu_A

and Cu_B). Complex IV acts as the terminal electron acceptor, with cytochrome a_3 and Cu_B forming a binuclear centre for oxygen reduction. These catalytic sites bind oxygen in a reaction which utilises four electrons, resulting in four substrate protons being converted to two molecules of water (Faxen et al., 2005).

1.1.3.5 Complex V

Complex V (ATP synthase) has an approximate molecular mass of 500 kDa, and it is made up of 16 different subunits, two of which are mitochondrially-encoded (von Ballmoos et al., 2009). These subunits assemble into two main structural units: F_o which is embedded in the inner mitochondrial membrane, and the catalytic domain F_1 which is located in the matrix compartment. F_1 is composed of 5 different subunits α, β, γ, δ and ε, with three α and three β subunits arranged alternately to form a cylinder $(\alpha\beta)_3$ around γ (Abrahams et al., 1994; Yoshida et al., 2001). The F_o structure has an $a_1b_2c_{12}$ stoichometry, with a cylindrical arrangement of *c* subunits bounded peripherally by subunits *a* and *b*. The subunit *c* ring is also linked to the γ subunit of the F_1 core both directly, and indirectly through mutual contacts with the ε subunit. The current working model of ATP synthase is based on a rotor-stator mechanism, with the rotor consisting of a c_{12} oligomer, and the stator arm being formed by an ab_2 complex and subunit δ (Gibbons et al., 2000; Ko et al., 2000). The translocation of protons by complexes I, III and IV creates an electrochemical gradient across the inner mitochondrial membrane, and this proton motive force is used to generate F_o rotatory movements. The generated torque alters the conformational states of the β subunits, via the rotor and stator, eventually resulting in the synthesis of ATP molecules within F_1 (Yoshida et al., 2001).

Table 1. The constituent subunits of the mitochondrial respiratory chain complexes.

Respiratory Chain Complex	Gene Location	
	MtDNA	**Nuclear DNA**
Complex I	7	~ 39
Complex II	0	4
Complex III	1	10
Complex IV	3	10
Complex V	2	14

1.2 Mitochondrial Genetics

1.2.1 Mitochondrial DNA

Mitochondria are unique in having their own genome in the form of a circular, double-stranded molecule 16,569 base pairs long (**Figure 3**) (Anderson et al., 1981; Andrews et al., 1999; Strachan and Read, 1999). It is a high-copy genome, with 2-10 mtDNA molecules in each mitochondrion, and hundreds to thousands of mitochondria per cell depending on their specific energy requirements (Chinnery et al., 1999a; Kirkman et al., 2008). MtDNA replicates continuously and this process is independent of the nuclear genome, occurring in both mitotic and post-mitotic cells. The mitochondrial genome codes for 2 ribosomal RNAs (12S and 16S rRNA), 22 transfer RNAs (tRNAs), and 13 polypeptide subunits of the respiratory chain complexes (Taylor and Turnbull, 2005). The majority of these genes are located on the H-strand, with only *MTND6* and eight tRNA genes found on the L-strand. MtDNA is a very compact molecule, with

overlapping gene regions, and the absence of introns. As a result of these distinct characteristics, tRNA genes often act as punctuation markers during the processing of polycistronic mRNA transcripts (Ojala et al., 1980b; Montoya et al., 1981; Ojala et al., 1981). However, the mitochondrial genome does contain a non-coding region, known as the D-loop, which is involved in mtDNA transcription and replication. This 1.1 Kb region also contains the regulatory elements for mtDNA expression, with binding sites for mitochondrial transcription factor A (TFAM), three conserved sequence blocks (CSB), and a termination associated sequence (TAS) (Sbisa et al., 1997; Roberti et al., 1998).

1.2.2 Mitochondrial Haplogroups

The mitochondrial genome accumulates mutations at a significantly faster rate compared to the nuclear genome (Howell et al., 1996; Jazin et al., 1998). Several factors contribute to this higher mutational rate: the absence of protective histones, the lack of effective repair mechanisms, and the close proximity of mtDNA molecules to the respiratory chain complexes where they are exposed to high levels of reactive oxygen species (ROS) (Raha and Robinson, 2000). The mutational rate varies between different mtDNA regions, and it is much faster within the two hypervariable regions (HVR I and II) of the D-loop where a mutation is estimated to occur every 30 maternal generations (Parsons et al., 1997; Siguroardottir et al., 2000). MtDNA is therefore highly polymorphic, and during human evolution, a number of relatively benign mitochondrial sequence variants have become fixed in different populations. As mtDNA is maternally inherited, these polymorphisms have accumulated sequentially along radiating female lineages, following the pattern of human migration from Africa into the various continents some 150,000 years ago (Cann, 2001). The human

phylogenetic tree contains 18 major mtDNA haplogroups, and these comprise a total of 497 haplogroup-defining polymorphic variants (Torroni and Wallace, 1994; Herrnstadt and Howell, 2004). Individuals of European ancestry belong to one of nine haplogroups: H, I, J, K, T, U, V, W and X, with haplogroup H accounting for nearly half of all cases (Torroni and Wallace, 1994; Herrnstadt et al., 2002).

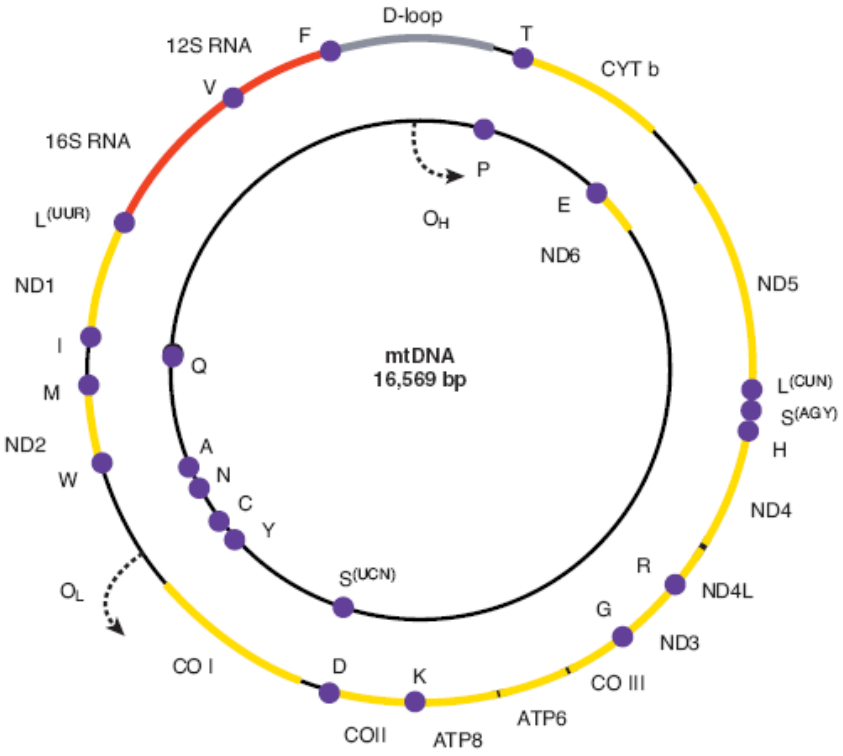

Figure 3. The human mitochondrial genome. Protein coding (yellow), rRNA (red), and tRNA (purple) genes are depicted on the heavy (H-, outer) and light (L-, inner) strands. The 22 tRNAs are indicated by their cognate amino acid letter code and the 2 rRNAs by their sedimentation coefficients (12S and 16S). The origins of mtDNA replication and the direction of synthesis are denoted by O_H for the H-strand, and O_L for the L-strand. Reproduced from Kirkman et al (2008).

1.2.3 Mitochondrial DNA Mutations

The first pathogenic mutations linked to human disease were identified in 1988. In two landmark papers, Holt and colleagues found mtDNA deletions in nine patients with mitochondrial myopathy (Holt et al., 1988), and Wallace and colleagues confirmed the m.11778G>A point mutation as the causative genetic defect in families with Leber hereditary optic neuropathy (LHON) (Wallace et al., 1988). Over the next two decades, a large number of pathogenic mtDNA variants have been identified, both point mutations and large-scale rearrangements: deletions, insertions, duplications and inversions (DiMauro and Schon, 2003; Taylor and Turnbull, 2005). The distribution of these pathogenic mutations is not uniform, and several, so-called "mutational hotspots" have been identified, which have been associated with specific clinical phenotypes. For example, *MTND1* and *MTND6* harbour the primary m.3460G>A and m.14484T>C LHON mutations respectively, but several, other LHON-causing mutations have also been identified in these two gene regions (Yu-Wai-Man et al., 2009; Fraser et al., 2010). The genetic heterogeneity and phenotypic variability of primary mtDNA disorders represent significant diagnostic challenges, and these are detailed further in later sections of this review.

1.2.4 Mitochondrial Heteroplasmy

There are ~ 10,000 mtDNA molecules per cell, with each mitochondrion containing multiple copies. Two possible situations can therefore arise, known as homoplasmy and heteroplasmy (Lightowlers et al., 1997; Chinnery, 2002). In the heteroplasmic state, two or more mtDNA variants are present at a specific nucleotide position, and the same phenomenon can also occur for mtDNA re-arrangements. Using different molecular assays, heteroplasmy can be quantified in a tissue both at the homogenate

and at the single-cell level, and it is not unusual for significant variations to be observed between different tissues from the same individual (Chinnery et al., 1999c; Krishnan et al., 2007; Greaves et al., 2010). These regional differences are thought to partly contribute to the variable clinical features observed in patients with mitochondrial disorders (Chinnery et al., 1999c; McFarland et al., 2002).

1.2.5 Threshold Effect

Most pathogenic mtDNA mutations are heteroplasmic, a feature which supports the concept of a mutational threshold for pathogenicity (Chinnery et al., 2000a; Taylor and Turnbull, 2005). The relationship between mutational load and respiratory chain activity has been extensively investigated in different tissues, and the deleterious consequences of most mtDNA mutations on OXPHOS usually become apparent when the proportion of the mutant species exceeds 60% to 80% (Shoubridge et al., 1990; Bua et al., 2006; Durham et al., 2007). There are mutation- and tissue-specific variations in this biochemical threshold (Corral-debrinski et al., 1992; Wang et al., 2001; Nekhaeva et al., 2002), and although these could account for the pattern of organ involvement and clinical severity associated with a particular genetic defect, the molecular mechanisms are much more complex. A notable example is LHON, which is the most common of the primary mtDNA diseases (Man et al., 2003; Schaefer et al., 2004; Schaefer et al., 2008). The majority of patients (90-95%) harbour one of three mtDNA point mutations: m.3460G>A (*MTND1*), m.11778G>A (*MTND4*), and m.14484T>C (*MTND6*), but only ~ 50% of male carriers, and ~ 10% of female carriers will experience visual loss during their lifetime (Yu-Wai-Man et al., 2009; Fraser et al., 2010). Although the risk of blindness is minimal if the mutational load is less than 60% (Chinnery et al., 2001), the majority of unaffected carriers (85-90%) are homoplasmic

for the primary LHON mutation (Smith et al., 1993; Harding et al., 1995; Man et al., 2003), clearly implicating other precipitating factors for optic nerve degeneration.

1.3 Mitochondrial Inheritance

1.3.1 Maternal Transmission

MtDNA is maternally inherited as sperm mitochondria are destroyed by an efficient ubiquitin-dependant mechanism following successful oocyte fertilization (Giles et al., 1980; Sutovsky et al., 1996). Although this fact remains central to genetic counselling of families with primary mtDNA disorders, there is one confirmed report of paternal inheritance in the literature (Schwartz and Vissing, 2002). In this unique case, a 28-year-old man with mitochondrial myopathy was found to harbour a novel 2-base pair (bp) mtDNA deletion in *MTND2*, and remarkably, the mutation was shown to be of paternal origin. It was heteroplasmic, present at levels of 90% in the patient's muscle mtDNA, but was absent in other tissues including blood, hair roots, and cultured fibroblasts. Subsequent studies of larger patient cohorts did not reveal evidence of paternal mtDNA transmission, confirming that this is a rare molecular occurrence (Taylor et al., 2003c; Schwartz and Vissing, 2004).

1.3.2 Mitochondrial Bottleneck

Mothers harbouring heteroplasmic mtDNA variants can transmit a wide range of heteroplasmy levels to their offpsring, and these rapid shifts in mitochondrial allele frequencies have been explained by a "mitochondrial bottleneck" in the germline (Jenuth et al., 1996; Jenuth et al., 1997). This bottleneck is thought to be a protective mechanism which allows the rapid removal of deleterious mtDNA mutations from the genetic pool (Khrapko, 2008; Cree et al., 2009). A pathogenic mtDNA variant would

either be lost during transmission to the next generation, or it would quickly reach suprathreshold levels within an oocyte, resulting in developmental arrest and its likely elimination. Even if a mature oocyte carrying a high proportion of the mutant species is successfully fertilised and a live birth results, there is a high probability that the affected individual's fertility will be subnormal, which again serves to limit the transmission of mtDNA mutations.

A fertilised oocyte contains ~ 250,000 copies of the mitochondrial genome, and from a population genetic perspective, the rate of genetic drift for such a large pool of mtDNA molecules would be very slow (Khrapko, 2008; Cree et al., 2009). Based on recent experimental evidence, different models have been put forward to explain how the rapid segregation of new genotypes ascribed to the mitochondrial bottleneck could arise. Although there is still some controversy regarding the actual mechanisms and developmental timing, some broad observations can still be made (Khrapko, 2008; Samuels et al., 2010). Following fertilisation, rapid cell division occurs in the absence of mtDNA replication, and the mtDNA pool is simply distributed to the daughter cells in these initial phases of mammalian embryogenesis. At around day 7.25 post conception (E7.25) in mice, and E20 in humans, the first committed germ-line precursor cells in mammals become identifiable, know as primordial germ cells (PGCs). In three independent studies in mice, mtDNA copy number was determined at different stages of germ cell formation and different results were obtained (Cao et al., 2007; Cree et al., 2008; Wai et al., 2008). In one study by Cao and colleagues, the mtDNA content of PGCs at E7.5 was estimated at ~ 2,000 copies (Cao et al., 2007). Although this represents a significant decrease in the mtDNA population, it was still ten times higher than the predicted value of ~ 200 required to generate the rapid shifts in allele frequencies previously observed in heteroplasmic mice transmitting neutral

mtDNA polymorphisms. The authors of that study concluded that the actual segregating units were not individual mtDNA molecules, but were in fact aggregates of several mtDNA molecules packaged together in structures known as nucleoids (Cao et al., 2007). In a second study by Cree and colleagues, which used different experimental techniques for the detection and isolation of PGCs, the median mtDNA content of individual PGCs at E7.5 was 203 (Cree et al., 2008). The rapid genetic drift in the germ line could therefore be explained by this dramatic reduction in copy number, without the need for nucleoids. In a third study, a completely different conclusion was reached by Wai and colleagues, and the genetic bottleneck was proposed to occur during postnatal folliculogenesis, and not during prenatal embryonic oogenesis (Wai et al., 2008). A better understanding of the mitochondrial genetic bottleneck is essential to current attempts at preventing the transmission of pathogenic mtDNA mutations, and additional studies are currently underway to clarify these fundamental research questions (Khrapko, 2008).

Irrespective of the actual nature of the mitochondrial genetic bottleneck, the fate of a specific mtDNA variant also depends on whether mtDNA segregation is a truly stochastic event, or whether there is active selective pressures either for or against the variant. A number of studies in both mice (Jenuth et al., 1996; Jenuth et al., 1997), and humans (Chinnery et al., 2000b; Brown et al., 2001), support random genetic drift for both neutral and pathogenic mtDNA variants in the female germline. However, two recent reports have led to a re-evaluation of the role of selection in the transmission of mtDNA variants. The mutator mouse is homozygous for a knock-in allele expressing a proofreading-deficient mtDNA polymerase gamma (*PolgA*), as a result of which it accumulates high levels of somatic mtDNA point mutations. In this mouse model, transmission of heteroplasmic mtDNA point mutations were subject to strong purifying

selection, with the rapid loss of non-synonymous mutations involving protein coding genes, compared to those located within tRNA and rRNA genes (Stewart et al., 2008). Similarly, in a trans-mitochondrial mouse model, a homoplasmic *MTND6* point mutation, with a marked effect on complex I activity, was rapidly eliminated from the maternal germline, whereas a milder *MTCOI* mutation was successfully transmitted over successive generations (Fan et al., 2008). As for most biological phenomena, the situation is likely to be more complex, and it is possible that random genetic drift and selection operate in different settings, influenced by mutation-, tissue-, and species-specific factors (Cree et al., 2009; Fukui and Moraes, 2009).

1.4 Mitochondrial DNA Maintenance

1.4.1 Mitochondrial Nucleoids

MtDNA molecules do not exist in isolation but they are packaged within intricate replicative structures known as nucleoids (Gilkerson, 2009). The components of these nucleoid structures are being actively investigated, and the mitochondrial protein TFAM has been identified as a major nucleoid-organizing protein (Wang and Bogenhagen, 2006; Bogenhagen et al., 2008; Kasashima et al., 2008). Several proteins with critical roles in mtDNA replication are also associated with mammalian nucleoids, and these include: (i) the mitochondrial polymerase-γ (POLG), (ii) the twinkle $5' \rightarrow 3'$ helicase, which unwinds double-stranded mtDNA at the replication fork, (iii) the mitochondrial single-stranded binding (mtSSB) protein, and (iv) the accessory topoisomerase and ligase proteins (Tyynismaa et al., 2004; Hudson and Chinnery, 2006). Other potential nucleoid-associated proteins include ATAD3, which is thought to bind to the mitochondrial D-Loop, regulating both mtDNA transcription

and replication (He et al., 2007), and the pro-apoptotic p53 protein, which mediates important signalling pathways activated by DNA damage and cellular hypoxia (Yoshida et al., 2003). Although this core list is constantly being updated, there is a degree of controversy, centred on the purification methods and the cell lines used, about which of these proteins truly represent *bona fide* nucleoid-associated proteins. Additional studies are needed to clarify the processes which control the assembly and structural organization of mitochondrial nucleoids.

1.4.2 Mitochondrial DNA Replication

MtDNA replication is independent of the phase of the cell cycle, occurring in both mitotic and post-mitotic tissues, albeit at different rates (Wang et al., 1997). Two main models have been proposed for replication of the mitochondrial genome: an asynchronous, strand-displacement mode of replication (Clayton, 1982), and a synchronous, strand-coupled mode of replication (Holt et al., 2000). This area of mitochondrial research remains highly controversial, and there is currently no agreement on which model actual operates *in vivo*.

According to the Clayton model, replication is initiated within the D-Loop at the heavy-strand origin of replication (O_H), and it results in the formation of a "leading" H-strand (Lee and Clayton, 1998; Brown et al., 2005). The extension of this single DNA strand proceeds in a clockwise direction until the light-strand origin of replication origin (O_L) becomes exposed (**Figure 4a-c**). Replication of the "lagging" L-strand then starts in the opposite direction, with replication of the "leading" H strand being nearly two-thirds complete. Due to the asynchronous nature of this mechanism, one of the two daughter mtDNA molecules will be completed first, with the entire replication cycle estimated at about two hours.

A modification of the Clayton model has recently been proposed termed RITOLS for ribonucleotide incorporation throughout the lagging strand (Yasukawa et al., 2006). This RITOLS model is similar to the original strand-displacement mode of replication, with a significant delay between leading and lagging strand synthesis. However, the defining feature of RITOLS is the incorporation of RNA, instead of DNA, throughout the lagging strand (**Figure 4**). During the maturation phase, these RNA segments are then converted to DNA, the conversion process starting in the vicinity of the O_L (Yang et al., 2002; Yasukawa et al., 2006).

The strand-coupled model of mtDNA replication is a more novel proposition put forward by Holt and colleagues (Holt et al., 2000; Holt et al., 2004). Using two-dimensional agarose gel electrophoresis (2D-AGE), this group of investigators studied the nature of partially single-stranded replication intermediates (RIs) detected in mammalian mitochondria. The predominant RIs they identified were consistent with a model of replication where synthesis of both the H- and L-strands occurred simultaneously, starting within a single zone of replication initiation (oriZ), and proceeding in parallel around the mitochondrial genome (**Figure 4d**). However, it is important to note that the original study by Holt and colleagues (Holt et al., 2000), outlining the synchronous OriZ mode of replication, does not preclude the joint occurrence, at the cellular level, of the more conventional strand-coupled model of mtDNA synthesis.

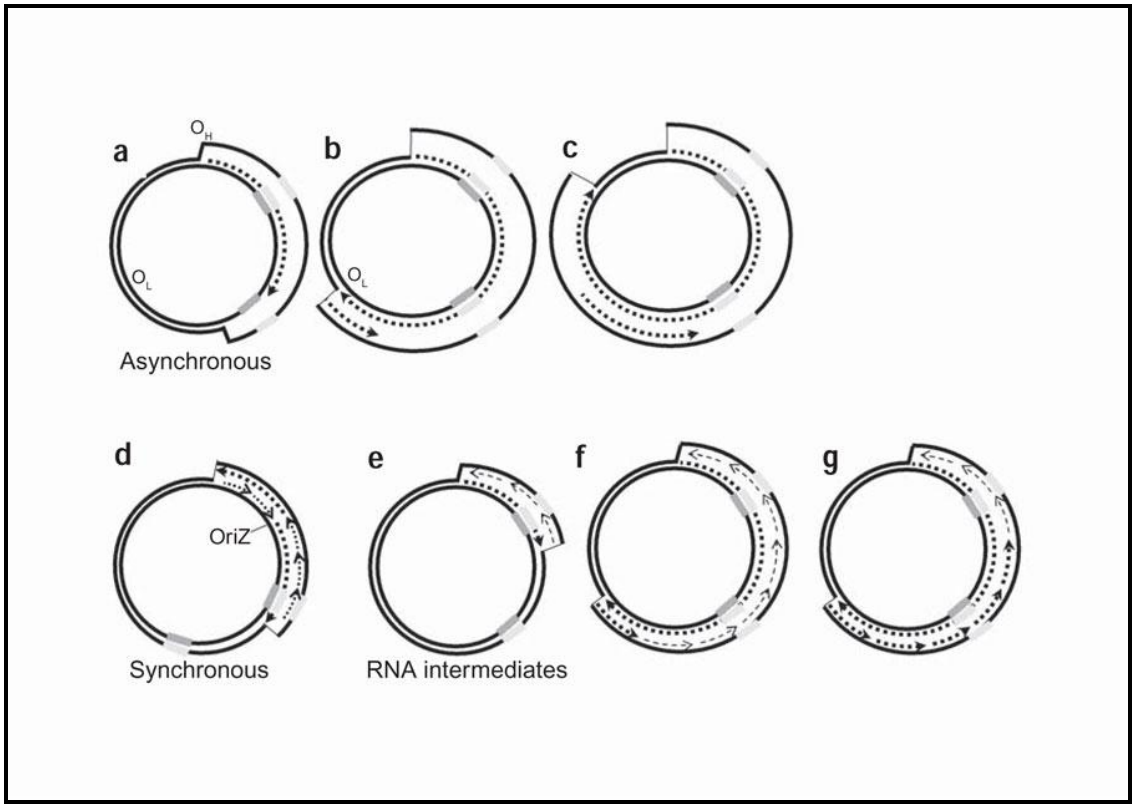

Figure 4. Proposed models of mtDNA replication: **(a-c)** Asynchronous model. Replication begins in the D loop at O_H and displaces the light strand from the heavy strand. The light strand is single stranded until synthesis of the nascent heavy strand exposes O_L. At this point, replication of the light strand begins in the opposite direction until both strands have been fully replicated; **(d)** Synchronous or strand-coupled model. Replication is initiated within a single zone of replication (oriZ) and proceeds bi-directionally via conventional coupled leading- and lagging-strand synthesis; **(e-g)** RITOLS model. Replication starts in the non-coding region close to or at O_H. The RITOLS model is similar to the asynchronous model of mtDNA replication, but RNA intermediates are produced (dashed lines) on the light strand before being converted to DNA. Reproduced from Krishnan et al (2008).

1.4.3 Mitochondrial Nucleotide Pools

The faithful replication of the mitochondrial genome is critically dependent upon a balanced supply of deoxyribonucleotides (dNTPs), the ‘‘building blocks’’ of mtDNA. Mitochondria are unable to synthesize dNTPs *de novo* and there is a constant need for the right balance of dNTPs to sustain mtDNA replication, which occurs throughout the cell cycle (Alberio et al., 2007). It is therefore not surprising that over the past decade, several nuclear genes have been identified (*TYMP*, *DGUOK*, *TK2*, *SUCLA2*, *SUCLG1*, and *RRM2B*), which cause mitochondrial depletion syndromes (MDS) due to their deleterious consequences on the synthesis and maintenance of these intramitochondrial nucleotide pools (**Figure 5**) (Ashley et al., 2007; Spinazzola and Zeviani, 2009).

