Identifying and Defining Clinical Phenotypes in Mitochondrial Disease

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Author's Declaration

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy. The research was performed at the Wellcome Trust Centre for Mitochondrial Research between the years 2013-2016, under the supervision of Dr Robert McFarland, Professor Sir Douglass M. Turnbull and Professor Robert W. Taylor.

I can certify that the material offered in this thesis has not been previously submitted by me for a degree or qualification in this, or any other university.

Acknowledgements

Where has the time gone? I still remember vividly the first visit I made to the Wellcome Trust Centre for Mitochondrial Research, Newcastle. I received a warm welcome from Doug, Bobby, Grainne, Andy, Rob and my predecessor of the clinical research associate post, Victoria. I felt their enthusiasm and passion in clinical care and research, and I instantly realised that I could learn a lot from them and blend in well with the team. The feeling must have been mutual, I think, as I was offered the post within two days. I successfully applied for out of programme for research and my journey in the mitochondrial world began in August 2013.

The beginning of my research was a bit of a struggle. Learning to use a pipette and perform real-time PCR was a challenging task for me in the first three months. I was, and still am, very grateful to my supervisors (Bobby, Doug and Rob) for having a frank discussion that laboratory work was not my strength at the earliest stage of my PhD; and thanks to their vision and experience, I have enjoyed and thrived in clinical research. Bobby and Doug have also given me invaluable opportunities to deliver plenary talks on their behalf at international meetings.

I worked closely with John Grady to redesign many aspects of the Mitochondrial Disease Patient Cohort (MitoCohort) database over the last two years. The outcome has been very positive, and the functionality of the database is vastly improved. Of course, I am thankful to have worked with other fantastic MitoCohort team members; including Julia, clinical trial manager, Alex, research nurse, current and former data managers including Doug, Christine, Eimear and Rebecca.

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千里之行，始于足下

A journey of a thousand miles begins with a single step.

To my parents, patients and their families

Proposal Summary

Mitochondrial disease is an umbrella term that encompasses many clinically, biochemically and genetically heterogeneous conditions, which develop as a consequence of impaired oxidative phosphorylation. The diagnosis of mitochondrial disease requires coordinated multi-disciplinary input, starting with a high index of clinical suspicion and meticulous assessment, followed by systematic biochemical and genetic investigations. More recently, the advent of next generation sequencing has rapidly identified novel disease genes and mechanisms in many previously undiagnosed cases. However, the aspects of natural history, disease progression and prognostication associated with many of these genetic variants require further elucidation. Furthermore, genotype-specific treatment guidance and surveillance strategy for complications are lacking in a routine clinical setting.

The MRC Mitochondrial Disease Patient Cohort (UK) is a national collaborative project initiated by three centres of NHS Highly Specialised Service for Rare Mitochondrial Disorders: Newcastle, London and Oxford. Newcastle is the largest centre and has enrolled over 55% of patients (~800), to date. My aim has been to utilise the longitudinal data collected for this national cohort database to facilitate deep phenotyping of common and rare genetic variants, investigate phenotype-genotype correlations and identify treatable complications of mitochondrial disease. In this thesis, I show that sudden adult death syndrome and severe intestinal pseudo-obstruction are two previously under-recognized clinical entities associated with the m.3243A>G mutation, the most common pathogenic heteroplasmic mitochondrial DNA mutation in adult patients. The significant implications of these findings for clinical practice are highlighted.

Extrapyramidal movement disorders are identified in 5% of patients followed up in Newcastle. I sought to determine the spectrum of extrapyramidal movement disorders in children and adults, and interrogate the genotype-phenotype correlation that may provide screening guidance for generalists.

Mutations in *RMND1* (Required for Meiotic Nuclear Division protein 1) and *YARS2* (mitochondrial tyrosyl-tRNA synthetase) genes both result in combined mitochondrial respiratory chain deficiencies, but their clinical phenotypes are distinctive. Survival in patients with RMDN1 deficiency is influenced by the presence of kidney disease, as shown in the multi-centre collaborative study that I have drawn together. Myopathy, lactic acidosis and sideroblastic anaemia (MLASA) is a classic syndrome associated with mutations in *YARS2*.

However, such a syndromic presentation is not always evident in all patients, and I sought to demonstrate that cardio-respiratory insufficiency is a prominent, potentially treatable, complication in patients who harbour mutations in *YARS2* without having the complete MLASA phenotype.

Understanding the spectrum of clinical phenotypes and providing appropriate, accurate genetic counselling for heteroplasmic mitochondrial DNA point mutations is challenging under normal circumstances, but this is particularly difficult when the specific mutation is rare and phenotypic data are scant. I sought to perform a detailed analysis of patients harbouring the m.13094T>C mutation in *MT-ND5*.

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Abbreviations

AAV	Adeno-associated virus vector
AD	Autosomal dominant
ADP	Adenosine diphosphate
AFG3L2	ATPase Family Gene 3-Like 2
AHSCT	Allogenic haematopoietic stem cell transplantation
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
ANT	Adenine nucleotide translocator
AR	Autosomal recessive
ATP	Adenosine triphosphate
bp	Base pair
BCL2	B-cell lymphoma 2
CK	Creatinine kinase
CMT	Charcott-Marie-Tooth neuropathy
CNS	Central nervous system
CoQ	Coenzyme Q10
CSB2	Conserved sequence block 2
CSF	Cerebrospinal fluid
CVS	Chorionic villous sampling
COX	Cytochrome *c* oxidase
CPEO	Chronic progressive external ophthalmoplegia
DARS2	Mitochondrial aspartyl-tRNA synthetase

DM	Diabetes mellitus
DNA2	DNA replication helicase 2
DRP1	Dynamin-related protein 1
dNTP	Deoxynucleotide pool
EARS2	Mitochondrial glutamyl-tRNA synthetase
ECG	Electrocardiogram
EEG	Electroencephalogram
EMG	Electromyogram
EPC	Epilepsia partialis continua
ETF	Electron transfer flavoprotein
ETFDH	Electron transfer flavoprotein dehydrogenase
$FADH_2$	Reduced flavin adenine dinucleotide
Fe-S	Iron-sulphur
FLAIR	Fluid attenuation inversion recovery
FMN	Flavin mononucleotide
FGF21	Human fibroblast growth factor 21
FVC	Forced vital capacity
GTPase	Guanosine triphosphatase
H	Heavy strand
HSP1	Heavy-strand promoter 1
HSP2	Heavy-strand promoter 2
HCM	Hypertrophic cardiomyopathy
IOSCA	Infantile onset spinocerebellar ataxia

IPO	Intestinal pseudo-obstruction
IVF	*in vitro* fertilisation
KSS	Kearns-Sayre syndrome
L	Light strand
LBSL	Leukoencephalopathy with brainstem and spinal cord involvement and high lactate
LTBL	Leukoencephalopathy with thalamus and brainstem involvement and high lactate
LHON	Leber hereditary optic neuropathy
LS	Leigh syndrome
LSP	Light-strand promoter
MADD	Multiple acyl-CoA dehydrogenase
MCU	Mitochondrial calcium uniporter
MDS	Mitochondrial depletion syndrome
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MEMSA	Myoclonic epilepsy myopathy sensory ataxia
MERRF	Myoclonic epilepsy with ragged red fibres
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MGME1	Mitochondrial genome maintenance exonuclease-1
MILS	Maternally inherited Leigh syndrome
MIDD	Maternally inherited diabetes and deafness
MIRAS	Mitochondrial recessive ataxia syndrome

MitoCohort	MRC Mitochondrial Disease Patient Cohort
MLASA	Myopathy, lactic acidosis and sideroblastic anaemia
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mt-LSU	Mitochondrial ribosomal large subunit
mt-SSB	Mitochondrial single-stranded binding protein
mt-SSU	Mitochondrial ribosomal small subunit
nDNA	Nuclear DNA
NAD+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
NCS	Nerve conduction studies
NCG	National commissioning group
NMDAS	Newcastle mitochondrial disease adult scale
NPMDS	Newcastle paediatric mitochondrial disease scale
$O2^{\bullet-}$	Superoxide
O_H	Origin of heavy strand
O_L	Origin of light strand
Opa1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation system
PDH	Pyruvate dehydrogenase

PDH	Pyruvate dehydrogenase complex
PGC	Primordial germ cell
PGC1-α	Proliferator-activated receptor gamma coactivator
PGD	Preimplantation genetic diagnosis
PPAR-α	Peroxisome proliferator-activated receptor alpha
PCR	Polymerase chain reaction
PINK1	PTEN-induced putative kinase protein 1
POLG	Polymerase gamma
POLRMT	Mitochondrial RNA polymerase
PS	Pearson syndrome
RET	Reverse electron transport
RITOL	Ribonucleotide incorporation throughout the lagging strand
RMND1	Required for Meiotic Nuclear Division protein 1
RNASEH1	Ribonuclease H1
RRF	Ragged-red fibre
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SANDO	Sensory ataxia neuropathy dysarthria and ophthalmoplegia
SD	Single, large-scale mtDNA deletion
SDH	Succinate dehydrogenase
SDM	strand-displacement model
SLE	Stroke-like episodes
SADS	Sudden adult death syndrome

SPG28	Spinocerebellar ataxia type 28
SUCLA2	Beta subunit of succinyl-CoA ligase
SUCLG1	Alpha subunit of succinyl-CoA ligase
TALEN	Transcription activator-like effector nucleases
TFAM	Transcription factor A
TFB2M	Mitochondrial transcription factor B2
TK2	Thymidine kinase 2
tRNA	Transfer ribonucleic acid
VUS	Variant of unknown significance
WES	Whole exome sequencing
WPW	Wolff-Parkinson-White syndrome
YARS2	Mitochondrial tyrosyl-tRNA synthetase
ZFN	Zinc finger nuclease

Chapter 1. Introduction

Mitochondrial disease is one of the most common metabolic diseases with an estimated prevalence of 1 in 4300 (Gorman *et al.*, 2015) and can affect all age groups from neonatal to adult onset. It is a collective term that encompasses a wide array of conditions that are biochemically, genetically and clinically heterogeneous. Currently, there remains no cure for mitochondrial disease, and the main focuses of management are monitoring for complications, supportive treatment and genetic counselling. Therefore, natural history studies and a better understanding of disease progression of individual genotypes are essential steps in improving the accuracy of prognostication, evaluating treatments and tailoring future therapeutic strategies.

1.1 Mitochondrial molecular biology

1.1.1 Origin

The most prevailing hypothesis to explain the origin of mitochondria is the endosymbiosis theory, which was popularised by Lynn Margulis (married name Sagan, 1938-2011) (Sagan, 1967). Essentially, the proposed theory suggests that an ancestor of eukaryotic cells (host cell) engulfed an aerobic prokaryote, which the latter became an endosymbiont (i.e., a cell living within another cell). The heterotrophic and anaerobic host benefited from endosymbionts that could undergo aerobic respiration and generate ATP more efficiently (Reece, 2011). Most of the genes encoding endosymbiont were transferred into the host nucleus over the course of formation of mitochondria (Dolezal *et al.*, 2006). The phylogenetic studies subsequently provided evidence to show that mitochondria originated from the α-alpha proteobacteria and only arose once in evolution more than a billion years ago (Gray *et al.*, 2001). Furthermore, most of the proteins that function in mitochondria carry short N-terminal targeting sequences that are highly conserved across the species (Dolezal *et al.*, 2006).

1.1.2 Mitochondrial structure and oxidative phosphorylation

Mitochondria are cellular organelles found in all nucleated cells (except mature red blood cells) in humans. Each mitochondrion has two membranes (outer and inner), inter-membrane space and matrix. The mitochondrial matrix contains multiple copies of mitochondrial DNA (mtDNA) molecules and enzymes involved in various biochemical pathways. It is clear that mitochondria have many functions, but probably the most crucial one is to generate energy in the form ATPs (adenosine triphosphate) via oxidative phosphorylation using glucose and fatty acid as fuel. The oxidative phosphorylation system (OXPHOS) is located in the inner membrane

and it consists of five multimeric protein complexes: complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase, COX) which are collectively called the mitochondrial respiratory chain and complex V (ATP synthase). In addition, there are two electron carriers (coenzyme Q10 and cytochrome *c*).

In aerobic respiration, glucose is metabolised to pyruvate, which is then transported to the mitochondria and converted to acetyl-CoA followed by a series of chemical reactions called tricarboxylic acid (TCA or Kreb) cycle. The outcome of TCA cycle is the formation of carbon dioxide (CO_2), reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$). The beta-oxidation of fatty acid produces $FADH_2$ and acetyl-CoA which the latter enters the common TCA pathway (**Figure 1**) (Taylor and Turnbull, 2005). Electron transfer flavoprotein (ETF) transfers electrons from beta-oxidation to co-enzyme Q10 via the ETF dehydrogenase (ETFDH). The mitochondrial respiratory chain oxidises both NADH and $FADH_2$ and pumps the derived protons across the inner membrane to the inter-membrane space creating an electrochemical gradient while at the same time coenzyme Q10 and cytochrome *c* transfer the electrons to the molecule oxygen (O_2) to form water. Complex V utilises this gradient to phosphorylate adenosine diphosphate (ADP) and produces ATP (**Figure *2***) (Schon *et al.*, 2012). Anaerobic respiration is a much simpler pathway where pyruvate is converted to lactate generating four ATPs in the cytosol. This is of particular relevance as one of the major consequences associated with mitochondrial respiratory chain dysfunction is lactic acidosis.

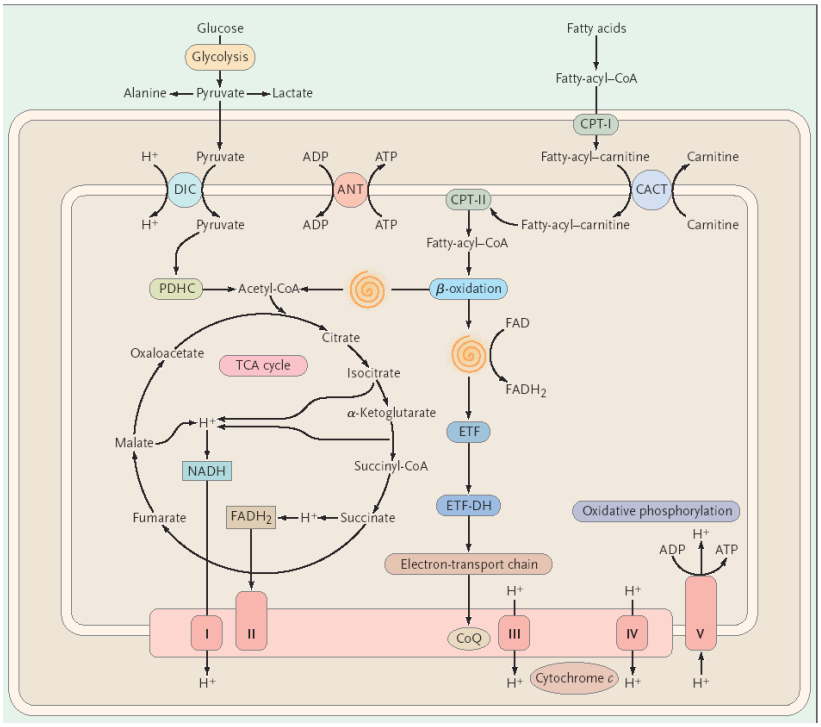

Figure 1. Tricarboxylic acid cycle and beta-oxidation. ADP= adenosine diphosphate, ATP= adenosine triphosphate, ANT= adenine nucleotide translocator, CACT = carnitine-acylcarnitine translocase, CoQ= coenzyme Q, CPT= carnitine palmitoyltransferase, DIC= dicarboxylate carrier, ETF= electron-transfer flavoprotein, ETF-DH= electron-transfer dehydrogenase, FAD= flavin adenine dinucleotide, $FADH_2$= reduced FAD, NADH= reduced nicotinamide adenine dinucleotide, PDHC= pyruvate dehydrogenase complex, TCA= tricarboxylic acid. Reproduced from (Di Mauro and Schon, 2003)

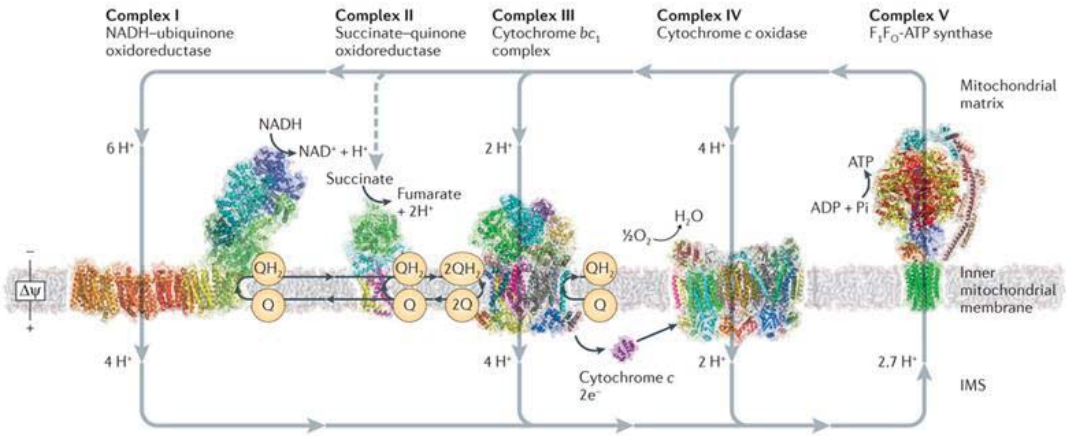

Figure 2. Mitochondrial electron transport chain (Complex I-IV) and ATP synthase (complex V). Electron transport between complexes is mediated by membrane-embedded ubiquinone (Q) and soluble cytochrome c. Complex I is the entry point for electrons from NADH, which are used to reduce Q to ubiquinol (QH2). QH2 is subsequently used by complex III to reduce cytochrome c in the intermembrane space (IMS), and complex IV uses cytochrome c to reduce molecular oxygen, which is the ultimate electron acceptor. For each NADH molecule oxidised, 10 protons are translocated across the membrane from the matrix to the IMS. Reproduced from (Sazanov, 2015)

1.1.2.1 Complex I

Complex I, also known as NADH: ubiquinone oxidoreductase, is the largest complex in the mitochondrial respiratory chain. There are 45 subunits in the mammalian complex I of which 14 core subunits (NDUFS1, NDUFV1, NDUFV2, NDUFS2, NDUFS3, NDUFS8, NDUFS7, ND1-6 and ND4L) are strictly conserved and shared across species; 31 accessory subunits have been acquired over the course of evolution (Sazanov, 2015; Zhu *et al.*, 2016).

Complex I is an L-shaped molecule with two arms; a hydrophilic peripheral arm (N module and Q module) and a hydrophobic membrane embedded (P module) arm. The peripheral arm consists of two functional modules (N module and Q module) and all of the necessary cofactors (one FMN, flavin mononucleotide; and eight iron-sulphur (Fe-S) clusters). The oxidisation of NADH occurs in the NDUFV1 of the N module followed by the transfer of electrons along a chain of Fe-S clusters to the terminal cluster (N2) and Q-module (Zhu *et al.*, 2016), where the ubiquinone is reduced to ubiquinol. The proton translocation activity in the membrane arm (P module) is triggered by the reduction of ubiquinone (**Figure 3**) (Wirth *et al.*, 2016). All the core subunits in the membrane arm are encoded by the mtDNA. The core subunits are clearly involved in the bioenergetics function. The precise roles of individual accessory subunits are less well-characterized but are thought to be important in the assembly of complex I, maintenance of the structural stability and some of them may play other metabolic roles (Zhu *et al.*, 2016). Using the gene editing tools (TALENS and CRISPR/Cas9), an Australian research group demonstrated 25 accessory subunits are required for assembly of a functional complex I and NDUFAB1 is an essential subunit for cell viability (Stroud *et al.*, 2016).

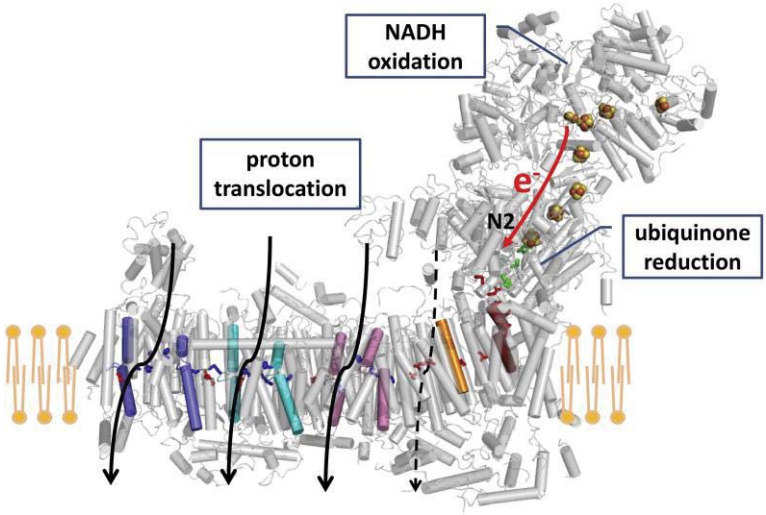

Figure 3. NADH oxidation, ubiquinone reduction and proton translocation in Complex I. Reproduced with permission from (Wirth et al., 2016)

The integration of subunits and formation of individual OXPHOS complexes are dependent on the specific assembly factors, and at least 13 assembly factors have been characterised in complex I (Giachin *et al.*, 2016). Given the numbers of structural subunits and assembly factors in complex I, it is unsurprising that complex I deficiency is the most common biochemical defect causing mitochondrial disease (Kirby *et al.*, 1999; Skladal *et al.*, 2003a).

1.1.2.2 Complex II

Complex II (succinate: ubiquinone oxidoreductase), is the smallest enzymatic complex of OXPHOS which only comprises of four nuclear-encoded subunits (SDHA, SDHB, SDHC and SDHD). SDHA and SDHB make up the hydrophilic domain of the complex II while SDHC and SDHD are anchored to the inner membrane (Briere *et al.*, 2005). SDHAF2 and SDHAF2 are the two known assembly factors of complex II.

Complex II catalyses the oxidation of succinate to fumarate in the TCA cycle with the reduction of ubiquinone to ubiquinol (Rustin *et al.*, 2002). It has a powerful reducing property and ability to trigger reverse electron flow through complex I, resulting in the production of NADH (Rustin *et al.*, 2002). Mutations in the SDH subunits causing primary mitochondrial disease have been reported (Bourgeron *et al.*, 1995; Alston *et al.*, 2012; Jackson *et al.*, 2014) but they are rare. Intriguingly, heterozygous mutations in the SDH subunits (SDHB, SDHC and SDHD) and assembly factors account for at least 15% of all paraganglioma and phaeochromocytoma (Baysal *et al.*, 2002; Gill *et al.*, 2010). The precise mechanism of SDH defect and the formation of specific neuro-endocrine tumours remains elusive although the activation of hypoxia-inducible factors involving in pseudo-hypoxia pathway has been implicated (Briere *et al.*, 2005; Pasini and Stratakis, 2009).

1.1.2.3 Ubiquinone

A human isoform of ubiquinone, Coenzyme Q10 (CoQ10), is composed of a benzoquinone ring with ten isoprene units attached to it (Alcázar-Fabra *et al.*, 2016). Most of CoQ is synthesised endogenously, but some of them are derived from the diet (Alcázar-Fabra *et al.*, 2016). Ubiquinone shuttles electrons from complexes I and II to complex III. In addition, it can also be reduced to ubiquinol (by receiving electrons) by other dehydrogenases that involve different metabolic pathways such as the electron transport flavoprotein dehydrogenases family (ETFDH) from fatty oxidation, dihydroorotate dehydrogenase that is involved in the pyrimidine nucleotide biosynthesis pathway and glycerol 3-phosphate

dehydrogenase that involves in the lipid metabolism (Alcázar-Fabra *et al.*, 2016). Primary ubiquinone deficiency is caused by mutations in the nuclear-encoded proteins involving in the CoQ biosynthesis pathway (Acosta *et al.*, 2016). Secondary deficiency has been observed in other conditions as a consequence of either defect in mitochondrial DNA such as mitochondrial depletion syndrome, mitochondrial tRNA point mutation or fatty acid oxidation defect, for example, mutations in the *ETFDH* gene (Alcázar-Fabra *et al.*, 2016).

1.1.2.4 Complex III

Mammalian complex III (ubiquinone: cytochrome *c* oxidoreductase) is a multi heteromeric enzyme that has 11 subunits, only one of them is encoded by mtDNA (*MT-CYB*). The number of subunits in complex III varies between species, but three of them are highly conserved: cyt *b*, cyt c_1 and the iron sulphur protein (Xia *et al.*, 2013). The assembly of mammalian complex III appears to be similar to the yeast mitochondria, and defects in five assembly factors have been identified to cause human diseases to date. However, the detail on intermediate steps of human complex III assembly is currently lacking (Fernandez-Vizarra and Zeviani, 2015). Complex III oxidises ubiquinol and transfers the electrons to cytochrome *c* coupled with proton pumping across the inner membrane via a Q-cycle mechanism which involves the generation of a semiquinone molecule (Trumpower, 1990; Xia *et al.*, 2013).

1.1.2.5 Cytochrome c

Mammalian cytochrome *c* (Cytc), encoded by the *CYCS* gene, shuttles electrons from Complex III to complex IV. It is a positively charged 12.3kDA protein and located in the mitochondrial intermembrane space. Cytc has also been identified to play important role in signalling apoptotic pathway in cells (also see **Section 1.1.4.1**) (Liu *et al.*, 1996; Cai *et al.*, 1998).

1.1.2.6 Complex IV

Complex IV (cytochrome *c* oxidase, COX) is the rate-limiting step of ATP synthesis as it is the final recipient of electrons in the OXPHOS. It comprises 13 subunits of which the three largest subunits (COX I-III) are encoded by the mtDNA, and the remaining subunits are encoded in the nucleus. The mtDNA-encoded subunits perform the catalytic function, and ten other subunits have the regulatory roles on oxygen consumption and proton translocation (Kadenbach and Huttemann, 2015). There are six isoforms subunits in mammals, which allow

the expression of tissue-specific functions and cell-signalling (Huttemann *et al.*, 2012). NDUFA4 has been proposed as the 14th subunit of complex IV based on the immunoprecipitation (Balsa *et al.*, 2012) but some researchers remain sceptical about this (Kadenbach and Huttemann, 2015). Currently, there are over 20 accessory proteins involve in complex IV assembly (Soto *et al.*, 2012).

Cytochrome *c* transfers four electrons to the haem a3-CuB binuclear centre of complex IV where two water molecules are formed by the reduction of an oxygen molecule, and proton translocation occurs (Sazanov, 2015). The rate of respiration is primarily controlled by the allosteric ATP-inhibition of the phosphorylated COX via feedback inhibition by ATP, depending on the ATP/ADP-ratio (Kadenbach and Huttemann, 2015).

1.1.2.7 Complex V

Complex V, also known as ATP synthase, comprises 15 different subunits which form two functional domains: F_0 and F_1 (Christoph von *et al.*, 2009). F_0 is located in the inner membrane, and it contains subunits c, a, b, d, F6, oligomycin sensitivity-conferring protein (OSCP) and the accessory subunits e, f, g and A6L. F_1 is made up five different subunits (three α, three β, and one γ, δ and ε) and is located in the matrix. The two rotatory components are formed by the F_0 subunit c (stator) and F_1 subunits γ, δ and ε (rotor). The central stalk of ATP synthase contains F1 subunits γ, δ and ε. The peripheral stalk consists of F_0 subunits b, d, F6 and OSCP. The rotational catalysis is accepted as the model to explain ATP synthesis (von Ballmoos *et al.*, 2009). Essentially, the electrochemical proton gradient drives the protons from intermembrane space to matrix via F_0, and the energy is transferred to F_1, leading to the phosphorylation of ADP to become ATP (Jonckheere *et al.*, 2012). All the subunits are nuclear encoded, except two F_0 subunits, subunit a and subunit A6L, are encoded by the *MTATP6* and *MTAT8* genes, respectively (Jonckheere *et al.*, 2012).The assembly of complex V is less well understood compared to other complexes in the OXPHOS; defects in three assembly factors (ATP11, ATP12 and TMEM70) have been recognised in human disease (Jonckheere *et al.*, 2012).

1.1.3 Reactive oxygen species

Complex I and complex III are two major sites of reactive oxygen species (ROS) production in the mitochondrial electron transport chain. Complex I produce large amounts of superoxide ($O2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in isolated mitochondria in two modes. First, in the

presence of a high NADH/NAH+ ratio in the matrix (Kudin *et al.*, 2004); second, during reverse electron transport (RET) when high $CoQH_2$/CoQ ratio and mitochondrial protonmotive force are evident (Δp; Δp consists of two components: the membrane potential (Δψ) and the pH gradient (ΔpH)). The production of $O2^{\bullet-}$ by complex I during RET was at least 3-fold more sensitive to the pH gradient than to the membrane potential (Lambert and Brand, 2004). Under physiological conditions, ROS production from complex III is thought to be negligible when compared to complex I in vitro (Murphy, 2009).

Excessive ROS production can lead to significant oxidative damage to mitochondrial proteins, membranes and DNA (Murphy, 2009). As a result, the ATP generation and various metabolic pathways such as the TCA cycle, fatty acid oxidation, the urea cycle, iron-sulphur biogenesis and others are compromised. Consequently, mitochondrial inner membrane proteins, such as cytochrome *c*, are released to the cytosol which activates caspase 3 and the cascade of apoptosis (see **Section 1.1.4.1**). In addition, mitochondrial ROS may function as modulatable redox signal from the organelle to the rest of cell (Murphy, 2009).

1.1.4 Non-OXPHOS functions

Mitochondria have many other important functions in mammalian cells such as apoptosis, iron-sulphur biosynthesis, calcium handling, urea cycle and others. Examples of these non-OXPHOS functions are briefly discussed as follows.

1.1.4.1 Intrinsic pathway of apoptosis

Apoptosis is a tightly regulated process that causes cellular demolition and programmed cell death without perturbing neighbouring cells (Taylor *et al.*, 2008). There are two major pathways involved in apoptosis, extrinsic and intrinsic pathways (Ichim and Tait, 2016) (**Figure 4**). Mitochondria regulate the intrinsic pathway, which can be initiated by a number of stimuli such as DNA damage, metabolic stress and endoplasmic reticular stress (Taylor *et al.*, 2008). This is followed by an increase in the permeability of the mitochondrial outer membrane, which allows the release of cytochrome c to the cytosol, and formation of the apoptosome. Caspase-9 is activated by apoptosome, in turn, activates caspase-3 and caspase-7, leading to apoptosis. Mitochondrial outer membrane permeabilization is mediated by the B-cell lymphoma 2 (BCL-2) protein family (Czabotar *et al.*, 2014).

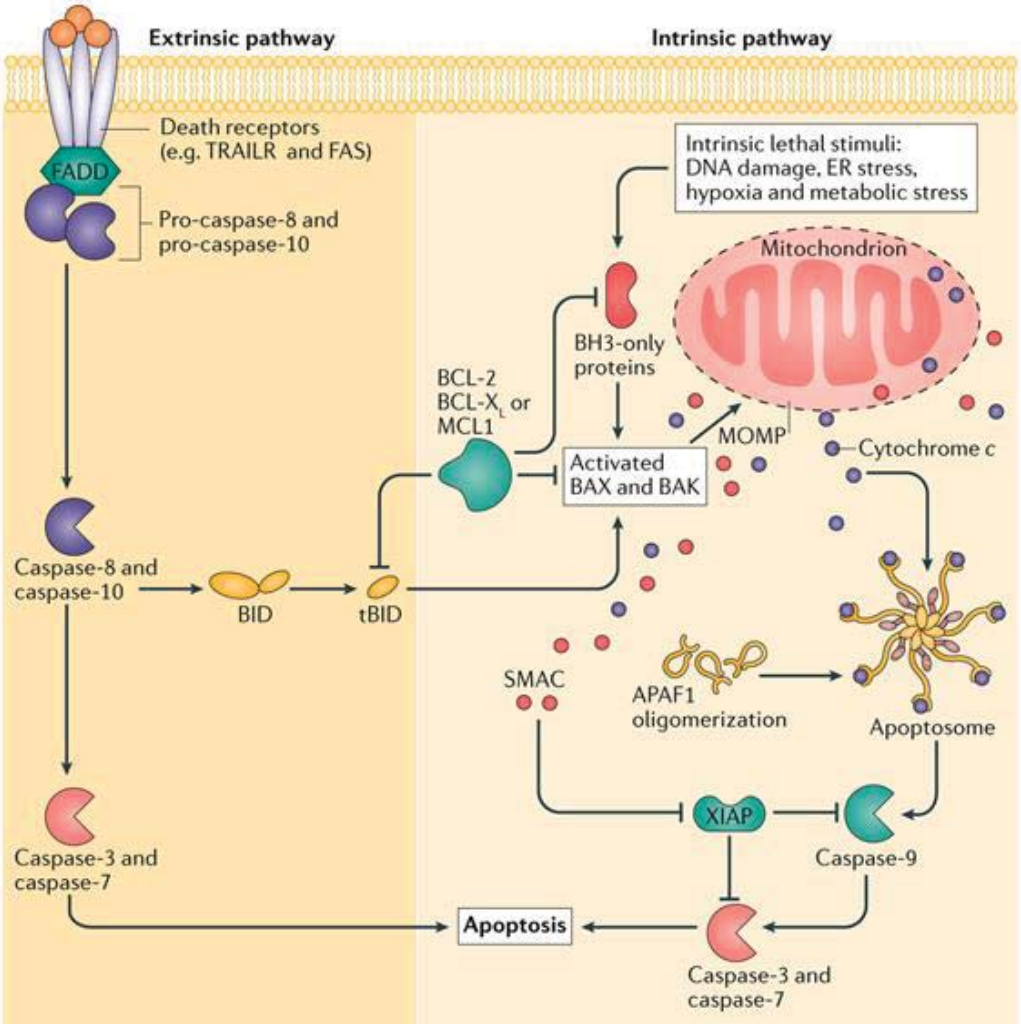

Figure 4. Extrinsic and intrinsic pathways in apoptosis. In the extrinsic apoptotic pathway, upon binding to their cognate ligand, death receptors such as tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) and FAS can activate initiator caspases (caspase-8 and caspase-10) through dimerization mediated by adaptor proteins such as FAS-associated death domain protein (FADD). Active caspase-8 and caspase-10 then cleave and activate the effector caspase-3 and caspase-7, leading to apoptosis. The intrinsic (or mitochondrial) pathway of apoptosis requires mitochondrial outer membrane permeabilization (MOMP). Cell stresses engage BCL-2 homology domain 3 (BH3)-only protein activation, leading to BAX and BAK activity that triggers MOMP. Anti-apoptotic BCL-2 family proteins counteract this. Following MOMP, mitochondrial intermembrane space proteins such as second mitochondria-derived activator of caspases (SMAC) and cytochrome c are released into the cytosol. Cytochrome c interacts with apoptotic protease activating factor 1 (APAF1), triggering apoptosome assembly, which activates caspase-9. Active caspase-9, in turn, activates caspase-3 and caspase-7, leading to apoptosis. Mitochondrial release of SMAC facilitates apoptosis by blocking the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). Caspase-8 cleavage of the BH3-only protein BH3-interacting death domain agonist (BID) enables crosstalk between the extrinsic and intrinsic apoptotic pathways. ER, endoplasmic reticulum; MCL1, myeloid cell leukaemia 1; tBID, truncated BID. Reproduced with permission from (Ichim and Tait, 2016)

1.1.4.2 Iron-sulphur biosynthesis

Iron-sulphur (Fe-S) clusters are inorganic cofactors that can facilitate electron transport, by accepting or donating single electrons. In mammalian cells, mitochondria are a major site of Fe-S cluster biogenesis: 12 Fe-S clusters of respiratory chain complexes I-III and those of the citric acid cycle enzymes aconitase and SDH (Rouault, 2015). However, it is unclear if the building blocks of Fe-S clusters of cytosolic proteins are dependent on mitochondria as there is little evidence that Fe-S clusters can cross the inner membrane to exit from mitochondria (Rouault, 2015). The first human disease linked to a defect in Fe-S cluster synthesis was Friedreich ataxia, the most common form of recessive ataxia with multi-system involvement (1 in 50,000), caused by a homozygous guanine-adenine-adenine (GAA) trinucleotide repeat in the *FXN* gene (Koeppen, 2011). Other defects in the Fe-S biogenesis have also been identified to cause human disease, including myopathy, cardiomyopathy, neonatal lactic acidosis and sideroblastic anaemia (Rouault, 2015).

1.1.4.3 Calcium handling

It is increasingly recognised that mitochondrial calcium handling plays significant roles in several cellular functions, including modulating energy production, buffering cytosol calcium concentration under pathophysiological states and regulation of an intrinsic apoptotic pathway (Rizzuto *et al.*, 2012; Williams *et al.*, 2013). Calcium transports across the mitochondrial inner membrane to the matrix via a mitochondrial calcium uniporter (MCU). Genes encoding structural subunits of MCU have been identified, and more recently genetic defects in MCU have been reported to cause predominantly childhood-onset myopathy and extrapyramidal movement disorder (Logan *et al.*, 2014). Altered calcium homoeostasis and mitochondrial dysfunction have been implicated in neuronal death and neurodegenerative disorders such as in Alzheimer's disease and Huntingdon's disease (Celsi *et al.*, 2009).

1.1.5 Mitochondrial dynamics

An early observation of mitochondrial dynamics including variable sizes, shapes and networks in chick embryo (Lewis and Lewis, 1914) first provided evidence that mitochondria are dynamic organelles that do not conform to discrete morphology. Mitochondrial dynamic comprises two processes, fusion and fission, which are regulated by a number of highly conserved guanosine triphosphatase (GTPase) proteins as illustrated in **Figure 5**.

Mitochondrial fusion is mediated by Mitofusin 1 and 2 (Mfn1 and 2) in the outer membrane and Optic atrophy 1 (Opa1) in the inner membrane. The main result of fusion is the formation

of an interconnected network which enables mitochondria to exchange content such as mtDNA, substrates, metabolites and lipid, which helps to maintain healthy mitochondrial population (Detmer and Chan, 2007). Mitochondrial fission, in contrast, creates smaller mitochondria which in turn facilitates mitophagy of damaged mitochondria (see 1.1.5 Mitophagy), mitochondrial transport or accelerating cell proliferation (Archer, 2013). Dynamin-related protein 1 (DRP1) is one of the key proteins that promotes fission. Studies of human mutations in *MFN2*, *OPA1* and *PINK1* genes have further elucidated our understanding of mitophagy (Burte *et al.*, 2015). Furthermore, dysfunctional mitochondrial dynamics has also been implicated in other neurodegenerative disorders, lung cancer and pulmonary arterial hypertension (Archer, 2013; Ranieri *et al.*, 2013).

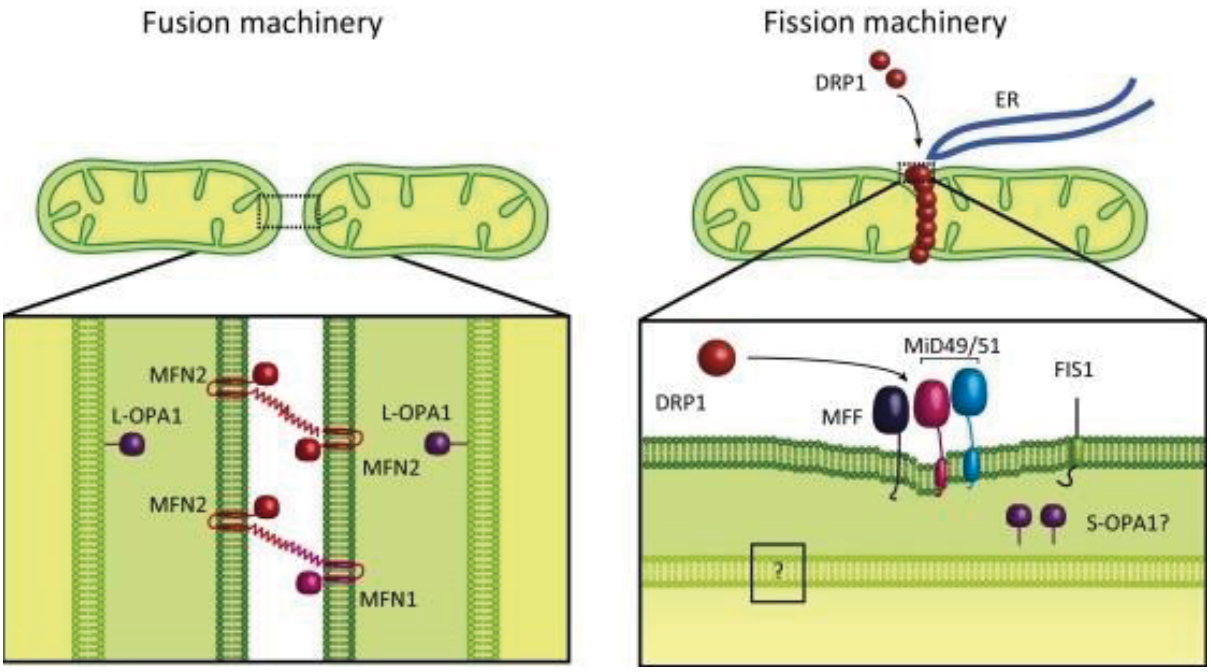

Figure 5. Regulation of Mitochondrial Fusion and Fission. Mitochondrial fusion is mediated by homo- and heterotypic interactions between MFN1 and MFN2 (red) at the mitochondrial outer membrane (OM, dark green) and L-OPA1 (purple) at the inner mitochondrial membrane (IM, light green). MitoPLD (green) is required for OM fusion. Receptor-mediated recruitment of Dynamin-related protein 1 (DRP1; red) from the cytosol to the OM by FIS1, MFF, and MiD49/51 to sites of division marked by endoplasmic reticulum (ER, blue) drives mitochondrial fission. IM fission machinery is unknown. Adapted from (Wai and Langer, 2016)

1.1.6 Mitophagy

The term ‘autophagy’ (‘self-eating’) was first coined in 1966 (De Duve and Wattiaux, 1966). It is a process of cellular catabolism in eukaryotic cells by which cytoplasmic materials such as protein and organelles are sequestered, forming double-membrane vesicles (autophagosomes) and are delivered to the lysosome for degradation (autolysosomes) (Yang and Klionsky, 2010; Youle and van der Bliek, 2012). There are two primary forms of autophagy: firstly, a non-selective process of releasing free amino acids and other nutrients in response to starvation or nutrient deprivation; secondly, a more specific process involving removal of damaged or excessive protein and organelles (Youle and van der Bliek, 2012), such as ribosomes, peroxisomes and mitochondria. Mitophagy is a degradation of mitochondria through autophagy that was initially observed in rat hepatocytes (Lemasters, 2005; Rodriguez-Enriquez *et al.*, 2006). Mitochondria go through constant cycles of fusion and fission, with fission events generate two subsets of daughter mitochondria with either increased or decreased membrane potential. The daughter mitochondria with higher membrane potential would proceed to fusion, whereas the depolarised daughter mitochondria are unable to proceed to the fusion process and were removed by mitophagy (Twig *et al.*, 2008). The kinase PTEN-induced putative kinase protein 1 (PINK1) accumulates in the damaged or depolarised mitochondria, leading to the recruitment E3 ubiquitin ligase parkin from the cytosol, ubiquitination of mitochondrial proteins and eventually the degradation of mitochondria by lysosomes (**Figure 6**) (Youle and Narendra, 2011). In addition to quality control of mitochondria, mitophagy also plays a crucial role in the maturation of reticulocytes (Zhang *et al.*, 2009; Dengjel and Abeliovich, 2016).

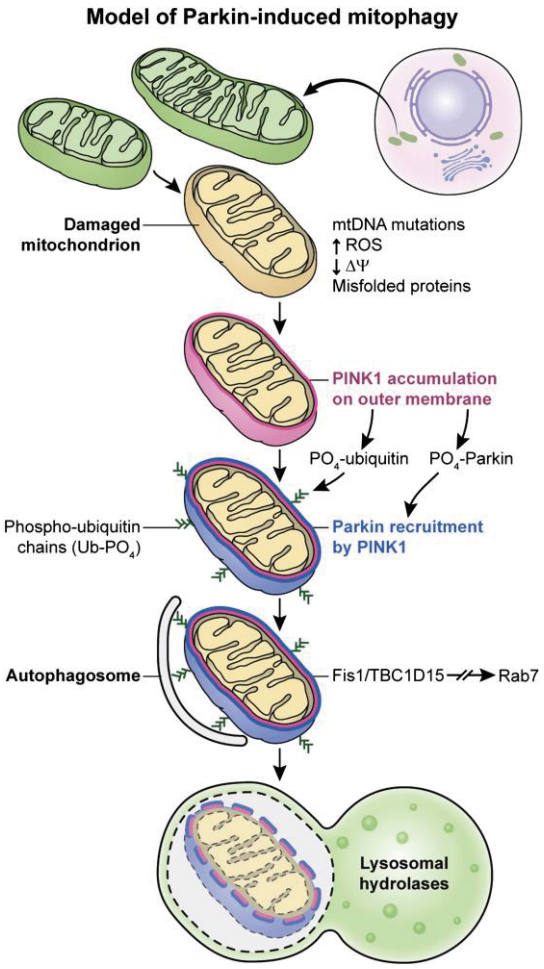

Figure 6. Model of Parkin-induced mitophagy. Damaged mitochondrion (yellow) fail to import and degrade PINK1, leading to accumulation of PINK1 on the mitochondrial outer membrane. PINK1 ubiquitylates Parkin to activate the Parkin's E3 ligase activity. Fis1 is a receptor on the outer membrane that binds two proteins, TBC1D15 and TBC1D17, to govern the developing LC3 isolation membrane to generate the autophagosome around the damaged mitochondria. The autophagosome is then delivered to the lysosome for degradation. Reproduced with permission (Pickrell and Youle, 2015)

1.2 Human Mitochondrial Genetics

1.2.1 Mitochondrial genome

The mitochondrial genome comprises of multiple copies of mtDNA molecules. Each mtDNA molecule is double stranded (inner light strand (L) and outer heavy strand (H)) and has 16,569 base pairs (bp) (**Figure 7**). There are 37 genes in the mitochondrial DNA, of which 24 of them are required for mitochondrial DNA translation (22 transfer RNAs and 2 ribosomal RNAs) and 13 encode polypeptides that form the subunits of the mitochondrial respiratory chain namely, 7 subunits of complex I (ND1-ND6, ND4L), 1 subunit of complex III (CYTB), 3 subunits of complex IV (COX I-III) and 2 subunits of ATPase (ATP6 and ATP8) (Anderson *et al.*, 1981). 27 of these mtDNA-encoded genes are located on the H-strand, and nine are on the L-strand

(Chinnery and Hudson, 2013). The mtDNA molecule is highly compact as there are no introns within mtDNA genes and almost no intergenic noncoding nucleotides exist, except the displacement loop (D-loop), which contains transcriptional promoters and at least one of the proposed replication origins (O_H) (Anderson *et al.*, 1981; Tuppen *et al.*, 2010).

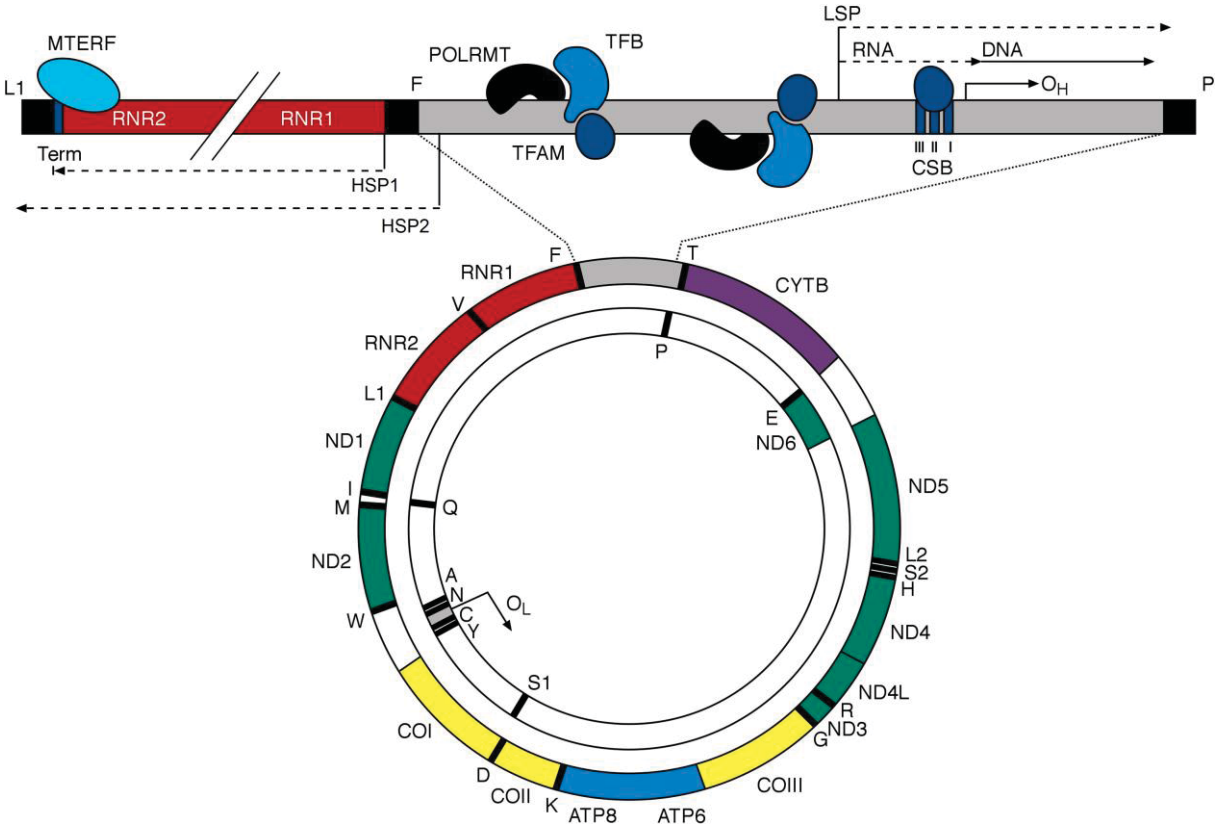

Figure 7. Human mitochondrial DNA. The outer circle represents heavy strand (H) and the inner circle is light strand (L). There are 37 genes in the mtDNA: 7 genes encode for complex I subunits (ND1-ND6, ND4L), 1 gene for complex III subunit (Cyt b), 3 genes for subunits of complex IV (COI-III), 2 genes for subunits of complex V (ATP7 and ATP8), 22 tRNA (denoted with single letter abbreviation) and 2 rRNA (RNR1 and RNR2). There are two non-coding regions, 1.1kb D-loop that is located in the heavy strand and the origin of L strand replication (O_L). The origin of H-strand replication is indicated within the D-loop (O_H). H-strand transcription is initiated either from HSP1, generating a short transcript that terminates at the RNR2/MTTL1 boundary (Term) under the guidance of the transcription termination factor MTERF, or from HSP2, generating polycistronic transcripts of the entire H-strand. LSP denotes the L-strand initiation point that produces polycistronic transcripts for this strand and also generates RNA precursors for H-strand replication initiation. Conserved sequence blocks (CSBs I-III) are conserved regions in human, mouse and rat that participate in the formation of RNA primers for replication. Transcription from all promoters requires the upstream binding of transcriptional activator TFAM, together with a single subunit RNA polymerase (POLRMT), which forms a heterodimeric complex with the transcription factor TFB2M (depicted as TFB). TFAM also binds to other regions of the D-loop; however, only binding to the CSB region is shown. Adapted from (Tuppen *et al.*, 2010)

1.2.2 Unique features associated with mitochondrial DNA

Mitochondrial genome has several distinctive features that differ from nuclear genome (Taylor and Turnbull, 2005): (1) maternal inheritance (Giles *et al.*, 1980); (2) variable copy number of mtDNA molecules ranging from hundreds to thousands depending on the energy demand of the cell type; (3) heteroplasmy and threshold level; (4) bottleneck effect creating rapid random drift of mutation in successive generations; (5) heteroplasmy shift in dividing cells due to vegetative segregation has been observed in some heteroplasmic mtDNA mutations such as m.3243A>G (Matthews *et al.*, 1995; Rajasimha *et al.*, 2008; Stewart and Chinnery, 2015); (6) replication of mtDNA is independent of cell cycle (Bogenhagen and Clayton, 1977); (7) In the mtDNA, codons AUA and AUG code for methionine, UGA codes for tryptophan (not a stop codon, as in the nuclear genome), and AGA and AGG are read as stop codons (not arginine, as in the nuclear genome) (Schon *et al.*, 2012).