There are distinct nucleotide pools for the nuclear and mitochondrial genomes. The mitochondrial dNTPs are formed by the salvage of deoxyribonucleosides, a process mediated by four different deoxynucleoside kinases (Rampazzo et al., 2004). Thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) are located in the cytosol, and their products are imported into the mitochondrial matrix in order to become available for mtDNA synthesis. Thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) are present within the mitochondrial matrix, and they catalyze the salvage pathways for pyrimidine and purine nucleosides, respectively. These mitochondrion-specific salvage pathways are very important as they represent the major source of dNTP precursors in stable tissues such as liver, brain, and muscle. Consistent with these critical functions, two *Tk2*-defective mouse models have recently been reported, characterised by mtDNA depletion in a range of tissues, and death within two to four weeks secondary to multi-organ failure (Akman et al., 2008; Zhou et al., 2008).

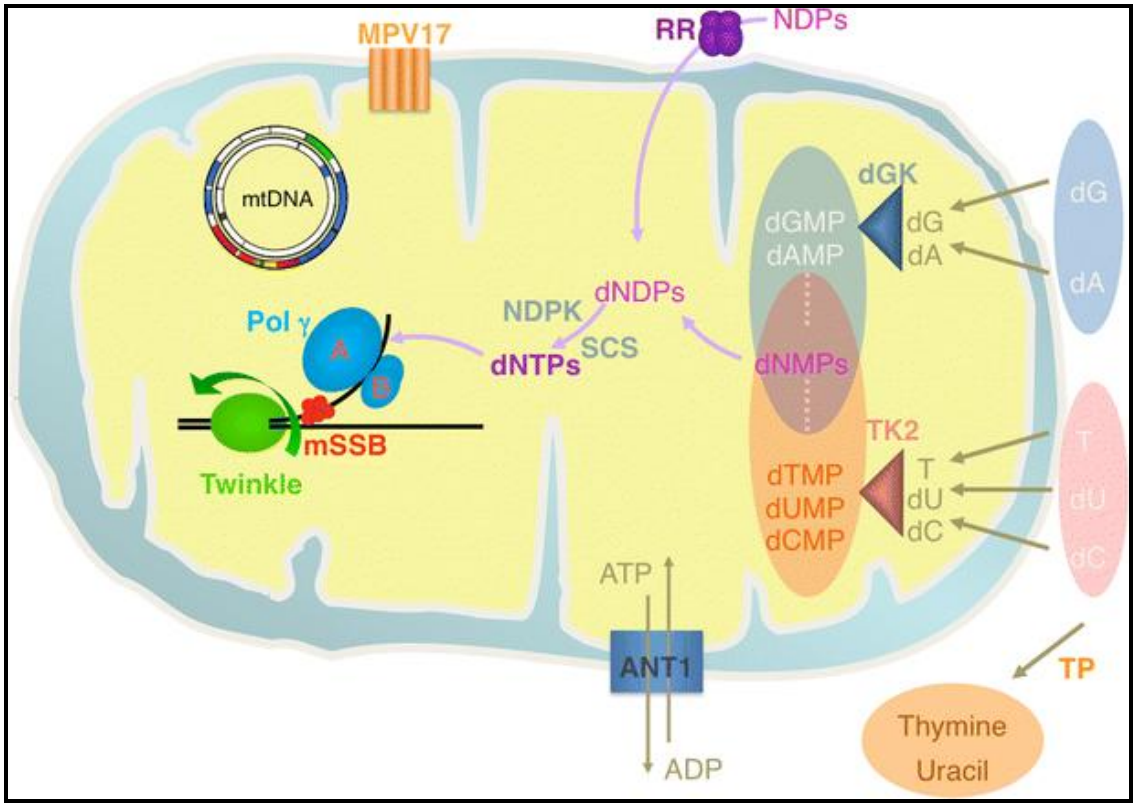

Figure 5. The major metabolic and enzyme pathways responsible for maintaining the intramitochondrial nucleotide pools. ANT1: adenine nucleotide translocator 1; dNMP, dNDP, and dNTP: mono-, di- and tri-phosphate deoxynucleotides; mtSSB: mitochondrial single-stranded binding protein; NDPK: nucleoside diphosphate kinase; RR: ribonucleotide reductase; SCS: succinate CoA synthase isoforms encoded by *SUCLA2* and *SUCLG1*; TP: thymidine phosphorylase; T, dU, dC, dG, and dA: deoxynucleosides. Reproduced from Spinazzola et al (2009).

RRM2B codes for the small p53-inducible subunit of ribonucleotide reductase (RR), a heterotetrameric cytosolic enzyme involved in the terminal steps of *de novo* dNTP synthesis. The ribonucleotide reductase enzyme converts ribonucleoside diphosphates

into the corresponding deoxyribonucleoside diphosphates during the S phase of the cell cycle (Tanaka et al., 2000). SCS-A and SCS-G, which are encoded by *SUCLA2* and *SUCLG1*, are also involved in the last step of the mitochondrial dNTP salvage pathway (Alberio et al., 2007; Spinazzola and Zeviani, 2009). Both proteins are associated with nucleoside diphosphate kinase (NDPK), a ubiquitous protein kinase which catalyzes the exchange of terminal phosphates between tri- and di-phosphoribonucleosides, maintaining the homeostasis of ribonucleotides and deoxyribonucleotides within mitochondria (Parks et al., 1973).

1.4.4 Regulation of MtDNA Copy Number

The mitochondrial genome is a high-copy number genome, and changes in mtDNA copy number are closely linked with disease state, with mitochondrial depletion representing one end of the spectrum, and mitochondrial proliferation often observed as a compensatory cellular mechanism (Spinazzola and Zeviani, 2009). Relatively little is currently known about the mechanisms which "sense" mtDNA copy number, and the nuclear-mitochondrial interactions involved still need to be clarified (Moraes, 2001). Although an over-simplification, the main players which control mtDNA copy number have been divided into three main groups: (i) structural nucleoid proteins, for example TFAM (**Sections 1.4.1**), (ii) proteins directly involved in the mtDNA replication machinery (**Sections 1.4.2**), and (iii) proteins which regulate nucleotide metabolism (**Sections 1.4.3**). The central role played by the TFAM protein was confirmed in a series of experiments involving heterozygous and homozygous *Tfam* knockout mice (Larsson et al., 1998). Cardiac tissue from heterozygous *Tfam* mice had a reduced mtDNA content, which was associated with a respiratory chain defect, whereas the homozygous mutant embryos were embryonically lethal, dying at E10.5 due to severe

mtDNA depletion. Although speculative, some investigators have also argued for the existence of rate limiting steps in mtDNA replication, on the premise that certain key mediators are only present in fixed amounts, and therefore can only support a certain number of mtDNA replication cycles (Moraes, 2001).

1.5 Mitochondrial Transcription

Mitochondrial transcription is initiated at three major sites, located within 150 bp of each other in the D-Loop; one on the L-strand (IT_L) at nucleotide position 407, and two on the H-strand (IT_{H1} and IT_{H2}) at nucleotide positions 561 and 638, respectively (Montoya et al., 1981; Montoya et al., 1982; Martin et al., 2005). All three initiation sites contain a short consensus sequence, 15 bp long, which is essential for transcription (Chang and Clayton, 1984; Chang and Clayton, 1985). The two H-strand initiation sites allow for independent rRNA and mRNA synthesis. IT_{H1} is more frequently used, producing two tRNAs ($tRNA^{Phe}$ and $tRNA^{Val}$), and the two mitochondrial rRNAs, whereas initiation of transcription at IT_{H2} results in a large polycistronic mRNA molecule corresponding to almost all of the H-strand (Montoya et al., 1983). This primary mitochondrial transcript is interspersed by tRNAs, which act as punctuation marks, delineating the rRNA and protein-coding mRNA sequences, and allowing for accurate endodonucleolytic cleavage (Ojala et al., 1980a; Ojala et al., 1981; Rossmanith et al., 1995).

Mitochondrial transcription involves a highly intricate machinery and several key proteins have been identified. One of these is the mitochondrial RNA polymerase (POLRMT), which is recruited to the promoter sites by TFAM (Tiranti et al., 1997; Diaz and Moraes, 2008). TFAM itself is bound to regulatory elements upstream of the

three initiation sites (IT_{H1}, IT_{H2}, and IT_L) to form a protein-DNA complex (Fisher et al., 1987). Two other important mitochondrial transcription factors, TFB1M and TFB2M, must also be present for transcription to occur effectively (Falkenberg et al., 2002; Shoubridge, 2002; Ekstrand et al., 2004). TFB1M and TFB2M are thought to directly interact with POLRMT, forming a hetero-dimeric structure, which is then delivered to the promoter regions occupied by TFAM (Asin-Cayuela and Gustafsson, 2007). Termination of mtDNA transcription is dependent on the mitochondrial transcription terminator factor (MTERF), which occurs in both active and inactive forms within mitochondria (Asin-Cayuela et al., 2004). More recently, a new transcription terminator factor has been identified (MTERF3), which blocks the initiation of mtDNA transcription *in vivo* (Park et al., 2007).

1.6 Mitochondrial Translation

Mitochondrial translation is poorly understood, and the lack of a reliable *in vitro* system has severely limited progress in this area of research. The vast majority of subunits (~ 90) that make up the mitochondrial ribosomes are encoded by the nuclear genome, with only two subunits, 12S and 16S, being of mitochondrial origin (O'Brien, 2003). Translation of mitochondrial transcripts is initiated by $IF2_{mt}$, which promotes binding of $tRNA^{Met}$ to the small ribosomal subunit, and $IF3_{mt}$, which facilitates the formation of the ribosomal initiation complex (**Figure 6**) (Christian et al., 2009). The extension of the nascent polypeptide chain has so far been shown to involve three mitochondrial elongation factors: $EF\text{-}G1_{mt}$, $EF\text{-}Tu_{mt}$, and $EF\text{-}Ts_{mt}$, all of which have strong homology to their bacterial counterparts (Rorbach et al., 2007). Mitochondrial mRNAs contain four stop codons: UAA, UAG, AGA and AGG, but it is not clear how termination of mitochondrial translation is activated. One hypothesis is that when the

ribosomal machinery encounters a stop codon, release factors induce hydrolysis of the newly formed polypeptide (Rorbach et al., 2007; Christian et al., 2009). So far, only one of these putative release factors, $RF1a_{mt}$, has been conclusively shown to terminate mRNA translation, both *in vitro* and *in vivo* (Soleimanpour-Lichaei et al., 2007).

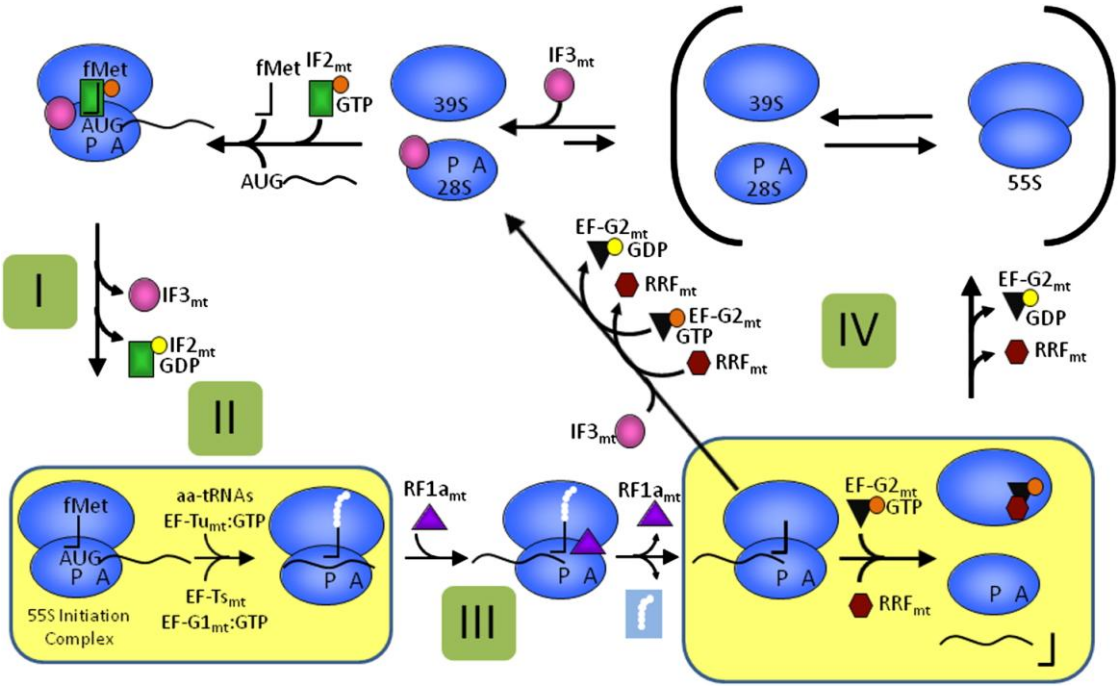

Figure 6. Current model of mitochondrial protein synthesis. (I) Initiation is carried out by $IF2_{mt}$ and $IF3_{mt}$; (II) Elongation of the polypeptide chain is regulated by $EF\text{-}Tu_{mt}$, $EF\text{-}Ts_{mt}$, and $EF\text{-}G1_{mt}$; (III) Termination of mitochondrial translation is facilitated by $RF1a_{mt}$; and (IV) Ribosome recycling requires RRF_{mt} and $EF\text{-}G2_{mt}$. A possible role for $IF3_{mt}$ is also indicated as an alternative mechanism. Reproduced from Christian et al. (2009).

1.7 Somatic MtDNA Abnormalities

1.7.1 Ageing

It is now well established that both mtDNA point mutations and deletions accumulate with age in a range of tissues including brain, liver, colon, skeletal and cardiac muscle (Khrapko et al., 1999; Fayet et al., 2002; Bender et al., 2006; Krishnan et al., 2008; Greaves and Turnbull, 2009). Although the underlying mechanisms remain unclear, deletions tend to accumulate in post-mitotic tissues, whereas point mutations show a predilection for rapidly-dividing mitotic tissues. These mtDNA defects are somatic in origin and they can accumulate to high levels within individual cells by a process of clonal expansion. When the proportion of the mutant species exceeds a critical threshold of 60-80% (Shoubridge et al., 1990; Bua et al., 2006; Durham et al., 2007), a biochemical defect becomes apparent, and this can be observed histochemically as cytochrome c oxidase (COX) deficiency. Most COX-deficient cells harbour high levels of a single point mutation or deletion, but clonal expansion of more than one mutant species can occur, possibly related to a higher *de novo* mutational rate (Reeve et al., 2008). The increase in COX-deficiency seen with normal ageing varies markedly between tissues, and these regional variations probably reflect the different rates and thresholds at which mtDNA defects reach phenotypic expression between cell types (Krishnan et al., 2008; Greaves and Turnbull, 2009).

A mitochondrial theory of human ageing has been proposed based on the hypothesis that the progressive accumulation of COX-deficient cells has a deleterious effect on overall tissue function, by accelerating the rate of cellular senescence. In support of this argument, high levels of mtDNA deletions have recently been identified in substantia nigra neurons isolated from both normal aged controls and patients with

Parkinson's disease, suggesting a contributory role in the process of neuronal loss (Bender et al., 2006; Kraytsberg et al., 2006). However, the role of these somatic mtDNA abnormalities in the development of Parkinson's disease and other neurodegenerative disorders require more rigorous functional studies to demonstrate a true causal effect on tissue dysfunction (Khrapko and Vijg, 2009; Kukat and Trifunovic, 2009).

1.7.2 *Polg* Mouse Models

To clarify the contribution of somatic mtDNA abnormalities to the ageing process, independent research groups have created transgenic mouse models carrying different proofreading-deficient versions of mtDNA polymerase gamma (*PolgA*) (Martin and Loeb, 2004; Trifunovic et al., 2004; Kujoth et al., 2005). Homozygous mutant mice ($PolgA^{mut}/PolgA^{mut}$) had a reduced lifespan and developed a premature ageing phenotype characterised by weight loss, reduced subcutaneous fat, alopecia, kyphosis osteoporosis, anaemia, cardiomyopathy, and infertility. The development of these striking features was linked to the accumulation of significantly higher levels of somatic mtDNA point mutations (~ 3-5 times) in these mice compared to their wild-type littermate controls (Trifunovic et al., 2004; Kujoth et al., 2005). Interestingly, there was a close correlation between this increased mutational load and the induction of pro-apoptotic markers, especially in tissues characterised by rapid cellular turnover (Kujoth et al., 2005). However, in a different mutator mouse model, Vermulst and colleagues did not replicate this causal link between the level of somatic mtDNA point mutations and a reduction in lifespan (Vermulst et al., 2007). In a subsequent study, these investigators showed that the main driving force behind the accelerated ageing process in their homozygous mutant ($PolgA^{mut}/PolgA^{mut}$) mice was the accumulation of

high levels of mtDNA deletions (Vermulst et al., 2008). Although there is no doubt that somatic mtDNA abnormalities accumulate at a faster rate in all of these mutator mouse models, we need to be cautious when extrapolating these findings to humans. Future studies will hopefully provide further insight into the role of somatic mtDNA point mutations, deletions, or both in the complex, multifactorial process which defines human ageing (Khrapko and Vijg, 2007; Khrapko and Vijg, 2009).

1.7.3 Cancer

The pathological hallmark of cancer is uncontrolled cellular growth which leads to local tissue dysfunction and distant metastatic foci when malignant transformation has occurred (Bianchi, 2010). Polyak and colleagues investigated ten human colorectal cell lines, and interestingly, somatic mtDNA point mutations were identified in seven of these cases (Polyak et al., 1998). The majority of these mutations were homoplasmic, and they were not present in healthy tissues, implicating a possible direct role in the carcinogenetic process. These mutations were postulated to confer a physiological growth advantage to the tumour cells, and with cellular replication occurring rapidly over thousands of generations, all wild-type mtDNA would eventually be replaced completely by the mutant species i.e. a state of homoplasmy. However, this theory of a replicative advantage was challenged by other research groups. Using *in silico* modelling experiments, these investigators argued instead that the high frequency of homoplasmic mtDNA mutations in human tumours could simply arise by random genetic drift, without the need for selective mechanisms (Coller et al., 2001; Chinnery et al., 2002). With the use of deep sequencing technology, which can reliably detect levels of heteroplasmy as low as 1%, it has now become apparent that the majority (~ 90%) of somatic mtDNA point mutations present in human tumours are actually

heteroplasmic (He et al., 2010). The significance of these heteroplasmic mtDNA variants to tumour development, malignant transformation, and phenotypic severity remains to be determined (Bianchi, 2010).

1.7.4 Nuclear Mitochondrial Disorders

Nuclear mitochondrial disorders are an important group of human diseases, and they often pose significant diagnostic challenges related to their genetic and phenotypic heterogeneity (**Section 1.10.2**). However, a common molecular feature shared by all these disorders is impaired mtDNA maintenance, which can lead to a reduction in mtDNA copy number, the accumulation of high levels of somatic mtDNA mutations, or both (Alberio et al., 2007; Chinnery and Zeviani, 2008; Spinazzola and Zeviani, 2009). The identification of these quantitative and qualitative mtDNA abnormalities in diagnostic specimens is therefore an important finding, suggesting an underlying nuclear defect, and helping to direct appropriate molecular genetic testing. MtDNA depletion is the pathological hallmark of several early-onset mitochondrial syndromes, and the clinical prognosis is often poor, due to the marked bioenergetic crisis caused by such a gross reduction in mtDNA copy number (Spinazzola et al., 2009). Interestingly, the observed mtDNA depletion can be highly tissue-specific, which partly explains the variability in disease presentation and severity.

A mosaic pattern of COX-deficient fibres is frequently observed in muscle biopsies of patients with nuclear-mitochondrial disorders (**Section 1.10.5**). For nuclear genetic defects involving *POLG1*, *POLG2*, *PEO1*, *SLC25A4*, *TYMP*, and more recently *OPA1*, the COX-defect is secondary to the accumulation of multiple mtDNA deletions, which have clonally expanded within individual cells to reach suprathreshold levels > 70% (Horvath et al., 2006; Amati-Bonneau et al., 2008; Hudson et al., 2008; Spinazzola and

Zeviani, 2009). These deleted mtDNA species can be detected in homogenate DNA samples with Southern blot and long-range PCR, or more accurately quantified at the single-fibre level using real-time PCR assays (He et al., 2002; Taylor et al., 2004a; Bua et al., 2006). As most mtDNA deletions involve critical tRNA and protein-encoding genes, OXPHOS is adversely affected, and this eventually leads to apoptotic cells loss and tissue dysfunction.

Mutations in *POLG1* and *TYMP* have also been linked with the accumulation of somatic mtDNA point mutations. In two studies of patients with chronic progressive external ophthalmoplegia (CPEO) and confirmed pathogenic *POLG1* mutations, most of the identified mtDNA point mutations were present at relatively low heteroplasmic levels < 50% (Del Bo et al., 2003; Wanrooij et al., 2004). Importantly, these point mutations were found at significantly higher levels only in the mitochondrial D-Loop, but not in the protein-coding regions. Although still speculative and controversial, these point mutations could compromise the replication machinery located within the D-Loop, thereby contributing to the formation of mtDNA deletions.

Nishigaki and colleagues found multiple somatic mtDNA point mutations in fibroblasts obtained from patients with *TYMP* mutations and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), which were not detected in unaffected matrilineal relatives (Nishigaki et al., 2003). These point mutations showed a specific pattern, the majority being T to C transitions, and usually preceded by 5′-AA sequences. These secondary mtDNA abnormalities were thought to be generated because *TYMP* mutations disrupt the metabolism of thymidine, deoxythymidine (dThd) and deoxyuridine (dUrd), causing imbalances of the intramitochondrial dNTP pools. Similar to the findings in *POLG1*, these *TYMP*-associated mtDNA point mutations

were heteroplasmic (2-81%), with most being present at levels < 10%. The majority of these variants were also predicted to be neutral polymorphisms, precluding a direct pathological effect on mitochondrially-encoded subunits of the respiratory chain complexes. The functional significance of the increased frequency of somatic mtDNA point mutations seen with both *POLG1* and *TYMP* mutations remains unclear, and more sophisticated studies at the single cell level are required to address these important issues (Hudson and Chinnery, 2006; Chinnery and Zeviani, 2008). It is also fascinating that different mutations within the same gene, e.g. *POLG1* and *TYMP*, can result in such a varied spectrum of secondary mtDNA abnormalities. The clarification of the secondary factors which dictate whether depletion, deletion, or point mutations predominate will provide crucial insights into the underlying disease mechanisms in nuclear mitochondrial disorders.

1.8 Mitochondrial DNA Deletions

1.8.1 Formation

Most reported mtDNA deletions occur within the major arc of the mitochondrial genome between the two proposed origins of replication, O_H and O_L (Samuels et al., 2004; Bua et al., 2006; Krishnan et al., 2008) (http://www.mitomap.org/MITOMAP, Accessed 31st of August 2010). The majority of mtDNA deletions are flanked by short homologous direct repeat sequences, one of which is removed by the deletion process. Class I deletions (40-60%) have perfect repeats, class II deletions (30-40%) have imperfect repeats, and class III deletions (10-20%) have no flanking repeat sequences (Samuels et al., 2004; Bua et al., 2006; Krishnan et al., 2008). In a recent study, breakpoint sequencing was performed for mtDNA deletions identified in single

substantia nigra neurones from three different groups: normal aged controls, patients with sporadic Parkinson's disease, and a patient with a heterozygous *POLG1* mutation leading to parkinsonism in association with CPEO and multiple mtDNA deletions (Reeve et al., 2008). Interestingly, the deletions identified in these three distinct group of subjects shared remarkable similarities, irrespective of the actual breakpoint locations. There were no significant differences in the size distribution of these deletions, their breakpoint characteristics, the presence or absence of flanking repeat sequences, and the length of the repeat sequences. These observations strongly suggest a common mechanism for the formation of mtDNA deletions in both ageing and disease states.

A number of hypotheses have been proposed for the formation of mtDNA deletions, with the more established ones based on the occurrence of slippage errors during replication (Krishnan et al., 2008). Assuming that the Clayton strand-displacement model of mtDNA replication is correct (Clayton, 1982), a slipped-strand replication mechanism could explain the formation of these deleted mtDNA species (**Figure 7**). However, if replication is required for the generation of mtDNA deletions, mitotic tissues, which are thought to have faster mitochondrial turnover rates (Wang et al., 1997), would be expected to have higher deletion loads compared to post-mitotic tissues. This has not been found to be the case in rapidly dividing colonic crypt epithelial cells, which accumulate somatic mtDNA point mutations, but not deletions (Taylor et al., 2003a; Greaves et al., 2006). To account for these discrepancies, some investigators have proposed an alternative mechanism of deletion formation, which is based on oxidative damage causing double-stranded breaks within mtDNA molecules (Krishnan et al., 2008). These double-stranded breaks undergo 3′→5′ exonuclease degradation, and create neighbouring genomic regions with single DNA strands.

Subsequent attempts at repairing these damages are ineffective, and instead they contribute to the loss of the intervening mtDNA fragment (**Figure 8**).

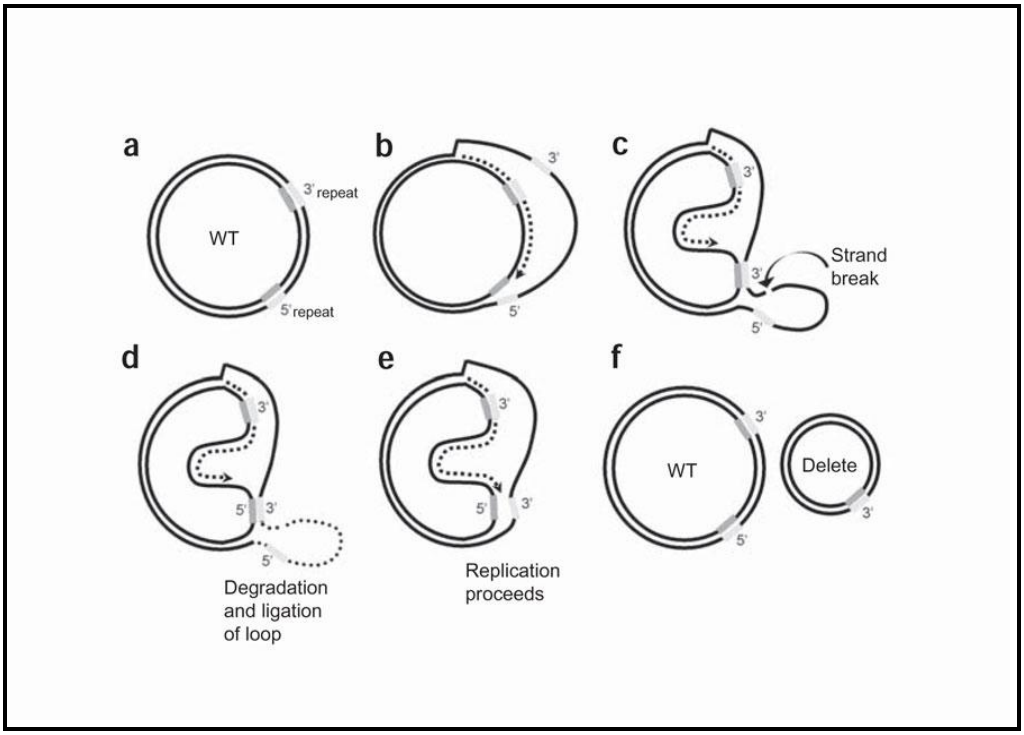

Figure 7. Proposed model of mtDNA deletion formation involving a slipped-strand model of replication. **(a)** MtDNA molecule showing two direct repeats labelled 5′ and 3′; **(b)** MtDNA replication begins in the D-Loop at O_H, displacing the light strand from the heavy strand; **(c)** The single-stranded 3′ repeat of the light strand mis-anneals to the newly exposed single-stranded 5′ repeat of the heavy strand. This generates a downstream, single-stranded, light-strand loop, which is susceptible to strand breaks; **(d)** The damaged loop is degraded until it reaches the double-stranded regions, and ligation of the free ends of the heavy strand occurs; **(e-f)** Replication resumes with the production of a wild type and a deleted mtDNA molecule. Reproduced from Krishnan et al (2008).

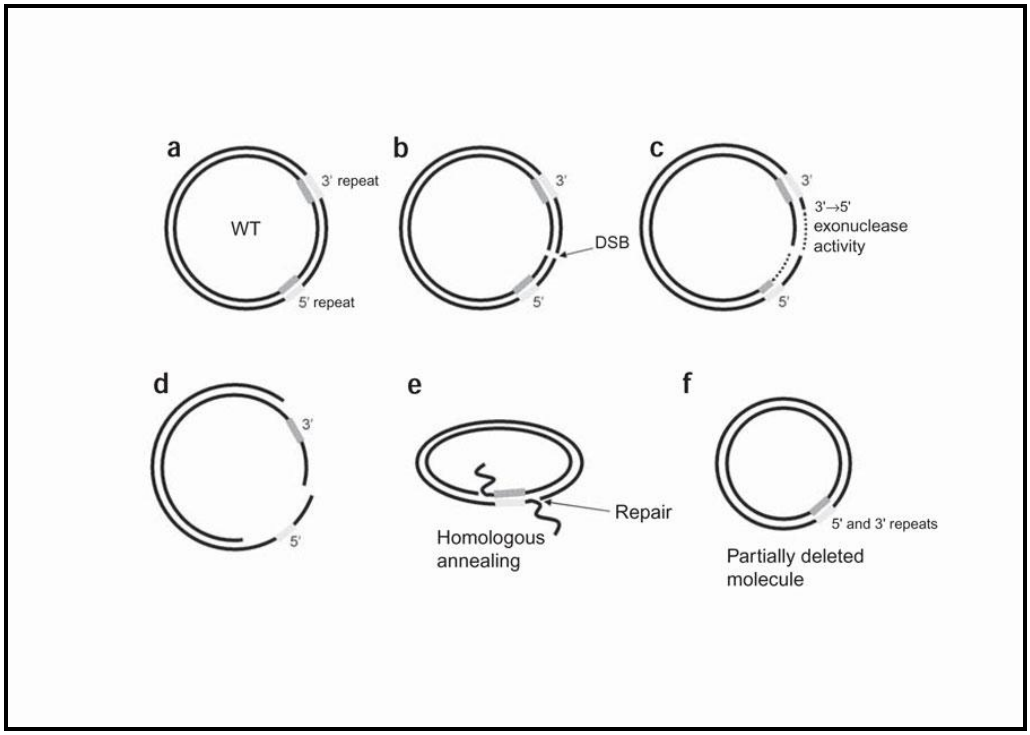

Figure 8. Generation of mtDNA deletions secondary to double-stranded breaks. **(a)** MtDNA molecule showing two direct repeats labelled 5′ and 3′; **(b)** Occurrence of a double-stranded break; **(c-d)** The double-stranded break is susceptible to 3′→5′ exonuclease activity, leading to the production of single strands; **(e)** The 5′- and 3′-repeat sequences can mis-anneal, leading to degradation of the unbound single strands, and ligation of the double strands; **(f)** This results in the production of a deleted mtDNA molecule, which contains copies of both the 5′ and 3′ repeats. Reproduced from Krishnan et al (2008).

1.8.2 Clonal Expansion

Once a mtDNA deletion has been generated, it has to reach suprathreshold levels within the cell in order to precipitate a biochemical defect. This process of clonal expansion within cells is analogous to genetic drift within populations, and two main

mechanisms have been proposed. These invoke either random intracellular processes (Chinnery and Samuels, 1999; Elson et al., 2001), or a replicative advantage for the deleted mtDNA species (Fukui and Moraes, 2009; Nicholas et al., 2009).

In the random genetic drift model, the mutant mtDNA allele becomes progressively fixed within individual cells, and over a period spanning several decades, the average mutational load gradually increases. The cell senses an imbalance caused by the accumulation of mutant mtDNA molecules, and mitochondrial proliferation is induced as a compensatory mechanism to maintain a critical level of wild-type genomes and normal respiratory chain function. This so-called *"maintenance of wild-type"* hypothesis is based on relaxed mtDNA replication, and does not require any selective pressures. Although mitochondrial proliferation helps to maintain an optimal level of wild-type mtDNA, when the mutational load exceeds a certain threshold level, further mitochondrial proliferation leads to replication of the mutant species at the expense of wild-type mtDNA, resulting in COX-deficiency (Chinnery and Samuels, 1999; Elson et al., 2001). It is important to note that the critical ratio of wild-type to mutated mtDNA molecules could be different for specific mutations, and it could also vary between tissues from the same individual (Durham et al., 2007; Yu-Wai-Man et al., 2010a).

In contrast to the random genetic drift model, some investigators favour positive selection as the predominant factor in the clonal expansion of mtDNA deletions (Fukui and Moraes, 2009; Nicholas et al., 2009). Deleted mtDNA species are smaller, and would therefore be expected to replicate faster than full-length, wild-type mtDNA molecules (Diaz and Moraes, 2008). A recent study has provided some important data supporting a replicative advantage for deleted mtDNA molecules in post-mitotic cells

(Fukui and Moraes, 2009). Fukui and Moraes developed a mouse model expressing an inducible restriction endonuclease (*PstI*), which resulted in double-stranded mtDNA breaks in neurones, and the formation of mtDNA deletions. Of major interest, mtDNA molecules with larger deletions were found to accumulate faster than those with smaller deletions (Fukui and Moraes, 2009). These results clearly imply a replicative advantage for smaller mtDNA species, which could play a powerful role in the accumulation of mtDNA deletions seen in both human ageing and mitochondrial maintenance disorders.

The process of clonal expansion is central to our understanding of mitochondrial genetic disorders, but the current evidence is still limited, with only few studies actually using pathological human samples (Krishnan et al., 2008; Nicholas et al., 2009). Random genetic drift and positive selection are not mutually exclusive, and other important factors could further influence the rate of clonal expansion of deleted mtDNA species; for example, the degree of compartmentalization of the mitochondrial network, which is critically dependent upon the balance between fusion and fission (**Section 1.9**), and the poorly understood mechanisms linked to autophagy which target the removal of specific populations of mitochondria (Twig et al., 2008a).

1.9 Mitochondrial Dynamics

1.9.1 Fusion and Fission

Mitochondria form a highly interconnected tubular network throughout the cell, and it is a dynamic process, with mitochondrial segments budding and fusing continuously (Chan, 2007; Detmer and Chan, 2007; Yu-Wai-Man et al., 2009). Over the past decade, key proteins have been identified which regulate this delicate balance between

fusion and fission, and several neurodegenerative disorders are now known to be due to disturbance in mitochondrial dynamics. The main pro-fusion proteins are mitofusin-1 (MFN1), mitofusin-2 (MFN2), and OPA1; and the main pro-fission proteins are DRP1 and hFIS1 (Chan, 2007; Knott et al., 2008; Twig et al., 2008b). An important structural similarity shared by all these proteins is a highly-conserved dynamin-related GTPase domain, which is essential for their normal function (Lenaers et al., 2009).

1.9.2 MFN1 and MFN2

MFN1 and MFN2 are the highly-conserved, mammalian orthologs of the yeast Fzo1 protein, and both proteins are localized to the mitochondrial outer membrane (**Figure 9**). The amino-terminal region contains the conserved GTPase domain, and the carboxy-terminal region, which faces the cytosol, consists of a coiled-coil structure. By forming homo- or hetero-typic complexes, these coiled-coil regions allow the tethering of neighbouring mitochondria, which is an important initial step in the fusional process (Knott et al., 2008; Suen et al., 2008). Disruption of outer membrane fusion leads to disease state, and *MFN2* mutations (1p36.2) have been identified in an autosomal dominant form of Charcot-Marie-Tooth disease (CMT-2A, OMIM 609260) (Zuchner et al., 2004). CMT-2A is characterised by axonal degeneration of the peripheral nerves, and affected patients experience progressive distal muscle weakness and sensory loss (Chung et al., 2006; Verhoeven et al., 2006). Interestingly, *MFN2* mutations have also been found in families with a different CMT subtype, hereditary sensory motor neuropathy type 6 (HSMN-6, OMIM 601152), where the peripheral neuropathy is complicated by the development of bilateral optic atrophy, emphasizing the important role played by MFN-2 in retinal ganglion cell (RGC) physiology (Zuchner et al., 2006).

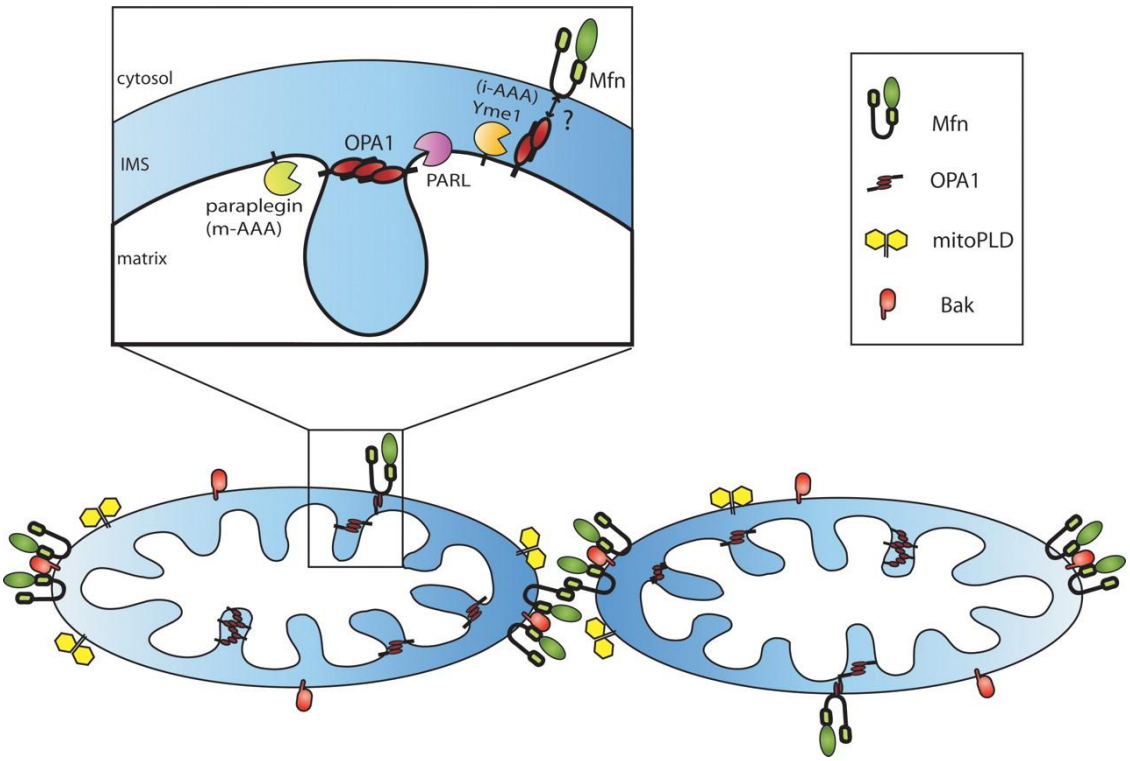

Figure 9. Major proteins regulating mitochondrial fusion. The insert shows the localization of proteases involved in OPA1 cleavage. mitoPLD: mitochondrial phospholipase D; PARL: presenilin-associated rhomboid-like (PARL) protease. Reproduced from Suen et al (2008).

Knockout mice lacking functional Mfn1 and Mfn2 have been developed and these models have provided important insights into the roles played by the mitofusin proteins (Chan, 2006; Chen and Chan, 2009). Homozygous mutant embryos died *in utero* due to placental insufficiency, clearly indicating that Mfn1 and Mfn2 are essential in the early stages of embryonic development (Chen et al., 2003). Cultured mouse embryonic fibroblasts (MEFs) showed a highly fragmented mitochondrial network due a severe reduction in mitochondrial fusion. This eventually led to a loss of membrane

potential in a subpopulation of fragmented mitochondria. The same group of investigators subsequently developed a conditional *Mfn2* knockout mouse model, using a cre-*loxP* recombinase strategy, which spared placental tissues, allowing normal embryonic development (Chen et al., 2007). The Meox2-Cre/*Mfn2*^{loxP} mutant mice showed marked cerebellar degeneration with an aberrant, fragmented mitochondrial morphology, which was associated with the loss of mtDNA nucleoids.

1.9.3 OPA1

OPA1 was originally characterised in the budding yeast *saccharomyces cerevisiae* in a screen for nuclear genes involved in mtDNA maintenance, and both the human and yeast (Mgm1) homologues show a high degree of evolutionary conservation. The *OPA1* gene (3q28–q29) consists of 30 exons spanning ~ 100Kb of genomic DNA and it codes for a 960 amino acid, dynamin-related GTPase protein located within the inner mitochondrial membrane (**Figure 9**) (Davies and Votruba, 2006). Alternative splicing of exons 4, 4b and 5b result in eight different mRNA isoforms, and their functional relevance and subcellular localisation are currently being investigated (Olichon et al., 2007a; Olichon et al., 2007b). *OPA1* mutations are the most common causes of autosomal dominant optic atrophy (DOA, OMIM 165500), and they account for about 60% of all clinically-diagnosed cases (Alexander et al., 2000; Delettre et al., 2000). Over 200 pathogenic mutations have now been identified and these cluster in two specific regions: the GTPase region (Exons 8-15), and the carboxy-terminal region which is the proposed site of the GTPase effector domain (GED) (Ferre et al., 2005; Ferre et al., 2009).

The native OPA1 protein has an amino-terminal domain with a mitochondrial targeting pre-sequence, which is cleaved by mitochondrial proteases following import into the

mitochondrial intermembrane space (Olichon et al., 2002; Lenaers et al., 2009). The transmembrane domain allows the protein to be firmly anchored to the inner mitochondrial membrane, in the narrow junctional regions enclosing the cristae spaces. The transmembrane domain is followed by a series of coiled-coil segments, which based on crystallographic studies, allow homo-polymerization of the OPA1 protein into a cylindrical tubular structure. Mitochondrial membrane proteases lead to further maturational processing (**Section 1.9.5**), which is relevant to the various cellular functions mediated by OPA1, in addition to inner membrane fusion (Davies and Votruba, 2006; Yu-Wai-Man et al., 2009). It is important to note that outer and inner membrane fusion are highly coordinated events, and in order to achieve this, OPA1 interacts closely with both MFN1 and MFN2, together with other accessory proteins. The latter are currently being defined in yeast models (Yaffe, 2003), and it is likely that mutations affecting the human orthologues will be associated with inherited neurodegenerative diseases.

1.9.4 DRP1 and hFIS1

Mitochondrial fission requires the recruitment of two dynamin-related GTPase proteins; DRP1 which is found within the cytosol (Pitts et al., 1999; Ishihara et al., 2009), and hFIS1, which is located within the mitochondrial outer membrane (Koch et al., 2005; Serasinghe and Yoon, 2008). DRP1 and hFIS1 are the mammalian orthologues of the yeast proteins Dnm1 and Fis1, respectively. The current working model of mitochondrial fission involves hFIS1 recruiting DRP1 to the mitochondrial outer membrane, with both proteins then assembling into rings and spirals that encircle and constrict the mitochondrial tubule (Sesaki and Jensen, 1999; Suen et al., 2008). Following GTPase hydrolysis and energy release, these helical structures twist and the

conformational changes result in fission (**Figure 10**). The actual sequence of events is likely to be more complex, and additional studies are needed to explore the intermediate steps involved, and whether the cytoskeleton plays a significant role in facilitating mitochondrial fission.

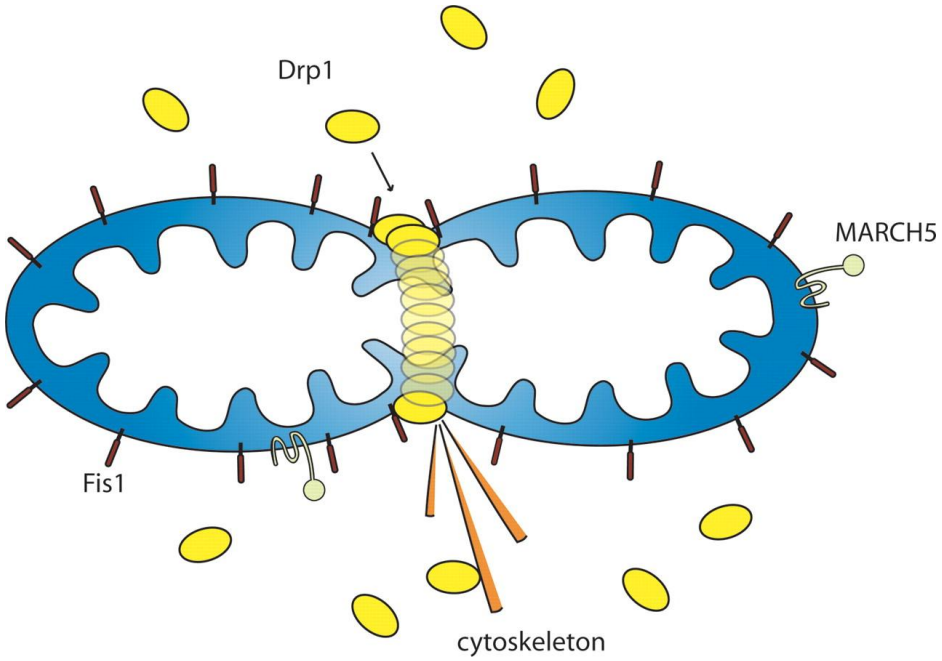

Figure 10. The mitochondrial fission machinery. Reproduced from Suen et al (2008).

The role of mitochondrial fission in maintaining cellular homeostatis was confirmed further when a heterozygous, dominant-negative, *DLP1* mutation was identified in a female infant with a complex neurodegenerative phenotype characterised by microcephaly, abnormal brain development, optic atrophy, and disturbed metabolic function (Waterham et al., 2007). The mitochondrial network in the patient's cultured

fibroblasts was elongated, forming tangled, tubular structures, which were clumped around the nucleus.

1.9.5 Mitochondrial Membrane Proteases

As a result of alternative splicing of exons 4, 4b and 5b, OPA1 exists as eight isoforms, and these are further modified by proteolytic cleavage mediated by the mitochondrial membrane proteases (Olichon et al., 2007a; Olichon et al., 2007b). These post-translational maturational steps are highly relevant to OPA1 function, and under physiological conditions, two specific splicing variants predominate, which differ for the absence (Variant 1), or presence (Variant 7) of exon 5b. Both OPA1 isoforms are first processed by the matrix metalloproteinases (MMP), which remove the mitochondrial targeting signal, and then by AAA (ATPases associated with a number of diverse cellular activities) proteases, which lead to the formation of long (L) and short (S) forms of OPA1 (Martinelli et al., 2009; Martinelli and Rugarli, 2010). On their own, the long and short forms of OPA1 have little functional activity, but when co-expressed, they complement each other, triggering mitochondrial network fusion (Duvezin-Caubet et al., 2006; Duvezin-Caubet et al., 2007; Song et al., 2007). The m-AAA proteases AFG3L2 and paraplegin cleave OPA1 at site S1 within exon 5, whereas the i-AAA protease YME1 cleaves OPA1 at S2 within exon 5b. Of note, paraplegin is encoded by *SPG7*, and mutations in this gene have been identified in a subgroup of patients with autosomal recessive hereditary spastic paraplegia (HSP-7, OMIM 607259) (Casari et al., 1998). These patients experience progressive weakness and spasticity of their lower limbs, due to degeneration of the corticospinal tract axons (Arnoldi et al., 2008). A fourth mitochondrial membrane protease related to the yeast orthologue Pcp1, the presenilin associated rhomboid-like (PARL) protease, is also

involved in OPA1 cleavage (Cipolat et al. 2006, Gottlieb 2006). The pool of soluble OPA1 isoforms generated within the intermembrane space by PARL is thought to exert a crucial anti-apoptotic influence (Gottlieb, 2006), especially under conditions of increased cellular oxidative stress (**Section 1.9.7**).

1.9.6 Reactive Oxygen Species

The shuttling of high-energy electrons along the mitochondrial respiratory chain complexes is a potent generator of ROS: superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Lee and Wei, 2007; Trifunovic and Larsson, 2008). These ROS molecules need to be contained by protective cellular mechanisms as they are powerful inducers of the apoptotic cascade. Superoxide dismutases (SODs) convert superoxide anions into hydrogen peroxide, which in turn can be transformed into water by catalase or members of the glutathione peroxidase enzyme family. The cell also contains nonenzymatic scavengers such as ascorbate, pyruvate, flavenoids, and carotenoids which are able to inactivate potentially damaging ROS (Trifunovic and Larsson, 2008).

There is no doubt that ROS can have a harmful influence on cell survival by causing oxidative damage to lipids, proteins and DNA within tissues. However, the links between increased ROS levels, normal ageing, and mitochondrial diseases remain controversial (Loeb et al., 2005; Trifunovic and Larsson, 2008). Yarosh and colleagues have recently established a *drosophila* model harbouring a specific *dOpa1* mutation (CG8479), and the ocular phenotypes associated with heterozygous and homozygous carriers were determined (Yarosh et al., 2008). Homozygous mutant flies developed a rough and glossy eye phenotype due to the loss of hexagonal lattice cells, and decreased lens and pigment deposition. The *dOpa1* mutation caused an increase in

ROS levels and mitochondrial fragmentation, which damaged both cone and pigment cells. In a series of elegant experiments, these investigators were then able to demonstrate that the rough and glossy eye phenotype could be partially reversed by dietary supplementation with SOD-1 and vitamin E, and by genetic over-expression of human *SOD1*. Heterozygous adult flies did not exhibit any ocular abnormalities, but similar to the homozygous mutants, they also demonstrated elevated ROS levels and a greater susceptibility to oxidative stress. The heteterozygous drosophila carriers showed irregular and dysmorphic mitochondria in their muscle, and they had a significantly shortened lifespan, which was only partially restored by antioxidant treatment (Shahrestani et al., 2009; Tang et al., 2009).

Other investigators have however challenged a direct cause and effect relationship between increased ROS and ageing (Kujoth et al., 2005; Trifunovic et al., 2005). The opposing arguments are based on the observations made on two *PolgA* mouse models, which developed a premature ageing phenotype due to an error-prone, mutant version of mtDNA polymerase gamma (**Section 1.7.2**). Although both *PolgA* mouse models showed marked respiratory chain dysfunction related to their high somatic mtDNA mutational loads, an increase in ROS levels was not observed in affected tissues, and there was no up-regulation in the mRNA expression of antioxidant ROS scavengers.