The clinical phenotypic heterogeneity in primary mitochondrial DNA disease is partly explained by these unique characteristics associated with the mitochondrial genome. The significance of heteroplasmy, threshold effect, segregation of mtDNA mutation and mitochondrial genetic bottleneck in clinical practice is further discussed in **Section 1.4**.

1.2.3 Mitochondrial DNA transcription and replication

There are three promoters involved in the mtDNA transcription, namely HSP1, HSP2 and LSP (**Figure 7**). Transcription from both the HSP2 and LSP cover all genes, except *RNR1*, *RNR2*, *MTTF* and *MTTV* which are generated by HSP1 (Montoya *et al.*, 1982; Tuppen *et al.*, 2010).

The transcription begins with the interaction of mitochondrial transcription factor A (TFAM) with the high-affinity binding site upstream of the transcription start site followed by recruitment of mitochondrial RNA polymerase (POLRMT). POLRM undergoes a conformational change, and the mitochondrial transcription factor B2 (TFB2M) binds to it, forming a complex to initiate replication. Other essential components of mtDNA replicating machinery include polymerase ɣ, TWINKLE helicase and mitochondrial single stranded binding protein (mt-SSB). TWINKLE helicase is only required for the synthesis of H-strand DNA.

The most well-established model to explain the replication of mitochondrial DNA is the strand-displacement model (SDM) (Robberson and Clayton, 1972; Berk and Clayton, 1974). In this model, the replication of mammalian mtDNA is thought to occur asymmetrically by using two separated unidirectional origins of replication, i.e. the origin of heavy (H)-strand

synthesis (O_H) and the origin of light (L)-strand synthesis (O_L). The replication initiates at the O_H and proceeds unidirectionally, displacing the parental H-strand as a single-stranded DNA (ssDNA). After the two-third of H-strand is synthesised, the OL is exposed, and the replication of L-strand begins in the opposite direction. The replication completes after the two events reach a full circle (**Figure 8**) (Brown *et al.*, 2005; Wanrooij and Falkenberg, 2010; Gustafsson *et al.*, 2016). Bootlace model (ribonucleotide incorporation throughout the lagging strand, RITOLS) has recently been proposed and challenged the conventional SDM (Reyes *et al.*, 2013; Holt and Jacobs, 2014).

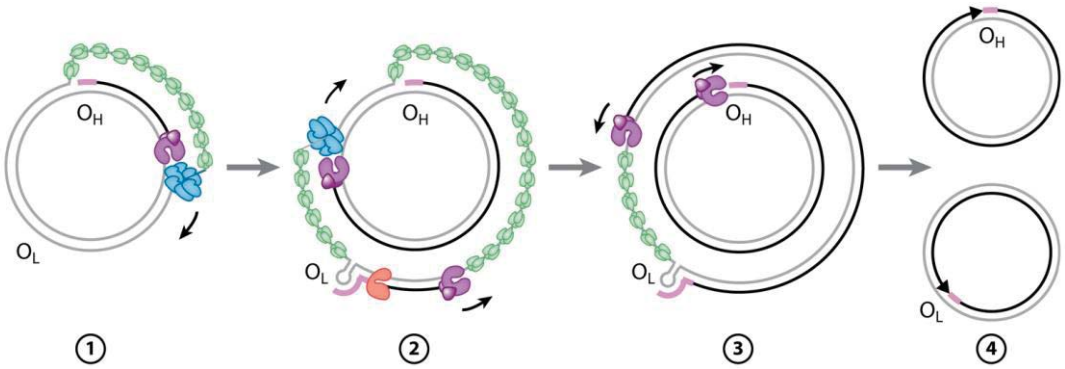

Figure 8. The strand-displacement model of mitochondrial DNA (mtDNA) replication. ① After initiation at the heavy-strand origin (O_H), the replisome (POLG (purple) and TWINKLE helicase (blue)) proceeds unidirectionally to produce the nascent H-strand. The displaced, parental H-strand is bound and stabilised by the mitochondrial single-stranded DNA-binding protein (mtSSB) (green). ② When the H-strand replication machinery passes the light-strand origin (O_L), a stem-loop structure is formed. The mitochondrial DNA-directed RNA polymerase POLRMT (orange) synthesises short primers at the stem loop, which are used to initiate L-strand DNA synthesis. ③,④After completion of mtDNA strand synthesis, replication is terminated at either O_H or O_L, depending on where DNA synthesis was initiated. Reproduced with permission from (Gustafsson *et al.*, 2016)

Ribonuclease H1 (RNASEH1) is thought to remove the RNA part of primer in the O_H region (from LSP to CSB2) after the initiation of mtDNA synthesis whilst the removal of the DNA part of the primer (from CSB2 to O_H) is by the mitochondrial genome maintenance exonuclease-1 (MGME1) (Gustafsson *et al.*, 2016).

1.2.4 Mitochondrial DNA translation

There are four major steps in mitochondrial translation: initiation, elongation, termination and recycling of the ribosome (**Figure 9**) (Smits *et al.*, 2010; Boczonadi and Horvath, 2014; Mai *et al.*, 2016). The translational process begins with the separation of the two mitochondrial ribosomal subunits (small subunit/28S, mt-SSU and large subunit/39S, mt-LSU) catalysed by the mitochondrial translation factor mtIF3, followed by the binding of mRNA, mtIF2 and formylmethionine-tRNA (fMET-tRNA^{Met}) to the peptidyl (P) site of the mitoribosome. The two mitochondrial ribosomal subunits then recombine and result in the release of initiation factors. The pairing of codon-anticodon between mRNA, elongation factor mtEFTu, GTP and amino-acylated tRNA takes place at the acceptor (A) site of mitoribosome. Following the decoding of mRNA, the elongation factor dissociates from the mitoribosome and the amino-acylated tRNA moves to the peptidyl (P) site of mitoribosome where the peptide bond formation is catalysed, adding one amino acid to the growing peptide (Smits *et al.*, 2010). mtEFTs is an elongation factor that converts mtEFTu to its active form (mtEFTu-GTP) whilst another elongation factor, mtEFG1-GTP catalyzes the translocation step by conformational changes in both mtEFG1 and the mitoribosome, during which the A and P site tRNAs move to the P and exit (E) sites of the mitoribosome and mRNA is advanced by one codon (Smits *et al.*, 2010). This elongation step repeats itself until the stop codon (UAA, UAG, AGA or AGG) is encountered in the A-site (Boczonadi and Horvath, 2014). The mitochondrial release factor (mtRF1a) recognises the stop codon and causes the release of polypeptide attached to the last tRNA molecule in the P site. Other termination release factors such as mtRF1, C12orf65 and ICT1 are also thought to play an essential role in the termination (Boczonadi and Horvath, 2014).

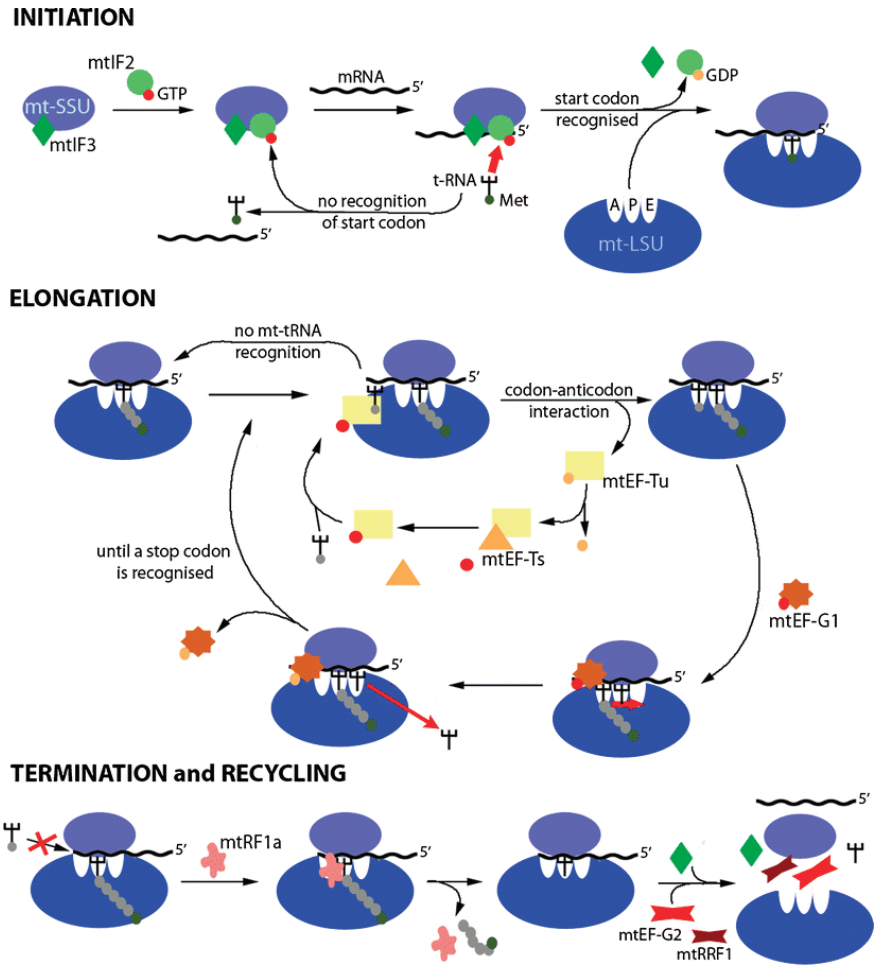

Figure 9. An illustration of mitochondrial translation and protein synthesis. Following ribosome recycling (top), the mitochondrial small subunit (mt-SSU in blue) remains bound to initiation factor mtIF3 (dark green). Initiation commences as mtIF2 (light green) bound to a GTP molecule (red) joins this complex. Once successful recruitment of mRNA has been achieved and fMet-tRNA^{Met} in the P-site anchors to the start codon, GTP is hydrolysed to GDP (orange), the initiation factors are released, and the mitochondrial small subunit (mt-LSU in darker blue) can associate, forming the monosome. During elongation (centre), the nascent polypeptide chain is bound to a P-site tRNA, while the A-site is sampled by charged mt-tRNAs delivered by mitochondrial elongation factor-Tu (mtEF-Tu in yellow), until the correct codon-anticodon pair forms. GTP hydrolysis and mtEF-Tu release follow together with the exchange of the GDP (light orange) for a new GTP molecule mediated by mtEF-Ts (orange). The charged A-site mt-tRNA changes its conformation juxtaposing its amino acid to that of the extending nascent chain within the peptidyl-transferase centre. This facilitates peptide bond formation transferring the polypeptide chain onto the A-site mt-tRNA. The elongation factor mtEF-G1 (dark orange) promotes the ribosome movement that repositions the mt-mRNA within the 55S and the mt-tRNAs from the A- and P-sites to the P- and E-sites. The E-site mt-tRNA leaves the monosome in anticipation of a new round of elongation. This cycle continues until the polypeptide is complete and a stop codon is presented in the A-site. Termination (bottom) described the recognition of the stop codon by a release factor protein (mtRF1a in pink), which then adopts a modified conformation that promotes hydrolysis of the ester bond anchoring the nascent chain to the final mt-tRNA. Once the polypeptide chain is released, the two recycling factors, mtRRF1 (dark red) and mtRRF2 (red), promote the dissociation of the ribosomal subunits and premature re-association is prevented by the formation of a mtIF3/mt-SSU complex. Reproduced with permission from (Mai *et al.*, 2016)

1.3 Prevalence of mitochondrial disease

Mitochondrial disease was once thought rare, until two separate studies estimated the prevalence is approximately 1 in 5000 in the population (Skladal *et al.*, 2003a; Schaefer *et al.*, 2008). Indeed, the prevalence of mitochondrial disease is comparable, if not, higher than other more recognisable genetic neurological disorders such as Charcot-Marie-Tooth (CMT) neuropathies (~1/10,000) (Foley *et al.*, 2012; Bargiela *et al.*, 2015), myotonic dystrophy (3-15/100,000) (Turner and Hilton-Jones, 2010), Huntingdon disease (1 in 10,000) (Roos, 2010) and limb-girdle muscular dystrophy (2.3/100,000) (Bargiela *et al.*, 2015).

More recently, an epidemiological study from the North East of England has further revised the overall prevalence of mitochondrial disease figure to 1 in 4300. This is the first attempt to analyse data of combined mtDNA and well-characterized nuclear gene mutations in the adult population (>18 years). Seventy-seven percent of adult mitochondrial disease is due to mutations in the mtDNA (**Figure 10**). The single most common genetic cause of the mitochondrial disease is the m.3243A>G mutation (28%). Collectively, three LHON mutations contribute to 29% of all cases (m.11778G>A, 16%; m.3460G>A, 11%; m.14484T>C, 2%). Single, large-scale mtDNA deletions which are sporadic mutations account for 12% cases. A rather surprising finding is recessive mutations in the *SPG7* gene, encoding for paraplegin matrix AAA peptidase subunit, has emerged as one of the most common nuclear defects causing a mitochondrial disease in the adult population.

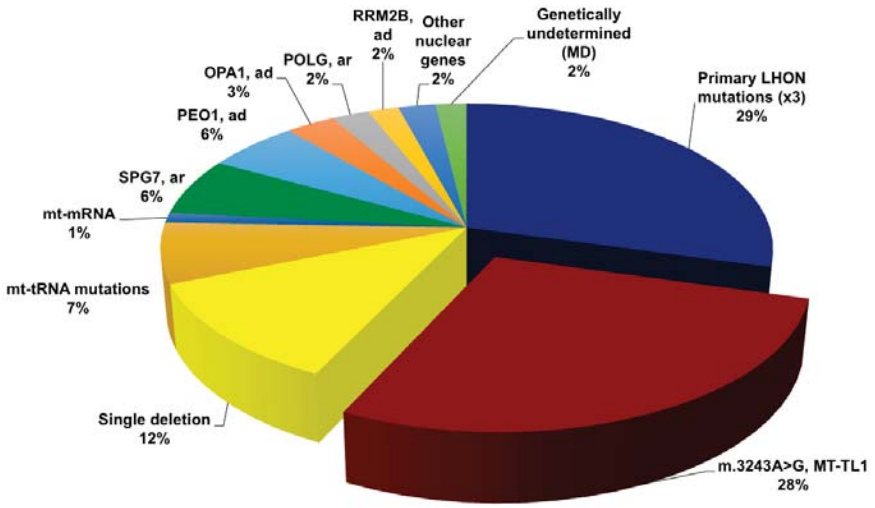

Figure 10. Genetic causes of mitochondrial disease and their frequencies in the North East of England (n=204). Created based on (Gorman *et al.*, 2015)

A study testing more than 3000 neonatal cord blood samples revealed that 1 in 200 newborns harbours one of the ten common mtDNA point mutations (Elliott *et al.*, 2008). While the m.3243A>G mutation is the most prevalent mtDNA mutation (~1 in 400), it is worth pointing out that many individuals carry a very low heteroplasmy level (at least when tested in the blood) and likely remain asymptomatic throughout their life. This observation is supported by the findings of a much smaller number of patients that are clinically manifesting by two separate studies (4.4 in 100,000 in the North East England (Gorman *et al.*, 2015); 18.4 in 100,000 in Finland (Uusimaa *et al.*, 2007)).

The prevalence of mitochondrial disease and frequencies of individual genotypes are clearly dependent on the studied populations and ethnicities (Gorman *et al.*, 2016). For example, LS has been reported to be ten times more prevalent in Saguenay-Lac-St-Jean, Canada compared to other populations, due to a founder mutation in *LRPPRC* (Mootha *et al.*, 2003); mutations in *SUCLA2* are associated with mitochondrial depletion and LS, and the estimated prevalence of disease caused by a founder mutation is 1 in 2500 in Faroes Island (Carrozzo *et al.*, 2007). The consanguineous marriages in some ethnicities, such as Lebanese ancestry, has been shown to have a much higher prevalence of recessive form mitochondrial disease compared to the general population (71/100,000 vs. 6.2/100,000) in Southeastern Australia (Skladal *et al.*, 2003a; Lim *et al.*, 2014). A high level of parental consanguinity has also been observed in patients of North African origin and complex IV deficiency (von Kleist-Retzow *et al.*, 1998).

The epidemiology of the paediatric mitochondrial disease is less well understood compared to the adult population. Mutations in mtDNA are likely only accounted for only 30% of the paediatric cases. Until now, genetic diagnosis has been very challenging to establish in the paediatric cases due to the overlapping genotype-phenotype and inherent limitations with the diagnostic set up such as the need for invasive biopsy and laborious candidate gene sequencing approach (Skladal *et al.*, 2003b; Scaglia *et al.*, 2004; Garcia-Cazorla *et al.*, 2005; Debray *et al.*, 2007). Furthermore, another group of paediatric patients that is difficult to capture is those with prenatal or neonatal onset with the rapidly fatal disease, because investigations are significantly limited with the poor health state of these babies, and the diagnosis of mitochondrial disease is often only achieved at post-mortem or not at all. However, the utilisation of next generation sequencing technology and multi-centre collaboration is changing the speed of securing a genetic diagnosis for these patients. Such developments will hopefully lead to a more accurate estimation of the epidemiology in the paediatric population.

1.3.1 Mortality in mitochondrial disease

Information on mortality data in adult mitochondrial disease is limited (Barends *et al.*, 2016). The findings from several m.3243A>G mutation case series (Hammans *et al.*, 1995; Klopstock *et al.*, 1999; Majamaa-Voltti *et al.*, 2008; Liu *et al.*, 2012) suggest that premature deaths in those with significant disease burden are usually due to neurological complications (stroke-like episodes (SLE) and status epilepticus), cardiac involvement (cardiomyopathy and arrhythmia), respiratory failure due to neuromuscular weakness and precipitated by chest sepsis. The aetiology of death and age among those with milder phenotypes are not known.

1.4 Primary mitochondrial DNA (mtDNA) mutations and disease

Mitochondrial DNA is susceptible to mutation compared to nuclear DNA due to its close location to the respiratory chain that produces free oxygen radicals and lacks protective histones (Sastre *et al.*, 2000). Mutations in the mtDNA can be classified into point mutations that are usually maternally inherited and mtDNA rearrangements (deletion and insertion) which mostly develop sporadically and have a low risk of transmission (Chinnery and Hudson, 2013). More than 300 mtDNA variants have been reported to cause human disease since 1988. Intriguingly, over half of these mutations are identified in tRNA genes, although tRNAs only contribute around 10% of the total coding capacity of the mtDNA. In contrast, 40% of the mutations are found in the polypeptide-encoding genes, comprising approximately 70% of the mtDNA, and there are very few mutations identified in two rRNA genes (Schon *et al.*, 2012). However, many of these mtDNA variants (~50%) have only identified in small number of pedigrees or single patients, and their pathogenicity has not always been robustly validated using the gold standard methods (DiMauro and Schon, 2001): trans-mitochondrial cybrid studies and single muscle fibre study of heteroplasmic mtDNA variant (comparison of mutant heteroplasmy levels between COX-positive and COX-negative fibres). The challenge of determining the pathogenicity of novel mitochondrial tRNA variant could be addressed by applying a rigorous pathogenicity scoring system in the diagnostic and clinical practice (McFarland *et al.*, 2004; Yarham *et al.*, 2010). A prediction tool combining machine-learning algorithm and biological data (segregation, histochemistry and biochemistry) has recently been developed and might serve as an alternative for determining the probability of pathogenicity of a given novel mtDNA variant particularly when the experimental data is not available (Niroula and Vihinen, 2016).

The majority of mitochondrial DNA disease, especially in adult patients, is caused by one of the following mutations: m.3243A>G, three common mutations associated with Leber

hereditary optic neuropathy (LHON) and large-scale, single deletion (as discussed in section 1.3).

1.4.1 Heteroplasmy, threshold level and tissue segregation

Multiple copies of mtDNA molecule are found in cells and mutations in mtDNA usually co-exist with the wild-type (normal) mtDNA and this is called heteroplasmy. Homoplasmy arises when all the mtDNA molecules are identical (either all wild-type or mutated). The expression of respiratory chain deficiency at the cellular level is only evident when a certain level of heteroplasmy is achieved, and this is described as a threshold effect. Many in-vitro studies using transmitochondrial cybrid cell lines (King and Attardi, 1989) have shown that many mutations are functionally recessive and threshold level varies considerably with a range of 60% to 90% (Chomyn *et al.*, 1992; Trounce *et al.*, 1994; Rossignol *et al.*, 2003; Taylor and Turnbull, 2005). Lower tissue threshold level (70-80%) is observed in mitochondrial rearrangement (mainly single deletion) (Sciacco *et al.*, 1994) compared to some tRNA mutations such as 90% in m.8344A>G mutation (*MTTK*) (Yoneda *et al.*, 1995). Strong correlation of disease phenotype and tissue heteroplasmy has been observed in some mutations such as m.8993 T>G mutation (*MTATP6*) (Santorelli *et al.*, 1993) and more recently muscle heteroplasmy level and deletion size in patients with single mtDNA deletions have been used to elucidate the disease severity and prediction of disease progression (Grady *et al.*, 2014)

Ideally, the threshold level should be established by examining an individual tissue type directly. However, this is not always practical due to very limited access to certain tissues, for example, brain and other internal organs such as heart. As such, skeletal muscle is often used as an alternative in estimating the heteroplasmy level of other post-mitotic tissues with an assumption that mutation load is similarly distributed and this practice is supported by post-mortem findings (Shoji *et al.*, 1993; Tanno *et al.*, 1993; Howell *et al.*, 1994). However, clinical observations have revealed that the relationship of tissue heteroplasmy and clinical manifestation of disease is not always consistent, for example in the most common pathogenic mtDNA mutation, m.3243A>G (further elaboration in **Section 1.4.3.1.2**).

Although threshold effect and variable segregation of mutations in different provide a reasonable explanation on heterogeneity in the clinical presentation of many heteroplasmic mtDNA mutations, they are clearly not applicable for homoplasmic mutations such as patients with LHON. Three common LHON mutations (m.3440A>G, m.11778A>G and m.14484T>C) are frequently detected in homoplasmy level (80% to 90% of the families) yet only 50% of male

carriers and 10% of female carriers develop visual loss at their 2nd and 3rd decade of life (Man *et al.*, 2003). Several factors such as mitochondrial haplogroup, the interplay between the LHON mutations and the X-linked chromosome, protective effect of female sex hormone, environmental triggers (e.g., smoking) have been implicated (Yu-Wai-Man *et al.*, 2014). It remains elusive why retinal ganglion cells are particularly susceptible to these mutations (Man *et al.*, 2002) (further elaboration in **Section 1.4.3.3**).

1.4.2 Mitochondrial DNA transmission and bottleneck effect

The variations in mutant heteroplasmy level between generations are observed, and the degree of variations differs between the mutations. Such observation leads to the theory of the mitochondrial genetic bottleneck, which hypothesises that only a small proportion of the maternal mitochondrial genome is transmitted to the offspring (Stewart and Chinnery, 2015). Three models are currently available to provide insights on when and how the mitochondrial genetic bottleneck occurs. First, a bottleneck occurs at the stage of primordial germ cells (PGCs) due to the combination of a significant reduction in the mtDNA copy number and random genetic drift (Jenuth *et al.*, 1997; Cree *et al.*, 2008). Second, the bottleneck occurs during the folliculogenesis, early in the post-natal period, due to the preferential replication of a subgroup of mitochondrial genomes (Wai *et al.*, 2008). Third, multiple mtDNA molecules are grouped into individual nucleoids, which serve as the units of segregation. The bottleneck occurs due to the unequal partitioning of homoplasmic segregating units within the PGCs, without a reduction in the mtDNA copy number in PGCs (Cao *et al.*, 2007; Cao *et al.*, 2009) (**Figure 11**) (Stewart and Chinnery, 2015).

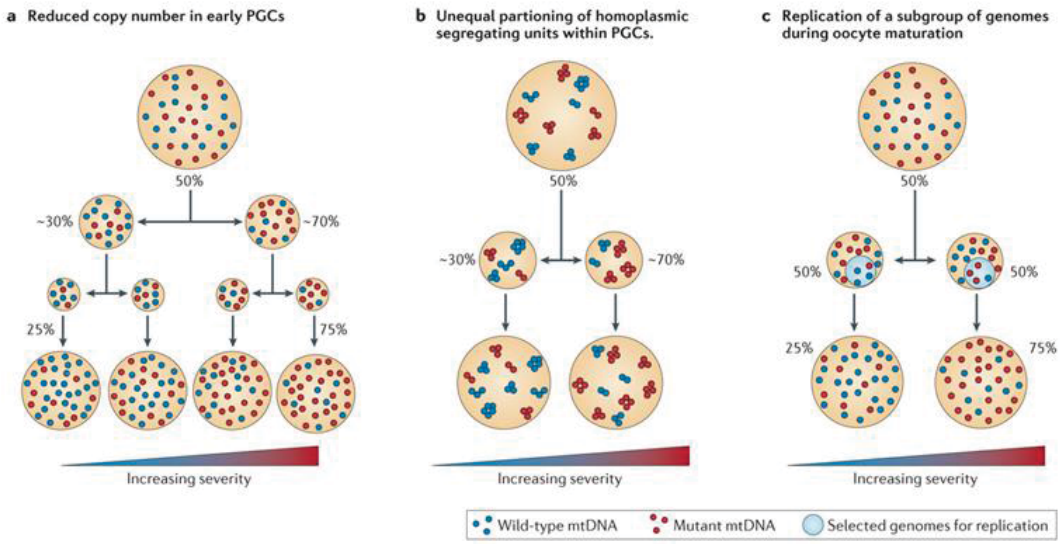

Figure 11. Three models for the mitochondrial DNA bottleneck. PGC= primordial germ cell. Reproduced with permission from (Stewart and Chinnery, 2015).