1.9.7 Apoptosis

By controlling the release of several pro-apoptotic effectors such as cytochrome *c*, smac/DIABLO, and AIF (Apoptosis inducing factor), mitochondria play a central role in regulating the apoptotic cascade which precipitates neuronal loss (**Figure 11**) (Hengartner, 2000; Lee and Wei, 2007; Wang and Youle, 2009). Although the mitochondrial apoptotic pathway is well established, it is only over the past few years

that a close link has emerged between mitochondrial dynamics and these key activation events. Cytochrome *c* molecules are sequestered within the mitochondrial cristae, and OPA1 is an integral component of the junctional complexes which prevent their release into the cytosolic compartment. OPA1 oligomers maintain the tightness of these cristae junctions, thereby counteracting the actions of BID, a pro-apoptotic BCL-2 family member (Cipolat et al., 2006). In addition, PARL, which is a major mitochondrial membrane protease, has been shown to mediate cristae remodelling via proteolytic cleavage of OPA1 (Cipolat et al., 2006).

Parl$^{-/-}$ knockout mice developed progressive, generalized cachexia, and they died prematurely secondary to multi-system atrophy (Cipolat et al., 2006; Gottlieb, 2006). Although *Parl*$^{-/-}$ null cells displayed normal mitochondrial morphology, they were sensitized to intrinsic apoptotic death stimuli triggered by etoposide, staurosporine, and hydrogen peroxide. Parl deficiency led to the unopposed leakage of cytochrome *c* from the cristae compartments, resulting in massive cellular apoptosis. The anti-apoptotic properties of OPA1 was further confirmed by Yamaguchi and colleagues, who showed that the pro-apoptotic peptides, Bid and Bim, induced cytochrome *c* release in mouse hepatocytes by triggering the disassembly of murine Opa1 complexes (Lee and Wei, 2007; Yamaguchi et al., 2008).

Co-immunoprecipitation studies indicate that OPA1 interacts directly with AIF, highlighting an important pathophysiological link between *OPA1* mutations and apoptotic RGC loss. Interestingly, both OPA1 and AIF are thought to be involved in complex I assembly and maintenance, reinforcing the overlapping properties of these two proteins (Vahsen et al., 2004; Zanna et al., 2008). The reliance of RGC survival on normal levels of AIF was elegantly demonstrated in the Harlequin (*Hq*) mouse model,

which contains a proviral insertion in *Aif*, resulting in a dramatic reduction (~80%) in gene expression (Klein et al., 2002). At three months of age, *Hq* mutant mice were exhibiting progressive RGC loss, and retinal degeneration became more severe by 11 months of age, eventually involving both the inner and outer plexiform layers. At that late stage, full field electroretinography confirmed complete loss of both rods and cones responses. Neuronal death in this *Hq* mouse model has been linked with increased oxidative stress, suggesting that AIF serves as a free radical scavenger under conditions of heightened cellular stress.

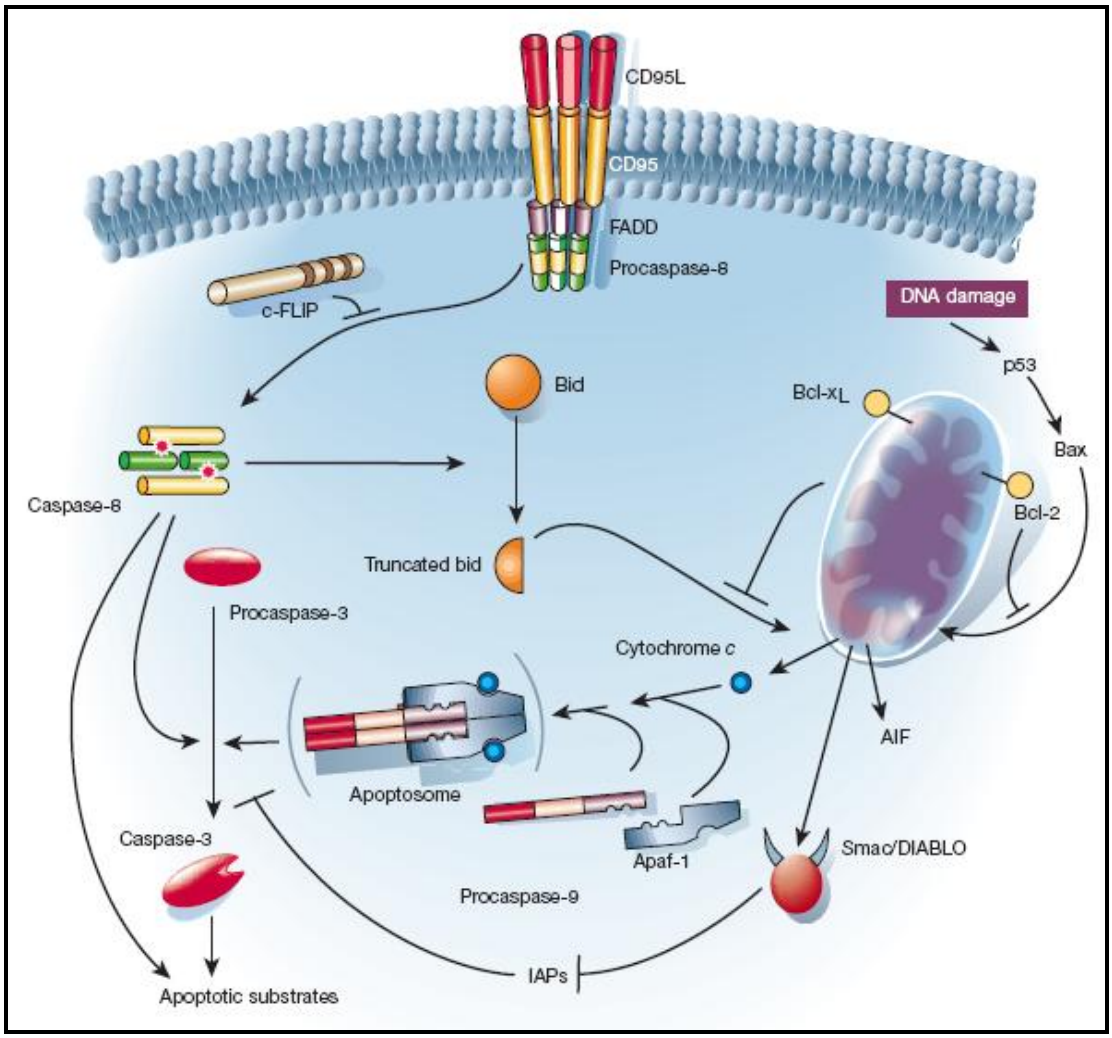

Figure 11. Schematic overview of the main mitochondrion-mediated and mitochondrion-independent apoptotic pathways. The death-receptor pathway is triggered by members of the death receptor superfamily such as CD95. Binding of the CD95 receptor ligand induces receptor clustering and formation of a death-inducing signalling complex. This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation. The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. Downstream of caspase-3, the apoptotic programme branches into a multitude of subprogrammes, the sum of which results in the ordered dismantling and removal of the cell. Reproduced from Hengartner et al (2000).

1.10 Mitochondrial Diseases

1.10.1 Epidemiology

Several epidemiological studies have confirmed that pathogenic mitochondrial DNA (mtDNA) mutations are major causes of human disease, with a minimum prevalence in the general population of 1 in 5000 (Schaefer et al., 2004; Schaefer et al., 2008). In a recent study of 3000 umbilical cord blood samples from a Cumbrian birth cohort in England, about 1 in 200 healthy neonates were found to harbour pathogenic mtDNA alleles, and these arose *de novo* almost every 1000 births (Elliott et al., 2008). In a tertiary diagnostic centre, the majority of patients with adult-onset mitochondrial disorders had primary mtDNA mutations (~ 75%), but this figure was significantly lower (~ 20%) among children with confirmed respiratory chain defects (Schaefer et

al., 2004; Schaefer et al., 2008). The remaining cases are likely to harbour nuclear genetic defects, and a growing number of these have been mapped and characterised over the past decade (McFarland et al., 2007; Zeviani and Carelli, 2007). With the exception of *POLG1* (Horvath et al., 2006), and *OPA1* (Yu-Wai-Man et al., 2010b), mutations in these nuclear genes are individually rare, but as a group, they are increasingly being recognised and diagnosed by clinicians. As molecular genetic testing becomes more widely available, and new disease genes are identified, it is very likely that nuclear mitochondrial disorders will prove to be at least as common as primary mitochondrial disorders.

1.10.2 Classification

Mitochondrial genetic disorders are classified into two major groups: primary mitochondrial disorders and nuclear mitochondrial disorders (**Tables 2 and 3**). In 2001, the first nuclear genes, *POLG1* and *PEO1*, were identified among families with autosomal dominant CPEO associated with multiple mtDNA deletions (Spelbrink et al., 2001; Van Goethem et al., 2001). Since then, the number of genes causing nuclear mitochondrial disorders has been expanding continuously, allowing significant progress to be made in elucidating the fundamental mechanisms that underpin mitochondrial physiology in both normal and disease states. As a result, we have gained a better understanding of the complex interactions between subunits of the respiratory chain complexes, and the crucial role played by accessory proteins in ensuring their proper assembly and stability along the inner mitochondrial membrane. These sometimes rare neurodegenerative and metabolic disorders have also provided important insights into the molecular components required for mtDNA maintenance, and the translational machinery that regulates intra-mitochondrial protein synthesis.

Table 2. Primary mitochondrial disorders

	Inheritance
Rearrangements (Large-scale partial deletions and duplications)	
Chronic progressive external ophthalmoplegia (CPEO)	S or M
Kearns-Sayre syndrome	S or M
Diabetes and deafness	S
Pearson marrow-pancreas syndrome	S or M
Sporadic tubulopathy	S
Point mutations	
Protein-encoding genes	
LHON (m.11778G>A, m.14484T>C, m.3460G>A)	M
NARP/Leigh syndrome (m.8993T>G/C)	M
tRNA genes	
MELAS (m.3243A>G, m.3271T>C, m.3251A>G)	M
MERRF (m.8344A>G, m.8356T>C)	M
CPEO (m.3243A>G, m.4274T>C)	M
Myopathy (m.14709T>C, m.12320A>G)	M
Cardiomyopathy (m.3243A>G, m.4269A>G, m.4300A>G)	M
Diabetes and deafness (m.3243A>G, m.12258C>A)	M
Encephalomyopathy (m.1606G>A, m.10010T>C)	M
rRNA genes	
Non-syndromic sensorineural deafness (m.7445A>G)	M
Aminoglycoside-induced non-syndromic deafness (m.1555A>G)	M

M = Maternal, S = Sporadic. MtDNA nucleotide positions refer to the L-chain, and are taken from the Cambridge reference sequence. LHON = Leber hereditary optic neuropathy, MELAS = Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, MERRF = Myoclonic epilepsy with ragged red fibres, NARP = Neurogenic weakness with ataxia and retinitis pigmentosa.

Table 3. Nuclear mitochondrial disorders

Mutations involving structural subunits of the mitochondrial respiratory chain
Leigh syndrome: with complex I deficiency – mutations in *NDUFS1, NDUFS4, NDUFS7, NDUFS8, NDUFV1*; with complex II deficiency – mutations in *SDHA*
Cardiomyopathy and encephalopathy with complex I deficiency – mutations in *NDFUS2*
Optic atrophy and ataxia with complex II deficiency – mutations in *SDHA*
Hypokalaemia and lactic acidosis with complex III deficiency – mutations in *UQCRB*
Mutations involving assembly factors of the mitochondrial respiratory chain
Leigh syndrome – mutations in *SURF* I and *LRPPRC*
Hepatopathy and ketoacidosis – mutations in *SCO1*
Cardiomyopathy and encephalopathy – mutations in *SCO2*
Leukodystrophy and renal tubulopathy – mutations in *COX10*
Hypertrophic cardiomyopathy – mutations in *COX15*
Encephalopathy, liver failure, and renal tubulopathy with complex III deficiency – mutations in *BCS1L*
Encephalopathy with complex V deficiency – mutations in *ATP12*
Nuclear genetic disorders of intra-mitochondrial protein synthesis:
Leigh syndrome, liver failure, and lactic acidosis – mutations in *EFG1*
Lactic acidosis, developmental failure, and dysmorphism – mutations in *MRPS16*
Myopathy and sideroblastic anaemia – mutations in *PUS1*
Leukodystrophy and polymicrogyria – mutations in *EFTu*
Encephalomyopathy and hypertrophic cardiomyopathy – mutations in *EFTs*
Edema, hypotonia, cardiomyopathy, and tubulopathy – mutations in *MRPS22*
Hypotonia, renal tubulopathy, and lactic acidosis – mutations in *RRM2B*

Nuclear genetic disorders of mitochondrial protein import

Mohr-Tranebjaerg syndrome or deafness-dystonia-optic neuronopathy (DDON) syndrome – mutations in *TIMM8A* (*DDP*)

Early-onset dilated cardiomyopathy with ataxia (DCMA) or 3-methylglutaconic aciduria, type V – mutations in *DNAJC19*

Nuclear genetic disorders of mitochondrial DNA maintenance

Chronic progressive external ophthalmoplegia – mutations in *POLG1, POLG2, PEO1, SLC25A4, RRM2B,* and *OPA1*

Mitochondrial neurogastrointestinal encephalomyopathy – mutations in *TYMP*

Alpers syndrome – mutations in *POLG1* and *MPV17*

Infantile myopathy and spinal muscular atrophy – mutations in *TK2*

Encephalomyopathy and liver failure – mutations in *DGUOK*

Hypotonia, movement disorder and/or Leigh syndrome with methylmalonic aciduria – mutations in *SUCLA2* and *SUCLG1*

Optic atrophy, deafness, chronic progressive external ophthalmoplegia, myopathy, ataxia, and peripheral neuropathy – mutations in *OPA1*

Miscellaneous

Co-enzyme Q10 deficiency – mutations in *PDSS2*, *APTX*, *COQ2*, and *ETFDH*

Barth syndrome – mutations in *TAZ*

Cardiomyopathy and lactic acidosis associated with mitochondrial phosphate carrier deficiency – mutations in *SLC25A3*

Alpers syndrome: epilepsy, cortical blindness, micronodular hepatic cirrhosis, episodic psychomotor regression; Barth syndrome: cardiomyopathy, hypotonia, weakness, and neutropenia.

1.10.3 Clinical Manifestations

Mitochondrial genetic disorders can affect any organ system and the heterogeneous clinical features seen are frequently compounded by marked inter- and intra-familial phenotypic variability (**Figure 12**). An underlying mitochondrial aetiology should therefore be considered in all patients with complex, multi-system presentations, especially those with neuromuscular, ocular and endocrine involvement. From a practical diagnostic standpoint, the possibility of a primary or nuclear mitochondrial genetic defect should be considered in the following three groups of patients (Chinnery et al., 1999a; McFarland et al., 2002; Kirkman et al., 2008). The first group includes patients with classic presentations which strongly suggest an underlying mitochondrial aetiology. For example, bilateral, sequential, and severe visual failure in a young adult male, with clear-cut evidence of optic nerve dysfunction is highly suggestive of LHON (Yu-Wai-Man et al., 2009). Similarly, a history of slowly progressive, bilateral, symmetrical ptosis, with marked restriction of extraocular movements in a middle-aged adult, is pretty characteristic for CPEO, and this diagnosis should be considered as part of the differential diagnosis. If CPEO develops at a younger age, before the age of twenty, in association with pigmentary retinopathy and cardiac conduction defects, a diagnosis of Kearns-Sayre syndrome can be made easily on clinical grounds (Fraser et al., 2010).

In the second group, patients do not fall into any specific mitochondrial syndromes, but instead, they develop a collection of clinical features which are highly suggestive of an underlying respiratory chain defect, such as a combination of proximal myopathy, cardiomyopathy, and sensorineural deafness. The third and most challenging group includes patients with vague symptoms such as reduced exercise tolerance and

myalgia, or presenting with isolated neurological features such as seizures, migraine, and peripheral neuropathy, all of which are relatively common in the general population. The presentation of mitochondrial genetic disorders in childhood can be even more non-specific, with neonatal hypotonia, feeding difficulties, and failure to thrive (McFarland et al., 2002; Chinnery and Schon, 2003). Unless there are other affected family members with more classical mitochondrial phenotypes, there are often significant delays in reaching a molecular diagnosis in this third group of patients.

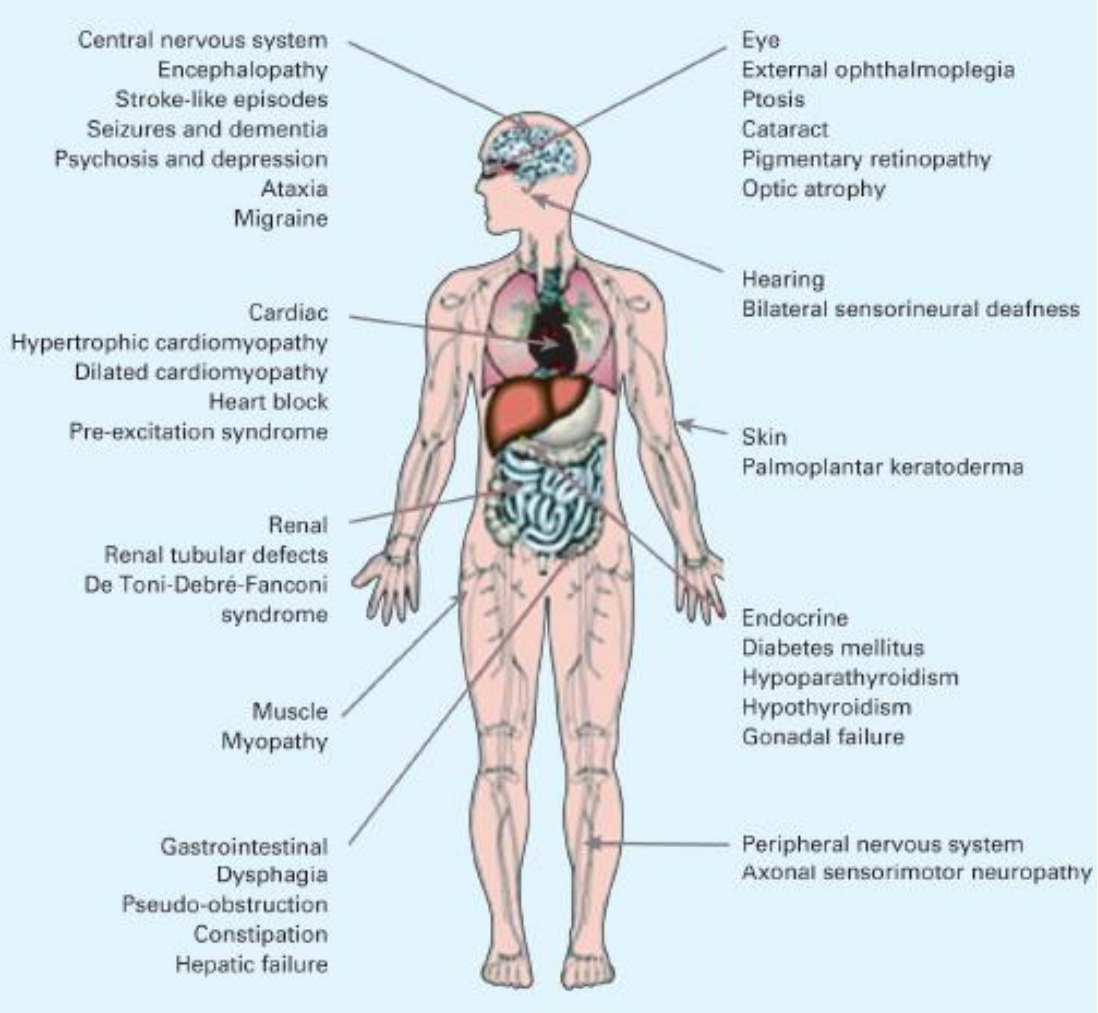

Figure 12. Clinical manifestations of mitochondrial genetic disorders. Reproduced from Chinnery and Schon (2003).

1.10.4 Diagnostic Investigations

The investigation of patients with suspected mitochondrial genetic disorders is a highly-specialised area, often requiring sophisticated laboratory assays to reach a molecular diagnosis (**Figure 13**). It is therefore crucial to have appropriately trained clinician scientists who are able to interpret the results and then guide the referring clinicians should further confirmatory studies be required. As a group, mitochondrial disorders are extremely heterogeneous, and given the frequent occurrence of ocular features in these patients, neuro-ophthalmologists should always consider this possibility in their differential diagnosis. A detailed history should be obtained, with particular attention paid to the family history, which can often be very revealing, but is often not volunteered by patients unless specifically asked for. The aims of ancillary testing fall into three broad categories (**Figure 14**): (i) to objectively delineate the severity and pattern of organ involvement, (ii) to exclude possible, treatable complications such as cardiac conduction defects and epilepsy, and (iii) to identify the nature of the underlying genetic defect with the specific use of histochemical, biochemical, and molecular studies (Chinnery et al., 1999a; McFarland et al., 2002; Kirkman et al., 2008).

1.10.5 Mitochondrial Histochemistry

A muscle biopsy is a powerful diagnostic tool when investigating a patient with a suspected mitochondrial aetiology (Taylor et al., 2004b). Open muscle biopsies are not routinely performed and the preferred method is a percutaneous approach, using either a conchotome or a modified biopsy needle (Dietrichson et al., 1987). The quadriceps and tibialis anterior muscles are the most frequently biopsied areas, and if possible, the biopsy of an affected muscle group is recommended.

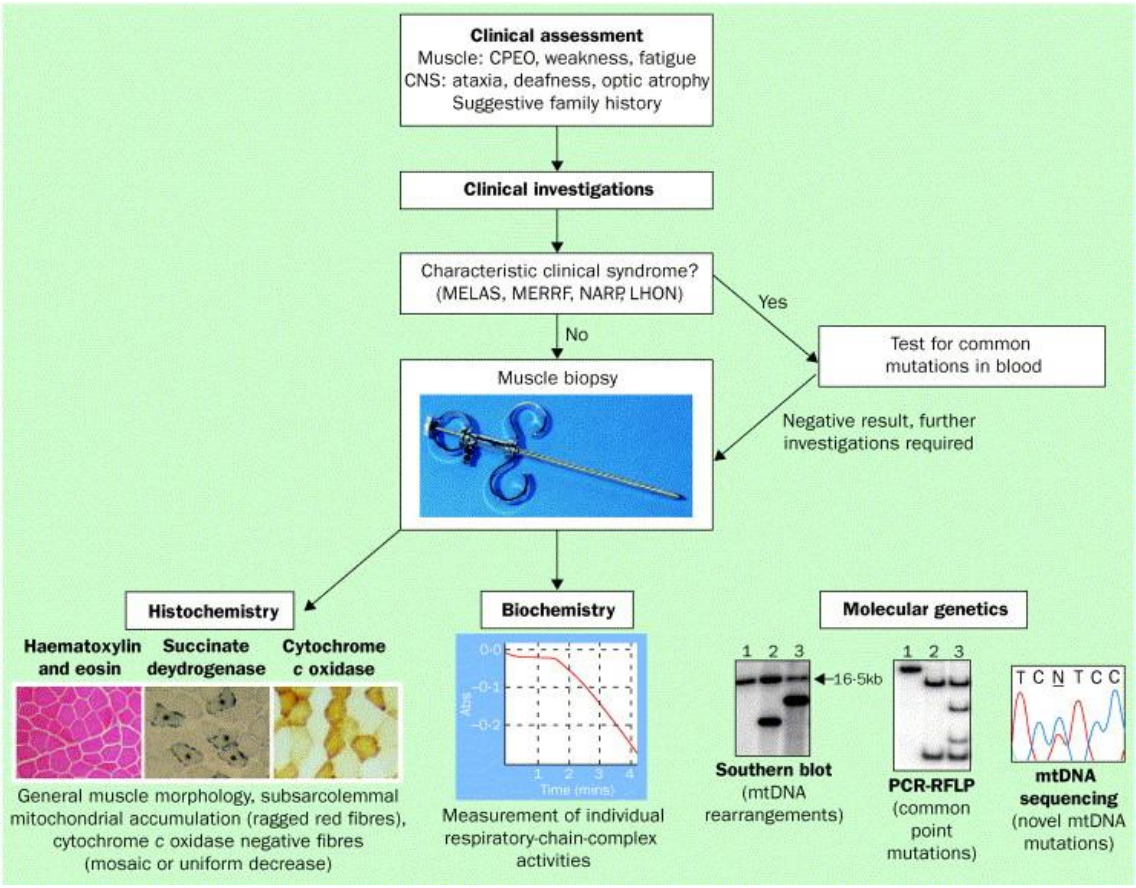

Figure 13. Histochemical, biochemical, and molecular genetic investigations of patients with suspected mitochondrial disease. Reproduced from McFarland et al (2002).

Once retrieved, the muscle specimen is placed in a melting isopentane bath (–150°C) to minimize freezing artefacts, before being stored at –80°C. Using a cryostat, serial muscle sections, usually 10-20 μm thick, are cut onto glass or membrane slides, before being stained using various reagents. Stains that are commonly performed in the investigation of patients with suspected mitochondrial disorders include: (i) haematoxylin and eosin (H&E), (ii) Gomori trichrome, (iii) COX, and (iv) succinate dehydrogenase (SDH) (**Figure 15**).

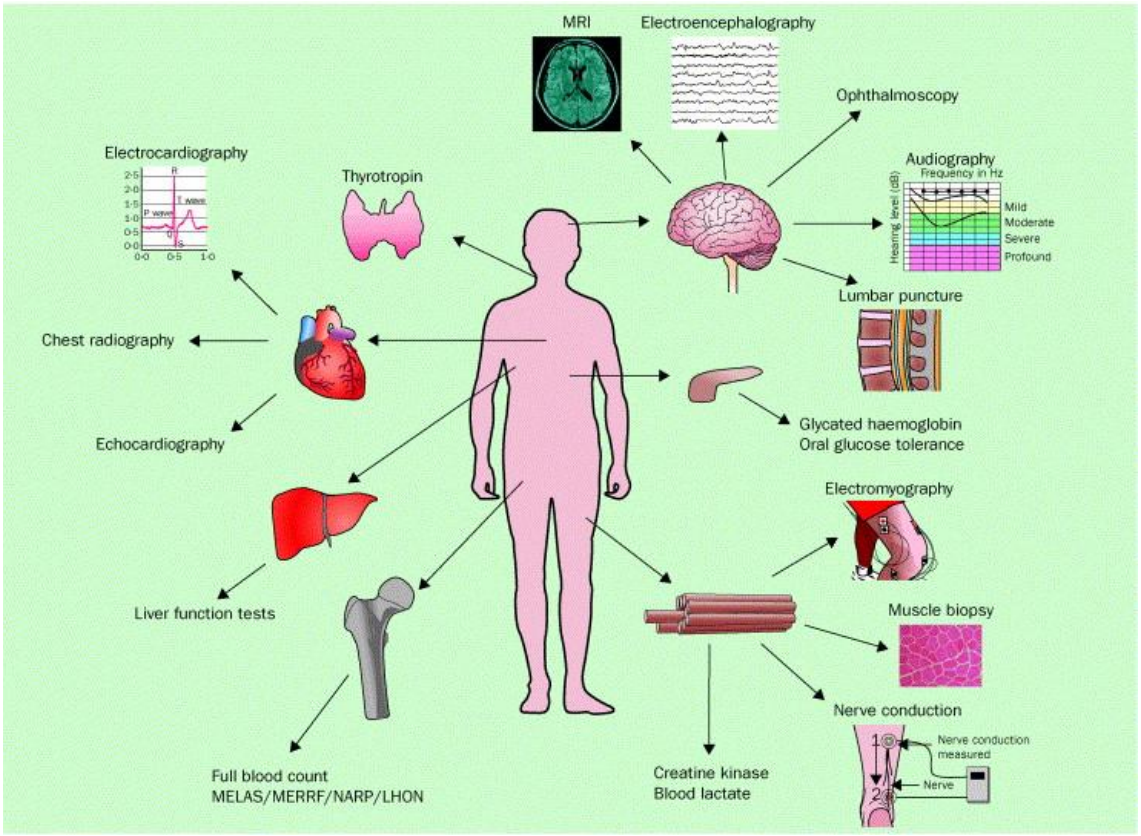

Figure 14. Additional investigations in patients with suspected mitochondrial disease. These are tailored according to the patient's presentation, family history, and underlying genetic defect if known. Reproduced from McFarland et al (2002).

When indicated, myofibrillar ATPase staining can also be performed to determine the distribution of Type I and Type II muscle fibres and identify pathological features such as fibre-type grouping, which is seen with denervation of the peripheral nerves (Johnson and Barron, 1996; Taylor et al., 2004b).

H&E provides useful basic information on muscle fibre morphology, whereas the Gomori trichrome stain highlights the abnormal accumulation of mitochondria in the

subsarcolemmal space, giving the classical appearance of "ragged red fibres" (RRFs) (**Figure 15**). COX and SDH specifically assess mitochondrial enzyme activity, with COX-stained fibres appearing brown, and SDH-stained fibres appearing blue. As complex II is entirely nuclear-encoded, SDH activity is usually preserved, unless a nuclear defect affects one of its four constituent subunits (Birch-Machin et al., 2000; Rutter et al., 2010). Pathological RRFs can also be detected with SDH, which has largely superseded the Gomori-trichrome stain in many diagnostic laboratories. There is a normal variation in COX staining in healthy skeletal muscle, oxidative Type I fibres appearing darker brown compared to glycolytic Type II fibres. Loss of COX activity is an important marker of mitochondrial dysfunction, and the frequency of these COX-negative fibres can be easily quantified with dual COX-SDH staining (**Figure 15**). RRFs are usually COX-negative, but COX-positive RRFs can also occur, especially with some mutations such as m.3243A>G.

The pattern of COX-negative muscle fibres can be a useful preliminary indicator of the underlying molecular genetic defect. A mosaic distribution of COX-positive and COX-negative fibres is usually seen with heteroplasmic mtDNA mutations, and in nuclear disorders linked with the accumulation of secondary mtDNA abnormalities (**Section 1.7.4**). On the other hand, a generalized decrease in overall COX activity is more suggestive of a homoplasmic mitochondrial tRNA mutation (McFarland et al., 2002; Taylor et al., 2003b), or a nuclear defect disrupting COX-assembly proteins, e.g. *SURF1* (Zhu et al., 1998). However, these are only broad generalizations, and patients harbouring these genetic defects have also been described whose muscle biopsies were completely normal, with no histochemical defect present.

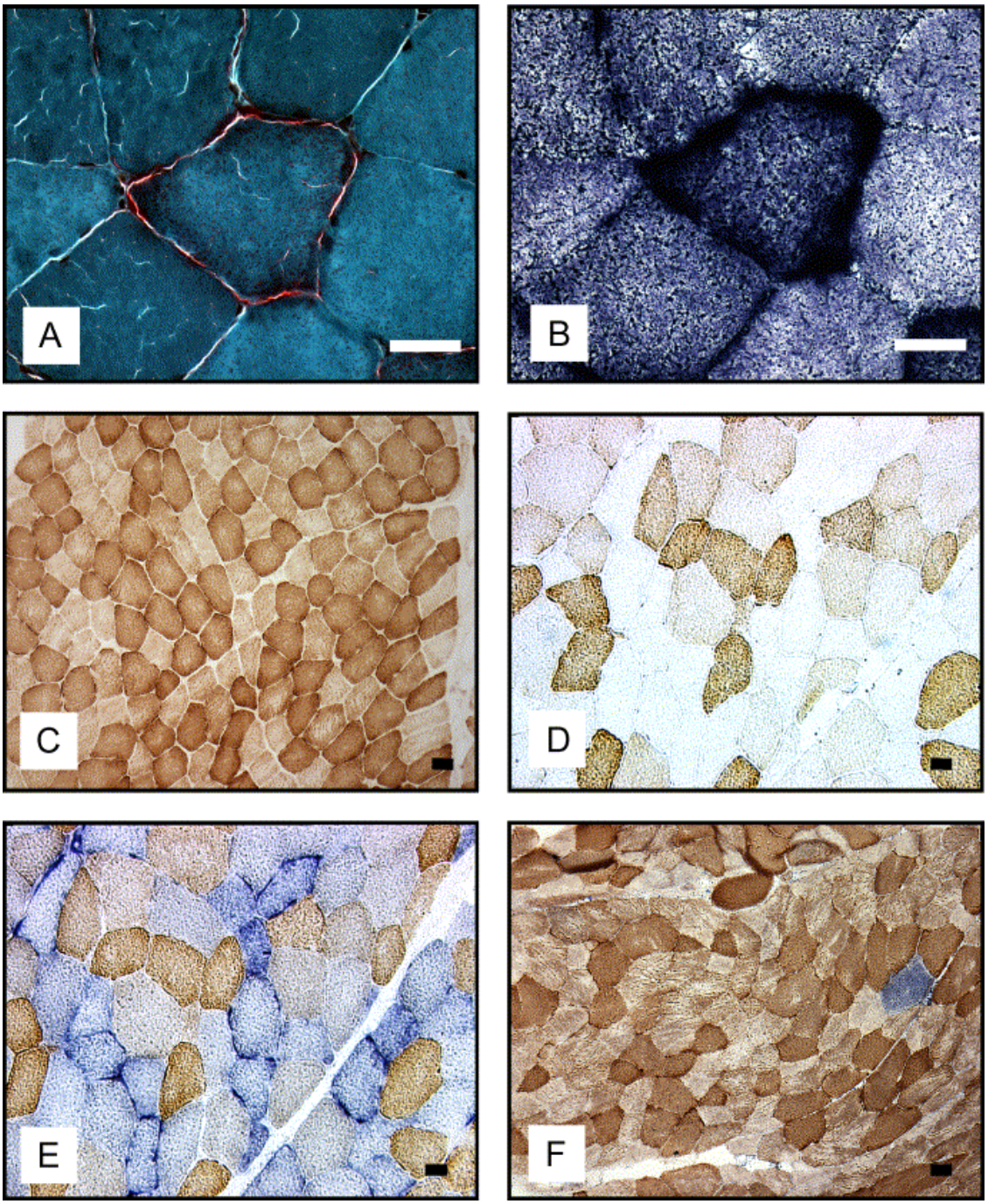

Figure 15. Mitochondrial histochemical studies. **(A)** Ragged-red muscle fibre identified using the modified Gomori trichome stain. The red component of the staining mixture is selectively sequestered by mitochondria, which have accumulated in the subsarcolemmal region, giving the fibre an irregular red outline; **(B)** Serial section of the same muscle fibre shown in (A) after SDH staining. This is a more specific assay for detecting the subsarcolemmal

accumulation of mitochondria, SDH being a specific marker of complex II activity; **(C)** Normal COX histochemistry with Type I and Type II muscle fibres in the cut section; **(D)** Abnormal COX histochemistry from a patient with CPEO, showing a large number of COX-deficient fibres; **(E)** Serial section of (D) following sequential COX-SDH histochemistry. The individual COX-deficient fibres are now highlighted blue due to preserved SDH activity; **(F)** Sequential COX-SDH histochemistry revealing a single COX-deficient muscle fibre in a section of ageing muscle, emphasizing that caution should be taken in the interpretation of muscle biopsies from elderly individuals. All scale bars 30 mm. Reproduced from Taylor et al (2004).

1.10.6 Biochemical Studies

Mitochondrial biochemical studies are fraught with difficulties, the specific protocols and normative ranges used varying widely between laboratories worldwide (Taylor et al., 2004b; Yu-Wai-Man et al., 2009). The results therefore need to be interpreted cautiously in the context of the clinical history and histochemical findings. Nevertheless, the measurement of respiratory chain complex activity can be very useful, especially in the investigation of children with complex metabolic and neurodegenerative disorders. Mitochondrial biochemical studies are usually performed on skeletal muscle, and although a fresh sample is preferable, this is not always possible when tissue biopsies need to be sent frozen to supra-regional centres. Fibroblast cultures are also frequently used, as a skin biopsy is a minimally invasive procedure which can be easily performed during a patient's outpatient clinic visit. The activities of the individual respiratory chain complexes are determined on

mitochondrially-enriched suspensions, and they are expressed relative to the activity of citrate synthase, a matrix marker enzyme (Janssen et al., 2003; Kirby et al., 2007). Depending on the specific genes involved, mtDNA point mutations and deletions can present with either an isolated or multiple complex defects. It is now also possible to reliably measure coenzyme Q_{10} (CoQ_{10}) levels directly in tissues (Musumeci et al., 2001; Sacconi et al., 2010).

Given the limitations of *in vitro* biochemical assays, some investigators have proposed the use of *in vivo* phosphorus magnetic resonance spectroscopy (^{31}P-MRS) to quantify mitochondrial oxidative function (Barbiroli et al., 1995; Lodi et al., 1997; Befroy et al., 2009). Magnetic resonance spectroscopy offers some distinct advantages, allowing more physiological measurements to be obtained both at rest and following a period of exercise. However, a major limitation is cost and it is not widely available as a diagnostic resource in most clinical centres.

1.10.7 Molecular Genetic Analysis

The identification of the underlying genetic defect has important ramifications both for the proband and other family members. The patient's clinical presentation, in addition to the preliminary histochemical and biochemical findings, often allow for a targeted molecular approach, thereby minimizing cost and diagnostic delays. In patients with classical presentations of a primary mtDNA disorder, e.g. LHON and MERFF, it is routine practice to first screen for the most common mutations linked with these disorders (McFarland et al., 2002). Several methods can be used including restriction fragment length polymorphism (RFLP), pyrosequencing, or direct PCR-based sequencing. Similarly, various protocols have been developed that provide accurate

quantification of heteroplasmy level, such as primer extension assay (Fahy et al., 1997), radioactive or fluorescent "last-cycle" PCR (Pyle et al., 2007).

Whole genome mitochondrial sequencing is labour intensive and the interpretation of the results can pose certain difficulties. It should therefore be reserved for patients with a high index of suspicion for an underlying primary mtDNA defect, but found to be negative for the more common mutations. As mentioned previously, mtDNA is highly polymorphic, and if a novel variant is identified, it is sometimes not possible to be definitive about its pathological significance. For a mtDNA variant to be considered pathogenic, certain "*canonical*" criteria need to fulfilled (Chinnery et al., 1999b; McFarland et al., 2002): (i) it must not have been previously reported as a neutral polymorphism in human mtDNA sequence databases, (ii) it must not be detected in healthy controls, (iii) pathogenic mtDNA variants are usually, but not always, heteroplasmic, (iv) it would normally be expected to alter a highly-conserved nucleotide or amino-acid, and (v) it should segregate with disease status, with higher heteroplasmy levels detected in affected family members and in affected tissues. For more complex situations, e.g. a possibly pathogenic homoplasmic tRNA variant, confirmation of a deleterious functional consequence at the biochemical or cellular level is required (Tuppen et al., 2008; Horvath et al., 2009).

It is important to note that although blood is a more easily obtained diagnostic sample, some mtDNA mutations, e.g. m.3243A>G, are only present at low levels in circulating leukocytes, with the levels often decreasing significantly with age (Pyle et al., 2007; Rajasimha et al., 2008). Depending on the specific clinical setting, screening of muscle homogenate DNA is therefore strongly recommended to avoid false negative results. Large-scale mtDNA rearrangements; single deletions, duplications and multiple

mtDNA deletions, can also be detected in homogenate muscle DNA. Southern blot analysis is often considered the "gold" standard when screening for these large-scale rearrangements, and it has the advantage of being quantitative (Taylor et al., 2000). MtDNA depletion can also be detected if the Southern blot is hybridised with a probe corresponding to a nuclear gene, such as the 18S rRNA, in addition to the mitochondrial probe, usually to the D-Loop region. However, Southern blot has some disadvantages, requiring the use of radioactive material and a relatively large starting quantity of genomic DNA, but more importantly, it has a detection threshold of ~ 5%. A second technique increasingly being used in diagnostic laboratories is long-range PCR, which only requires small amounts of genomic DNA. Being PCR-based, this assay will preferentially amplify smaller amplicons, and it will detect low levels of mtDNA deletions (Lightowlers et al., 1999). This can lead to some pitfalls, and it is important to consider the patient's age when only a few deletion bands are identified on the electrophoresis gel, in the presence of a relatively strong, wild-type band, corresponding to the full-length PCR product. These could represent normal ageing changes, as healthy individuals over the age of 60 can harbour low frequency of COX-deficient fibres in their muscle of ~ 1% (**Section 1.10.5**) (Brierley et al., 1996; Brierley et al., 1997). A third technique, fluorescence-based real-time PCR, has been optimized for the identification of mtDNA deletions at the single-cell level. It allows very accurate quantification of both wild-type and deleted mtDNA copy number, and by measuring deletion levels in laser-microdissected COX-positive and COX-negative single cells, it is possible to determine segregation with the histochemical defect (He et al., 2002; Durham et al., 2007).

Screening for an underlying nuclear defect is usually dictated by the patient's age, the clinical phenotype, and features pointing towards an underlying mtDNA maintenance

disorder in affected tissues. For example, in a patient presenting with a classical *POLG* phenotype characterised by CPEO, ataxia, epilepsy, and multiple mtDNA deletions, many diagnostic labs will initially screen for the c.1399G>A (p.A467T) and c.2243G>C (p.W748S) *POLG1* mutations (Horvath et al., 2006; Chinnery and Zeviani, 2008). If these nucleotide changes are not present, the entire *POLG1* genomic region can then be sequenced, followed by other nuclear genes linked with multiple mtDNA deletion if still negative. The available "nuclear panel" will largely dependent on the diagnostic facility, and this will be influenced by cost-issues both for the laboratory and the patient. A primary nuclear defect is frequently not identified in patients with multiple mtDNA deletions, even among those with clear-cut autosomal pattern of inheritance. In two case series, *POLG1*, *PEO1*, and *SLC25A4* were sequenced in patients with sporadic CPEO and multiple mtDNA deletions (Di Fonzo et al., 2003; Hudson et al., 2006). No *PEO1* or *SLC25A4* mutations were identified in these cohorts, and the positive hit rate for *POLG1* was only 10-25%. The investigation of suspected nuclear mitochondrial disorder poses significant challenges, and genetic testing is still not widely available. Hopefully, these practical difficulties will be resolved with the development of cheaper, high-throughput sequencing platforms, which will also play a major role in accelerating the pace of discovery for as yet unidentified nuclear mitochondrial genes.

1.11 Genetic Counselling

Male carriers harbouring mtDNA point mutations can be reassured that their children are not at risk of inheriting their genetic defect. Female carriers will transmit the mutation to all their offspring, but if the mutation is heteroplasmic, it is not possible to reliably predict the mutational level that will be transmitted (Yu-Wai-Man et al., 2009;

Fraser et al., 2010). As discussed previously, significant variations in heteroplasmy levels can occur as a result of the mitochondrial genetic bottleneck (**Section 1.3.2**). The use of amniocentesis or chorionic villus sampling for prenatal testing is therefore limited in this situation, as the mutational load detected in amniocytes and chorionic villus cells could differ from other foetal tissues, especially those at greater risk from a specific mtDNA point mutation (Brown et al., 2006). For mtDNA single deletions, the risk of maternal transmission is relatively low, and this has been estimated at ~ 1 in 24 births i.e. ~ 4%. There is also no evidence that increased maternal age is associated with an increased risk of having an affected child with a mtDNA deletion disorder (Chinnery et al., 2004). The risks of transmission for nuclear mitochondrial disorders follow the laws of Mendelian inheritance, but if a specific nuclear defect has not been identified, only an approximate risk can be provided based on the family history. As a result of the marked inter- and intra-familial phenotypic variability seen with mitochondrial disorders, genetic counselling for patients and their families remains a challenging area of practice.

1.12 Treatments for Mitochondrial Disease

1.12.1 Supportive Measures

The treatment options for patients with mitochondrial genetic disorders are currently limited (Chinnery et al., 2006). However, there are several practical steps that can be taken as part of a multi-disciplinary team to improve a patient's quality of life, and minimize long-term morbidity (Yu-Wai-Man et al., 2009; Fraser et al., 2010). Depending on their needs, these patients should be provided with access to facilities such as low visual aids, physiotherapy, occupational therapy, and clinicians can help

with financial assistance through their local social services. It is also important to aggressively manage related medical problems such as diabetes and epilepsy, and clinicians need to be vigilant to the development of new complications such as cardiomyopathy and sensorineural deafness, which could be amenable to therapeutic intervention. Patients with mitochondrial disorders should be strongly advised not to smoke and to minimise their alcohol intake, not only as a general health measure, but smoking, and to a lesser extent excessive alcohol intake, have been linked with an increased risk of visual loss among LHON carriers (Kirkman et al., 2009). Patients with CPEO often get significant benefit from simple conservative measures such as ptosis props or Fresnel prisms for symptomatic ocular misalignments (Richardson et al. 2005). In some cases, strabismus and ptosis surgery are indicated but these should be performed by experienced surgeons (Shorr et al., 1987; Ahn et al., 2008), because of the increased risk of complications such as corneal exposure secondary to poor orbicularis oculi function and impaired Bell's phenomenon (Daut et al., 2000).

1.12.2 Disease-Modifying Treatments

On the basis of limited, mostly anecdotal evidence, various combinations of pharmacological agents have been used to treat patients with mitochondrial disorders including multi-vitamin supplements, CoQ_{10} and its derivatives, and putative free radical scavengers (Horvath et al., 2008; Tarnopolsky, 2008; Schon et al., 2010). There is relatively more clinical data on the use of CoQ_{10}, which has shown a clear benefit for patients with primary CoQ_{10} deficiency (Mancuso et al., 2010). In one randomised, double-blind, crossover study of 16 patients with mitochondrial cytopathies, a combination of CoQ_{10} with creatine monohydrate and α-lipoic acid, reduced the levels of resting plasma lactate and oxidative stress markers (Rodriguez et al., 2007).

Idebenone is a synthetic analogue of CoQ_{10}, and it is currently being investigated as a treatment option for neurodegenerative disorders such as Friedreich ataxia (FRDA) and LHON. Idebenone is able to operate under low oxygen tension and it is thought to have antioxidant properties, in addition to optimising ATP production by the respiratory chain complexes (Tonon and Lodi, 2008; Sacconi et al., 2010). Initial reports of idebenone therapy in FRDA showed promising results with an improvement in both cardiac and neurological status (Rustin et al., 2004; Di Prospero et al., 2007). However, preliminary data released from two recently completed randomised controlled trials have proven rather disappointing, with no significant difference in primary cardiac and neurological endpoints between the idebenone treated and placebo FRDA groups. (http://www.santhera.com/index.php?mid=6&vid=&lang=en, Accessed 31st of August 2010). In a retrospective study of 28 affected LHON patients, half of whom received a combination of idebenone, vitamin B2, and vitamin C for at least one year, the treated group showed a faster rate of visual recovery (Mashima et al., 2000). However, in a more recent report, megadoses of idebenone, vitamin C, and riboflavin did not prevent second eye involvement in two m.11778G>A LHON carriers treated after the onset of unilateral visual loss, and both patients showed no improvement in visual function (Barnils et al., 2007). The benefits of idebenone in LHON remains unclear, but as it is a safe drug, it is often recommended by clinicians or self-prescribed by patients. A randomised controlled trial of idebenone in LHON has recently been completed, and the preliminary results suggest a beneficial effect, especially among patients with recent disease onset, and relatively good visual acuity in the second eye to be affected. (http://www.santhera.com/index.php?docid=212&vid=&lang=en&newsdate=201006&newsid=1424223&newslang=en, Accessed 31st of August 2010).

The pathology seen in MNGIE is secondary to imbalances in the intramitochondrial dNTP pools, and the toxic accumulations of thymidine and deoxyuridine. Allogenic haematopoietic stem cell transplantation (HSCT) has shown some evidence of benefit in a small group of patients, with a partial restoration of buffy coat thymidine phosphorylase activity, and the lowering of plasma nucleosides levels (Hirano et al., 2006; Halter et al., 2010). However, this procedure entails significant risks and additional work is needed to determine whether correcting the biochemical abnormalities in blood will improve the prognosis for patients with MNGIE (Chinnery and Vissing, 2006).

1.12.3 Gene Therapy

MtDNA mutations are often present in the heteroplasmic state and most of them show a threshold effect, with a biochemical defect only becoming apparent above a mutational load of 60-80% (**Section 1.2.5**). Theoretically, by lowering the level of the pathogenic mutation to below this biochemical threshold, one could expect a functional improvement in tissue function. This forms the basis of "gene shifting" as a treatment modality for mitochondrial diseases, and different approaches have been adopted by research groups worldwide (Taylor et al., 1997a; Horvath et al., 2008; Fraser et al., 2010). A promising option is exercise training, which not only improved muscle strength and the quality of life for patients with single mtDNA deletions, but also decreased the number of COX-negative fibres in their muscle biopsies (Taivassalo et al., 2006; Taivassalo et al., 2007; Murphy et al., 2008). The mechanism is likely to involve the activation and replication of muscle satellite cells in response to myofibre damage. These myogenic precursors are thought to contain lower levels of the mutant mtDNA species (Clark et al., 1997), and as they differentiate into myocytes and merge

with existing myofibre segments, the overall mutational burden is reduced (Zeviani, 2008). Another technique for gene shifting is the use of mutation-specific restriction endonucleases, which successfully reduced the level of the m.8993T>G mutation in cybrid cell lines (Tanaka et al., 2002). Peptide nucleic acids (PNAs) have also been shown to be effective in inhibiting the replication of mutant, but not wild-type, mtDNA *in vitro* (Taylor et al., 1997b).

Classical gene therapy is proving very challenging for primary mitochondrial disorders, because the tools required for successful gene transfer into the mitochondrial genome is still not available (DiMauro and Mancuso, 2007; Kyriakouli et al., 2008; DiMauro and Rustin, 2009). The mitochondrial inner membrane represents a significant physical barrier that needs to be overcome, and given the large number of mitochondria per cell, a highly efficient vector will be required to achieve an adequate level of transfection. To bypass these difficulties, allotopic strategies have been developed where the gene of interest is transfected into the nuclear genome, usually by an adenovirus-associated virus (AAV) vector (Manfredi et al., 2002; Qi et al., 2007a). The protein product has a mitochondrial targeting sequence and as result, it gets imported into the mitochondrial compartment, either replacing the missing protein or complementing the dysfunctional mutant protein (**See also Section 1.13.2**).