It is increasingly evident that size of bottleneck varies between the mtDNA mutations and a recent simulation study based upon a compilation of heteroplasmy levels from family pedigrees published in the literature and unpublished data, clearly demonstrated that the rate of random genetic drift varies between mutations. However, there is no evidence to support a 'purifying'/selective process, at least in the common point mutations such as m.3243A>G, m.11778G>A, m.3460G>A, m.8344A>G and m.8993T>G/C mutations that account for the majority of patients (Wilson *et al.*, 2016). Tighter genetic bottleneck, such as in the case of m.8993T>G/C mutation, indicates a more rapid segregation of mtDNA heteroplasmy between generations, which explains a common scenario encountered in the clinical practice that a severely affected child with very high/near homoplasmic mutant heteroplasmy born to an asymptomatic mother who carries very low mutant load (Wilson *et al.*, 2016). The findings of a variable genetic bottleneck for different mutations are also corroborated by data derived from the Genomes of the Netherlands (GoNL) project (Li *et al.*, 2016).

Selection or purifying process appears to be a plausible biological explanation on how the inheritable mitochondrial DNA disease is only dominated by several common mutations in the population, given mtDNA is so susceptible to *de novo* mutations. This idea is supported by the observation from an animal study that the severe mtDNA mutation (*MTND6*) was negatively selected against the wild type in the mouse germline and was eliminated after four generations, whereas the milder mutation (*MTCO1*) was retained in multiple generations (Fan

et al., 2008). However, such observation has not been replicated in the human data presented by a recent Dutch study (Li *et al.*, 2016).

1.4.3 Classic syndromes of mitochondrial disease

Organs with high energy demand such as brain, skeletal muscle (including ocular muscle), cochlear, optic nerve, peripheral nerve, heart, and liver are particularly susceptible to mitochondrial dysfunction irrespective the genetic mutations (**Figure *12***). Neurological involvement by far is the most common presenting feature and majority of patients have central, peripheral or combined involvement, which frequently referred as mitochondrial encephalomyopathy (McFarland *et al.*, 2010; DiMauro *et al.*, 2013). The onset of clinical manifestation varies between different genes and often even within the group of patients sharing the same genetic mutation. All age groups including paediatric (antenatal period, neonatal onset, infancy and childhood (Goldstein *et al.*, 2013)) and adult can be affected.

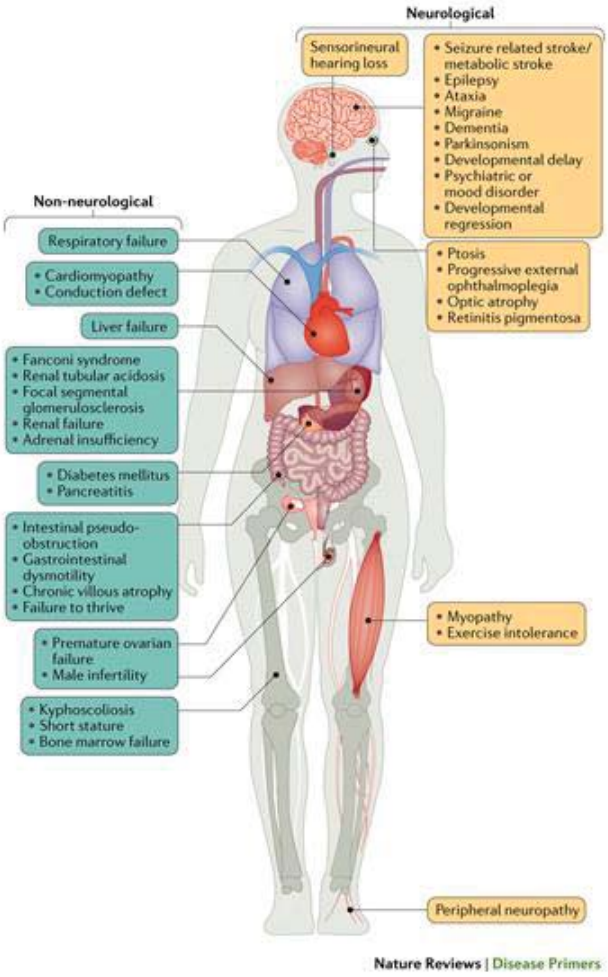

Figure 12. Clinical features of mitochondrial disease. Reproduced with permission from (Gorman *et al.*, 2016)

Historically, patients with suspected or biopsy-proven mitochondrial disease were classified into different clinical syndromes, primarily based on the clinical findings and occasionally, histopathological findings. This approach was effective in identifying groups of patients that were clinically homogeneous, eventually led to the findings of mitochondrial DNA mutations and nuclear gene mutations in several distinctive clinical syndromes such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF), maternally inherited diabetes and deafness (MIDD), Kearn-Sayre syndrome (KSS), Pearson syndrome, neuropathy, ataxia and retinitis pigmentosa (NARP), maternally inherited Leigh syndrome (MILS) and chronic progressive external ophthalmoplegia (CPEO). These syndromes are discussed individually as follows.

1.4.3.1 Mitochondrial encephalomyopathy, stroke-like episodes and lactic acidosis

1.4.3.1.1 Clinical and neuropathological characteristics

The acronym MELAS was coined in 1984 (Pavlakis *et al.*, 1984). Although the original case was presented at a paediatric neurology meeting in 1976, the full description of the original case of MELAS only became available 15 years later (Hirano *et al.*, 1992). The patient was a girl that had a normal birth but was noted to have short stature, low birth weight and developed progressive muscle symptoms from age two years onwards. The first presentation of the stroke-like episode occurred at age 5.5 years, with documentation of loss of consciousness for 30 minutes, multiple episodes of dimmed vision and lactic acidosis after a strenuous exercise. Subsequent neurological presentations included focal sensory disturbance, focal and generalised seizures, with post-ictal right hemiparesis that lasted up to 3 days, cortical blindness and dementia. She died from heart failure at age ten years. The key neuropathological findings were multiple small cortical infarcts found predominantly in occipital and posterior temporal lobes and loss of half the neurones in the medial dorsal nucleus of the thalamus. In addition, capillary proliferation and hypertrophy/dilatation of tunica media were observed.

The diagnostic criteria of MELAS was proposed based on the literature review of 69 cases (Hirano *et al.*, 1992): (1) stroke-like episode before age 40 years; (2) encephalopathy characterized by seizures, dementia, or both; (3) lactic acidosis, ragged red fibres, or both; (4) normal early development; (5) recurrent headache; (6) recurrent vomiting. Whilst such diagnostic criteria improve the recognition of the clinical syndrome; there are several limitations. Firstly, the early case reports or case series of MELAS focused on children and young adults, giving an impression that stroke-like episodes only occurred below the age of

40. However, it is becoming clear that stroke-like episodes could develop at any age, late presentation of stroke-like episodes has been increasingly recognised (Minamoto *et al.*, 1996; Aurangzeb *et al.*, 2014; Marques-Matos *et al.*, 2016). Indeed 30% of patients with MELAS syndrome had their first presentation of the stroke-like episode after 40 years old (unpublished data from Newcastle mitochondrial disease patient cohort). Secondly, there is no specific clinical and radiological definition of what constitutes of a stroke-like episode and transient neurological symptoms associated with a migrainous headache (<24 hours) is an important differential diagnosis; Thirdly, dementia is a late clinical feature that often develops after recurrent stroke-like episodes. Furthermore, lactic acidosis and ragged red fibres are non-specific laboratory findings of mitochondrial disease; these findings are supportive but may be absent in some patients with stroke-like episodes associated with the m.3243A>G mutation (de Vries *et al.*, 1994), which is the most common cause of MELAS and the mutation can be rapidly confirmed using blood, urine or other non-invasive methods (McDonnell *et al.*, 2004).

Stroke-like lesions often do not confine to the vascular territories, with the predilection of occipital, parietal and temporal lobes involvement. These unique characteristics have been consistently observed in both the imaging (Majamaa *et al.*, 1997; Stoquart-Elsankari *et al.*, 2008; Ito *et al.*, 2011) and neuropathological (Gilchrist *et al.*, 1996; Tanahashi *et al.*, 2000; Sparaco *et al.*, 2003; Betts *et al.*, 2006) studies. The precise pathogenesis remains debatable (Lax *et al.*, 2016) and the leading hypotheses are angiopathy/endothelial dysfunction (Hasegawa *et al.*, 1991; Koga *et al.*, 2005), neuronal hyperexcitability (Iizuka *et al.*, 2002) and inherent OXPHOS dysfunction caused combined neuronal and vascular dysfunction (Gilchrist *et al.*, 1996). The theory of angiopathy derives from the early observation of strongly SDH reactive vessels (the tunica media of vessel) with increase subsarcolemmal accumulation of mitochondria and subsequent postulation of impairment of vascular tone/vasodilatation leading to the development of stroke-like lesion. Findings from in vivo cerebral perfusion studies appear conflicting, and both under perfusion or hyperperfusion leading to cytotoxic/vasogenic oedema have been described (Gropen *et al.*, 1994; Tzoulis and Bindoff, 2009; Ito *et al.*, 2011). However, such variations likely represent the evolution of the underlying process, and stroke-like episodes are frequently under-recognized at the early phase. The theory of neuronal-hyper excitability advocates that the seizure increases the energy demand in the neurones with impaired function in the mitochondrial respiratory chain, leading to neuronal death and formation of a stroke-like lesion. Findings from clinical observation, EEG abnormalities and spreading nature of stroke-like lesions (Iizuka *et al.*, 2003), appear to corroborate this hypothesis.

1.4.3.1.2 The m.3243A>G mutation and heteroplasmy

The most common genetic defect causing MELAS syndrome is the m.3243A>G mutation, accounting for 80% (n=32/40) of cases when first reported by Japanese researchers in 1992 (Goto *et al.*, 1992). They found that the common clinical features associated with stroke-like episodes were a headache with vomiting (100%), unconsciousness (85%), focal and generalised seizures (85%), and visual disturbance (53%). Unilateral limb weakness was only present in less than a quarter of patients. Lactic acidosis was almost invariably present. Abnormal biochemical defects were only detected in 60% of the muscle biopsies, and complex I deficiency (either isolated or combined) was present in 43% of cases. Strongly SDH-reactive blood vessels (SSVs) were identified in 88% of cases.

The genetic defect in muscle from m.3243A>G related MELAS causes a decrease in levels of mtDNA-encoded polypeptides, with normal corresponding transcripts (Moraes *et al.*, 1992). The threshold level for RRF formation was around 85% based on the single fibre analysis, and it appeared that the percentage of mutant mtDNA heteroplasmy correlated with the percentage of RRF. The reduction in COX activity in muscle fibres of patients with MELAS was less severe compared to the muscle fibres of patients with MERRF or KSS. There was no apparent change in the steady-state rRNA:mRNA levels, suggesting that transcription termination is not crucial in the pathogenesis of MELAS.

Hammans and colleagues showed that the m.3243A>G mutation could be detected in blood, suggesting that a more rapid screening for the mutation was achievable without the requirement of muscle biopsy (Hammans *et al.*, 1991). Subsequently, they found that the m.3243A>G was not detectable in 17% of patients (6/36) who were older than 37 years (Hammans *et al.*, 1995). It is now well-recognized that testing the m.3243A>G mutation in blood alone could yield a false negative result (Rahman *et al.*, 2001; Shanske *et al.*, 2004).

The spectrum of clinical features associated with the m.3243A>G mutation continued to expand with the detection of the mutation in patients with CPEO (Moraes *et al.*, 1993) and MIDD (Guillausseau *et al.*, 2004). The findings from cohort-based natural history studies have further enriched our understanding on the phenotypic heterogeneities associated with the m.3243A>G mutation (de Laat *et al.*, 2012; Mancuso *et al.*, 2013a; Nesbitt *et al.*, 2013) and disease progression (Kaufmann *et al.*, 2011). Interestingly, there is an anecdotal observation of intrafamilial clustering of MELAS and CPEO in the early studies (Hammans *et al.*, 1995; Mariotti *et al.*, 1995). However, this might be a selection bias and require further validation from prospective, large-scale studies.

The presence of tissue threshold and mutant mtDNA heteroplasmy in vitro has generated a lot of interest on studying the correlation of disease severity, phenotypes and the m.3243A>G heteroplasmy. An early study suggested that there was a limitation of using m.3243A>G heteroplasmy level in blood for genetic counselling or prognostication because of a considerable overlap in the level between affected individuals and asymptomatic carriers (Hammans *et al.*, 1995). In contrast, a study based on the published literature showed statistically significant correlations between several clinical features such as stroke-like episodes and the m.3243A>G heteroplasmy in muscle, suggested the mutation load in muscle may have a prognostic value particularly among those with the level of >90% (Chinnery *et al.*, 1997). However, a significant level of uncertainty remains for the majority of patients who harbour the intermediate level of heteroplasmy.

1.4.3.2 Syndromes associated with single, large-scale deletion

Single, large-scale mtDNA deletions were the first genetic defect in mtDNA linked to mitochondrial disease in humans (Holt *et al.*, 1988). The phenotypic manifestation of single, large-scale mtDNA deletions ranges from severe multi-system involvement as in Pearson syndrome, Kearns-Sayre syndrome (KSS) to insidious chronic progressive external ophthalmoplegia (CPEO), of which the clinical findings are often confined to skeletal and ocular muscles in the latter (Pitceathly *et al.*, 2012b).

Kearns-Sayre syndrome was first described in the late 1950s (Kearns and Sayre, 1958) and the triad of clinical features are retinitis pigmentosa, progressive external ophthalmoplegia with the onset below 20 years plus one or more of the following findings: heart block, cerebellar ataxia and raised CSF protein (> 1g/L) (Pitceathly *et al.*, 2012b). Additional features may include deafness, short stature, diabetes mellitus, elevated CK, lactic acidosis and Fanconi syndrome (Yamashita *et al.*, 2008).

Pearson syndrome (PS) is characterised by exocrine pancreatic failure and sideroblastic anaemia that present predominantly in early childhood with an extremely poor prognosis (Rotig *et al.*, 1989). Other clinical features associated with PS include renal tubulopathy and poor growth due to gastrointestinal dysfunction. The heteroplasmy of mtDNA deletion is higher in the blood than other tissues, and due to the high replication in blood cells, the resolution of bone marrow abnormalities has been observed in those individuals who survived the initial manifestation. However, the mtDNA deletion accumulates in post-mitotic tissues leading to the progressive nature of multi-system involvement. Some cases with the initial

manifestation of PS survived and evolved into clinical features compatible with KSS (Larsson *et al.*, 1990) or LS (Lee *et al.*, 2007).

The question regarding the timing of deletion formation has prompted a lot of research interest. It is intuitive to deduce that the single deletion would have occurred before the differentiation of three germ layers for patients who have multi-system involvement; whereas in patients presenting with isolated myopathy, the deletion could only occur after the formation of the mesoderm. An extensive study of 27 post-mortem tissues of a 59 year old man who had CPEO, mild ataxia, optic atrophy and cardiac involvement (Marzuki *et al.*, 1997) have provided some insights. The authors showed that the deletion was detectable in multiple tissues derived from ectodermal (CNS) and mesodermal (skeletal and ocular muscles) origins, with heteroplasmy levels higher in the muscle than the CNS tissues. It was suggested that the deletion occurred in the early embryonic development, before the differentiation and subsequently segregated to the ectoderm and mesoderm. After the identification of low levels of common mtDNA deletion 4,977 bp in human oocytes (0.1% per 100,000 mtDNA molecule per oocyte), a study suggested that the deletion may, in fact, occur as early as oogenesis (Chen *et al.*, 1995). Brenner and colleagues also showed that deletion was detectable in at least 1/3 of human oocytes that were studied, but more interestingly, the frequency of deletion was statistically significantly lower in embryos than oocytes. Whilst such findings have not fully supported the mtDNA bottleneck theory; these deletions might be eliminated by an unknown, nuclear-driven mechanism (Chen *et al.*, 1995). A theory hypothesises that single deletions may, in fact, occur in grandmother's oocyte that fertilised, and developed to the zygote of the mother of an affected individual. Due to the random genetic drift, segregation and amplification of the mtDNA with deletion, the mother's oocyte that contains high heteroplasmy of mtDNA deletion is fertilised, giving birth to an offspring that becomes clinically affected (Chinnery *et al.*, 2004). Based on the clinical observation, the risk of transmitting the deletion and causing a mitochondrial disease in an affected female is around 4% (95% confidence interval 1% – 12%) (Chinnery *et al.*, 2004). Such findings lead to a consensus that the single, large-scale mtDNA deletions are 'sporadic' mutations even though maternally inherited single deletion has rarely been reported (Bernes *et al.*, 1993; Shanske *et al.*, 2002).

1.4.3.3 Leber hereditary optic neuropathy

LHON is characterised by acute or subacute bilateral visual loss that predominantly involves central vision, usually in young, otherwise healthy men (Nikoskelainen, 1984). Clinically,

affected patients usually present with a painless visual loss that reaches a nadir (with visual acuity >6/60) 4-6 weeks after the onset. Central scotoma is a characteristic visual field defect, and impaired colour vision is evident in the early phase, but pupillary reflexes are usually intact. Fundoscopic examination reveals vascular tortuosity of the central retinal vessels and swelling of the retinal nerve fibre layer. Of note, ~ 20% of LHON cases have a normal looking optic disc in the acute phase (Man *et al.*, 2002).

Before the identification of genetic mutation, an early, comprehensive study reported the median age of onset was around 20 years for males and females and spontaneous recovery of at least one eye was observed in ~ 45% of patients. It was observed that the risk of offspring developing the visual loss was higher if their mothers were affected (M:F 50% vs 24-32%) compared to unaffected mothers (M:F 50% vs 8%), notably the risk for female offspring was invariably lower than males (van Senus, 1963). The puzzle of inheritance pattern in LHON was not solved until the discovery of the m.11778G>A mutation in *MTND4* gene in nine pedigrees (Wallace *et al.*, 1988). Two other common point mutations m.3460G>A in *MTND1* (Howell *et al.*, 1991) and m.14484T>C in *MTND6* (Johns *et al.*, 1992), together with the m.11778G>A, account for ~ 90% of LHON cases (Yu-Wai-Man).

The prevailing perception about LHON is a selective involvement of retinal ganglion cell caused by the mitochondrial dysfunction, leading to the clinical manifestation of isolated optic neuropathy. Interestingly, the association of LHON and cardiac arrhythmia (frequency ~ 8-10%) was observed in two case series (Nikoskelainen *et al.*, 1985; Nikoskelainen *et al.*, 1994; Mashima *et al.*, 1996) (Bower *et al.*, 1992). A study of 46 patients with LHON demonstrated that 59% of them have neurological abnormalities such as postural tremor (n=9), thoracic kyphosis (n=7), multiple sclerosis (MS) like disorder with periventricular white matter changes (n=2), parkinsonism with dystonia (n=1) and chronic motor tic disorder (n=1) (Nikoskelainen *et al.*, 1995). Furthermore, there are patients who harbour the 'LHON' mutations and manifest with severe neurological deficits without a visual loss (McFarland *et al.*, 2007). Harding and colleagues provided a compelling evidence about the link between LHON and multiple sclerosis by reporting a case series of eight women from different pedigrees who presented with classical LHON and subsequently various neurological features and widespread white matter lesions strongly suggestive of MS (Harding *et al.*, 1992). In a blinded review of cranial MRI of three group of patients, i.e. patients with LHON (n=31), LHON and MS-like illness (LMS) (n=11) and MS control (n=30), the white matter changes seen in all LMS and a quarter of LHON (n=8) patients were deemed indistinguishable from typical MS (Matthews *et al.*, 2014). The risk for female patients with LHON having

characteristic white matter lesions of MS was around eight times higher than males (95% CI 2.8 – 25.1, p<0.01). 17% of LHON mutation carriers in this study had asymptomatic white matter changes (n=7; two females, five males). It has also been suggested that the risk of MS among patients with LHON is around 5% (Vanopdenbosch *et al.*, 2000). These findings contradict the conclusion made by the other group that suggested the co-occurrence of LHON and MS was simply by chance (Pfeffer *et al.*, 2013a).

Incomplete penetrance and sex bias (males are at higher risk) in homoplasmic LHON mutations is biologically intriguing but imposes a great challenge in genetic counselling. Overall, for individuals who harbour homoplasmic mutations, the risk of disease is 30~50% in males and 5-15% in females (Man *et al.*, 2002). Heavy smoking has been shown to be an independent risk factor in these individuals (Kirkman *et al.*, 2009; Carelli *et al.*, 2016).

1.4.3.4 Myoclonic epilepsy and ragged-red fibres

Myoclonic epilepsy and ragged-red fibres, less well known as Fukuhara disease, was first considered as a type of mitochondrial encephalomyopathy, manifesting with overlapping features of myoclonus (dyssynergia cerebellaris myoclonica, Ramsay Hunt Syndrome), Friedreich ataxia and myopathy with association of ragged red fibres due to mitochondrial dysfunction in two Japanese patients in 1980 (Fukuhara *et al.*, 1980). The most striking clinical feature was childhood/teenage onset of myoclonus, accompanied by other features including epilepsy, mental deterioration, cerebellar ataxia, muscular atrophy and foot deformity. Ragged red fibres (>10%) were identified in the muscle biopsies, and the sural nerve biopsies showed marked fibrosis and depletion of myelinated fibres. The ragged red fibres were, in fact, prominent accumulations of abnormal mitochondrial especially in the subsarcolemmal regions when examined by electron microscopy.

The heteroplasmic m.8344A>G mutation in *MTTK* gene was identified as a cause of MERRF in 1990 (Shoffner *et al.*, 1990). Lipomas and hypertension appeared to be prominent features in addition to other neurological presentations in five generations of a large family pedigree (Austin *et al.*, 1998). The age of onset, severity of symptoms and biochemical defect did not correlate with the mtDNA mutation in muscle (Silvestri *et al.*, 1993). No skewed segregation was observed in various tissues on autopsies; no significant difference between muscle and blood was noted. Neuropathological findings of in another patient also had changes consistent with LS.

Cohort-based observational studies have provided a better understanding of the spectrum of clinical features associated with the m.8344A>G mutation over the last decade. The m.8344A>G mutation was present ~4% of all cases (n=42; 27 pedigrees) of mitochondrial disease in an Italian national cohort (~12% of all point mtDNA mutations) (Mancuso *et al.*, 2013b). The mean age of disease onset was 30 +/- 18 years (range 0-66; just under a third of patients presented under age 16 years). Features of neuromuscular dysfunction appear to be commoner than CNS involvement (77% vs. 56%). Muscle weakness was present in 59% of patients, exercise intolerance (44%), multiple lipomatosis (32%), ptosis (29%) with ophthalmoparesis only present in two cases (6%), muscle wasting (21%), generalized seizure (35%), ataxia (24%), myoclonus (24%), cardiac involvement (18%), peripheral neuropathy (15%) and diabetes mellitus (12%). Lactic acidemia was present in around 2/3 of cases (65%). The authors suggested that the origin of myoclonus might derive from the cerebellar dysfunction due to the association of myoclonus and ataxia (Fisher Exact test, $p = 0.025$), such association with epilepsy was lacking ($p=0.176$). Uncommon clinical features ($n<=2$) were hypothyroidism, hypogonadism, dysphagia, psychiatric involvement and cataract. Ragged red fibres were identified in 96% of muscle biopsies (26/27), including four who were not myopathic. No statistical difference in the heteroplasmy (both blood and urine) level between carriers and affected individuals was noted. Their review of the literature (n=321) showed that four core features of MERRF, namely myoclonus, seizures, ataxia and ragged red fibres were identified in 61%, 55%, 62% and 69% of reported cases (Mancuso *et al.*, 2013b).

A case series with smaller numbers of patients with the m.8344AG mutation (n=15; 7 Pedigrees) also showed muscle weakness (53%) was a commoner feature compared to myoclonus (40%), generalised seizure (40%) and ataxia (13%) (Catteruccia *et al.*, 2015). Two third of the patients from this case series had a restrictive pattern of respiratory involvement and 47% of patients required ventilatory support. Cardiomyopathy and arrhythmia were present in 47% and 40% of the cases, respectively. EEG abnormalities (73%) were identified even in patients with predominant myopathic phenotype. Significant numbers of RRFs (10-55%) were present in all but one patient. Glutei and posterior compartments of both thigh and leg appeared to be more affected compared to anterior compartments in five patients who underwent MRI muscle. Complex IV deficiency was the most severe biochemical defect (9/15). Mutation load in muscle was relatively homogeneous, and it did not correlate with clinical manifestations. The authors suggested that the variation in the frequencies of different clinical features in their case series might be due to the study design.

A case series from the German nationwide, multi-centre cohort study mitoNET (n=34; 13 pedigrees) has recently been published (Altmann *et al.*, 2016). More detailed clinical evaluation using NMDAS was performed on 16 patients. The mean age of disease onset was 24.5 +/- 10.9 (range 6-48 years; 75% had onset 18 years or older). Deafness is the most common symptoms (72%), followed by cerebellar ataxia (70%), myoclonus (59%), migraine (52%), proximal muscle weakness (58%), muscle atrophy (39%) and others. Common investigations were EEG abnormalities (15/17, 88%), lactic acidemia (54%), raised CK (46%) and cerebral/cerebellar atrophy (43%). Muscle biopsy was performed in 8 patients, and RRFs were identified in 5. They found a significant association between myoclonus and seizures (Fisher exact test, n=23, p = 0.0128), in contrast to the Italian findings where the myoclonus was associated with ataxia. No correlation between heteroplasmy level and age of onset; also mutation level in blood did not correlate with disease severity. Interestingly, the lowest heteroplasmy level in an asymptomatic individual was 7% in the blood. The authors believed that the discrepancies in the frequency of several clinical features (e.g., hearing loss, ataxia, psychiatric disorder) were likely due to differences in the methods of assessment and data collection.