1.12.4 Preventing Transmission

Following successful fertilisation, two distinct structures can be observed within the fertilised oocyte, known as the male and female pronuclei (Brown et al., 2006). Given the lack of treatment for mitochondrial disorders, pronuclear transfer is currently being investigated as a method to prevent the transmission of mtDNA mutations in human embryos (Tachibana et al., 2009; Craven et al., 2010). This strategy involves the

replacement of the entire mitochondrial population, by transferring karyoplast containing the pronuclei from a donor zygote to an enucleated recipient zygote. In a landmark study using abnormally fertilized human zygotes, Craven and colleagues have shown that pronuclear transfer resulted in minimal carry-over of donor zygote mtDNA, and it was compatible with successful progression to the blastocyst stage (Craven et al., 2010).

1.13 Animal Models of Mitochondrial Disease

1.13.1 Autosomal Dominant Optic Atrophy

Two DOA mouse models have recently been developed which harbour pathogenic mutations in exon 8 (c.1051C>T) and intron 10 (c.1065+5g>a) of the *Opa1* gene (Alavi et al., 2007; Davies et al., 2007). These mutations are truncative in nature and they result in a 50% reduction in the overall expression of the Opa1 protein. In both models, homozygous mutant mice (*Opa1*-/-) died *in utero* during embryogenesis, highlighting the central role played by the Opa1 protein in early development. Heterozygous *Opa1*+/- mice faithfully replicated the human phenotype exhibiting a slowly progressive optic neuropathy and demonstrating objective reduction in visual function on psychophysical testing. Visual evoked potential (VEP) measurements showed significantly reduced amplitudes, but no change in latencies, supporting an ascending progress of degeneration from the soma towards the axon (Heiduschka et al., 2010). Histological and retrograde labelling experiments confirmed a gradual loss of RGCs and an associated thinning of the retinal nerve fibre layer. The surviving optic nerve axons had an abnormal morphology with swelling, distorted shapes, irregular areas of demyelination and myelin aggregates. These features of optic nerve

degeneration were seen as early as nine months, but they were much more visible by 24 months. An increased number of autophagosomes was also noted in the RGC layer of heterozygous *Opa1*+/- mice at these later time points, which is probably due the accumulation of dysfunctional mitochondria (White et al., 2009). Mitochondria within these axons showed disorganized cristae structures on transmission electron microscopy and cultured fibroblasts showed increased fragmentation of their mitochondrial network. A third *Opa1* mouse model is currently being characterised harbouring a heterozygous c.2708-2711delTTAG mutation in exon27. Heterozygous mutant mice are showing signs of optic nerve degeneration from the age of 6 months, with subnormal VEPs, and abnormal mitochondrial distribution, especially in the lamina cribosa region (Dr Guy Lenaers, personal communication). All these *Opa1* mouse models only manifest pure optic atrophy, and it will be important to create a DOA+ mouse model in order to dissect the mechanisms which contribute to multi-system involvement.

The majority of families with DOA harbour pathogenic *OPA1* mutations, but *OPA3* mutations have also been described in two French families with a dominantly inherited phenotype characterised by optic atrophy and early-onset cataracts (Reynier et al., 2004). Interestingly, *OPA3* mutations were originally described in Iraqi Jewish patients with Costeff's syndrome, an autosomal recessive, relentlessly progressive neurodegenerative disorder associated with 3-methylglutaconic aciduria (Anikster et al., 2001). Davies and colleagues have recently reported an *Opa3* mouse model carrying a c.365T>C (p.L122P) mutation (Davies et al., 2008). Heterozygous *Opa3*+/- mice were not compromised, whereas homozygous *Opa3*-/- mice developed multi-system organ failure with cachexia, dilated cardiomyopathy, extrapyramidal features, and a reduced lifespan of less than four months. These mice had severely impaired

visual function, and although all the retinal layers were affected, cell loss was much more prominent within the RGC layer, further reinforcing the selective vulnerability of this specific cell type.

1.13.2 Leber Hereditary Optic Neuropathy

There is still no animal model where the primary LHON mutations have been successfully introduced into the mitochondrial genome. In spite of these technical difficulties, four experimental techniques have been developed which can replicate the optic nerve degeneration seen in LHON: (i) intravitreal injection of a respiratory chain poison such as rotenone (Zhang et al., 2002), (ii) stereotactic injection of biodegradable rotenone-loaded microspheres into the optical layer of the superior colliculus (Marella et al., 2010), (iii) downregulation of nuclear-encoded complex I subunits (e.g. NFUFA1) with specific mRNA-degrading ribozymes (Qi et al., 2003), and (iv) allotopic expression of mutant subunits (e.g. MTND4) which are then imported into the mitochondria (Qi et al., 2007b; Ellouze et al., 2008). These disease models will be indispensable when testing the feasibility of gene therapy in LHON, and different strategies are currently being pursued. It has been shown both *in vitro* and *in vivo* that the loss of RGCs could be dramatically reduced by transfecting them with an AAV vector containing the human *SOD2* gene (Qi et al., 2004; Qi et al., 2007a). The increased expression of the superoxide dismutase enzyme is thought to improve RGC survival by minimizing free radical damage, and decreasing the cell's susceptiblity to undergo apoptosis. Allotopic rescue, with the replacement of the defective mitochondrial complex subunit, is another attractive option for gene therapy in LHON (**Section 1.12.3**). Proof-of-principle for this approach has already been demonstrated in a rat model expressing a defective *ND4* gene containing the m.11778A>G primary

LHON mutation (Ellouze et al., 2008). The loss of visual function in these rats was reversed by transfecting RGCs with the wild-type *ND4* gene, using an *in vivo* electroporation technique instead of an AAV vector. The transgene became stably integrated within the nuclear genome and the level of expression achieved was sufficient for successful RGC neuroprotection. These early studies of allotopic rescue in LHON are promising, but the results need to be replicated in larger animals, and long-term safety data is essential before human clinical trials can be contemplated (Friedmann, 2000; Miller, 2008). These issues of safety and efficacy are especially relevant given the significant concerns that have been raised recently about the limitations of allotropic rescue in the treatment of primary mtDNA disorders (Perales-Clemente et al., 2010). As yet, there is no conclusive experimental evidence that allotopically-expressed mitochondrial subunits can be properly integrated into fully-assembled OXPHOS complexes. Mitochondrially-encoded complex I subunits are also highly hydrophobic and if a proportion of these polypeptides are not imported, they could have deleterious consequences by physically aggregating into the cytosol or triggering an inappropriate immune response.

1.13.3 Chronic Progressive External Ophthalmoplegia

The development of a faithful mouse model with the classical ocular features of CPEO has proven difficult to achieve (Tyynismaa and Suomalainen, 2009; Wallace and Fan, 2009). In an *Ant1* mouse model, extraocular muscles (EOMs) from homozygous mutant *Ant1*-/- mice showed pathological mitochondrial changes which were similar to those seen in skeletal muscle from patients with CPEO (Yin et al., 2005). EOM fibres had an increase in intracellular mitochondrial content with characteristic subsarcolemmal accumulation of mitochondria. However, none of the *Ant1*-/- mice

developed marked ptosis or restriction in ocular motility (Yin et al., 2005). Two transgenic *Twinkle* mice colonies have also been created harbouring dominant mutations previously shown to be pathogenic in humans: dup352-364 and A359T (Tyynismaa et al., 2005). COX-deficiency was observed in skeletal muscle and distinct brain regions of heterozygous *Twinkle*^{dup} and *Twinkle*^{AT} mice, with Southern blot and long PCR confirming the accumulation of multiple mtDNA deletions in these tissues. EOMs from these mice were not studied, but similar to the *Ant1* mice, the *Twinkle* mice did not develop features suggestive of CPEO. It is not entirely clear why these mouse models fail to replicate the prominent ocular phenotype seen in patients harbouring *SLC25A4* and *PEO1* mutations, despite clear histological and molecular evidence of mitochondrial dysfunction in post-mitotic tissues. Inter-species differences in EOM physiology such as fatigue resistance could be important, but there also technical limitations, which could have resulted in subtle deficits in EOM function being missed. It will be very interesting to document the EOM features present in a future DOA+ mouse model, and determine whether CPEO eventually develops during the course of the disease process.

Chapter 2

Research Focus

The aim of my PhD project was to further our understanding of mitochondrial disorders with ocular manifestations, both from the clinical and molecular genetic perspectives. The published work presented in this thesis are focused on the following main research areas:

2.1 Autosomal Dominant Optic Atrophy

In the initial phase of this project, I set about defining the epidemiology and natural history of DOA in a defined geographical region encompassing the North of England. A detailed neuro-ophthalmological assessment was performed on all patients, with the collection of additional data such as retinal nerve fibre layer thickness with optical coherence tomography (OCT), and *in vivo* mitochondrial oxidative function with phosphorus magnetic resonance spectroscopy (^{31}P-MRS). A relatively high proportion of affected *OPA1* carriers developed DOA+ features, and in a subsequent multi-centre study, I collected a large dataset which confirmed the expanding phenotypic spectrum linked with *OPA1* disease. In collaboration with research groups at the University of Tübingen (Germany) and Emory University (USA), we further studied the frequency of *OPA1* and *OPA3* mutations in patients with suspected inherited optic neuropathies. During the course of these clinical studies , it became evident that the same *OPA1* mutation could cause both pure and syndromal forms of DOA. In order to explore the basic disease mechanisms that influenced the development of these additional neuromuscular features, I investigated the mitochondrial changes present in tissues from two *Opa1* mouse models, and patients with both pure DOA and DOA+ phenotypes.

2.2 Leber Hereditary Optic Neuropathy

LHON carries a poor visual prognosis and in a multi-centre study using the VF-14 questionnaire, the quality of life of a large group of affected and unaffected LHON carriers was formally assessed. The marked gender bias and incomplete penetrance in LHON is still not fully understood, and using the same patient cohort, the contribution of possible environmental triggers was studied further.

2.3 Chronic Progressive External Ophthalmoplegia

CPEO is a frequent manifestation of mitochondrial genetic disorders, being observed in at least a third of all affected patients. Interestingly, it often develops in isolation, and even when other neurological features are present, the severity of ptosis and ophthalmoplegia often predominates. In order to investigate this selective involvement, we first documented the mitochondrial changes seen in EOMs during normal ageing, relating our findings to those seen in skeletal muscle. We then performed a comprehensive histological and molecular genetic study comparing the pathological features seen in EOM and skeletal muscle samples from patients with CPEO.

Chapter 3

DOA – Clinical Phenotype and Mutational Spectrum

3.1 *OPA1* in multiple mitochondrial DNA deletion disorders

Stewart JD, Hudson G, **Yu-Wai-Man P**, Horvath R, Maddison P, Wright A, Blakeley EL, He LP, Griffiths PG, Turnbull DM, Taylor RW, Chinnery PF. *OPA1* in multiple mitochondrial DNA deletion disorders. Neurology. 2008:71(22):1829-31.

3.2 Genomic rearrangements in *OPA1* are frequent in autosomal dominant optic atrophy

Fuhrmann N, Alavi MV, Bitoun P, Woernle S, Auburger G, Leo-Kottler B, **Yu-Wai-Man P**, Chinnery PF, Wissinger B. Genomic rearrangements in OPA1 are frequent in autosomal dominant optic atrophy. Journal of Medical Genetics. 2009;46(2):136-44.

3.3 *OPA1* increases the risk of normal but not high tension glaucoma

Yu-Wai-Man P, Stewart JD, Hudson G, Andrews RM, Griffiths PG, Birch MK, Chinnery PF. *OPA1* increases the risk of normal but not high tension glaucoma. Journal of Medical Genetics. 2010;47(2):120-5.

3.4 Multi-system neurological disease is common in patients with *OPA1* mutations

Yu-Wai-Man P, Griffiths PG, Gorman GS, Lourenco CM, Wright AF, Auer-Grumbach M, Toscano A, Musumeci O, Valentino ML, Caporali L, Lamperti C, Tallaksen CM, Duffey P, Miller J, Whittaker RG, Baker MR, Jackson MJ, Clarke MP, Dhillon B, Czermin B, Stewart JD, Hudson G, Reynier P, Bonneau D, Marques W Jr, Lenaers G, McFarland R, Taylor RW, Turnbull DM, Votruba M, Zeviani M, Carelli V, Bindoff L, Horvath R, Amati-Bonneau P, Chinnery PF. Brain. 2010;133(3):771-86.

3.5 The prevalence and natural history of dominant optic atrophy due to *OPA1* mutations

Yu-Wai-Man P, Griffiths PG, Burke A, Sellar PW, Clarke MP, Gnanaraj L, Ah-Kine D, Hudson G, Czermin B, Taylor RW, Horvath R, Chinnery PF. Ophthalmology. 2010;117(8):1538-1546.

3.6 Genetic Screening for *OPA1* and *OPA3* Mutations in Patients with Suspected Inherited Optic Neuropathies

Yu-Wai-Man P, Shankar SP, Biousse V, Miller NR, Bean LJH, Coffee B, Hegde M, Newman NJ. Ophthalmology. 2010; *Epub ahead of print*.

3.7 Pattern of retinal ganglion cell loss in dominant optic atrophy due to *OPA1* mutations

Yu-Wai-Man P, Bailie M, Atawan A, Chinnery PF, Griffiths PG. Eye. *In press*.

Chapter 4

DOA – Animal Models and Disease Mechanisms

4.1 Secondary mtDNA defects do not cause optic nerve dysfunction in a mouse model of dominant optic atrophy

Yu-Wai-Man P, Davies VJ, Piechota MJ, Cree LM, Votruba M, Chinnery PF. Investigative Ophthalmology and Visual Science. 2009;50(10):4561-6.

4.2 Subtle neurological and metabolic abnormalities in an *Opa1* mouse model of autosomal dominant optic atrophy

Alavi MV, Fuhrmann N, Nguyen HP, **Yu-Wai-Man P**, Heiduschka P, Chinnery PF, Wissinger B. Experimental Neurology. 2009;220(2):404-9.

4.3 *OPA1* mutations cause cytochrome *c* oxidase deficiency due to loss of wild-type mtDNA molecules.

Yu-Wai-Man P, Sitarz KS, Samuels DC, Griffiths PG, Reeve AK, Bindoff L, Horvath R, Chinnery PF. Human Molecular Genetics. 2010;19(15):3043-52.

4.4 *OPA1* mutations impair mitochondrial function in both pure and complicated dominant optic atrophy

Yu-Wai-Man P, Trenell MI, Hollingsworth KG, Griffiths PG, Chinnery PF. Brain. 2010; *Epub ahead of print*.

Chapter 5

Leber Hereditary Optic Neuropathy

5.1 Leber hereditary optic neuropathy presenting in a 75-year-old man.

Yu-Wai-Man P, Bateman DE, Hudson G, Griffiths PG, Chinnery PF. Journal of Neuro-ophthalmology. 2008;28(2):155.

5.2 Gene-environment interactions in Leber hereditary optic neuropathy

Kirkman MA, **Yu-Wai-Man P**, Korsten A, Leonhardt M, Dimitriadis K, De Coo IF, Klopstock T, Chinnery PF. Brain. 2009;132(9):2317-26.

5.3 Quality of life in patients with Leber hereditary optic neuropathy

Kirkman MA, Korsten A, Leonhardt M, Dimitriadis K, De Coo IF, Klopstock T, Griffiths PG, Hudson G, Chinnery PF, **Yu-Wai-Man P**. Investigative Ophthalmology and Visual Science. 2009;50(7):3112-5.

5.4 Genetic variation in the methylenetetrahydrofolate reductase gene (MTHFR) does not alter the risk of visual failure in Leber hereditary optic neuropathy.

Hudson G, **Yu-Wai-Man P**, Zeviani M, Chinnery PF. Molecular Vision. 2009;15:870-5.

Chapter 6

Extraocular Muscles – Mitochondrial Ageing and Disease

6.1 Somatic mitochondrial DNA deletions accumulate to high levels in aging human extraocular muscles

Yu-Wai-Man P, Lai-Cheong JE, Borthwick GM, He L, Taylor GA, Greaves LC, Taylor RW, Griffiths PG, Turnbull DM. Investigative Ophthalmology and Visual Science. 2010;51(7):3347-53.

6.2 Mitochondrial DNA defects and selective extraocular muscle involvement in CPEO

Greaves LC, **Yu-Wai-Man P**, Blakely EL, Krishnan KJ, Beadle NE, Kerin J, Barron MJ, Griffiths PG, Dickinson AJ, Turnbull DM, Taylor RW. Investigative Ophthalmology and Visual Science. 2010;51(7):3340-6.

Chapter 7

Other Neuro-Ophthalmological Disorders

7.1 Vertigo and vestibular abnormalities in spinocerebellar ataxia type 6 (SCA6)

Yu-Wai-Man P, Gorman G, Bateman DE, Leigh RJ, Chinnery PF. Journal of Neurology. 2009;256(1):78-82.

7.2 Complex I deficiency in recessive spastic paraparesis due to methylenetetrahydrofolate reductase deficiency

Bathgate D, **Yu-Wai-Man P**, Webb B, Taylor RW, Fowler B, Chinnery PF. Journal of Neurology, Neurosurgery and Psychiatry. *Epub ahead of print*.

Chapter 8

Review Articles

8.1 Leber hereditary optic neuropathy

Yu-Wai-Man P, Chinnery PF. In: Pagon RA, Bird TC, Dolan CR, Stephens K, editors. Gene Reviews. 2008; online access at: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=lhon

8.2 Leber hereditary optic neuropathy

Hudson G, **Yu-Wai-Man P**, Chinnery PF. Expert Opinion on Medical Diagnostics. 2008;2(7):789-99.

8.3 The clinical spectrum of mitochondrial genetic disorders

Kirkman MA, **Yu-Wai-Man P**, Chinnery PF. Clinical Medicine. 2008;8(6):601-6.

8.4 Inherited mitochondrial optic neuropathies

Yu-Wai-Man P, Griffiths PG, Hudson G, Chinnery PF. Journal of Medical Genetics. 2009;46(3):145-58.

8.5 Inherited optic neuropathies – Disease mechanisms and therapeutic strategies

Yu-Wai-Man P, Griffiths PG, Chinnery PF. Progress in Retinal and Eye Research.

Epub ahead of print.

Chapter 9

References

Abrahams JP, Leslie AGW, Lutter R, Walker JE. Structure at 2.8-Angstrom Resolution of F1-Atpase from Bovine Heart-Mitochondria. Nature 1994; 370: 621-628.

Acin-Perez R, Bayona-Bafaluy MP, Fernandez-Silva P, Moreno-Loshuertos R, Perez-Martos A, Bruno C, et al. Respiratory complex III is required to maintain complex I in mammalian mitochondria. Molecular Cell 2004; 13: 805-815.

Ahn J, Kim NJ, Choung HK, Hwang SW, Sung M, Lee MJ, et al. Frontalis sling operation using silicone rod for the correction of ptosis in chronic progressive external ophthalmoplegia. British Journal of Ophthalmology 2008; 92: 1685-1688.

Akman HO, Dorado B, Lopez LC, Garcia-Cazorla A, Vila MR, Tanabe LM, et al. Thymidine kinase 2 (H126N) knockin mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance. Human Molecular Genetics 2008; 17: 2433-2440.

Alavi MV, Bette S, Schimpf S, Schuettauf F, Schraermeyer U, Wehrl HF, et al. A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy. Brain 2007; 130: 1029-42.

Alberio S, Mineri R, Tiranti V, Zeviani M. Depletion of mtDNA: Syndromes and genes. Mitochondrion 2007; 7: 6-12.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Energy conversion: mitochondria and chloroplasts. In: Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD, editors. Molecular Biology of the Cell. New York: Garland Publishing, 1994: 655-717.

Alexander C, Votruba M, Pesch UEA, Thiselton DL, Mayer S, Moore A, et al. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nature Genetics 2000; 26: 211-215.

Amati-Bonneau P, Valentino ML, Reynier P, Gallardo ME, Bornstein B, Boissiere A, et al. OPA1 mutations induce mitochondrial DNA instability and optic atrophy plus phenotypes. Brain 2008; 131: 338-351.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981; 290: 457-65.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature Genetics 1999; 23: 147-147.

Anikster Y, Kleta R, Shaag A, Gahl WA, Elpeleg O. Type III 3-methylglutaconic aciduria (optic atrophy plus syndrome, or Costeff optic atrophy syndrome): Identification of the OPA3 gene and its founder mutation in Iraqi Jews. American Journal of Human Genetics 2001; 69: 1218-1224.

Arnoldi A, Tonelli A, Crippa F, Villani G, Pacelli C, Sironi M, et al. A clinical, genetic, and biochemical characterization of SPG7 mutations in a large cohort of patients with hereditary spastic paraplegia. Human Mutation 2008; 29: 522-531.

Ashley N, Adams S, Slama A, Zeviani M, Suomalainen A, Andreu AL, et al. Defects in maintenance of mitochondrial DNA are associated with intramitochondrial nucleotide imbalances. Human Molecular Genetics 2007; 16: 1400-1411.

Asin-Cayuela J, Gustafsson CM. Mitochondrial transcription and its regulation in mammalian cells. Trends in Biochemical Sciences 2007; 32: 111-117.

Asin-Cayuela J, Helm M, Attardi G. A monomer-to-trimer transition of the human mitochondrial transcription termination factor (mTERF) is associated with a loss of in vitro activity. Journal of Biological Chemistry 2004; 279: 15670-15677.

Barbiroli B, Montagna P, Cortelli P, Iotti S, Lodi R, Barboni P, et al. Defective Brain and Muscle Energy-Metabolism Shown by in-Vivo P-31 Magnetic-Resonance Spectroscopy in Nonaffected Carriers of 11778-Mtdna Mutation. Neurology 1995; 45: 1364-1369.

Barnils N, Mesa E, Munoz S, Ferrer-Artola A, Arruga J. Response to idebenone and multivitamin therapy in Leber's hereditary optic neuropathy. Arch Soc Esp Oftalmol 2007; 82: 377-80.

Befroy DE, Petersen KF, Rothman DL, Shulman GI. Assessment of in Vivo Mitochondrial Metabolism by Magnetic Resonance Spectroscopy. Methods in Enzymology 2009; 457: 373-393.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nature Genetics 2006; 38: 515-517.

Bianchi NO. Mitochondrial Genome Instability in Cancer. Cytogenetic and Genome Research 2010; 128: 66-76.

Birch-Machin MA, Taylor RW, Cochran B, Ackrell BAC, Turnbull DM. Late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II gene. Annals of Neurology 2000; 48: 330-335.

Bogenhagen DF, Rousseau D, Burke S. The layered structure of human mitochondrial DNA nucleoids. Journal of Biological Chemistry 2008; 283: 3665-3675.

Brierley EJ, Johnson MA, Bowman A, Ford GA, Subhan F, Reed JW, et al. Mitochondrial function in muscle from elderly athletes. Annals of Neurology 1997; 41: 114-116.

Brierley EJ, Johnson MA, James OFW, Turnbull DM. Effects of physical activity and age on mitochondrial function. Qjm-Monthly Journal of the Association of Physicians 1996; 89: 251-258.

Brown DT, Herbert M, Lamb VK, Chinnery PF, Taylor RW, Lightowlers RN, et al. Transmission of mitochondrial DNA disorders: possibilities for the future. Lancet 2006; 368: 87-89.

Brown DT, Samuels DC, Michael EM, Turnbull DM, Chinnery PF. Random genetic drift determines the level of mutant mtDNA in human primary oocytes. American Journal of Human Genetics 2001; 68: 533-536.

Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA. Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. Genes & Development 2005; 19: 2466-2476.

Bua E, Johnson J, Herbst A, Delong B, McKenzie D, Salamat S, et al. Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. American Journal of Human Genetics 2006; 79: 469-480.

Cann RL. Genetic clues to dispersal in human populations: retracing the past from the present. Science 2001; 291: 1742-8.

Cao LQ, Shitara H, Horii T, Nagao Y, Imai H, Abe K, et al. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. Nature Genetics 2007; 39: 386-390.

Casari G, De Fusco M, Ciarmatori S, Zeviani M, Mora M, Fernandez P, et al. Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease. Cell 1998; 93: 973-983.

Chan DC. Dissecting mitochondrial fusion. Developmental Cell 2006; 11: 592-594.

Chan DC. Mitochondrial dynamics in disease. New England Journal of Medicine 2007; 356: 1707-1709.

Chang DD, Clayton DA. Precise Identification of Individual Promoters for Transcription of Each Strand of Human Mitochondrial-DNA. Cell 1984; 36: 635-643.

Chang DD, Clayton DA. Priming of Human Mitochondrial-DNA Replication Occurs at the Light-Strand Promoter. Proceedings of the National Academy of Sciences of the United States of America 1985; 82: 351-355.

Chen H, Chan DC. Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. Human Molecular Genetics 2009; 18: R169-R176.

Chen H, McCaffery JM, Chan DC. Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell 2007; 130: 548-562.

Chen HC, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. Journal of Cell Biology 2003; 160: 189-200.

Chinnery PF. Modulating heteroplasmy. Trends in Genetics 2002; 18: 173-176.

Chinnery PF, Andrews RM, Turnbull DM, Howell NN. Leber hereditary optic neuropathy: Does heteroplasmy influence the inheritance and expression of the G11778A mitochondrial DNA mutation? American Journal of Medical Genetics 2001; 98: 235-43.

Chinnery PF, DiMauro S, Shanske S, Schon EA, Zeviani M, Mariotti C, et al. Risk of developing a mitochondrial DNA deletion disorder. Lancet 2004; 364: 592-596.

Chinnery PF, Howell N, Andrews RM, Turnbull DM. Clinical mitochondrial genetics. Journal of Medical Genetics 1999a; 36: 425-36.

Chinnery PF, Howell N, Andrews RM, Turnbull DM. Mitochondrial DNA analysis: Polymorphisms and pathogenicity. Journal of Medical Genetics 1999b; 36: 505-510.

Chinnery PF, Johnson MA, Wardell TM, Singh-Kler R, Hayes C, Brown DT, et al. The epidemiology of pathogenic mitochondrial DNA mutations. Annals of Neurology 2000a; 48: 188-93.

Chinnery PF, Majamaa K, Turnbull D, Thorburn D. Treatment for mitochondrial disorders. Cochrane Database of Systematic Reviews 2006.

Chinnery PF, Samuels DC. Relaxed replication of mtDNA: A model with implications for the expression of disease. American Journal of Human Genetics 1999; 64: 1158-1165.

Chinnery PF, Samuels DC, Elson J, Turnbull DM. Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? Lancet 2002; 360: 1323-1325.

Chinnery PF, Schon EA. Mitochondria. Journal of Neurology Neurosurgery and Psychiatry 2003; 74: 1188-1199.

Chinnery PF, Thorburn DR, Samuels DC, White SL, Dahl HHM, Turnbull DM, et al. The inheritance of mitochondrial DNA heteroplasmy: random drift, selection or both? Trends in Genetics 2000b; 16: 500-505.

Chinnery PF, Vissing J. Treating MNGIE - Is reducing blood nucleosides the first cure for a mitochondrial disorder? Neurology 2006; 67: 1330-1332.

Chinnery PF, Zeviani M. 155th ENMC workshop: polymerase gamma and disorders of mitochondrial DNA synthesis, 21-23 September 2007, Naarden, The Netherlands. Neuromuscul Disord 2008; 18: 259-67.

Chinnery PF, Zwijnenburg PJG, Walker M, Howell N, Taylor RW, Lightowlers RN, et al. Nonrandom tissue distribution of mutant mtDNA. American Journal of Medical Genetics 1999c; 85: 498-501.

Christian B, Haque E, Spremulli L. Ribosome Shifting or Splitting: It Is All Up To the EF-G. Molecular Cell 2009; 35: 400-402.

Chung KW, Kim SB, Park KD, Choi KG, Lee JH, Eun HW, et al. Early onset severe and late-onset mild Charcot-Marie-Tooth disease with mitofusin 2 (MFN2) mutations. Brain 2006; 129: 2103-2118.

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, et al. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell 2006; 126: 163-175.

Clark KM, Bindoff LA, Lightowlers RN, Andrew RM, Griffiths PG, Johnson MA, et al. Reversal of a mitochondrial DNA defect in human skeletal muscle. Nature Genetics 1997; 16: 222-224.

Clayton DA. Replication of Animal Mitochondrial-DNA. Cell 1982; 28: 693-705.

Coller HA, Khrapko K, Bodyak ND, Nekhaeva E, Herrero-Jimenez P, Thilly WG. High frequency of homoplasmic mitochondrial DNA mutations in human tumors can be explained without selection. Nature Genetics 2001; 28: 147-150.

Corral-debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF, Wallace DC. Mitochondrial-DNA Deletions in Human Brain - Regional Variability and Increase with Advanced Age. Nature Genetics 1992; 2: 324-329.

Craven L, Tuppen HA, Greggains GD, Harbottle SJ, Murphy JL, Cree LM, et al. Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. Nature 2010; 465: 82-U89.

Cree LM, Samuels DC, Chinnery PF. The inheritance of pathogenic mitochondrial DNA mutations. Biochimica Et Biophysica Acta-Molecular Basis of Disease 2009; 1792: 1097-1102.

Cree LM, Samuels DC, Lopes S, Rajasimha HK, Wonnapinij P, Mann JR, et al. A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nature Genetics 2008; 40: 249-254.

Daut PM, Steinemann TL, Westfall CT. Chronic exposure keratopathy complicating surgical correction of ptosis in patients with chronic progressive external ophthalmoplegia. American Journal of Ophthalmology 2000; 130: 519-521.

Davies V, Votruba M. Focus on molecules: the OPA1 protein. Experimental Eye Research 2006; 83: 1003-4.

Davies VJ, Hollins AJ, Piechota MJ, Yip W, Davies JR, White KE, et al. Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. Human Molecular Genetics 2007; 16: 1307-18.

Davies VJ, Powell KA, White KE, Yip W, Hogan V, Hollins AJ, et al. A missense mutation in the murine Opa3 gene models human Costeff syndrome. Brain 2008; 131: 368-380.

Del Bo R, Bordoni A, Sciacco M, Di Fonzo A, Galbiati S, Crimi M, et al. Remarkable infidelity of polymerase gamma A associated with mutations in POLG1 exonuclease domain. Neurology 2003; 61: 903-908.

Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, et al. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nature Genetics 2000; 26: 207-210.

Detmer SA, Chan DC. Functions and dysfunctions of mitochondrial dynamics. Nature Reviews Molecular Cell Biology 2007; 8: 870-879.

Di Fonzo A, Bordoni A, Crimi M, Sara G, Del Bo R, Bresolin N, et al. POLG mutations in sporadic mitochondrial disorders with multiple mtDNA deletions. Hum Mutat 2003; 22: 498-9.

Di Prospero NA, Baker A, Jeffries N, Fischbeck KH. Neurological effects of high-dose idebenone in patients with Friedreich's ataxia: a randomised, placebo-controlled trial. Lancet Neurology 2007; 6: 878-886.

Diaz F, Moraes CT. Mitochondrial biogenesis and turnover. Cell Calcium 2008; 44: 24-35.

Dietrichson P, Coakley J, Smith PEM, Griffiths RD, Helliwell TR, Tedwards RH. Conchotome and Needle Percutaneous Biopsy of Skeletal-Muscle. Journal of Neurology Neurosurgery and Psychiatry 1987; 50: 1461-1467.

DiMauro S, Mancuso M. Mitochondrial diseases: Therapeutic approaches. Bioscience Reports 2007; 27: 125-137.

DiMauro S, Rustin P. A critical approach to the therapy of mitochondrial respiratory chain and oxidative phosphorylation diseases. Biochimica Et Biophysica Acta-Molecular Basis of Disease 2009; 1792: 1159-1167.

DiMauro S, Schon EA. Mechanisms of disease: Mitochondrial respiratory-chain diseases. New England Journal of Medicine 2003; 348: 2656-2668.

Durham SE, Samuels DC, Cree LM, Chinnery PF. Normal levels of wild-type mitochondrial DNA maintain cytochrome c oxidase activity for two pathogenic mitochondrial DNA mutations but not for m.3243A -> G. American Journal of Human Genetics 2007; 81: 189-195.

Duvezin-Caubet S, Jagasia R, Wagener J, Hofmann S, Trifunovic A, Hansson A, et al. Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. Journal of Biological Chemistry 2006; 281: 37972-37979.

Duvezin-Caubet S, Koppen M, Wagener J, Zick M, Israel L, Bernacchia A, et al. OPA1 processing reconstituted in yeast depends on the subunit composition of the m-AAA protease in mitochondria. Molecular Biology of the Cell 2007; 18: 3582-3590.

Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Human Molecular Genetics 2004; 13: 935-944.

Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF. Pathogenic mitochondrial DNA mutations are common in the general population. American Journal of Human Genetics 2008; 83: 254-60.

Ellouze S, Augustin S, Bouaita A, Bonnet C, Simonutti M, Forster V, et al. Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction. American Journal of Human Genetics 2008; 83: 373-387.

Elson JL, Samuels DC, Turnbull DM, Chinnery PF. Random intracellular drift explains the clonal expansion of mitochondrial DNA mutations with age. American Journal of Human Genetics 2001; 68: 802-806.

Fahy E, Nazarbaghi R, Zomorrodi M, Herrnstadt C, Parker WD, Davis RE, et al. Multiplex fluorescence-based primer extension method for quantitative mutation analysis of mitochondrial DNA and its diagnostic application for Alzheimer's disease. Nucleic Acids Research 1997; 25: 3102-3109.

Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM. Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nature Genetics 2002; 31: 289-294.

Fan WW, Waymire KG, Narula N, Li P, Rocher C, Coskun PE, et al. A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. Science 2008; 319: 958-962.

Faxen K, Gilderson G, Adelroth P, Brzezinski P. A mechanistic principle for proton pumping by cytochrome c oxidase. Nature 2005; 437: 286-289.

Fayet G, Jansson M, Sternberg D, Moslemi AR, Blondy P, Lombes A, et al. Ageing muscle: clonal expansions of mitochondrial DNA point mutations and deletions cause focal impairment of mitochondrial function. Neuromuscular Disorders 2002; 12: 484-493.

Ferre M, Amati-Bonneau P, Tourmen Y, Malthiery Y, Reynier P. eOPA1: An online database for OPA1 mutations. Human Mutation 2005; 25: 423-428.

Ferre M, Bonneau D, Milea D, Chevrollier A, Verny C, Dollfus H, et al. Molecular screening of 980 cases of suspected hereditary optic neuropathy with a report on 77 novel OPA1 mutations. Hum Mutat 2009; 30: E692-705.

Fisher RP, Topper JN, Clayton DA. Promoter Selection in Human Mitochondria Involves Binding of a Transcription Factor to Orientation-Independent Upstream Regulatory Elements. Cell 1987; 50: 247-258.

Fraser JA, Biousse V, Newman NJ. The Neuro-ophthalmology of Mitochondrial Disease. Survey of Ophthalmology 2010; 55: 299-334.

Frey TG, Mannella CA. The internal structure of mitochondria. Trends in Biochemical Sciences 2000; 25: 319-324.

Friedmann T. Medical ethics - Principles for human gene therapy studies. Science 2000; 287: 2163-+.

Fukui H, Moraes CT. Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. Human Molecular Genetics 2009; 18: 1028-1036.

Gabaldon T, Huynen MA. Shaping the mitochondrial proteome. Biochimica Et Biophysica Acta-Bioenergetics 2004; 1659: 212-220.

Gibbons C, Montgomery MG, Leslie AGW, Walker JE. The structure of the central stalk in bovine F-1-ATPase at 2.4 angstrom resolution. Nature Structural Biology 2000; 7: 1055-1061.

Giles RE, Blanc H, Cann HM, Wallace DC. Maternal Inheritance of Human Mitochondrial-DNA. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 1980; 77: 6715-6719.

Gilkerson RW. Mitochondrial DNA nucleoids determine mitochondrial genetics and dysfunction. International Journal of Biochemistry & Cell Biology 2009; 41: 1899-1906.

Gottlieb E. OPA1 and PARL keep a lid on apoptosis. Cell 2006; 126: 27-29.

Gray MW, Burger G, Lang BF. Mitochondrial evolution. Science 1999; 283: 1476-1481.

Greaves LC, Preston SL, Tadrous PJ, Taylor RW, Barron MJ, Oukrif D, et al. Mitochondrial DNA mutations are established in human colonic stem cells, and mutated clones expand by crypt fission. Proceedings of the National Academy of Sciences of the United States of America 2006; 103: 714-719.

Greaves LC, Turnbull DM. Mitochondrial DNA mutations and ageing. Biochimica Et Biophysica Acta-General Subjects 2009; 1790: 1015-1020.

Greaves LC, Yu-Wai-Man P, Blakely EL, Krishnan KJ, Beadle NE, Kerin J, et al. Mitochondrial DNA Defects and Selective Extraocular Muscle Involvement in CPEO. Investigative Ophthalmology & Visual Science 2010; 51: 3340-3346.

Grigorieff N. Structure of the respiratory NADH : ubiquinone oxidoreductase (complex I). Current Opinion in Structural Biology 1999; 9: 476-483.

Halter J, Schupbach WM, Casali C, Elhasid R, Fay K, Hammans S, et al. Allogeneic hematopoietic SCT as treatment option for patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): a consensus conference proposal for a standardized approach. Bone Marrow Transplant 2010; *Epub ahead of print*.

Harding AE, Sweeney MG, Govan GG, Riordan-Eva P. Pedigree analysis in Leber hereditary optic neuropathy families with a pathogenic mtDNA mutation. American Journal of Human Genetics 1995; 57: 77-86.

Hatefi Y, Galante YM. Isolation of Cytochrome-B560 from Complex Ii (Succinate-Ubiquinone Oxidoreductase) and Its Reconstitution with Succinate-Dehydrogenase. Journal of Biological Chemistry 1980; 255: 5530-5537.

He JY, Mao CC, Reyes A, Sembongi H, Di Re M, Granycome C, et al. The AAA(+) protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. Journal of Cell Biology 2007; 176: 141-146.

He LP, Chinnery PF, Durham SE, Blakely EL, Wardell TM, Borthwick GM, et al. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. Nucleic Acids Research 2002; 30.

He YP, Wu J, Dressman DC, Iacobuzio-Donahue C, Markowitz SD, Velculescu VE, et al. Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. Nature 2010; 464: 610-U175.

Heiduschka P, Schnichels S, Fuhrmann N, Hofmeister S, Schraermeyer U, Wissinger B, et al. Electrophysiological and Histologic Assessment of Retinal Ganglion Cell Fate in a Mouse Model for OPA1-Associated Autosomal Dominant Optic Atrophy. Investigative Ophthalmology & Visual Science 2010; 51: 1424-1431.

Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407: 770-776.

Herrnstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, et al. Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups. American Journal of Human Genetics 2002; 70: 1152-1171.

Herrnstadt C, Howell N. An evolutionary perspective on pathogenic mtDNA mutations: haplogroup associations of clinical disorders. Mitochondrion 2004; 4: 791-798.

Hirano M, Marti R, Casali C, Tadesse S, Uldrick T, Fine B, et al. Allogeneic stem cell transplantation corrects biochemical derangements in MNGIE. Neurology 2006; 67: 1458-1460.

Hirst J, Carroll J, Fearnley IM, Shannon RJ, Walker JE. The nuclear encoded subunits of complex I from bovine heart mitochondria. Biochimica Et Biophysica Acta-Bioenergetics 2003; 1604: 135-150.

Holt I, Yasukawa T, Reyes A, Yang M, Bowmaker M. Replication of mitochondrial DNA. Biochimica Et Biophysica Acta-Bioenergetics 2004; 1657: 12-12.

Holt IJ, Harding AE, Morganhughes JA. Deletions of Muscle Mitochondrial-DNA in Patients with Mitochondrial Myopathies. Nature 1988; 331: 717-719.

Holt IJ, Lorimer HE, Jacobs HT. Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. Cell 2000; 100: 515-524.

Horvath R, Gorman G, Chinnery PF. How can we treat mitochondrial encephalomyopathies? Approaches to therapy. Neurotherapeutics 2008; 5: 558-568.

Horvath R, Hudson G, Ferrari G, Futterer N, Ahola S, Lamantea E, et al. Phenotypic spectrum associated with mutations of the mitochondrial polymerase gamma gene. Brain 2006; 129: 1674-1684.

Horvath R, Kemp JP, Tuppen HAL, Hudson G, Oldfors A, Marie SKN, et al. Molecular basis of infantile reversible cytochrome c oxidase deficiency myopathy. Brain 2009; 132: 3165-3174.

Howell N, Kubacka I, Mackey DA. How rapidly does the human mitochondrial genome evolve? American Journal of Human Genetics 1996; 59: 501-9.

Hudson G, Amati-Bonneau P, Blakely EL, Stewart JD, He LP, Schaefer AM, et al. Mutation of OPA1 causes dominant optic atrophy with external ophthalmoplegia, ataxia, deafness and multiple mitochondrial DNA deletions: a novel disorder of mtDNA maintenance. Brain 2008; 131: 329-337.

Hudson G, Chinnery PF. Mitochondrial DNA polymerase-gamma and human disease. Human Molecular Genetics 2006; 15: R244-R252.

Hudson G, Deschauer M, Taylor RW, Hanna MG, Fialho D, Schaefer AM, et al. POLG1, C10ORF2, and ANT1 mutations are uncommon in sporadic progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. Neurology 2006; 66: 1439-1441.

Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, et al. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nature Cell Biology 2009; 11: 958-U114.

Janssen AJM, Smeitink JAM, van den Heuvel LP. Some practical aspects of providing a diagnostic service for respiratory chain defects. Annals of Clinical Biochemistry 2003; 40: 3-8.

Jazin E, Soodyall H, Jalonen P, Lindholm E, Stoneking M, Gyllensten U. Mitochondrial mutation rate revisited: hot spots and polymorphism. Nature Genetics 1998; 18: 109-110.

Jenuth JP, Peterson AC, Fu K, Shoubridge EA. Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nature Genetics 1996; 14: 146-151.

Jenuth JP, Peterson AC, Shoubridge EA. Tissue-specific selection for different mtDNA genotypes in heteroplasmic mice. Nature Genetics 1997; 16: 93-95.

Johnson MA, Barron MJ. Muscle biopsy analysis. In: Lane RJM, editor. Handbook of muscle disease New York, USA: Marcel Dekker, 1996: 61-79.

Kadenbach B, Jarausch J, Hartmann R, Merle P. Separation of Mammalian Cytochrome-C-Oxidase into 13 Polypeptides by a Sodium Dodecyl-Sulfate Gel-Electrophoretic Procedure. Analytical Biochemistry 1983; 129: 517-521.

Kasashima K, Sumitani M, Satoh M, Endo H. Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids. Experimental Cell Research 2008; 314: 988-996.

Khrapko K. Two ways to make an mtDNA bottleneck. Nature Genetics 2008; 40: 134-135.

Khrapko K, Bodyak N, Thilly WG, van Orsouw NJ, Zhang XM, Coller HA, et al. Cell-by-cell scanning of whole mitochondrial genomes in aged human heart reveals a significant fraction of myocytes with clonally expanded deletions. Nucleic Acids Research 1999; 27: 2434-2441.

Khrapko K, Vijg J. Mitochondrial DNA mutations and aging: a case closed? Nature Genetics 2007; 39: 445-446.

Khrapko K, Vijg J. Mitochondrial DNA mutations and aging: devils in the details? Trends in Genetics 2009; 25: 91-98.

Kirby DM, Thorburn DR, Turnbull DM, Taylor RW. Biochemical assays of respiratory chain complex activity. Mitochondria, 2nd Edition. Vol 80, 2007: 93-119.

Kirkman MA, Yu-Wai-Man P, Chinnery PF. The clinical spectrum of mitochondrial genetic disorders. Clinical Medicine 2008; 8: 601-606.

Kirkman MA, Yu-Wai-Man P, Korsten A, Leonhardt M, Dimitriadis K, De Coo IF, et al. Gene-environment interactions in Leber hereditary optic neuropathy. Brain 2009; 132: 2317-2326.

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, et al. The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 2002; 419: 367-74.

Knott AB, Perkins G, Schwarzenbacher R, Bossy-Wetzel E. Mitochondrial fragmentation in neurodegeneration. Nature Reviews Neuroscience 2008; 9: 505-518.

Ko YH, Hullihen J, Hong S, Pedersen PL. Mitochondrial F(0)F1 ATP synthase - Subunit regions on the F-1 motor shielded by F-0, functional significance, and evidence for an involvement of the unique F-0 subunit F-6. Journal of Biological Chemistry 2000; 275: 32931-32939.

Koch A, Yoon Y, Bonekamp NA, McNiven MA, Schrader M. A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Molecular Biology of the Cell 2005; 16: 5077-5086.

Koopman WJH, Verkaart S, Visch HJ, van Emst-de Vries S, Nijtmans LGJ, Smeitink JAM, et al. Human NADH: ubiquinone oxidoreductase deficiency: radical changes in mitochondrial morphology? American Journal of Physiology-Cell Physiology 2007; 293: C22-C29.

Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, Khrapko K. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nature Genetics 2006; 38: 518-520.

Krishnan KJ, Greaves LC, Reeve AK, Turnbull DM. Mitochondrial DNA mutations and aging. In: Rattan SISAS, editor. Biogerontology: Mechanisms and Interventions. Vol 1100, 2007: 227-240.

Krishnan KJ, Reeve AK, Samuels DC, Chinnery PF, Blackwood JK, Taylor RW, et al. What causes mitochondrial DNA deletions in human cells? Nature Genetics 2008; 40: 275-279.

Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science 2005; 309: 481-484.

Kukat A, Trifunovic A. Somatic mtDNA mutations and aging - Facts and fancies. Experimental Gerontology 2009; 44: 101-105.

Kyriakouli DS, Boesch P, Taylor RW, Lightowlers RN. Progress and prospects: gene therapy for mitochondrial DNA disease. Gene Therapy 2008; 15: 1017-1023.

Lancaster CRD, Kroger A. Succinate: quinone oxidoreductases: new insights from X-ray crystal structures. Biochimica Et Biophysica Acta-Bioenergetics 2000; 1459: 422-431.

Larsson NG, Wang JM, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, et al. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nature Genetics 1998; 18: 231-236.

Lee DY, Clayton DA. Initiation of mitochondrial DNA replication by transcription and R-loop processing. Journal of Biological Chemistry 1998; 273: 30614-30621.

Lee HC, Wei YH. Oxidative stress, mitoichondrial DNA mutation, and apoptosis in aging. Experimental Biology and Medicine 2007; 232: 592-606.

Lenaers G, Reynier P, ElAchouri G, Soukkarieh C, Olichon A, Belenguer P, et al. OPA1 functions in mitochondria and dysfunctions in optic nerve. International Journal of Biochemistry & Cell Biology 2009; 41: 1866-1874.

Lightowlers RH, Jacobs HT, Kajander OA. Mitochondrial DNA - all things bad? Trends in Genetics 1999; 15: 91-93.

Lightowlers RN, Chinnery PF, Turnbull DM, Howell N. Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. Trends in Genetics 1997; 13: 450-5.

Lodi R, Taylor DJ, Tabrizi SJ, Kumar S, Sweeney M, Wood NW, et al. In vivo skeletal muscle mitochondrial function in Leber's hereditary optic neuropathy assessed by P-31 magnetic resonance spectroscopy. Annals of Neurology 1997; 42: 573-579.

Loeb LA, Wallace DC, Martin GM. The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. Proceedings of the National Academy of Sciences of the United States of America 2005; 102: 18769-18770.

Man PY, Griffiths PG, Brown DT, Howell N, Turnbull DM, Chinnery PF. The epidemiology of Leber hereditary optic neuropathy in the North East of England. American Journal of Human Genetics 2003; 72: 333-9.

Mancuso M, Orsucci D, Volpi L, Calsolaro V, Siciliano G. Coenzyme Q10 in Neuromuscular and Neurodegenerative Disorders. Current Drug Targets 2010; 11: 111-121.

Manfredi G, Fu J, Ojaimi J, Sadlock JE, Kwong JQ, Guy J, et al. Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus. Nature Genetics 2002; 30: 394-399.

Marella M, Seo BB, Thomas BB, Matsuno-Yagi A, Yagi T. Successful amelioration of mitochondrial optic neuropathy using the yeast NDI1 gene in a rat animal model. PLoS One 2010; *Epub ahead of print*.

Margulis L. Symbiosis and evolution. Scientific American 1971; 225: 48-57.

Martin GM, Loeb LA. Ageing - Mice and mitochondria. Nature 2004; 429: 357-+.

Martin M, Cho JY, Cesare AJ, Griffith JD, Attardi G. Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. Cell 2005; 123: 1227-1240.

Martin W, Muller M. The hydrogen hypothesis for the first eukaryote. Nature 1998; 392: 37-41.

Martinelli P, La Mattina V, Bernacchia A, Magnoni R, Cerri F, Cox G, et al. Genetic interaction between the m-AAA protease isoenzymes reveals novel roles in cerebellar degeneration. Human Molecular Genetics 2009; 18: 2001-2013.

Martinelli P, Rugarli EI. Emerging roles of mitochondrial proteases in neurodegeneration. Biochimica et Biophysica Acta 2010; 1797.

Mashima Y, Kigasawa K, Wakakura M, Oguchi Y. Do idebenone and vitamin therapy shorten the time to achieve visual recovery in Leber hereditary optic neuropathy? Journal of Neuro-Ophthalmology 2000; 20: 166-170.

McFarland R, Taylor RW, Turnbull DM. The neurology of mitochondrial DNA disease. Lancet Neurology 2002; 1: 343-351.

McFarland R, Taylor RW, Turnbull DM. Mitochondrial disease - Its impact, etiology, and pathology. Mitochondrion in the Germline and Early Development. Vol 77, 2007: 113-+.

Miller JW. Preliminary results of gene therapy for retinal degeneration. New England Journal of Medicine 2008; 358: 2282-2284.

Montoya J, Christianson T, Levens D, Rabinowitz M, Attardi G. Identification of Initiation Sites for Heavy-Strand and Light-Strand Transcription in Human

Mitochondrial-DNA. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 1982; 79: 7195-7199.

Montoya J, Gaines GL, Attardi G. The Pattern of Transcription of the Human Mitochondrial Ribosomal-RNA Genes Reveals Two Overlapping Transcription Units. Cell 1983; 34: 151-159.