1.4.3.5 Neurogenic weakness, ataxia, retinopathy pigmentosa

Neurogenic weakness, ataxia and retinitis pigmentosa (NARP) syndrome was first associated with the m.8993T>G mutation in the *MTATP6* gene (Holt *et al.*, 1990). The m.8993T>G heteroplasmy was detected at the level of 82% and 88% in the proband and similarly affected family member, respectively. The highest level (97%) was identified in the most severely affected individual who presented with development delay, short stature, upper motor neurone signs in the lower limbs, ataxia, cerebellar and brainstem atrophy (Holt *et al.*, 1990). The same mutation was reported to cause Leigh syndrome (LS) in a patient who harboured high mutant level (>95% in multiple tissues) (Tatuch *et al.*, 1992). The m.8993T>G mutation changes a conserved leucine to a positively charged arginine in the subunit a, resulting in the reduction of ATP synthesis (Tatuch and Robinson, 1993; Baracca *et al.*, 2000). Furthermore, the correlation of mutant heteroplasmy level and ATP synthesis have also been demonstrated (Sgarbi *et al.*, 2006). Generally speaking, the threshold of clinical expression of LS is thought to be >90%; individuals with 60-90% are less severely affected, often with NARP or are oligo-symptomatic (Uziel *et al.*, 1997). However, late-onset Leigh syndrome (at age 43) has been reported in a Japanese patient who harboured >90% in multiple tissues (Nagashima *et al.*, 1999). The m.8993T>G heteroplasmy level remains relatively constant over time, and the

skewed tissue segregation is uncommon. Furthermore, de novo mutations have been reported in at least 20% of families (White *et al.*, 1999).

The m.8993T>C mutation was first described in a family with Leigh syndrome (de Vries *et al.*, 1993). The mutation changes a leucine to non-charge proline, causes a milder functional defect on the ATP synthase compared to the m.8993T>G mutation in the biochemical studies of fibroblasts (Vazquez-Memije *et al.*, 1998). Childhood and adult onset NARP has also been observed in this mutation (Sciacco *et al.*, 2003; Rantamaki *et al.*, 2005).

Homoplasmic m.9176T>C mutation can cause infantile Leigh syndrome (Dionisi-Vici *et al.*, 1998) and a mixed clinical picture of adult-onset progressive spastic paraparesis and axonal neuropathy (Verny *et al.*, 2011). In contrast to the m.8993T>C/G mutations, the differences in disease onset, clinical phenotype and severity could not be explained by the mutation alone, the influences of mtDNA haplogroup and nuclear background have thus been suggested (Jonckheere *et al.*, 2012).

Another interesting point mutation in *MTATP6* gene is the m.9185T>C mutation. Distal motor neuropathy and ataxia are prominent features associated with the mutation (Pfeffer *et al.*, 2012a; Pitceathly *et al.*, 2012a). A study has shown that ~1% of CMT2 (distal hereditary motor neuropathy) is caused by this mutation (Pitceathly *et al.*, 2012a). Progressive motor neurone syndrome, mimicking spinal muscular atrophy, has also been described (Brum *et al.*, 2014). LS has also been reported in this mutation (Childs *et al.*, 2007).

1.4.3.6 Leigh syndrome

Leigh syndrome, also known as subacute necrotising encephalopathy, is one of the most common presentations of mitochondrial disease among the paediatric patients with an estimated prevalence of 1 in 40,000 live births (Rahman *et al.*, 1996). Denis Archibald Leigh reported the first case in London in 1951. It was about a 7-month old boy who had a normal birth and early development for six weeks, subsequently presented with a constellation of neurological signs and symptoms including developmental regression, poor feeding, optic atrophy, and limb spasticity. A post-mortem examination revealed bilateral symmetrical subacute necrotic lesions in thalami, brainstem (pons and inferior olives) and the posterior columns of the spinal cord with relatively sparing the caudate and lentiform nuclei (Leigh, 1951; Baertling *et al.*, 2014). Leigh made an interesting observation that these pathological findings were very similar to patients with Wernicke's encephalopathy. The core clinical features of LS are abnormal motor functions and intellectual retardation, usually with

regression, and signs of brainstem dysfunction, including respiratory failure, autonomic dysfunction, ophthalmoparesis/strabismus and nystagmus. Other neurological features may also present such as epilepsy, cerebellar syndrome and optic neuropathy (Rahman *et al.*, 1996). In a multi-centre retrospective study (n=130) involving a genetically heterogeneous group of patients (22% with mtDNA mutations of which *MTATP6* was most common; 38% with mutations in the nuclear genes of which the common nuclear genes were *SUCLA2*, *SLC19A3*, *SURF1* and *PDHA1*; genetically undetermined 40%), the median age of disease onset was 7 months and only 20% of patients only manifested after the age of 2 years (Sofou *et al.*, 2014). The median age at death was 2.4 years (40% of the cohort, range:1 month – 21 years).The most common presenting clinical feature was abnormal motor findings (99%) such as tone abnormality, muscle weakness, abnormal reflexes and extrapyramidal movement disorders (99%), followed by abnormal ocular findings (61%) (which include strabismus/ ophthalmoplegia, ptosis, nystagmus, optic atrophy and visual impairment), epilepsy (39%), respiratory dysfunction (38%) and mental retardation (37%). Acute exacerbation was reported in 57% of patients. The predictors of poor survival were the age of onset below six months, failure to thrive, brainstem lesions and intensive care admission.

The definitive diagnosis of Leigh disease could only be confirmed neuropathologically (i.e., post-mortem) with the findings of gliosis, vacuolation, capillary proliferation with relative neuronal preservation predominantly affecting the brainstem and basal ganglia in the past (Lake *et al.*, 2015). The advancement of or neuroimaging and genetic sequencing technology have revolutionised and increased the rate of premortem diagnosis (Baertling *et al.*, 2014). Typical MRI head findings associated with Leigh syndrome are bilateral, symmetrical T2 hyperintensities in the basal ganglia and brainstem (Arii and Tanabe, 2000; Baertling *et al.*, 2014). Lentiform nuclei appear to be most frequently identified on the MR imaging, followed by symmetrical signal abnormalities in the caudate nuclei and thalami in the cerebral deep grey matter (Sofou *et al.*, 2013; Bonfante *et al.*, 2016). Other less frequent imaging changes may include cerebellum, spinal cord and cerebral white matter. Spinal cord lesion has been less frequently reported on the neuroimaging of patients with Leigh syndrome, compared to the prominent findings in the early post-mortem studies, and this may just reflect the scanning practice that focuses on the brain in the clinical practice (Lake *et al.*, 2015). Lower brainstem lesions (especially in the periaqueductal gray matter and reticular formation in the lower medulla) were frequently observed when patients lost respiratory drive (Arii and Tanabe, 2000) and indeed a larger cohort study confirms that presence of brainstem lesion on imaging is one of the poor prognostic factors (Sofou *et al.*, 2014).

The first mtDNA mutation that linked to LS was the m.8993T>G mutation in *MTATP6* (Tatuch *et al.*, 1992). Intriguingly, LS appears to be more frequently caused by mutations in genes encoding for structural subunits such as *MTATP6* and *MTND1-5*, compared to mitochondrial encoded tRNA genes in the mtDNA (Lake *et al.*, 2016). LS caused by defects in nuclear gene is discussed in **Section 1.5.3**

1.5 Nuclear genes and mitochondrial disease

There are currently more than 200 nuclear genes linked to human mitochondrial disease (Koopman *et al.*, 2012; Mayr *et al.*, 2015) and this number continues to grow because there are at least 1500 mitochondrial proteins estimated by the proteomic studies (Meisinger *et al.*, 2008). The nuclear-related mitochondrial disease can be classified based upon our understanding of the protein function, secondary defects in mtDNA and downstream biochemical defects in the OXPHOS (Schapira, 2012; Chinnery and Hudson, 2013). Isolated complex deficiencies are usually secondary to the defects in the structural subunits or assembly factors; in stark contrast, combined mitochondrial respiratory chain deficiencies are associated with multiple genes and pathways, as illustrated in **Figure 13**. Mutations in nuclear genes that are responsible for mitochondrial DNA replication and maintenance may result in secondary qualitative (multiple deletions) or quantitative changes (depletion, i.e., reduced mtDNA copy number) in mtDNA and combined respiratory chain deficiencies, for example, *POLG*, *RRM2B* and *TWNK* (aka *PEO1*, *C10ORF2*). Isolated respiratory deficiency is usually caused by mutations in genes encoding for OXPHOS structural subunits such as *NDUFB4* (complex I subunit) or assembly factor, for example, *SURF1* (assembly factor for complex IV). Recessive nuclear gene mutations have a more severe phenotype with multi-organ involvement and paediatric onset compared to dominant mutations such as in *POLG* and *RRM2B* and *TWNK* genes, which display milder phenotype and later onset in life. However, a recent study reported infantile onset and severe myopathy associated with *de novo* mutations in the *SUCLA4* gene, suggesting that the frequency of de no mutations may be under-recognized in early-onset mitochondrial disorder (Thompson *et al.*, 2016).

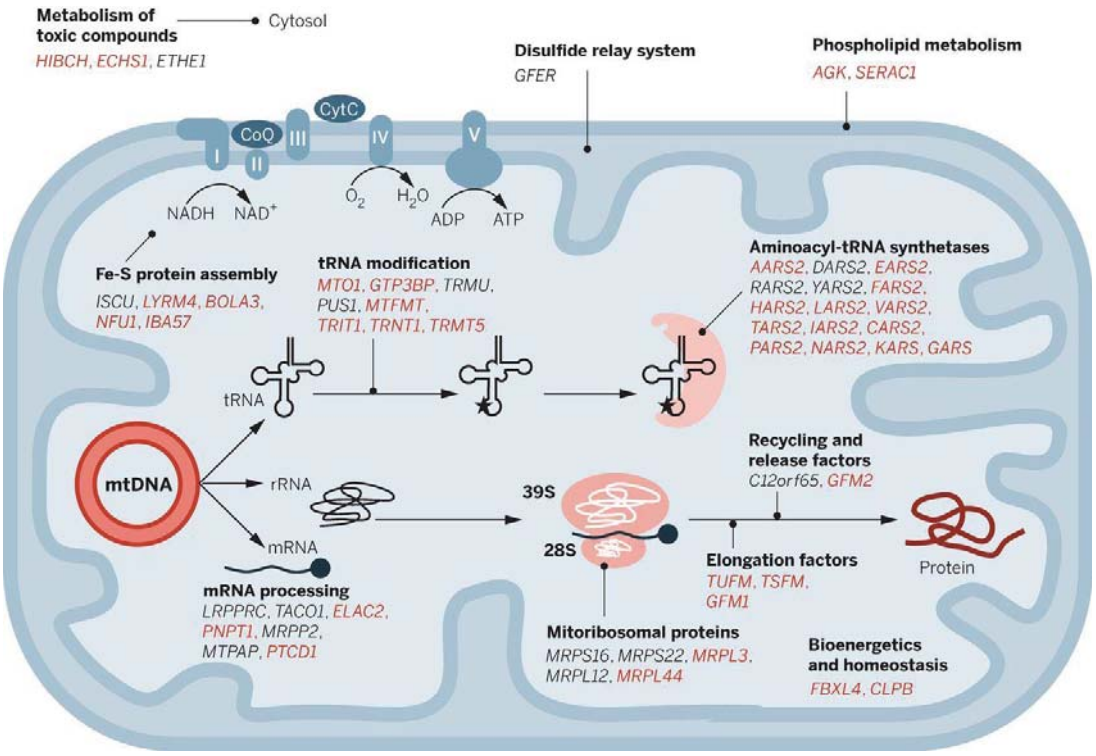

Figure 13. Nuclear gene defects causing multiple mitochondrial OXPHOS abnormalities. Schematic showing mitochondrial genes and pathways, many involved in key component steps of mitochondrial translation, in which human mitochondrial diseases associated with combined OXPHOS activity deficiencies have been identified. Those shown in red indicate disease genes identified by next-generation, largely WES studies. Reproduced with permission from (Lightowlers *et al.*, 2015)

1.5.1 Multiple deletions in mtDNA

Multiple deletions in the mtDNA are an important finding of adult onset CPEO and mitochondrial myopathy, and the dominant inheritance was first reported back in the late 1980s (Zeviani *et al.*, 1989; Zeviani *et al.*, 1990a; Zeviani *et al.*, 1990b). A decade later, single heterozygous mutations in *SLC25A4* gene (encoding for adenine nucleotide translocase type 1, ANT1) were identified as the first nuclear gene that linked to multiple mtDNA deletions. *ANT1* gene encodes for the heart and skeletal muscle isoform of adenine nucleotide translocator which is the most abundant protein in the mitochondrial matrix (Kaukonen *et al.*, 2000). Subsequently, a number of nuclear genes involved in the mtDNA replication and maintenance have been linked to adult onset CPEO with multiple deletions in the muscle biopsy (**Table 1**) (Sommerville *et al.*, 2014). Interestingly, a single heterozygous mutation in *MFN2* was identified in a large family with childhood onset optic atrophy, adult onset axonal neuropathy and myopathy, multiple deletions in muscle biopsy but without CPEO (Rouzier *et al.*, 2012).

1.5.2 Mitochondrial depletion syndrome

MtDNA depletion was first recognised as a cause of mitochondrial disease in 1991 (Moraes *et al.*, 1991). Moraes and colleagues identified marked COX-deficient fibres and reduction of the mtDNA copy number in muscle (range 3-17% of control) of two infants who presented with fatal encephalomyopathy and in the liver (range 12% of control) of an infant that died from liver failure. Tissue-specific expression of mtDNA depletion was noted.

Mitochondrial depletion syndrome (MDS) has since emerged as an important group of mitochondrial disorder and is clinically and genetically heterogeneous. Broadly speaking, there are four clinical phenotypes associated with mtDNA depletion, which includes hepatocerebral syndrome, encephalomyopathy, pure myopathy and neurogastrointestinal involvement (Nogueira *et al.*, 2014). The underlying molecular mechanisms are related to either a defect in the mtDNA replication (*POLG* and *TWNK*) or mitochondrial deoxynucleotide (dNTP) pool regulation (*TK2*, *DGUOK*, *RRM2B* and *TYMP*) (Suomalainen and Isohanni, 2010). Succinyl-CoA ligase is an enzyme involved in the Kreb cycle and mutations in the alpha subunit (*SUCLG1*) and beta subunit (*SUCLA2*) cause mtDNA depletion, although the precise mechanism is unclear. Similarly, the function of *MPV17* is not completely characterised. More recently, *POLG2* (Varma *et al.*, 2016)and *TFAM (Stiles et al., 2016)* mutations have been associated with infantile-onset liver failure and added to the expanding list of gene in MDS. The clinical features of these genes associated with MDS are summarised in **Table 1**.

Recessive mutations in the *TWNK* cause infantile onset spinocerebellar ataxia (Nikali *et al.*, 2005) and hepatocerebral syndrome (Hakonen et al. 2007). The refractory nature of epilepsia partialis continua, hypotonia and liver dysfunction in those cases described to have infantile onset spinocerebellar ataxia, in fact, are reminiscent of Alpers syndrome caused by *POLG* mutations (Hakonen *et al.*, 2007). A systematic review of *POLG* mutations associated with epilepsy is discussed in **Section 3.2**.

For the CNS involvement, common features of MDS are hypotonia, dystonia, seizure and encephalopathy. However, Leigh or Leigh-like disease appears to be very uncommon in MDS (Suomalainen and Isohanni, 2010), except in *SUCLA2 (Carrozzo et al., 2007; Ostergaard et al., 2007)* and *SUCLG1*. Given we know about the primary function of Succinyl-CoA synthase in Krebs cycle, the precise mechanism of SUCLA2 causing mtDNA depletion is not known. Cardiac manifestation is rare in most of the maintenance genes except in a case series that showed 14% of patients with mutations in *SUCLG1* developed hypertrophic cardiomyopathy (Carrozzo *et al.*, 2016).

Gene	Protein	MtDNA depletion	Clinical presentation/ tissue specificity				Other features related to MDS	MtDNA multiple deletions	CPEO
			CNS	Liver	Muscle	GI			
DGUOK	Deoxyguanosine kinase	+	Dystonia, nystagmus	+	+		Hypoglycaemia	Not reported	N/A
MPV17	MPV17	+	Demyelination in CNS & PNS	+			Navajo neurohepatopathy, neuropathy, hypoglycaemia	Not reported	N/A
TWNK	Mitochondrial Twinkle helicase	+	Alpers-like; IOSCA	+			Valproate toxicity, athetoid movement	Yes	+ (*ad*)
POLG	Catalytic subunit of polymerase gamma	+	Alpers syndrome	+		+	Valproate toxicity; 5% with MNGIE-like feature	Yes	+ (*ad*, *ar*) and other clinical phenotypes*
POLG2	Accessory subunits of polymerase gamma	+		+				Yes	+ (*ad*)
RRM2B	p53-R2	+	Hypotonia		+	Rare	Neuropathy, renal tubulopathy, MNGIE-like	Yes	+ (*ad*)
SUCLA2	Beta subunit of succinyl-CoA ligase	+	LS, dystonia		+		Deafness; methylmalonic aciduria; prevalent in Faroes Island	Not reported	N/A
SUCLG1	Alpha subunit of succinyl-CoA ligase	+	LS, dystonia				Deafness; methylmalonic aciduria; cardiomyopathy (14%)	Not reported	N/A
TK2	Thymidine kinase 2	+	Seizure (uncommon)	Uncommon	Rapidly progressive myopathy		Raised CK level, SMA-like phenotype, type II respiratory failure	Yes	+
TFAM	Mitochondrial transcription factor A	+		+			Hypoglycaemia, IUGR	Not reported	N/A
TYMP	Thymidine phosphorylase	+	Encephalopathy			+	MNGIE syndrome, demyelinating neuropathy, high thymidine in blood, low TP enzyme activity	Yes	+ (ar) and MNGIE

DNA2	DNA replication helicase 2	Not reported	N/A	N/A	N/A	N/A	Yes	+ (*ad*); Seckel syndrome (*ar*)
MGME1	Mitochondrial genome maintenance exonuclease 1	+	Mental retardation, spinal deformities		+	Respiratory failure (NIV), low BMI, renal colics	Yes	+ (*ar*)
RNASEH1	Ribonuclease H1	Not reported	N/A	N/A	N/A	N/A	Yes	+ (*ad*)
SPG7	Paraplegin	Not reported	N/A	N/A	N/A	N/A	Yes	+ (*ad*, *ar*) with spastic ataxia
AFG3L2	Subunit of m-AAA proteases	Not reported	N/A	N/A	N/A	N/A	Yes	+ (*ad*), SPG28
FBXL4	F-box/LRR-repeat protein 4	+	Hypotonia, seizure			+ Dysmorphic features, RTA,	Not reported	N/A

Table 1. Mutations in the mtDNA maintenance nuclear genes associated with mitochondrial depletion syndrome and/or multiple deletions. BG= basal ganglia, CK= creatinine kinase, CNS= central nervous system, CPEO= chronic progressive external ophthalmoplegia, GI = gastrointestinal system, IUGR= intrauterine growth restriction, IOSCA= infantile onset spinocerebellar ataxia, LS= Leigh syndrome, MDS= mitochondrial depletion syndrome, MNGIE= mitochondrial neurogastrointestinal encephalopathy, N/A= not applicable, PNS= peripheral nervous system, RTA= renal tubular acidosis, SMA= spinomuscular atrophy, SPG28= spinocerebellar ataxia type 28, (*ad*) = autosomal dominant, (*ar*)= autosomal recessive, *= Other clinical phenotypes associated with mutations in *POLG* include myoclonic epilepsy myopathy sensory ataxia (MEMSA)/ spinocerebellar ataxia with epilepsy (SCAE) and ataxia neuropathy syndrome/ sensory ataxia neuropathy dysarthria and ophthalmoplegia (SANDO)/ mitochondrial recessive ataxia syndrome (MIRAS)

1.5.3 Leigh syndrome

Pyruvate dehydrogenase deficiency secondary to mutations in the *PDHA1* gene on the X chromosome was the first nuclear gene mutation associated with LS (Matthews *et al.*, 1993). Overall, the complex I deficiency was thought to be the cause of Leigh syndrome in at least one-third of patients (Rahman *et al.*, 1996). This is unsurprising given that complex I is the largest enzymatic complex of OXPHOS, with 45 subunits and increasing number of assembly factors implicated in the biosynthesis of the complex (Zhu *et al.*, 2016). Isolated complex IV deficiency is the next common biochemical defect associated with LS (Gerards *et al.*, 2016). Complex II and III deficiencies infrequently result in the development of LS. Currently, the most frequently reported genetic causes (with >50 cases reported in the literature) are *SURF1* (Wedatilake *et al.*, 2013), *PDHA1*-related pyruvate dehydrogenase deficiency (Patel *et al.*, 2012), *LRPPRC* (which frequently regarded as French-Canadian Leigh disease) (Debray *et al.*, 2011), followed by *NDUFS4, NDUFS1, NDUFV1* (>20 cases reported in the literature) (Lake *et al.*, 2016; Ortigoza-Escobar *et al.*, 2016), *SUCLA2* (estimated homozygote frequency of 1 in 2500 in the population of Faroes Island) (Carrozzo *et al.*, 2007) and *MTFMT* (~20 cases*) (Tucker et al., 2011; Neeve et al., 2013; Haack et al., 2014)*. To date, over 75 genes have been shown to cause LS (Lake *et al.*, 2016). However, there remains a significant proportion of patients (>50%) that are genetically undiagnosed (Wortmann *et al.*, 2015).

1.6 Making a diagnosis of mitochondrial disease

1.6.1 Roles of neuroimaging

Imaging of brain and spinal cord is a useful diagnostic tool when investigating suspected cases of mitochondrial disease. Symmetrical calcification in basal ganglia is a common abnormality identified on CT head, but it is a non-specific finding and can be seen in other conditions such as hypoparathyroidism (Sachs *et al.*, 1982) and Fahr's disease (Wang *et al.*, 2012). MRI head is a preferred radiological modality and often helps to characterise CNS involvement and evolution of the underlying pathological process in mitochondrial disease (Saneto *et al.*, 2008) (**Figure 14**). For example, symmetrical, striatal T2/FLAIR hyperintensities are radiological hallmarks of Leigh syndrome and additional signal abnormalities in the brainstem may be identified in these patients when presenting with acute crisis and cranial nerve palsies. Acute stroke-like lesions associated with MELAS typically show restricted diffusion with mixed ADC map changes (i.e., mixed cytotoxic and vasogenic oedema) in parietal, temporal and occipital lobes; these lesions may resolve over time. Extensive white matter changes have been recognised in patients with KSS, and these changes

may be associated with cerebral folate deficiency (Allen *et al.*, 1983; Serrano *et al.*, 2010). Delayed myelination may be present in paediatric patients that manifest with developmental delay and hypotonia. Progressive radiological features of neurodegeneration irrespective of genotype are often evident on serial brain imaging (Valanne *et al.*, 1998; Barragán-Campos *et al.*, 2005; Scaglia *et al.*, 2005; Tschampa *et al.*, 2013; Bindu *et al.*, 2015).

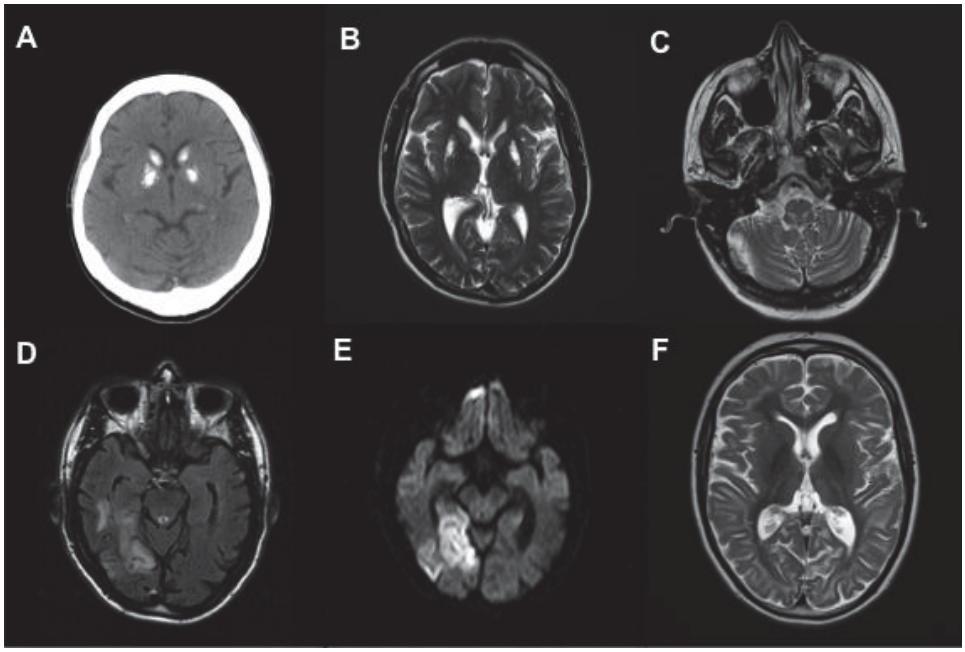

Figure 14. Radiological findings associated with mitochondrial disease. (A) CT head shows bilateral calcification in basal ganglia. **(B)** Axial T2-weighted MRI head shows symmetrical signal abnormalities in bilateral globus pallidi, typically seen in Leigh syndrome. **(C)** Axial T2-weighted MRI shows hyperintensities in the medulla in a young adult who presented with acute Leigh crisis. **(D**) FLAIR sequence of MRI head shows acute stroke-like lesions in the right temporal and occipital lobes of a patient who harbours the m.3243A>G mutation, corresponding with changes of restricted diffusion seen on DWI sequence **(E)**. **(F)** Axial T2 weighted MRI shows diffuse white matter signal abnormalities identified in an adolescent with single, large-scale mtDNA deletion and Kearns-Sayre syndrome.

Interestingly, characteristics radiological changes have been observed in mitochondrial disease caused by mutations in several mitochondrial tRNA synthetases, which may help direct genetic testing. Leukoencephalopathy with brainstem and spinal cord involvement and high lactate (LBSL) is caused by mutations in *DARS2* (Steenweg *et al.*, 2011) whilst leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) has been observed in *EARS2* mutations (Steenweg *et al.*, 2012). On the other hand, pontocerebellar hypoplasia type 6 has been linked to *RARS2* mutations (Edvardson *et al.*, 2007). However, clinical phenotypes associated with these genetic mutations are expanding,

and cases with additional imaging findings (Biancheri *et al.*, 2015) or lacking typical imaging abnormalities (Nishri *et al.*, 2016) previously described are increasingly being reported.

1.6.2 Current diagnostic approach

As discussed in the previous sections, making a diagnosis of mitochondrial disease remains formidable due to the genotypic and phenotypic heterogeneity. It requires a collaborative effort from clinicians and scientists from different disciplines namely histopathology, biochemistry, molecular genetics and increasingly bioinformatics. Comprehensive diagnostic criteria and algorithms of investigating mitochondrial disease have been published by the field experts to provide a framework for clinicians (Bernier *et al.*, 2002; Taylor *et al.*, 2004; Taylor and Turnbull, 2005; McFarland *et al.*, 2010; Parikh *et al.*, 2015; Gorman *et al.*, 2016) (**Figure 15**). Classic syndromes such as MERRF, MELAS and LHON can be diagnosed by testing common mtDNA point mutations in blood or urine. However, it is important that clinicians are cognizant of the fact that the m.3243A>G heteroplasmy level declines in blood with age, probably due to a negative selection against the mutant mtDNA (Rahman *et al.*, 2001; Frederiksen *et al.*, 2006) and such a diagnostic caveat is clearly outlined by a study that showed the m.3243A>G mutation was not detectable in blood, but present in the urine samples of 13% of their subjects (Shanske *et al.*, 2002).

Many mitochondrial diseases do not have pathognomonic features that point towards a particular genetic diagnosis and biopsy of the affected tissue, most commonly skeletal muscle is advocated as a gold standard investigation. Routine light microscopy study of the muscle biopsy may identify RRFs, best appreciated with modified Trichomori stain, as a non-specific finding frequently associated with mitochondrial disease; though RRF is often absent in paediatric cases (Koenig, 2008; Gerards *et al.*, 2016). COX-deficient fibres can be better demonstrated by using sequential COX/SDH histochemistry (**Figure 16**). More recently, a novel quadruple immunofluorescent technique with high reproducibility and sensitivity has been developed in Newcastle. This sensitive assay allows an objective measurement of key structural subunits of complex I and IV, and has a potential to be translated into the diagnostic setting (Rocha *et al.*, 2015). Measurement of respiratory chain complex activities and ubiquinone (Coenzyme Q10) quantifies the extent of complex deficiencies (isolated or combined deficiency, ubiquinone deficiency) and helps to target candidate genes employing Sanger sequencing technique. Other methods of molecular genetics include real-time PCR (quantitative measurement of mtDNA copy number), long-range PCR (to detect mtDNA rearrangement- single, large-scale deletion or multiple deletions), pyrosequencing

(quantifying heteroplasmy level) and sequencing of whole mtDNA (to detect rare or novel point mutations in mtDNA).

Currently, the diagnostic process is laborious and time-consuming after primary mtDNA mutations have been excluded. This is especially true in cases of combined respiratory chain deficiencies as highlighted in that the diagnostic yield of individual nuclear gene sequencing can be less than 5% (Kemp *et al.*, 2011).

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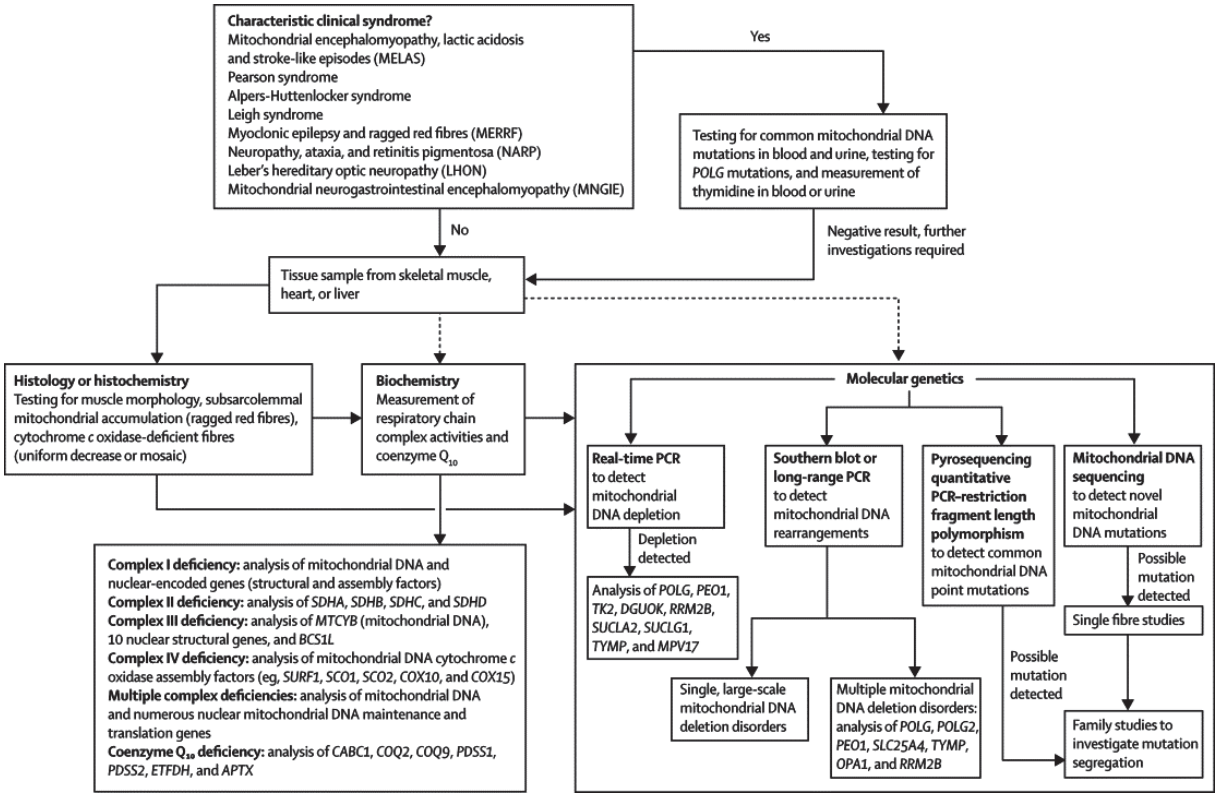

Figure 15. Current pathway of investigating mitochondrial disease in the UK.
Reproduced with permission from (McFarland *et al.*, 2010)

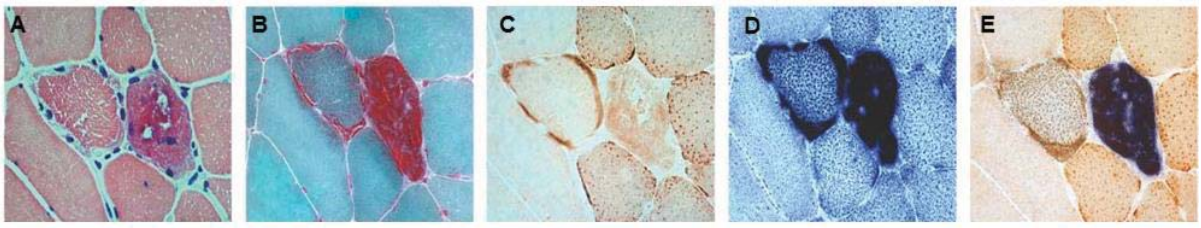

Figure 16. Common histopathological abnormalities in muscle biopsies observed in mitochondrial disease. The sections highlight two fibres stained or reacted for the following: (A) haematoxylin and eosin (H&E) staining to show general muscle morphology, in this case identifying abnormal, granular fibres showing basophilic rims; (B) modified Gomori trichrome stain highlighting these as classical ragged-red muscle fibres; (C) COX histochemistry showing that these fibres are COX-deficient to differing degrees; (D) the SDH reaction which reveals the subsarcolemmal accumulation of mitochondrial activity is exclusively found in mitochondria; (E) sequential COX/SDH histochemistry highlighting COX-deficient fibres, which retain SDH activity. Adapted from (Greaves et al., 2012)

1.6.2.1 Predictive genetic testing for the mtDNA mutations

Pre-test genetic counselling for currently asymptomatic maternal family members regarding the primary mitochondrial DNA disease is challenging due to the marked variability regarding the threshold effect of individual mutations, disease onset, clinical manifestation, disease progression and prognosis frequently observed within families. The implications of predictive genetic testing are outlined as follows:

(1) Surveillance for complications associated with the underlying mtDNA mutations

Cardiac involvement is common in many mtDNA mutations (Bates *et al.*, 2012) and is associated with increased morbidity and mortality (Malfatti *et al.*, 2013; Mancuso *et al.*, 2013b; Catteruccia *et al.*, 2015; Wahbi *et al.*, 2015). Left ventricular hypertrophy is the most common type of cardiac abnormality and, though often subclinical, may progress to decompensated heart failure in some individuals. Moreover, sudden cardiac death in the m.3243A>G mutation has also been reported (Majamaa-Voltti *et al.*, 2008; Kaufmann *et al.*, 2011). Predictive genetic testing should, therefore, be offered to at-risk maternal family members so that they receive regular cardiac surveillance and treatment. In addition, anticipatory guidance for pre-symptomatic individuals and health care personnel can be provided on recognising severe neurological complications such as stroke-like episodes, seizures and brainstem dysfunction that are frequently associated with certain mtDNA defects such as mutations in *MTTL1* and *MTND5*. Recognising other systematic involvement such as diabetes mellitus, renal

involvement and sensorineural deafness can be associated with mtDNA mutations could avoid unnecessary diagnostic work up. On the other hand, a negative genetic result would provide reassurance and avoid unnecessary medical follow up.

(2) Modification of environmental risk factors

Smoking has been established to significantly increase the risk of developing optic neuropathy among individuals affected by LHON (Kirkman *et al.*, 2009; Carelli *et al.*, 2016) and therefore advice on lifestyle modification is imperative for those asymptomatic family members.

(3) Family planning and reproductive options

The estimated prevalence of primary mtDNA disease derived from the most recent cohort based study is approximately 1 in 5000 (Gorman *et al.*, 2015). A population-based study showed that the prevalence of m.3243A>G mutation was over 1 in 400 (236 per 100,000) (Manwaring *et al.*, 2007), suggesting there is an under-diagnosis of a significant number of individuals especially those who are oligosymptomatic (Gorman *et al.*, 2016). It is also likely that some people may remain asymptomatic throughout their life due to low mutant heteroplasmy levels. However, female carriers, irrespective of their clinical state, are still at risk of transmitting the mtDNA mutation to their offspring who may be severely affected due to the unpredictable nature of mtDNA genetic bottleneck. Currently, reproductive options such as PGD and mitochondrial replacement therapy (aka mitochondrial donation) shortly (Herbert and Turnbull, 2017) offer the opportunity for reducing the risk of transmitting the mtDNA mutations to the offspring.

Similar to the Mendelian genetic disorders, presymptomatic and predictive genetic testing for the mtDNA mutations in the paediatric population is contentious because of its ethical implications such as removal of the child's autonomy on deciding for genetic testing especially mutations are associated with adult onset disease, and risk of stigmatisation (Valente *et al.*, 2008). Therefore, such testing should be limited to those mutations with a high risk of childhood onset complications after discussing with parents and highlighting the caveat that mutant heteroplasmy level cannot solely explain the variability of clinical phenotypes and potential disease burden in the future.

1.6.3 Novel biomarkers

1.6.3.1 Human fibroblast growth factor 21

Human fibroblast growth factor 21 (FGF21) is one of the 22 proteins that belongs to the FGF superfamily. It is expressed in the metabolically active organs which include liver, pancreas, white adipose tissue, thymus, and skeletal muscles (Long and Kharitonenkov, 2011). FGF21 plays important roles in glucose and fatty acid metabolism during the fasting and fed states. Peroxisome proliferator-activated receptor alpha (PPAR-α) induces the expression of FGF21 in hepatocytes as well as circulating level in response to fasting, resulting in hepatic fatty acid oxidation, ketogenesis and gluconeogenesis (Long and Kharitonenkov, 2011; Woo *et al.*, 2013). FGF21 can increase glucose uptake in adipocytes, increase insulin secretion and inhibit glucagon release in the pancreas, inhibit lipolysis and hence decrease free fatty acids (Woo *et al.*, 2013).The elevation of FGF21 level has been associated with several common diseases such as obesity, diabetes mellitus, ischaemic heart disease, non-alcoholic fatty liver disease, polycystic ovarian syndrome, chronic renal failure and diabetic nephropathy, and Cushing'syndrome (Woo *et al.*, 2013; Fisher and Maratos-Flier, 2016) and post-exercise in healthy subjects (Kim *et al.*, 2013).

The observation of increased FGF21 level in the mitochondrial myopathy was first reported in mitochondrial helicase TWINKLE mouse model with dominant mutation (Tyynismaa *et al.*, 2010). Suomalainen and co-workers proposed that FGF21 is a potentially helpful diagnostic biomarker in mitochondrial disease after investigating a cohort of 67 patients with mitochondrial disorders caused by different genetic mutations (22 adults and 12 children, 94% with genetic diagnosis), 34 disease controls (mixture of genetic muscle diseases and acquired neuromuscular disorders such as inclusion body myositis and motor neuron disease) and 74 healthy controls. They found that the sensitivity and specificity were close to 92% in adult and child muscle-manifesting mitochondrial disorders (Suomalainen *et al.*, 2011). Similar diagnostic sensitivity (91% with 95% CI 86-97%) in the adult patients (n=54, mean age 51, range 20-70) has also been replicated by the Australian researchers and them also identified that the level of elevation was higher among those who had proximal myopathy, compared to those who without muscle weakness in the disease group and control (Davis *et al.*, 2013). More recently, elevated FGF21 has been identified in myotonic dystrophy type 1, similar to patients with mitochondrial disease. Insulin resistance appears to be a plausible mechanism to explain such elevation (Lovadi *et al.*, 2016).

The diagnostic potential of FGF21 in the paediatric setting remains unknown. In general, children with mitochondrial disease are more frequently manifest CNS disease and less so

with myopathy when compared to the adult patients. The use of FGF21 in paediatric patients might be limited because the sensitivity of FGF21 in diagnosing adult patients with mitochondrial disease primarily manifesting with CNS dysfunction was merely 42% (Suomalainen *et al.*, 2011).

The FGF21 level appeared to correlate with disease burden (measured with the NMDAS) in patients harbouring the m.3243A>G mutation. However, its role in monitoring disease progression was thought to be limited (Koene *et al.*, 2014).

1.6.3.2 Growth differentiation factor 15

Growth differentiation factor 15 (GDF-15) belongs to the transforming growth factor beta superfamily, and it is ubiquitously expressed. The biological function of GDF-15 is not well characterized but it has been studied as biomarker or predictor of adverse outcome in several conditions, including chronic liver disease (Lee *et al.*, 2016), acute kidney injury (Heringlake *et al.*, 2016), white matter hyperintensities in cognitive impairment (Chai *et al.*, 2016) and chronic heart failure (Lok *et al.*, 2013). GDF-15 was first proposed as a potential biomarker in mitochondrial disease following a transcriptomic study of skeletal muscle from patients with *TK2* mutations (Kalko *et al.*, 2014). Overall, the serum GDF-15 concentration was elevated 2 to 11 folds in both paediatric and adult patients with the mitochondrial disease compared to the healthy and disease controls, as demonstrated by different studies (Yatsuga *et al.*, 2015; Davis *et al.*, 2016; Montero *et al.*, 2016). The sensitivity of GDF-15 varies between studies: the lowest being 68% (Montero *et al.*, 2016), the intermediate is 79% (Davis *et al.*, 2016) whilst the highest has been reported to be 98% (Yatsuga *et al.*, 2015). The much greater sensitivity reported by the Japanese group (Yatsuga *et al.*, 2015) is probably related to all the patients have confirmed genetic diagnosis with classic syndromes compared to the other two studies. The specificities, on the other hand, are very similar in these studies (>90%). The area under the curve (AUC) value using ROC curve analysis for GDF-15 has also been shown to be consistently higher than FGF-21 as well as other conventional biomarkers (e.g., lactate, CK level), suggesting its superiority as a diagnostic biomarker. Furthermore, the elevation of the GDF-15 level is not restricted by myopathic phenotype (Yatsuga *et al.*, 2015; Montero *et al.*, 2016), compared to FGF-21 (Suomalainen *et al.*, 2011).

However, there might be a potential limitation of GDF-15 when evaluating suspected cases with single organ involvement such as cardiomyopathy at the outset of presentation, because patients with isolated heart failure (disease control) had a comparable elevated GDF-15 level

compared to the mitochondrial disease group (Yatsuga *et al.*, 2015). Furthermore, the negative predictive value was reported to be only 61% in a study (Montero *et al.*, 2016), indicating that the exclusion of mitochondrial disease could not be based on this biomarker alone in day-to-day clinical practice.

1.6.4 Next generation sequencing

Next generation sequencing (NGS) is a new and high-throughput technique that allows sequencing of multiple candidate genes is simultaneously leading to a more rapid diagnosis and increase the diagnostic yield (Tucker *et al.*, 2010). The diagnostic yield of whole exome sequencing (WES) in mitochondrial disease has been reported to range from 17% and up to 55% (**Table 2**). New genes linked to mitochondrial respiratory chain defects are constantly identified via WES, with clinical phenotypes overlapping with other known genes. On the other hand, the spectrum of clinical phenotypes of known genes is also expanded with such unbiased diagnostic approach. One of the biggest challenges with the NGS is to provide proof of pathogenicity for novel variants in the known genes, and perhaps more so for the new genes that have not been previously linked to any disease. Segregation study of affected and unaffected family members would help to prioritise the analysis of variants of unknown significance (VUS). Detailed understanding of clinical phenotypes and identification of other affected individuals from different pedigrees are the pivotal step of validating the diagnosis (Calvo *et al.*, 2012). Multi-centre collaboration is often required to identify these patients because many of these VUS are rare. In the circumstance of private mutations which segregation study cannot be performed, further in vitro studies such as Western blotting, mutant cell characterization, rescue experiment and animal modelling are required (Legati *et al.*, 2016).

Biopsies of affected tissues and biochemical measurement of these samples are invaluable when interpreting the WES findings, and they would continue to have a major role in the diagnostic work up in mitochondrial disease for the foreseeable future. However, it is also increasingly recognized that a number of genetics or ‘acquired’ neuromuscular diseases could have secondary histopathological, biochemical, ultrastructural changes similar to the primary mitochondrial disorders (Joshi *et al.*, 2014; Pyle *et al.*, 2015; Rygiel *et al.*, 2016; Vincent *et al.*, 2016), again highlighting the complexity of investigating patients with evidence of ‘mitochondrial dysfunction’ in some cases.

Study	N	Inclusion criteria	Overall diagnosis	Candidate genes	Non-mito genetics
Calvo et al. 2012	42	Infants with clinical features and biochemical defects	23 (55%); 10 were known variants	13 (31%; pathogenicity proven in two genes)	N/A
Haack et al. 2012$^{\Delta}$	10	CI deficiency, onset age<3 yo	7 (70%); 2 patients had *MTFMT* mutations	N/A	N/A
Lieber et al. 2013	102	Clinical and biochemical defined with variable onset age	New diagnosis 8 (out of 84) and 17 (out of 18) previously diagnosed cases*	26 (25%; pathogenicity proven in the *ATP5A1* gene)	*3 (3%)* *DPYD* (CI, CIII deficiencies), *KARS, WFS1* (multiple deletions)
Ohtake et al. 2013	104	Neonatal and infantile-onset mitochondrial respiratory chain deficiencies	18 (17%)	27 (26%)	N/A
Taylor et al. 2014$^{\Delta}$	53	Combined respiratory chain deficiencies; age of onset: at birth – 17yo	28 (53%)	4 (8%)	N/A
Wortman et al. 2015$^{\Delta}$	109	Clinical scoring and biochemical defects (muscle biopsy, n=94; fibroblast, n=92)	42 (39%): HS - 24/42 IS - 10/44 LS – 8/23		Half of the diagnosis were non-mito genes (21;19%)
Khoda et al. 2016	142	Respiratory chain deficiencies, onset age <15yo	49 (39%); 3 novel genes with proven pathogenicity (*MRPS23, QRSL1, PNPLA4*)		7 (5%); *MECP2* (CI deficiency), *TNNI3* (CI deficiency) and pathogenic chromosomal deletions
Legati et al. 2016$^{\Delta}$	125	Clinical and biochemical, onset age: 78<1yo, the rest with mean age 18.6yo	15 (12%) and 6 (out of 10) had undergone WES	27 (22%)	4 *(CYP2U1, PREPL, E4F1, RANBP2)*

Table 2 Summary of reported clinical studies applying gene panels and WES (n=8). $^{\Delta}$= primary mtDNA mutations and single, large-scale deletions were excluded prior to the WES. *= the m.3243A>G mutation was not detected by the WES in a known case with 3% m.3243A>G heteroplasmy in blood. CI= complex I, CIII= complex III, N/A= not applicable, HS= high suspicion of mitochondrial disease, IS= intermediate suspicion of mitochondrial disease, LS= low suspicion of mitochondrial disease

1.7 Current management of mitochondrial disease

Patients with mitochondrial disease often develop multi-system involvement. Active surveillance and regular follow up are crucial to identifying specific deficits which may not be apparent at the outset of initial clinical manifestation. Although there remains no cure for mitochondrial disease, there is an organ-specific supportive measurement that may offer alleviation of symptoms, reduction of disease burden and potentially life-saving treatment. Specific treatments are available for several forms of mitochondrial disorders (Parikh *et al.*, 2009; Parikh *et al.*, 2015; Gorman *et al.*, 2016).

1.7.1 Supportive treatment

1.7.1.1 Acute neurological crises

The most severe, acute neurological complications in mitochondrial disease are decompensation of brainstem function frequently associated with LS and status epilepticus associated with or without stroke-like episodes. These events often are triggered by intercurrent illnesses such as infection and dehydration. Prompt recognition of acute brainstem dysfunction such as new onset cranial nerve palsies, altered breathing pattern or hypersomnolence is crucial to circumvent delay in treatment in the high dependency or intensive care unit. For stroke-like episodes, the mainstay of treatment remains aggressive seizure management whilst optimising nutritional support and treating the potential underlying triggers. L-arginine, a precursor of nitric oxide, has been advocated to treat metabolic stroke based on positive findings from anecdotal case reports (Koga *et al.*, 2002; Kubota *et al.*, 2004; Shigemi *et al.*, 2011) and a single open-labelled trial (Koga *et al.*, 2005; Koenig *et al.*, 2016). However, there might be a research bias because of the nature of open-labelled trial design. Further clinical trials are currently underway, and more conclusive results are awaited. An open-labelled trial showed that citrulline could mitigate baseline lactic acidosis in patients with MELAS (El-Hattab *et al.*, 2014). However, its effect on acute stroke-like episodes has not yet been demonstrated.

1.7.1.2 Chronic neurological problems

Input from a specialist physiotherapist and an occupational therapist is vital to managing chronic neurological complications such as tone abnormalities, myopathy and ataxia in patients affected by mitochondrial disease (McFarland *et al.*, 2010). In addition,

pharmacological treatments such as benzodiazepine, baclofen and Botulinum toxin injections may be beneficial for patients with dystonia and spasticity. Parkinsonism is common in certain genotypes such as mutations in *POLG* (Luoma *et al.*, 2004; Orsucci *et al.*, 2011) and dopamine agonists/replacement therapy may be considered as symptomatic therapy.

1.7.1.3 Hearing loss

Progressing hearing deficit with preferential involvement of high-frequency hearing loss is a common manifestation seen in many patients with mitochondrial disease. The underlying pathology has been localised to cochlear dysfunction (Chinnery *et al.*, 2000; Kullar *et al.*, 2016). Hearing aids could improve mild to moderate deafness whilst placement of a cochlear implant is an option for those with severe hearing loss (Rosenthal *et al.*, 1999; Santarelli *et al.*, 2015; Yamamoto *et al.*, 2015).

1.7.1.4 Ocular disease

Ptosis is a common finding in patients with CPEO. Corrective measures such as frontalis sling and ptosis props may improve both vision and cosmetic appearances significantly. However, caution should be exercised in those with facial weakness because of complications associated with over-correction (McFarland *et al.*, 2010).

But perhaps most promising, has been the licensing of the use of idebenone in patients with LHON by the European Medicine Agency (EMA) (further discussion in **Section 1.8.2.2**).

1.7.1.5 Malnutrition and gastrointestinal dysfunction

Malnutrition is commonly observed in patients with moderate to severe mitochondrial disease burden. This may be secondary to dysphagia due to bulbar/pseudo-bulbar dysfunction, myopathy or loss of appetite, or early satiety secondary to the underlying gut dysmotility. Swallowing and nutritional assessment should be considered early so that appropriate dietary advice may be given to minimise the risk of aspiration and optimisation of nutrition. For those with an unsafe swallow and significant weight loss, gastrostomy feeding may be a solution. Total parenteral nutrition (TPN) may be temporarily used in patients who have intestinal pseudo-obstruction (IPO), and occasionally prolonged use might be necessary due to a

protracted period of severe gut dysmotility. Monitoring for liver dysfunction is important, but particularly in the chronic use of TPN (Wang *et al.*, 2015).

1.7.1.6 Cardiac and respiratory management and surveillance

Cardiac involvement is evident in 30% of adult patients with mitochondrial disease (Wahbi *et al.*, 2015). Genotype-specific cardiac complications have been observed, for example, hypertrophic cardiomyopathy (HCM) and Wolf-Parkinson-White syndrome (WPW) in m.3243A>G and m.8344A>G-related mitochondrial disease; bradyarrhythmia including heart block in single, large-scale deletion (Bates *et al.*, 2012); Sengers syndrome, characterized by cardiomyopathy and congenital cataracts, caused by mutations in *AGK* (Haghighi et al., 2014). Early recognition provides a window opportunity to initiate pharmacological agents that can modulate cardiac remodelling and preserve cardiac function, ablation therapy for accessory pathways in WPW syndrome and insertion of a pacemaker could be life-saving in those who develop complete heart block (Bates *et al.*, 2012).

Advanced respiratory insufficiency necessitating non-invasive ventilation secondary to neuromuscular weakness is considered relatively uncommon in mitochondrial disease (Pfeffer *et al.*, 2015), except in patients with KSS due to single, large-scale deletion, m.8344A>G (Catteruccia *et al.*, 2015), and mutations in *TK2* (Alston et al., 2013). A more detailed study of 26 patients with CPEO (mixed genotypes) showed mild respiratory muscle weakness was evident, particularly in patients with limb-girdle weakness although forced vital capacity was within normal range (Smits *et al.*, 2011).