Montoya J, Ojala D, Attardi G. Distinctive Features of the 5'-Terminal Sequences of the Human Mitochondrial Messenger-RNAs. Nature 1981; 290: 465-470.

Moraes CT. What regulates mitochondrial DNA copy number in animal cells? Trends in Genetics 2001; 17: 199-205.

Murphy JL, Blakely EL, Schaefer AM, He L, Wyrick P, Haller RG, et al. Resistance training in patients with single, large-scale deletions of mitochondrial DNA. Brain 2008; 131: 2832-2840.

Musumeci O, Naini A, Slonim AE, Skavin N, Hadjigeorgiou GL, Krawiecki N, et al. Familial cerebellar ataxia with muscle coenzyme Q10 deficiency. Neurology 2001; 56: 849-855.

Nekhaeva E, Bodyak ND, Kraytsberg Y, McGrath SB, Van Orsouw NJ, Pluzhnikov A, et al. Clonally expanded mtDNA point mutations are abundant in individual cells of human tissues. Proceedings of the National Academy of Sciences of the United States of America 2002; 99: 5521-5526.

Nicholas A, Kraytsberg Y, Guo XH, Khrapko K. On the timing and the extent of clonal expansion of mtDNA deletions: Evidence from single-molecule PCR. Experimental Neurology 2009; 218: 316-319.

Nijtmans LGJ, Ugalde C, van den Heuvel LP, Smeitink JA. Function and dysfunction of the oxidative phosphorylation system. In: Bauer M and Koehler C, editors. Topics in Current Genetics: Biogenesis of mitochondria and associated diseases. Heidelberg: Springer, 2004: 149-176.

Nishigaki Y, Marti R, Copeland WC, Hirano M. Site-speciflic somatic mitochondrial DNA point mutations in patients with thymidine phosphorylase deficiency. Journal of Clinical Investigation 2003; 111: 1913-1921.

O'Brien T. Properties of human mitochondrial ribosomes. Iubmb Life 2003; 55: 505-513.

Ojala D, Merkel C, Gelfand R, Attardi G. The Transfer-RNA Genes Punctuate the Reading of Genetic Information in Human Mitochondrial-DNA. Cell 1980a; 22: 393-403.

Ojala D, Montoya J, Attardi G. Transfer-RNA Punctuation Model of RNA Processing in Human Mitochondria. Nature 1981; 290: 470-474.

Ojala DK, Montoya J, Attardi G. The Putative mRNA for Subunit-II of Human Cytochrome-C Oxidase Starts Directly at the Translation Initiator Codon. Nature 1980b; 287: 79-82.

Olichon A, Elachouri G, Baricault L, Delettre C, Belenguer P, Lenaers G. OPA1 alternate splicing uncouples an evolutionary conserved function in mitochondrial fusion from a vertebrate restricted function in apoptosis. Cell Death & Differentiation 2007a; 14: 682-92.

Olichon A, Emorine LJ, Descoins E, Pelloquin L, Brichese L, Gas N, et al. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. Febs Letters 2002; 523: 171-6.

Olichon A, Landes T, Arnaune-Pelloquin L, Emorine LJ, Mils V, Guichet A, et al. Effects of OPA1 mutations on mitochondrial morphology and apoptosis: relevance to ADOA pathogenesis. Journal of Cellular Physiology 2007b; 211: 423-30.

Park CB, Asin-Cayuela J, Camara Y, Shi YH, Pellegrini M, Gaspari M, et al. MTERF3 is a negative regulator of mammalian mtDNA transcription. Cell 2007; 130: 273-285.

Parks RE, Brown PR, Cheng Y, Agarwal KC, Kong CM, Agarwal RP, et al. Purine Metabolism in Primitive Erythrocytes. Comparative Biochemistry and Physiology 1973; 45: 355-364.

Parsons TJ, Muniec DS, Sullivan K, Woodyatt N, AllistonGreiner R, Wilson MR, et al. A high observed substitution rate in the human mitochondrial DNA control region. Nature Genetics 1997; 15: 363-368.

Perales-Clemente E, Fernandez-Silva P, Acin-Perez R, Perez-Martos A, Enriquez JA. Allotopic expression of mitochondrial-encoded genes in mammals: achieved goal, undemonstrated mechanism or impossible task? Nucleic Acids Research 2010; *Epub ahead of print*.

Perkins G, Renken C, Martone ME, Young SJ, Ellisman M. Electron tomography of neuronal mitochondria: Three-dimensional structure and organization of cristae and membrane contacts. Journal of Structural Biology 1997; 119: 260-272.

Pitts KR, Yoon Y, Krueger EW, McNiven MA. The dynamin-like protein DLP1 is essential for normal distribution and morphology of the endoplasmic reticulum and mitochondria in mammalian cells. Molecular Biology of the Cell 1999; 10: 4403-4417.

Polyak K, Li YB, Zhu H, Lengauer C, Willson JKV, Markowitz SD, et al. Somatic mutations of the mitochondrial genome in human colorectal tumours. Nature Genetics 1998; 20: 291-293.

Pyle A, Taylor RW, Durham SE, Deschauer M, Schaefer AM, Samuels DC, et al. Depletion of mitochondrial DNA in leucocytes harbouring the 3243A -> G mtDNA mutation. Journal of Medical Genetics 2007; 44: 69-74.

Qi XP, Lewin AS, Hauswirth WW, Guy J. Suppression of complex I gene expression induces optic neuropathy. Annals of Neurology 2003; 53: 198-205.

Qi XP, Lewin AS, Sun L, Hauswirth WW, Guy J. SOD2 gene transfer protects against optic neuropathy induced by deficiency of complex I. Annals of Neurology 2004; 56: 182-191.

Qi XP, Sun L, Hauswirth WW, Lewin AS, Guy J. Use of mitochondrial antioxidant defenses for rescue of cells with a Leber hereditary optic neuropathy-causing mutation. Archives of Ophthalmology 2007a; 125: 268-272.

Qi XP, Sun L, Lewin AS, Hauswirth WW, Guy J. The mutant human ND4 subunit of complex I induces optic neuropathy in the mouse. Investigative Ophthalmology & Visual Science 2007b; 48: 1-10.

Raha S, Robinson BH. Mitochondria, oxygen free radicals, disease and ageing. Trends in Biochemical Sciences 2000; 25: 502-508.

Rajasimha HK, Chinnery PF, Samuels DC. Selection against pathogenic mtDNA mutations in a stem cell population leads to the loss of the 3243A -> G mutation in blood. American Journal of Human Genetics 2008; 82: 333-343.

Rampazzo C, Ferraro P, Pontarin G, Fabris S, Reichard P, Bianchi V. Mitochondrial deoxyribonucleotides, pool sizes, synthesis, and regulation. Journal of Biological Chemistry 2004; 279: 17019-17026.

Reeve AK, Krishnan KJ, Elson JL, Morris CM, Bender A, Lightowlers RN, et al. Nature of mitochondrial DNA deletions in substantial nigra neurons. American Journal of Human Genetics 2008; 82: 228-235.

Reynier P, Amati-Bonneau P, Verny C, Olichon A, Simard G, Guichet A, et al. OPA3 gene mutations responsible for autosomal dominant optic atrophy and cataract. Journal of Medical Genetics 2004; 41.

Roberti M, Musicco C, Polosa PL, Milella F, Gadaleta MN, Cantatore P. Multiple protein-binding sites in the TAS-region of human and rat mitochondrial DNA. Biochemical and Biophysical Research Communications 1998; 243: 36-40.

Rodriguez MC, MacDonald JR, Mahoney DJ, Parise G, Beal MF, Tarnopolsky MA. Beneficial effects of creatine, CoQ(10), and lipoic acid in mitochondrial disorders. Muscle & Nerve 2007; 35: 235-242.

Rorbach J, Soleimanpour-Lichaei R, Lightowlers RN, Chrzanowska-Lightowlers ZMA. How do mammalian mitochondria synthesize proteins? Biochemical Society Transactions 2007; 35: 1290-1291.

Rossmanith W, Tullo A, Potuschak T, Karwan R, Sbisa E. Human Mitochondrial Transfer-RNA Processing. Journal of Biological Chemistry 1995; 270: 12885-12891.

Rustin P, Bonnet D, Rotig A, Munnich A, Sidi D. Idebenone treatment in Friedreich patients: One-year-long randomized placebo-controlled trial. Neurology 2004; 62: 524-525.

Rutter J, Winge DR, Schiffman JD. Succinate dehydrogenase - Assembly, regulation and role in human disease. Mitochondrion 2010; 10: 393-401.

Sacconi S, Trevisson E, Salviati L, Ayme S, Rigal O, Redondo AG, et al. Coenzyme Q(10) is frequently reduced in muscle of patients with mitochondrial myopathy. Neuromuscular Disorders 2010; 20: 44-48.

Samuels DC, Schon EA, Chinnery PF. Two direct repeats cause most human mtDNA deletions. Trends in Genetics 2004; 20: 393-398.

Samuels DC, Wonnapinij P, Cree LM, Chinnery PF. Reassessing evidence for a postnatal mitochondrial genetic bottleneck. Nature Genetics 2010; 42: 471-472.

Sbisa E, Tanzariello F, Reyes A, Pesole G, Saccone C. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene 1997; 205: 125-140.

Schaefer AM, McFarland R, Blakely EL, He L, Whittaker RG, Taylor RW, et al. Prevalence of mitochondrial DNA disease in adults. Annals of Neurology 2008; 63: 35-9.

Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders - past, present and future. Biochimica Et Biophysica Acta-Bioenergetics 2004; 1659: 115-120.

Schon EA, DiMauro S, Hirano M, Gilkerson RW. Therapeutic prospects for mitochondrial disease. Trends in Molecular Medicine 2010; 16: 268-276.

Schwartz M, Vissing J. Paternal inheritance of mitochondrial DNA. New England Journal of Medicine 2002; 347: 576-580.

Schwartz M, Vissing J. No evidence for paternal inheritance of mtDNA in patients with sporadic mtDNA mutations. Journal of the Neurological Sciences 2004; 218: 99-101.

Serasinghe MN, Yoon Y. The mitochondrial outer membrane protein hFis1 regulates mitochondrial morphology and fission through self-interaction. Experimental Cell Research 2008; 314: 3494-3507.

Sesaki H, Jensen RE. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. Journal of Cell Biology 1999; 147: 699-706.

Shahrestani P, Leung HT, Le PK, Pak WL, Tse S, Ocorr K, et al. Heterozygous Mutation of Drosophila Opa1 Causes the Development of Multiple Organ Abnormalities in an Age-Dependent and Organ-Specific Manner. Plos One 2009; 4.

Shorr N, Christenbury JD, Goldberg RA. Management of ptosis in chronic progressive external ophthalmoplegia. Ophthal Plast Reconstr Surg 1987; 3: 141-5.

Shoubridge EA. The ABCs of mitochondrial transcription. Nature Genetics 2002; 31: 227-228.

Shoubridge EA, Karpati G, Hastings KE. Deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fiber segments in mitochondrial disease. Cell 1990; 62: 43-9.

Siguroardottir S, Helgason A, Gulcher JR, Stefansson K, Donnelly P. The mutation rate in the human mtDNA control region. American Journal of Human Genetics 2000; 66: 1599-1609.

Smith KH, Johns DR, Heher KL, Miller NR. Heteroplasmy in Leber's hereditary optic neuropathy. Archives of Ophthalmology 1993; 111: 1486-90.

Soleimanpour-Lichaei HR, Khul I, Gaisne M, Passos JF, Wydro M, Rorbach J, et al. mtRF1a is a human mitochondrial translation release factor decoding the major termination Codons UAA and UAG. Molecular Cell 2007; 27: 745-757.

Song ZY, Chen H, Fiket M, Alexander C, Chan DC. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. Journal of Cell Biology 2007; 178: 749-755.

Spelbrink JN, Li FY, Tiranti V, Nikali K, Yuan QP, Tariq M, et al. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene LF-like protein localized in mitochondria. Nature Genetics 2001; 28: 223-231.

Spinazzola A, Invernizzi F, Carrara F, Lamantea E, Donati A, DiRocco M, et al. Clinical and molecular features of mitochondrial DNA depletion syndromes. Journal of Inherited Metabolic Disease 2009; 32: 143-158.

Spinazzola A, Zeviani M. Mitochondrial Diseases: A Cross-Talk Between Mitochondrial and Nuclear Genomes. In: Espinos CFVPF, editor. Inherited Neuromuscular Diseases: Translation from Pathomechanisms to Therapies. Vol 652, 2009: 69-84.

Stewart JB, Freyer C, Elson JL, Larsson NG. Purifying selection of mtDNA and its implications for understanding evolution and mitochondrial disease. Nature Reviews Genetics 2008; 9: 657-662.

Strachan T, Read AP. Organization of the human genome. In: Strachan T and Read AP, editors. Human Molecular Genetics. Oxford: BIOS Scientific Publishers, 1999: 139-165.

Suen DF, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. Genes & Development 2008; 22: 1577-1590.

Sutovsky P, Navara CS, Schatten G. Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilization. Biology of Reproduction 1996; 55: 1195-205.

Tachibana M, Sparman M, Sritanaudomchai H, Ma H, Clepper L, Woodward J, et al. Mitochondrial gene replacement in primate offspring and embryonic stem cells. Nature 2009; 461: 367-372.

Taivassalo T, Gardner J, Taylor R, Schaefer A, Haller R, Taylor D. Resistance exercise training in mitochondrial myopathy due to single, large-scale deletions: Implications for therapy. Neuromuscular Disorders 2007; 17: 829-829.

Taivassalo T, Gardner JL, Taylor RW, Schaefer AM, Newman J, Barron MJ, et al. Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions. Brain 2006; 129: 3391-3401.

Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 2000; 404: 42-49.

Tanaka M, Borgeld HJ, Zhang J, Muramatsu S, Gong JS, Yoneda M, et al. Gene therapy for mitochondrial disease by delivering restriction endonuclease Smal into mitochondria. Journal of Biomedical Science 2002; 9: 534-541.

Tang S, Le PK, Tse S, Wallace DC, Huang TS. Heterozygous Mutation of Opa1 in Drosophila Shortens Lifespan Mediated through Increased Reactive Oxygen Species Production. Plos One 2009; 4.

Tarnopolsky MA. The mitochondrial cocktail: Rationale for combined nutraceutical therapy in mitochondrial cytopathies. Advanced Drug Delivery Reviews 2008; 60: 1561-1567.

Taylor RW, Barron MJ, Borthwick GM, Gospel A, Chinnery PF, Samuels DC, et al. Mitochondrial DNA mutations in human colonic crypt stem cells. Journal of Clinical Investigation 2003a; 112: 1351-1360.

Taylor RW, Chinnery PF, Clark KM, Lightowlers RN, Turnbull DM. Treatment of mitochondrial disease. Journal of Bioenergetics and Biomembranes 1997a; 29: 195-205.

Taylor RW, Chinnery PF, Turnbull DM, Lightowlers RN. Selective inhibition of mutant human mitochondrial DNA replication in vitro by peptide nucleic acids. Nature Genetics 1997b; 15: 212-215.

Taylor RW, Giordano C, Davidson MM, d'Amati G, Bain H, Hayes CM, et al. A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. Journal of the American College of Cardiology 2003b; 41: 1786-1796.

Taylor RW, McDonnell MT, Blakely EL, Chinnery PF, Taylor GA, Zeviani M, et al. Genotypes from patients indicate no paternal mitochondrial DNA contribution. Annals of Neurology 2003c; 54: 521-524.

Taylor RW, Schaefer AM, Barron MJ, McFarland R, Turnbull DM. The diagnosis of mitochondrial muscle disease. Neuromuscul Disord 2004a; 14: 237-45.

Taylor RW, Schaefer AM, Barron MJ, McFarland R, Turnbull DM. The diagnosis of mitochondrial muscle disease. Neuromuscular Disorders 2004b; 14: 237-245.

Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nature Reviews Genetics 2005; 6: 389-402.

Taylor RW, Wardell TM, Blakely EL, Borthwick GM, Brierley EJ, Turnbull DM. Analysis of mitochondrial DNA mutations - Deletions. Aging Methods and Protocols 2000; 38: 245-264.

Tiranti V, Savoia A, Forti F, Dapolito MF, Centra M, Racchi M, et al. Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the expressed sequence tags database. Human Molecular Genetics 1997; 6: 615-625.

Tonon C, Lodi R. Idebenone in Friedreich's ataxia. Expert Opinion on Pharmacotherapy 2008; 9: 2327-2337.

Torroni A, Wallace DC. Mitochondrial DNA variation in human populations and implications for detection of mitochondrial DNA mutations of pathological significance. Journal of Bioenergetics & Biomembranes 1994; 26: 261-71.

Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, et al. Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. Proceedings of the National Academy of Sciences of the United States of America 2005; 102: 17993-17998.

Trifunovic A, Larsson NG. Mitochondrial dysfunction as a cause of ageing. Journal of Internal Medicine 2008; 263: 167-178.

Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 2004; 429: 417-423.

Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, ShinzawaItoh K, et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 angstrom. Science 1996; 272: 1136-1144.

Tuppen HAL, Fattori F, Carrozzo R, Zeviani M, DiMauro S, Seneca S, et al. Further pitfalls in the diagnosis of mtDNA mutations: homoplasmic mt-tRNA mutations. Journal of Medical Genetics 2008; 45: 55-61.

Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. Embo Journal 2008a; 27: 433-446.

Twig G, Hyde B, Shirihai OS. Mitochondrial fusion, fission and autophagy as a quality control axis: The bioenergetic view. Biochimica Et Biophysica Acta-Bioenergetics 2008b; 1777: 1092-1097.

Tyynismaa H, Mjosund KP, Wanrooij S, Lappalainen I, Ylikallio E, Jalanko A, et al. Mutant mitochondrial helicase Twinkle causes multiple mtDNA deletions and a late-onset mitochondrial disease in mice. Proceedings of the National Academy of Sciences of the United States of America 2005; 102: 17687-17692.

Tyynismaa H, Sembongi H, Bokori-Brown M, Granycome C, Ashley N, Poulton J, et al. Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. Human Molecular Genetics 2004; 13: 3219-3227.

Tyynismaa H, Suomalainen A. Mouse models of mitochondrial DNA defects and their relevance for human disease. Embo Reports 2009; 10: 137-143.

Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, et al. AIF deficiency compromises oxidative phosphorylation. EMBO Journal 2004; 23: 4679-89.

Van Goethem G, Dermaut B, Lofgren A, Martin JJ, Van Broeckhoven C. Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. Nature Genetics 2001; 28: 211-212.

Verhoeven K, Claeys KG, Zuchner S, Schroder JM, Weis J, Ceuterick C, et al. MFN2 mutation distribution and genotype/phenotype correlation in Charcot-Marie-Tooth type 2. Brain 2006; 129: 2093-2102.

Vermulst M, Bielas JH, Kujoth GC, Ladiges WC, Rabinovitch PS, Prolla TA, et al. Mitochondrial point mutations do not limit the natural lifespan of mice. Nature Genetics 2007; 39: 540-543.

Vermulst M, Wanagat J, Kujoth GC, Bielas JH, Rabinovitch PS, Prolla TA, et al. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. Nature Genetics 2008; 40: 392-394.

von Ballmoos C, Wiedenmann A, Dimroth P. Essentials for ATP Synthesis by F1F0 ATP Synthases. Annual Review of Biochemistry 2009; 78: 649-672.

Wai T, Teoli D, Shoubridge EA. The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. Nature Genetics 2008; 40: 1484-1488.

Wallace DC, Fan WW. The pathophysiology of mitochondrial disease as modeled in the mouse. Genes & Development 2009; 23: 1714-1736.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 1988; 242: 1427-30.

Wang CX, Youle RJ. The Role of Mitochondria in Apoptosis. Annual Review of Genetics 2009; 43: 95-118.

Wang GJ, Nutter LM, Thayer SA. Insensitivity of cultured rat cortical neurons to mitochondrial DNA synthesis inhibitors - Evidence for a slow turnover of mitochondrial DNA. Biochemical Pharmacology 1997; 54: 181-187.

Wang Y, Michikawa Y, Mallidis C, Bai Y, Woodhouse L, Yarasheski KE, et al. Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication. Proceedings of the National Academy of Sciences of the United States of America 2001; 98: 4022-4027.

Wang YS, Bogenhagen DF. Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. Journal of Biological Chemistry 2006; 281: 25791-25802.

Wanrooij S, Luoma P, van Goethem G, van Broeckhoven C, Suomalainen A, Spelbrink JN. Twinkle and POLG defects enhance age-dependent accumulation of mutations in the control region of mtDNA. Nucleic Acids Research 2004; 32: 3053-3064.

Waterham HR, Koster J, van Roermund CWT, Mooyer PAW, Wanders RJA, Leonard JV. A lethal defect of mitochondrial and peroxisomal fission. New England Journal of Medicine 2007; 356: 1736-1741.

White KE, Davies VJ, Hogan VE, Piechota MJ, Nichols PP, Turnbull DM, et al. OPA1 Deficiency Associated with Increased Autophagy in Retinal Ganglion Cells in a

Murine Model of Dominant Optic Atrophy. Investigative Ophthalmology & Visual Science 2009; 50: 2567-2571.

Yaffe MP. The cutting edge of mitochondrial fusion. Nature Cell Biology 2003; 5: 497-499.

Yamaguchi R, Lartigue L, Perkins G, Scott RT, Dixit A, Kushnareva Y, et al. Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak oligomerization. Molecular Cell 2008; 31: 557-569.

Yang MY, Bowmaker M, Reyes A, Vergani L, Angeli P, Gringeri E, et al. Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. Cell 2002; 111: 495-505.

Yarosh W, Monserrate J, Tong JJ, Tse S, Le PK, Nguyen K, et al. The molecular mechanisms of OPA1-mediated optic atrophy in Drosophila model and prospects for antioxidant treatment. Plos Genetics 2008; 4.

Yasukawa T, Reyes A, Cluett TJ, Yang MY, Bowmaker M, Jacobs HT, et al. Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. Embo Journal 2006; 25: 5358-5371.

Yin H, Stahl JS, Andrade FH, McMullen CA, Webb-Wood S, Newman NJ, et al. Eliminating the Ant1 isoform produces a mouse with CPEO pathology but normal ocular motility. Investigative Ophthalmology & Visual Science 2005; 46: 4555-4562.

Yoshida M, Muneyuki E, Hisabori T. ATP synthase - A marvellous rotary engine of the cell. Nature Reviews Molecular Cell Biology 2001; 2: 669-677.

Yoshida Y, Izumi H, Torigoe T, Ishiguchi H, Itoh H, Kang DC, et al. p53 physically interacts with mitochondrial transcription factor A and differentially regulates binding to damaged DNA. Cancer Research 2003; 63: 3729-3734.

Yu-Wai-Man P, Griffiths PG, Burke A, Sellar PW, Clarke MP, Gnanaraj L, et al. The prevalence and natural history of dominant optic atrophy due to *OPA1* mutations. Ophthalmology 2010a; *Epub Ahead of Print*.

Yu-Wai-Man P, Griffiths PG, Gorman GS, Lourenco CM, Wright AF, Auer-Grumbach M, et al. Multi-system neurological disease is common in patients with OPA1 mutations. Brain 2010b; 133: 771-786.

Yu-Wai-Man P, Griffiths PG, Hudson G, Chinnery PF. Inherited mitochondrial optic neuropathies. Journal of Medical Genetics 2009; 46: 145-158.

Yu CA, Tian H, Zhang L, Deng KP, Shenoy SK, Yu L, et al. Structural basis of multifunctional bovine mitochondrial cytochrome bc(1) complex. Journal of Bioenergetics and Biomembranes 1999; 31: 191-199.

Yue WH, Zou YP, Yu L, Yu CA. Crystallization of Mitochondrial Ubiquinol Cytochrome-C Reductase. Biochemistry 1991; 30: 2303-2306.

Zanna C, Ghelli A, Porcelli AM, Karbowski M, Youle RJ, Schimpf S, et al. OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. Brain 2008; 131: 352-67.

Zeviani M. Train, train, train No pain, just gain. Brain 2008; 131: 2809-2811.

Zeviani M, Carelli V. Mitochondrial disorders. Current Opinion in Neurology 2007; 20: 564-571.

Zhang X, Jones D, Gonzalez-Lima F. Mouse model of optic neuropathy caused by mitochondrial complex I dysfunction. Neuroscience Letters 2002; 326: 97-100.

Zhou XS, Solaroli N, Bjerke M, Stewart JB, Rozell B, Johansson M, et al. Progressive loss of mitochondrial DNA in thymidine kinase 2-deficient mice. Human Molecular Genetics 2008; 17: 2329-2335.

Zhu ZQ, Yao JB, Johns T, Fu K, De Bie I, MacMillan C, et al. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nature Genetics 1998; 20: 337-343.

Zuchner S, De Jonghe P, Jordanova A, Claeys KG, Guergueltcheva V, Cherninkova S, et al. Axonal neuropathy with optic atrophy is caused by mutations in mitofusin 2. Annals of Neurology 2006; 59: 276-281.

Zuchner S, Mersiyanova IV, Muglia M, Bissar-Tadmouri N, Rochelle J, Dadali EL, et al. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nature Genetics 2004; 36: 449-451.

Publications