1.7.1.7 Solid organ transplant

Several genotypes manifest with tissue-specific involvement and may progress to end-stage organ failure. The role of solid organ transplantation is increasingly recognised as a therapeutic strategy in mitochondrial disease. Hepatic failure is a common feature associated with mitochondrial depletion syndromes such as mutations in *DGUOK* and *MPV17*; liver transplantation has been performed in these cases, with improved clinical outcome associated with milder neurological involvement (Dimmock *et al.*, 2008; Uusimaa *et al.*, 2014). Decompensated cardiomyopathy has been shown to benefit from heart transplantation in *ACAD9* (Collet *et al.*, 2016) and m.3243A>G-related mitochondrial disorders (Bhati *et al.*, 2005). Successful kidney transplantation has also been reported in patients with both

m.3243A>G (Jansen *et al.*, 1997; Lederer *et al.*, 2010) and single, large-scale mtDNA deletions (Rötig *et al.*, 1995). A recent retrospective study of 35 patients from 17 centres clearly demonstrated liver, kidney and cardiac transplants were well-tolerated by patients with mitochondrial disease (mixed genotypes) with improved survival, except in patients with mutations in *POLG* (Parikh et al., 2016). However, better survival following liver transplantation in cases of *POLG* mutations has been reported in another study (Hynynen *et al.*, 2014).

1.7.2 Vitamin and cofactor supplements

Vitamin and cofactor supplements, either in isolation or combination (cocktail), are commonly used in patients with various forms of mitochondrial disease (Parikh *et al.*, 2009). While there is no robust evidence for many of these supplements (Pfeffer *et al.*, 2012b), significant benefits have been observed in subgroups of patients. For example, riboflavin (Vitamin B2) is beneficial in patients with multiple acyl-CoA dehydrogenase (MADD) deficiency caused by mutations in *ETFDH* (Olsen *et al.*, 2007; Zhu *et al.*, 2014) and *SLC25A32* (encoding for mitochondrial flavin adenine dinucleotide transporter) (Schiff *et al.*, 2016), as well as mutations in *ACAD9* (encoding for flavin adenine dinucleotide-containing flavoprotein) (Gerards *et al.*, 2011).

1.7.3 Exercise therapy

Aerobic training (Taivassalo *et al.*, 1998) (Jeppesen *et al.*, 2006; Taivassalo *et al.*, 2006; Bates *et al.*, 2013) has been shown to be safe and efficacious at improving exercise capacity as well as the quality of life in mitochondrial myopathies. However, there is no change in mitochondrial respiratory chain function, mutant heteroplasmy level, mtDNA copy number and muscle fibre morphology (Jeppesen *et al.*, 2006; Taivassalo *et al.*, 2006). Resistance training has also been shown to be of benefit in patients with single, large-scale deletion through the activation of satellite cells and regeneration of myofibres (mitochondrial biogenesis) (Murphy *et al.*, 2008).

1.7.4 Targeted treatments for specific conditions

1.7.4.1 Mitochondrial neurogastrointestinal encephalomyopathy

MNGIE is caused by a deficiency of thymidine phosphorylase, encoded by *TYMP* gene (Nishino *et al.*, 1999). The main therapeutic aim is to restore enzymatic activity and decrease the levels of plasma thymidine and deoxyuridine. Two approaches have been attempted with clinical and biochemical improvements observed: erythrocyte-encapsulated thymidine phosphorylase (n=1) (Bax *et al.*, 2013) and allogeneic haematopoietic stem cell transplantation (AHSCT). However, there is a significant safety concern regarding AHSCT because of its associated high morbidity and mortality (Halter *et al.*, 2015). Liver transplantation (n=1) has recently been proposed to be a new, attractive alternative to AHSCT (De Giorgio *et al.*, 2016).

1.7.4.2 Primary CoQ10 deficiency

Primary CoQ10 deficiency is associated with five major phenotypes: severe, infantile multisystem disease, encephalomyopathy, nephrotic syndrome, cerebellar ataxia and isolated myopathy (Emmanuele *et al.*, 2012). Supplementation of high dose CoQ10 has ameliorated symptoms and disease progression in some patients (Pineda *et al.*, 2010; Emmanuele *et al.*, 2012).

1.7.4.3 Ethylmalonic encephalopathy

Ethylmalonic encephalopathy is characterised by early onset encephalopathy, microangiopathy, haemorrhagic diarrhoea, accumulation of sulphide (H_2S) and COX deficiency due to mutations in the *ETHE1* gene, which encodes for mitochondrial matrix sulphur dehydrogenase (Haack *et al.*, 2012 3441). Supplementation of combined N-acetyl cysteine and metronidazole appears to be an effective treatment for patients with ethylmalonic encephalopathy (Viscomi *et al.*, 2010).

1.7.5 Prevention of transmission

Genetic counselling on the risk of disease transmission is a major focus in clinical management. Mitochondrial disease caused by defects in nuclear gene follows the rules of Mendelian inheritance, and their risk assessment is relatively straightforward. In contrast, the risk prediction of primary mitochondrial DNA mutations is complicated due to the

mitochondrial genetics bottleneck (**Section 1.4.2**). Even for homoplasmic mtDNA mutations, it remains very difficult to quantify the risk of disease due to incomplete penetrance and environmental factors (Gorman *et al.*, 2016) (**Section 1.4.3.3**).

Prenatal testing for nuclear gene disorders is well established and currently includes preimplantation genetic testing (PGD), chronic villous sampling (CVS) and amniocentesis. CVS and amniocentesis are associated with a risk of miscarriage. Prenatal testing of primary mtDNA mutations was first introduced into the UK in 2007 (Nesbitt *et al.*, 2014). PGD is now also offered to women with mtDNA mutations. It involves *in vitro* fertilisation (IVF), and biopsy of developing embryos (at the one or two blastomeres stage) with high-quality embryos with the lowest mutant mtDNA heteroplasmy levels implanted, and if successful, results in pregnancy (Richardson *et al.*, 2015; Gorman *et al.*, 2016). One of the most challenging aspects of PGD in mtDNA mutations is to determine the threshold level of clinical expression for the individual mutation (Hellebrekers *et al.*, 2012; Sallevelt *et al.*, 2013). Moreover, PGD is not an effective option for patients with homoplasmic mtDNA mutations (Lightowlers *et al.*, 2015; Richardson *et al.*, 2015).

1.8 Emerging, potential therapeutic strategies

There are a number of major developments in creating animal models and finding novel therapies for different types of mitochondrial disease over the last decade (Rai *et al.*, 2015). Currently, the research of potential therapeutic strategies could be divided into two broad categories (**Figure 17**): first, targeted therapy aimed at the underlying genetic defect; second, generic treatment targeting improving mitochondrial function irrespective of the genetic mutations. The first approach could be highly efficacious and potentially leads to a cure although their application is genotype-specific and may be very costly to develop. On the other hand, although generic treatment is not curative, it may be more cost-effective and could potentially benefit more patients and have wider applications in other neurodegenerative disorders.

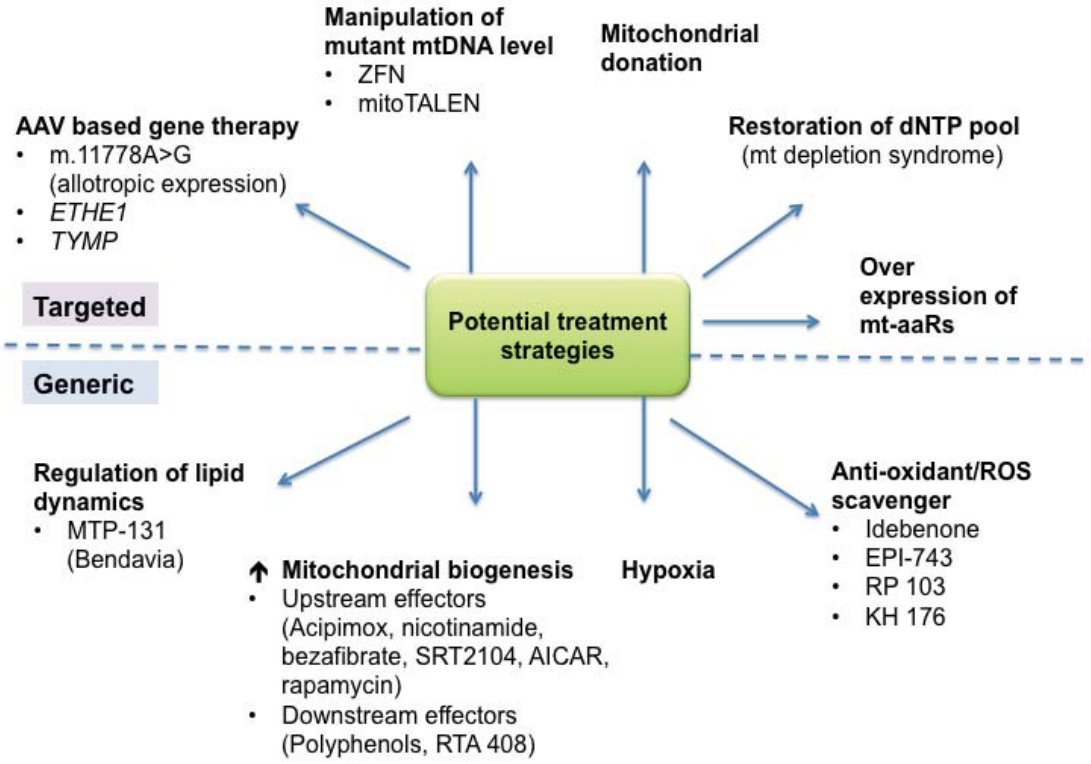

Figure 17. Emerging, potential therapeutic options for patients with mitochondrial disease. AICAR= 5-Aminoimidazole-4-carboxamide ribotide, dNTP= deoxynucleotide pool, *ETHE1*= mutations in this gene cause ethylmalonic encephalopathy, mt-aaRs= mitochondrial amino-acyl tRNA synthetases, ROS= reactive oxygen species *TYMP*= mutations in this gene cause MNGIE, TALEN= transcription activator-like effector nucleases, ZFN= zinc finger nuclease. Adapted from (Lightowlers *et al.*, 2015; Rai *et al.*, 2015)

1.8.1 Targeted treatment

1.8.1.1 Mitochondrial donation

Mitochondrial donation is emerging as a promising strategy to prevent transmission of primary mtDNA disease, especially for those women with very high heteroplasmic or homoplasmic mutations. It is an IVF technique and requires healthy donor eggs. The transfer of a patient's nuclear genome can be performed on the fertilised oocyte (pronuclear transfer) or before the egg is fertilised (spindle transfer) (**Figure 18**) (Richardson *et al.*, 2015). New regulations to allow mitochondrial donation was debated and approved in the UK in February 2015, after more than ten years of extensive preparations including public consultation and several independent scientific and ethical reviews (Kmietowicz, 2015). A recent study provides further preclinical evidence on the safety profile of pronuclear transfer and its potential for reducing the risk of manifesting with the disease. However, offspring born to this

technique may still harbour mtDNA mutations. Therefore, the prevention of mutant mtDNA carryover is not guaranteed (Hyslop *et al.*, 2016).

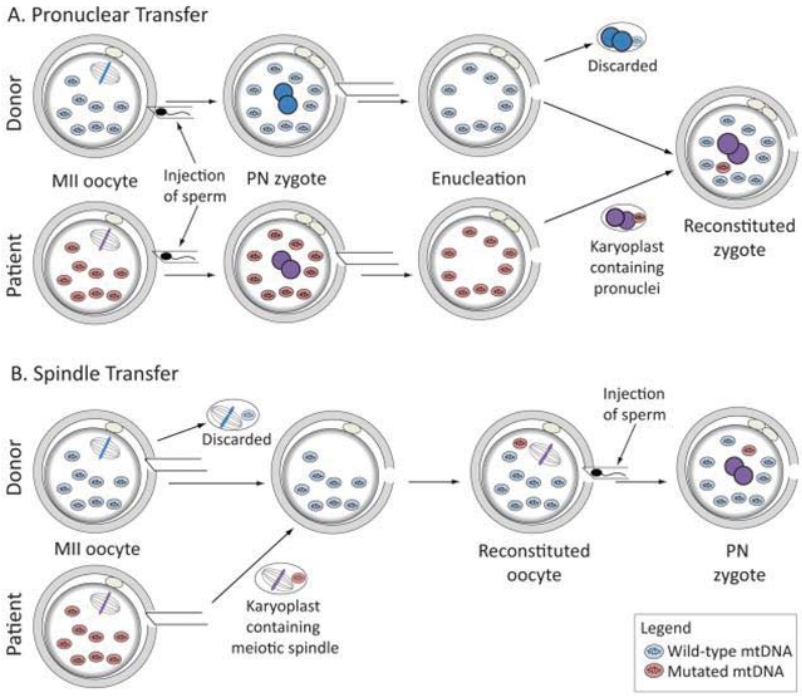

Figure 18. Mitochondrial donation. (A): Pronuclear transfer: MII-arrested oocytes obtained from the affected woman and a healthy donor are fertilized and the pronuclei are transferred in a karyoplast from the affected woman's fertilised egg to the enucleated donor egg. **(B):** Spindle transfer: oocytes obtained from an affected woman and a healthy donor are enucleated by removal of the MII spindle and its chromosomes in a karyoplast. The karyoplast from the affected woman is fused with the enucleated oocyte from the healthy donor. Reconstituted oocytes are then fertilised and undergo the second meiotic division followed by formation of the male and female pronuclei. Abbreviations: MII, second meiotic division; PN, pronuclei. Reproduced from (Richardson *et al.*, 2015).

1.8.1.2 Manipulation of mutant mtDNA heteroplasmy

Zinc fingers nucleases (ZFN) (Gammage *et al.*, 2014) and transcription activator-like effector nucleases (TALEN) (Bacman *et al.*, 2013) have been used experimentally to manipulate the ratio of mutant and wild-type mtDNA in cell lines and have shown an impressive reduction of mutant heteroplasmy level below the phenotypic expression threshold. Furthermore, use of MitoTALEN has been attempted in the mouse germ line and provided proof of concept of its potential efficacy in preventing mtDNA transmission (Reddy *et al.*, 2015). However, neither technique would be applicable to homoplasmic mtDNA mutations; nor substantial reduction in the mtDNA copy number in cell lines with subsequent recovery raises a severe concern of safety in vivo (Nightingale *et al.*, 2016).

1.8.1.3 Gene therapy

Another form of targeted approach is gene therapy based on adeno-associated virus vector (AAV). For example, delivery of AAV-*ETHE1* to the liver of a mouse model has restored enzymatic activity and improved survival (Di Meo *et al.*, 2012), which may be translated as an effective treatment for patients with ethylmalonic encephalopathy. Allotropic expression of synthetic *ND4* gene for the m.11778G>A mutation (which is the most common cause of LHON) appears to be a promising treatment from the in vitro studies (Ellouze *et al.*, 2008). Currently, a randomized, double-blind, sham-controlled clinical trial is recruiting patients with visual loss associated with the m.11778G>A mutation to evaluate the effectiveness of gene therapy (ClinicalTrials.gov identifier: NCT02652767)

1.8.1.4 Supplementation of deoxyribonucleotide

Supplementation of deoxyribonucleotide or inhibition of its catabolism has been shown to increase mtDNA copy number and mitochondrial respiratory function in cell lines with either deoxyguanosine kinase (mutations in *DGUOK*) or thymidine phosphorylase (mutations in *TYMP*) deficiency (Camara *et al.*, 2014). Moreover, supplementation of deoxycytidine and deoxythymidine monophosphates (dCMP and dTMP) in TK2 knockout mice has shown positive phenotypic and biochemical improvement (Garone *et al.*, 2014).

1.8.2 Generic treatment

1.8.2.1 Increase mitochondrial biogenesis

Mitochondrial biogenesis refers to an increase in the copy number of mitochondria in cells. It is regulated by the peroxisome proliferator-activated receptor gamma coactivator (PGC1-α), which is frequently known as the key regulator of cellular metabolism and mitochondrial biogenesis because of its interactions with multiple transcription factors (Liang and Ward, 2006). Stimulation of mitochondrial biogenesis is an attractive treatment strategy that may improve underlying mitochondrial dysfunction and therefore potentially ameliorate clinical symptoms. Several existing drugs that are licenced for other treatment purposes have been shown to modulate PGC1-α via different pathways. Bezafibrate is an agonist for the peroxisome proliferator-activated receptor (PPAR) and has been primarily used as a lipid-lowering agent. Currently, there is phase II clinical trial investigating the efficacy of bezafibrate in mitochondrial myopathy (m.3243A>G mutation) (ClinicalTrials.gov identifier: NCT02398201) in Newcastle. Resveratrol is another mitochondrial proliferator that increases

NAD+ (oxidised nicotinamide adenine dinucleotide) which subsequently activates PGC1-α (Nightingale *et al.*, 2016); in an open-labelled trial, high dose Resveratrol appeared to improve the clinical rating scale. However, no improvement was observed in cardiac function or patient-reported outcome neurological function in patients with Friedreich ataxia (Yiu *et al.*, 2015). Another agent, 5- Aminoimidazole -4-carboxamide ribonucleotide (AICAR) was initially designed as a cardiac-protective drug, and has been shown to increase mitochondrial biogenesis by activating the AMP-activated protein kinase (AMPK).

Mammalian target of rapamycin (mTOR) is a kinase that involves in mitochondrial oxidative through a YY1-PGC-1alpha transcriptional complex (Cunningham *et al.*, 2007). Rapamycin, an mTOR inhibitor, has been experimented in a mouse model of LS (*NDUFS4* knockout mouse) and demonstrated significant improvements in neurological function and survival (Johnson *et al.*, 2013). This is rather intriguing because mTOR is important for mitochondrial biogenesis and the downstream effects of inhibiting mTOR are not yet well characterized. It has been postulated that the rescue of phenotype might be related to the metabolic shift towards amino acid catabolism and away from glycolysis. However, the side effects such as immunosuppression and growth restriction may preclude its clinical use, particularly in the paediatric population.

Maintaining the ratio of NAD+ and NADH is important in oxidative phosphorylation. Supplementation of NAD+ with nicotinamide riboside (Vitamin B3) has shown increased mitochondrial biogenesis in two mouse models (Cerutti *et al.*, 2014; Khan *et al.*, 2014). The mechanism has been attributed to the activation of sirtuins SIRT1 and SIRT3 (Lightowlers *et al.*, 2015). Acipimox, another NAD+ precursor, holds potentials in the treatment of mitochondrial myopathy, as shown by a recent study were it boosts mitochondrial function in the skeletal muscle of patients with type II diabetes mellitus (van de Weijer *et al.*, 2015).

1.8.2.2 Antioxidant

Accumulation of ROS could deplete the pool of antioxidants such as glutathione in cells which further compromises mitochondrial and cellular functions (Rahman, 2015). Many past and present therapeutic studies have focused on finding anti-oxidants that could ameliorate the damaging effects of ROS. Co-enzyme Q10 (CoQ10) is the most widely used supplement in clinical practice due to its theoretical benefit of improving electron transfer in the mitochondrial respiratory chain and its anti-oxidative effect. Benefits of supplementing

CoQ10 in patients with primary ubiquinone deficiency have been observed (**Section 1.7.4.2**). However, the wider application of CoQ10 in mitochondrial myopathy has not yielded any positive results in several clinical trials (Pfeffer *et al.*, 2012b).

Idebenone is a potent, synthetic CoQ10 analogue and has been trialled in patients with LHON. The primary end point for the LHON trial did not reach statistical significance, but post-trial ad hoc analysis suggests its use may benefit patients with discordant visual acuities (i.e. those who are at high risk of losing sight in the other eye) (Klopstock *et al.*, 2011).

EPI-743 is a synthetic analogue of Vitamin E, which is an anti-oxidant and in vitro studies have shown that it has a potency that is a thousand fold higher than CoQ10 and idebenone. Two open-label trials of EPI-743, with a small number of paediatric patients with mixed phenotypes and genotypes, have shown some clinical improvement (Enns *et al.*, 2012; Martinelli *et al.*, 2012). A randomised, controlled trial of EPI-743 in Leigh syndrome has since been completed but the formal report of trial primary and secondary outcomes is still awaited (ClinicalTrials.gov identifier: NCT01721733).

Other novel, small molecular therapies with an anti-oxidative activity that are currently tested in clinical trials including RP103 cysteamine bipartite (ClinicalTrials.gov identifier: NCT02023866) and KH176 (Koopman *et al.*, 2010) (ClinicalTrials.gov identifier: NCT02909400).

1.9 Natural history and cohort studies in mitochondrial disease

Improvement in the diagnostic strategies with the application of NGS has solved the diagnostic conundrum of many cases of mitochondrial disease. Furthermore, our understanding of the biochemical defects and molecular mechanisms of mitochondrial disease has benefited greatly from in vitro studies of patient cell lines and animal models over the last two decades. However, risk stratification and surveillance for complications, prediction of disease progression and prognostication are extremely challenging in the clinical setting. The limitation in the longitudinal and natural history data creates significant barriers to developing medical management guidance, determining the timing for therapeutic trial and outcome measures, which are patient-centred and clinically relevant. The m.3243A>G mutation is a great example to illustrate these points. It is well-known to cause MELAS syndrome, which manifests as recurrent SLEs, yet the clear natural history of these SLEs such as clinical and radiological evolution, the interval between the SLEs and extent of recovery following each

SLE is largely lacking, and only reported in small case series or single cases. On the other hand, only ~20% of m.3243A>G mutation carriers develop MELAS syndrome yet currently there is no predictive tool to provide accurate prognostication for other maternal family members especially those who are currently asymptomatic. Phenotypic heterogeneities are often also observed even within the same family pedigrees: CPEO, renal disease, MIDD, exercise intolerance and cardiomyopathy, to name a few examples. These factors have to be considered when devising surveillance programme for complications, inclusion criteria of clinical trial and outcome measures. More stringent patient selection would limit the patient recruitment from a single source, and multi-centre collaboration would be imperative to achieve sufficient sample size especially for randomized-controlled trials (Pfeffer *et al.*, 2013b).

1.9.1 The MRC Mitochondrial Disease Patient Cohort

The clinical and diagnostic service for mitochondrial disease is centralised to three centres in the UK: Newcastle upon Tyne, London and Oxford. Mitochondrial Disease Patient Cohort (MitoCohort), is a collaborative project initiated by these three centres and funded by the Medical Research Council (MRC) since 2009. Two main objectives of MitoCohort are: first, to improve our understanding of natural histories of mitochondrial disease caused by different genetic mutations based on prospective and longitudinal observation; second, to promote trial readiness of a large group of well-characterized patients. The current recruitment criteria are: 1) individual should be alive when enrolled to the cohort; 2) biochemical evidence or genetic confirmation of mitochondrial disease; 3) asymptomatic carriers of genetic mutation are included. There is no restriction on age.

Newcastle is the largest national centre and is actively followed up 57% of patients (~800) recruited to the MitoCohort (**Figure 19**). Patients are comprehensively assessed using Newcastle Mitochondrial Disease Adult Scale (NMDAS), a validated clinical assessment tool (Schaefer et al., 2006) specific to adult patients with mitochondrial disease. A baseline NMDAS is performed at the patient's first visit and repeated on subsequent follow-up, typically every 12-24 months. Results of past and present clinical investigations such as routine blood tests, ECG, echocardiography, brain imaging, neurophysiological studies (electroencephalogram, nerve conduction studies and electromyography) and muscle biopsy findings are also collected systematically on the MitoCohort database.

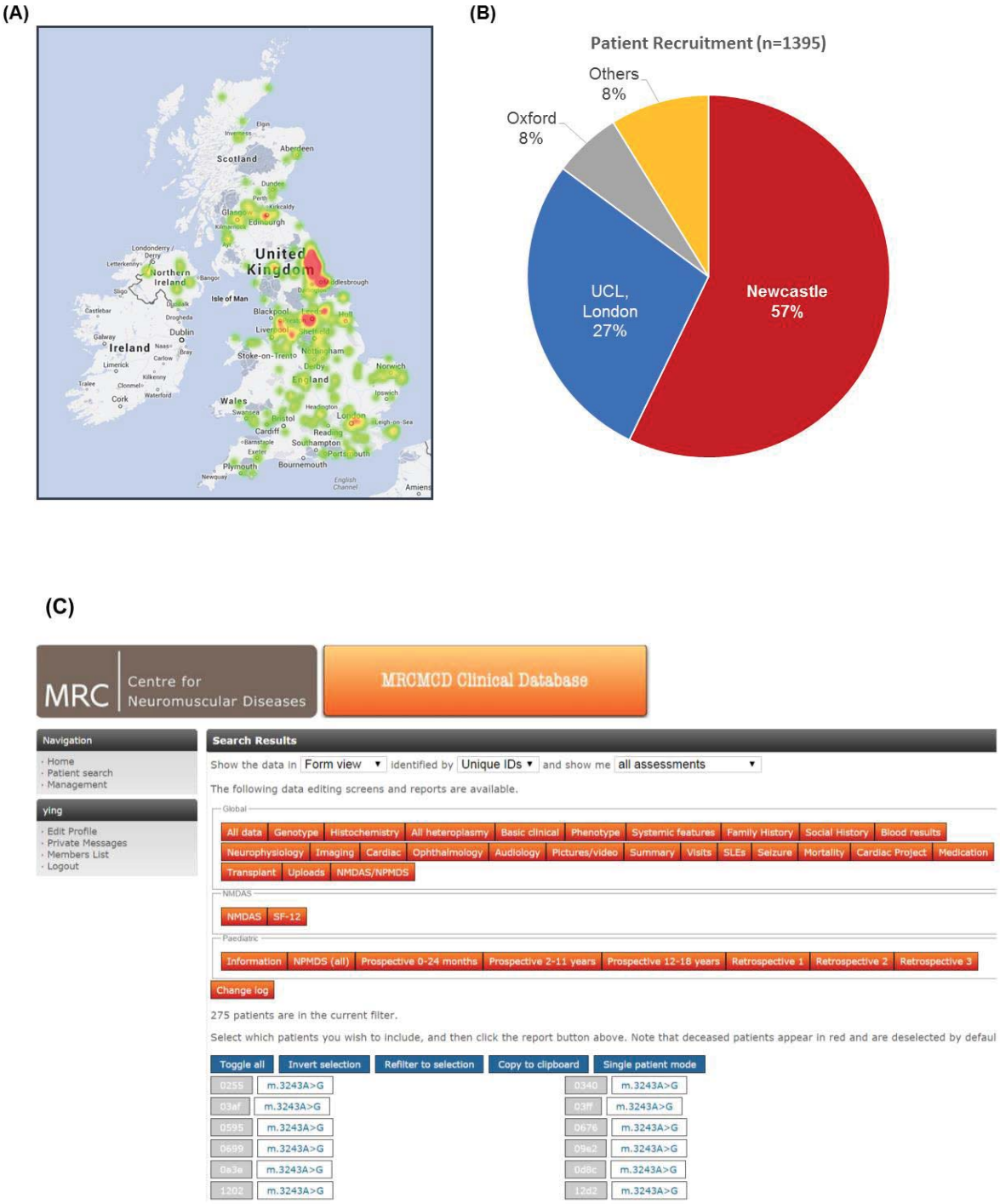

Figure 19. The MRC Mitochondrial Disease Patient Cohort. (A) Heat map constructed based on patients' post codes. Patients with mitochondrial disease are actively recruited in the North East England, where Newcastle is situated. (B) Pie chart shows a total number of patients and percentage of patient recruited by the individual centre (updated on 28th October 2016). (C) The layout of cohort database.

Chapter 2. Aims and scope

2.1 Aims and scope

The main aims of this thesis were: (1) to delineate the clinical and genetic spectrum of mitochondrial DNA and nuclear gene disorders in the Mitochondrial Disease Patient Cohort, Newcastle; (2) to identify potential prognostic factors that may aid risk stratification, surveillance and conception of treatment guidelines that may be implemented into clinical practice (**Figure *20***).

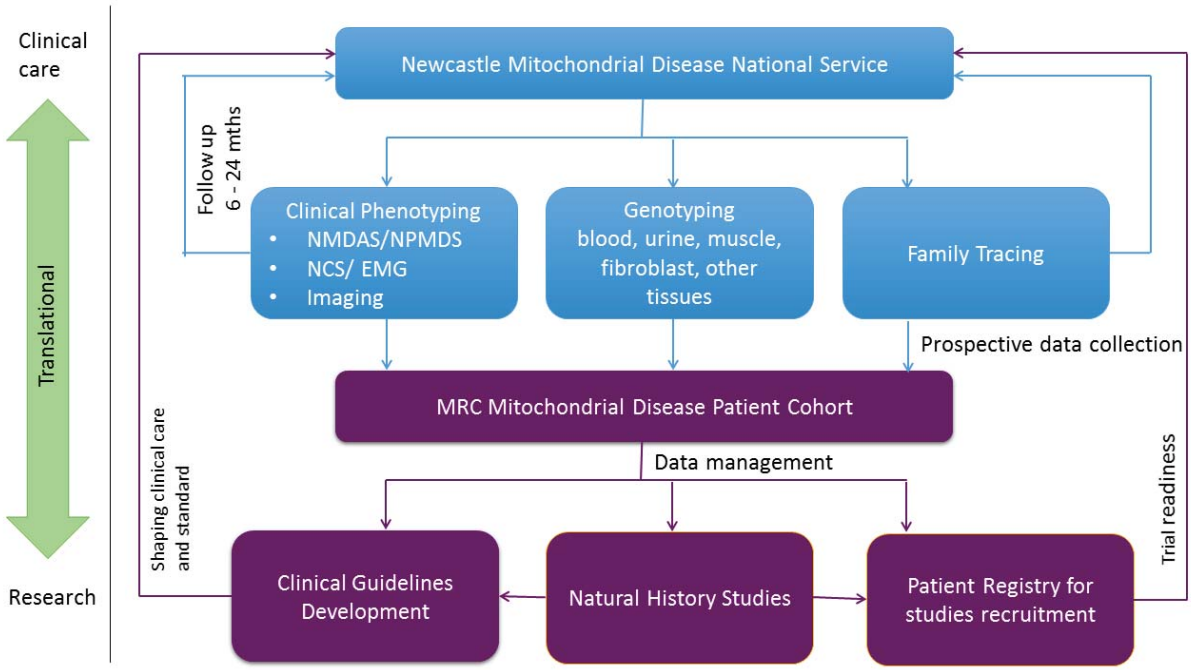

Figure 20. Utilisation of the MRC Mitochondrial Disease Patient Cohort and translation of research findings into clinical practice.

2.2 Overview of Mitochondrial Disease and Neurological Presentations

In the invited review (co-authored with Professor Doug Turnbull), I briefly discussed the genetics and epidemiology of mitochondrial disease and emphasised the crucial step of diagnosing mitochondrial disease is still dependent on meticulous clinical assessment and interpretation of family history. I highlighted the acute and chronic clinical presentations of mitochondrial disease that 'jobbing' neurologists may encounter in the clinical setting and gave an overview of the current evidence for specific and supportive treatments, surveillance for complications, genetic counselling and reproductive options.

Recessive *POLG* mutations remain one of the most common nuclear gene-related forms of mitochondrial disease seen in both paediatric and adult populations. In a systematic review (joint first author with Maria-Eleni Anagnostou), I performed a comprehensive literature

search and analysis of all *POLG* related epilepsy cases published to date (n=372) and detailed the associated spectrum of clinical, electrophysiological and radiological features, neuropathological findings and molecular genetics. I determined that there is a bimodal distribution of age of disease onset with females presenting later than males. Seizures, especially in cases of epilepsia partialis continua, were often refractory to multiple pharmacological agents in vast majority of cases. I confirmed that the clinical outcome of *POLG*-related epilepsy was influenced, in part, by the location of pathogenic variants in the gene and presence of hepatic dysfunction. These findings provide greater insight into disease trajectory and prognosis and highlight the need for an improved or alternative treatment strategy.

2.3 Interrogating the clinical manifestations of m.3243A>G-related mitochondrial disease

The m.3243A>G mutation is the most common pathogenic mutation in adult patients, manifesting with marked heterogeneity in clinical manifestations, between individuals and within pedigrees; as well as between different tissues. Cardiac involvement, in the form of hypertrophic cardiomyopathy or Wolff-Parkinson-White syndrome, is recognised clinical entities associated with this mutation. However, following two unrelated cases of sudden adult death syndrome (SADS), I interrogated their clinical and autopsy findings and reviewed the cause of death in other family pedigrees. Initially, the most striking finding was that both individuals were asymptomatic carriers of the m.3243A>G mutation with high mtDNA heteroplasmy. Review of other family pedigrees led to the identification of six other SADS cases. Such findings affirmed the importance of cardiac surveillance even among the 'apparently' asymptomatic mutation carriers. My findings helped devise a prediction tool to identify patients who are at risk of developing cardiac complications of m.3243A>G-related disease. Development of this risk stratification tool has facilitated more extensive surveillance on high-risk patients, as advised in the manuscript and has led to a revision of the Newcastle best practice cardiac guidelines.

While I was analysing the cardiac involvement in patients with the m.3243A>G mutation, I noted that severe gut dysmotility, manifesting as intestinal pseudo-obstruction (IPO) was often recorded. I also observed a few patients required emergency hospital admission for treatment. Intestinal pseudo-obstruction (IPO) is a typical feature associated with MNGIE caused by mutations in the *TYMP* gene, however, it has only been reported in case reports or

small, retrospective case series of patients with the m.3243A>G mutation. I sought to study the clinical features and radiological characteristics of severe IPO (mimicking an acute surgical abdomen) in patients harbouring the m.3243A>G mutation, to document the clinical outcome especially among those who underwent surgical interventions, and to determine its prevalence. I identified risk factors for the development of severe IPO in patients with m.3243A>G and these findings have informed the derivation of gastrointestinal screening and management guidelines for adult patients (these guidelines are available at http://www.newcastle-mitochondria.com/clinical-professional-home-page/clinical-publications/clinical-guidelines/).

2.4 Investigating The Spectrum of Extrapyramidal Movement Disorders in Mitochondrial Disease

Descriptions of extrapyramidal features associated with mitochondrial disease are often based on individual genetic mutations. In addition, there are limited data on the treatment options of extrapyramidal movement disorders and their efficacy in mitochondrial disease. I aimed to determine the frequency and spectrum of extrapyramidal movement disorders seen in children and adults and review their response to conventional treatments. My findings suggest that the genotype-phenotype correlates in this study may provide guidance on targeting genetic screening. I observed that reduction in dopaminergic uptake on nuclear imaging did not appear to predict treatment response to dopaminergic drugs in the adult patients (data on paediatric patients were too limited to make any meaningful conclusions). Further research of extrapyramidal movement disorders in mitochondria disease is warranted, given the debilitating nature of these symptoms and limited understanding of their pathogenesis.

2.5 Characterising Nuclear Genes in - Combined mitochondrial respiratory chain deficiencies

Combined mitochondrial respiratory chain deficiencies are associated with heterogeneous groups of genetic mutations and clinical phenotypes. Mutations in the *RMND1* (Required for Meiotic Nuclear Division protein 1) gene were initially identified as a cause of multiple respiratory chain defects in infantile encephalomyopathy using WES. Subsequently, milder cases with sensorineural deafness and renal disease were also reported. In a multi-centre collaboration project, I sought to define the clinical, biochemical and molecular genetic spectrum associated with the mutations in *RMND1* gene. I performed survival analysis to

identify possible favourable factors associated with better clinical outcome. I also highlighted the importance of screening for renal complications, given it was identified in two-third of patients. The recognition of renal involvement is important, as there are effective supportive treatment strategies such as anti-hypertensive medication and correction of electrolyte imbalance, and potentially life-saving management such as kidney transplant.

Intriguing clinical manifestations with unusual combinations of organ involvement have been reported in defects of mitochondrial amino-acyl tRNA synthetases. The *YARS2* gene encodes for mitochondrial tyrosyl-tRNA synthetase and mutations in this gene have been linked to myopathy, lactic acidosis and sideroblastic anaemia (MLASA) syndrome particularly in individuals of Middle-Eastern ancestry. I identified six cases from four unrelated family pedigrees (three Scottish families). The first two pedigrees were identified via WES, and direct *YARS2* gene sequencing was performed on two families based on the interpretation of clinical phenotypes. A final genetic diagnosis was provided (with the supporting evidence from Yeast modelling; work of Dr Claudia Donnini and her team), to the original case of MLASA that was first described in the mid-1970s (Rawles and Weller, 1974). These findings expand the clinical spectrum in *YARS2*-related mitochondrial disease and also emphasise the importance of cardiac and respiratory surveillance in these patients. Testing for the *YARS2* gene should be considered in patients with exercise intolerance and multiple respiratory chain deficiencies in muscle biopsy, irrespective of ethnicity or absence of any of the individual clinical features MLASA syndrome.

2.6 Expanding clinical phenotypes of an uncommon, heteroplasmic mutation in *MT-ND5*

Rare pathogenic mtDNA variants impose challenges on genetic counselling and often there are only single case reports to inform clinical decision. This case series began with a female patient in her late twenties who presented with progressive ataxia and found to have the m.13094T>C mutation. I was intrigued by the presence of a unilateral thalamic lesion on her brain imaging. Such finding was rather unusual in mitochondrial disease, and the patient's clinical feature was not similar to three other previously reported cases. I sought to determine the spectrum of clinical and imaging findings of this mutation. Altogether, I identified 15 patients from eight pedigrees from our diagnostic database and collaboration with other centres outside the UK. I delineated the heterogeneities in the clinical presentation associated with the m.13094T>C mutation and more importantly, I also showed that thalamic lesion without striatal abnormality is a common finding in this mutation.

Chapter 3. Overview of Mitochondrial Disease and Neurological Presentations

3.1 Mitochondrial disease: genetics and management

Ng, Y.S. and Turnbull, D.M. (2016) 'Mitochondrial disease: genetics and management', *J Neurol*, 263(1), pp. 179-91.

3.2 Epilepsy due to mutations in the mitochondrial polymerase gamma (*POLG*) gene: A clinical and molecular genetic review

Anagnostou, M.E., **Ng, Y.S.**, Taylor, R.W. and McFarland, R. (2016) 'Epilepsy due to mutations in the mitochondrial polymerase gamma (*POLG*) gene: A clinical and molecular genetic review', *Epilepsia*, 57(10), pp. 1531-1545.

Chapter 4. Interrogating the clinical manifestations of m.3243A>G-related mitochondrial disease

4.1 Sudden adult death syndrome in m.3243A>G-related mitochondrial disease: an unrecognized clinical entity in young, asymptomatic adults

Ng, Y.S., Grady, J.P., Lax, N.Z., Bourke, J.P., Alston, C.L., Hardy, S.A., Falkous, G., Schaefer, A.G., Radunovic, A., Mohiddin, S.A., Ralph, M., Alhakim, A., Taylor, R.W., McFarland, R., Turnbull, D.M. and Gorman, G.S. (2016b) 'Sudden adult death syndrome in m.3243A>G-related mitochondrial disease: an unrecognized clinical entity in young, asymptomatic adults', *Eur Heart J*, 37(32), pp. 2552-9.

4.2 Pseudo-obstruction, stroke, and mitochondrial dysfunction: A lethal combination

Ng, Y.S., Feeney, C., Schaefer, A.M., Holmes, C.E., Hynd, P., Alston, C.L., Grady, J.P., Roberts, M., Maguire, M., Bright, A., Taylor, R.W., Yiannakou, Y., McFarland, R., Turnbull, D.M. and Gorman, G.S. (2016) 'Pseudo-obstruction, stroke, and mitochondrial dysfunction: A lethal combination', *Ann Neurol*. doi: 10.1002/ana.24736

Chapter 5. Investigating the spectrum of extrapyramidal movement disorders in mitochondrial disease using the MitoCohort

5.1 Clinical, Genetic, and Radiological Features of Extrapyramidal Movement Disorders in Mitochondrial Disease.

Martikainen, M.H., **Ng, Y.S**., Gorman, G.S., Alston, C.L., Blakely, E.L., Schaefer, A.M., Chinnery, P.F., Burn, D.J., Taylor, R.W., McFarland, R. and Turnbull, D.M. (2016) 'Clinical, Genetic, and Radiological Features of Extrapyramidal Movement Disorders in Mitochondrial Disease', *JAMA Neurol*, 73(6), pp. 668-74.

Chapter 6. Characterising nuclear genes in combined mitochondrial respiratory chain deficiencies

6.1 The clinical, biochemical and genetic features associated with *RMND1*-related mitochondrial disease

Ng, Y.S., Alston, C.L., Diodato, D., Morris, A.A., Ulrick, N., Kmoch, S., Houstek, J., Martinelli, D., Haghighi, A., Atiq, M., Gamero, M.A., Garcia-Martinez, E., Kratochvilova, H., Santra, S., Brown, R.M., Brown, G.K., Ragge, N., Monavari, A., Pysden, K., Ravn, K., Casey, J.P., Khan, A., Chakrapani, A., Vassallo, G., Simons, C., McKeever, K., O'Sullivan, S., Childs, A.M., Ostergaard, E., Vanderver, A., Goldstein, A., Vogt, J., Taylor, R.W. and McFarland, R. (2016) 'The clinical, biochemical and genetic features associated with *RMND1*-related mitochondrial disease', *J Med Genet*. doi: 10.1136/jmedgenet-2016-103910

6.2 Clinical features, molecular heterogeneity and prognostic implications in *YARS2*-related mitochondrial myopathy

Sommerville, E.W.,* **Ng, Y.S**.,* Alston, C.L.,* Dallabona, C., Gilberti, M., He, L., Knowles, C., Chin, S.L., Schaefer, A.M., Falkous, G., Murdoch, D., Longman, C., de Visser, M., Bindoff, L.A., Rawles, J.M., Dean, J.C.S., Petty, R.K., Farrugia, M.E., Haack, T.B., Prokisch, H., McFarland, R., Turnbull, D.M., Donnini, C., Taylor, R.W., Gorman, G.S.

(* Joint first author, accepted in *JAMA Neurology* on 25 Aug 2016)

Chapter 7. Additional Material

7.1 Refractory seizures, spinocerebellar ataxia and optic atrophy caused by the m.13094T>C mutation

Ng, Y.S., Lax, N.Z., Maddison, P., Alston, C.L., Blakely, E.L., Hepplewhite, P.D., Riordan, G., Meldau, S., Chinnery, P.F., Pierre, G., Chronopoulou, E., Du, A., Hughes, I., Morris, A.M., Kamakari, S., Chrousos, G., Rodenburg, R.J., Saris, C.G.J., Feeney, C., Hardy, S.A., Schaefer, A.M., Mundy, H., Champion, M.P., Turnbull, D.M., Taylor, R.W., McFarland, R., Gorman, G.S.

(This additional material is relevant to thesis but awaiting publication.)

Discussion

Mitochondrial disease is clinically, biochemically and genetically heterogeneous and can affect any age group and any organ. A recent epidemiological study based on the North East England population has shown that the prevalence of mitochondrial disease is at least 1 in 4300 (Gorman *et al.*, 2015), making it as one of the most common forms of inherited muscle disease. The diagnostic challenge in mitochondrial disease is being addressed with better awareness and recognition of the clinical entity, and perhaps more so with significant advancements in diagnostic approaches, particularly with the application of next generation sequencing. However, there remain several outstanding, unmet needs for the patients. First, the natural history of disease progression and prognostic factors are not well characterised, and many uncertainties remain about genetic counselling and discussion on disease trajectory. Second, clinical management of many mitochondrial disorders vary between centres and countries; yet standards of medical care have not been established in a routine clinical setting. And lastly, effective treatment targeting mitochondrial dysfunction remains unavailable for most types of mitochondrial disorders.

Many classic syndromes have been described in mitochondrial disease. The distinctive nature of these syndromes improves the recognition and diagnosis of mitochondrial disease. However, it is increasingly clear that only small portions of patients develop full syndromic presentations (Liang *et al.*, 2014). Furthermore, our current understanding or perception of natural history in different genetic forms of mitochondrial disease may be biased towards the severe disease end or those with classic syndromes, which may be unhelpful or potentially misleading. A cohort-based observational study is, therefore, necessary to clarify the heterogeneities associated with the same mutation and may facilitate the interrogation of the natural history of individual mutations using standardised assessment and longitudinal data (Schaefer *et al.*, 2006). Based on data derived from the Mitocohort in Newcastle, I have expanded on our understanding of two clinical features, namely, sudden adult death syndrome (SADS) (Ng *et al.*, 2016c) and severe intestinal pseudo-obstruction (Ng *et al.*, 2016b), which have been under-recognized in m.3243A>G-related mitochondrial disease, the most common mitochondrial DNA mutation that accounts for nearly a third of all adult cases of mitochondrial disease (Gorman *et al.*, 2015). These findings helped inform and facilitate an update of the existing Newcastle cardiac guidelines, with the incorporation of cardiac risk stratification in individuals who harbour the m.3243A>G mutation. More extensive investigations such as cardiac MR and implantable cardiac monitoring (LinQ device) are being introduced as part of a pilot study for high-risk individuals.

The detrimental effects of chronic, excessive alcohol intake to the heart are well-established: cardiomyopathy (Piano, 2002), heart failure (Klatsky *et al.*, 2005), malignant ventricular arrhythmia (Guzzo-Merello *et al.*, 2015) and increased incidence of sudden cardiac death (Wannamethee and Shaper, 1992; Fauchier *et al.*, 2000; Wu *et al.*, 2015). Whilst acute alcohol intake have also been associated with sudden unexpected death (Penttila *et al.*, 1989; Perkiomaki *et al.*, 2016), the precise mechanism is less clear, and it is hypothesised that alcohol triggered the electrical instability in these patients (Perkiomaki *et al.*, 2016). Studies have shown that 13-17% of patients with the m.3243A>G mutation are at risk of developing ventricular pre-excitation and Wolff-Parkinson-White syndrome (WPW) (Sproule *et al.*, 2007; Malfatti *et al.*, 2013). WPW may deteriorate to malignant arrhythmia such as ventricular fibrillation in some individuals, therefore there is an increased lifetime risk of sudden death (Pappone *et al.*, 2012; Kim and Knight, 2016). It is tempting to speculate that our patients had an undiagnosed cardiac arrhythmia that was triggered by the combination of acute alcohol intake, dehydration and lactic acidemia; the increase of susceptibility to these factors might be related to the widespread mitochondrial dysfunction in the cardiomyocytes. More recently, sudden unexpected cardiac deaths were reported in some patients with the deficiency of the mitochondrial inorganic pyrophosphatase due to recessive mutations in *PPA2* and exquisite sensitivity to ethanol (levels of alcohol intake reported to be <0.1g and 10g respectively in two deaths; one unit of alcohol (UK) is equivalent of 8g of pure alcohol) was observed (Kennedy *et al.*, 2016). The link between alcohol metabolism and inorganic pyrophosphatase function has been implicated (Kennedy *et al.*, 2016).

The findings of SADS and the high number of cardiac deaths reported in the literature (Klopstock *et al.*, 1999; Majamaa-Voltti *et al.*, 2002; Kaufmann *et al.*, 2011; Malfatti *et al.*, 2013) provides compelling evidence regarding the importance of family tracing and the need for cardiac surveillance irrespective of disease status in the m.3243A>G mutation. Appropriate counselling prior to the predictive genetic testing for the m.3243A>G mutations should be offered to maternal family members. The implications of long-term surveillance for other complications such as gut dysmotility, sensorineural deafness, renal involvement and diabetes mellitus should be discussed. The identification of disease manifestation at the earliest stage would enable prompt medical interventions, therefore preserving overall health, delaying or circumventing the development of potentially fatal complications such as hypertrophic cardiomyopathy and intestinal pseudo-obstruction. From the perspective of natural history study, longitudinal follow up of these individuals would provide prospective data on the progression of asymptomatic stage to affected status, which has important

implications for health economics and resource planning. These data may also serve as one of the important end points for any future disease-modifying treatment.

Gastrointestinal symptoms are common among patients harbouring the m.3243A>G mutation (Mancuso *et al.*, 2013a; de Laat *et al.*, 2015) and severe gut dysmotility manifesting as severe IPO has been reported. My analysis of our cohort data showed that IPO affected 1 in 8 (13%) individuals carrying the m.3243A>G mutation (Ng *et al.*, 2016b). I estimated that the prevalence of combined IPO and m.3243A>G mutation is 1 in 200,000, and is far more common than the estimated prevalence of MNGIE which is typically associated with recurrent IPO, at 0.15 per 1,0000,000 (D'Angelo *et al.*, 2016). I identified several risk factors for the development of IPO including stroke-like episodes, cardiomyopathy, low body mass index and high urinary mutation load. Furthermore, I was able to establish that the prompt recognition and medical treatment of IPO were of paramount importance in clinical practice; with delayed treatment or inappropriate surgical intervention leading to prolonged metabolic crisis and even death. These findings have reshaped our clinical care and the development of Newcastle gastrointestinal screening and management guidelines.

Working at the Wellcome Trust Centre for Mitochondrial Research, one of the most challenging clinical queries that we often encounter is the management of super-refractory epilepsy associated with *POLG* mutations. Alpers syndrome, characterised by intractable seizures, psychomotor retardation and hepatic dysfunction, was commonly described as an infantile/early childhood disease caused by mutations in the *POLG* gene. However, we have been involved in the clinical management of several cases of teenage-onset Alpers syndrome with mixed outcomes over the last five years. Such observations motivated me to perform a systematic review of *POLG*-related epilepsy to determine any potential prognostic factors in these patients and current guidance on seizure management (Anagnostou *et al.*, 2016). We identified the onset of *POLG*-related epilepsy clustered in two age categories (<5 years and teenage); with females generally having a later disease onset (the median difference between genders is four years). Disease onset and severity was influenced by the location of pathogenic variants in the gene: homozygous p.Ala467Thr and homozygous p.Trp748Ser mutations were generally associated with longer survivals compared to compound heterozygous mutations (e.g. p.Ala467Thr and p.Gly848Ser). *POLG*-related epilepsy often has an explosive onset and is relentlessly progressive. The management of *POLG*-related epilepsy remains super-refractory to conventional pharmacology therapy, and anecdotal evidence of individual efficacious treatments from single case reports warrants further investigation. We suspect a more rapid escalation of anti-seizure management, even when

patients only present with focal seizures, may improve outcome. A new avenue such as transcranial direct stimulation may deserve further exploration because it may represent a safe adjunctive therapy in epilepsy (San-Juan *et al.*, 2015). I have been involved in a case recently in which transcranial direct stimulation was used to control refractory EPC in a teenage patient with mutations in *POLG*, with promising effect (Publication in preparation).

The MitoCohort provides a unique opportunity to investigate genotype-phenotype overlaps. For example, extrapyramidal movement disorders have been identified in just over 5% of patients in the MitoCohort with dystonia and Parkinsonism, the most common extrapyramidal findings in our paediatric and adult populations, respectively (Martikainen *et al.*, 2016). I identified dystonia was frequently associated with Leigh syndrome, which was caused by either mutation in mtDNA or nuclear genes. On the other hand, Parkinsonism was most commonly associated with mutations in the *POLG* gene. Although most of these extrapyramidal features were not apparent at the outset of the disease, they are progressively debilitating, and their response to standard pharmacotherapy such as dopamine was mixed, and often could not be predicted. These genotype-phenotype correlates may guide genetic testing for anyone with suspected mitochondrial disease in the non-specialist clinic; but also alert clinicians to actively screen and manage specific movement disorders associated with the relevant genotype. Further research into the mechanisms of these extrapyramidal movement disorders is necessary to find better treatment options in these patients.

The use of whole exome sequencing (WES) in the research setting has identified new genes and provided more genetic diagnoses in mitochondrial disease. For example, several intriguing clinical syndromes with apparent tissue-specific involvement have been described in various mitochondrial amino-acyl tRNA synthetases (Diodato *et al.*, 2014). However, the number of cases and families harbouring individual genetic mutations are often small, and further validation of these clinical entities and their natural histories is required with larger case series, through multi-centre collaboration. The *YARS2* gene encoding for mitochondrial tyrosyl-tRNA synthetase was proposed as a second genetic cause of myopathy, lactic acidosis and sideroblastic anaemia (MLASA) syndrome (Riley *et al.*, 2010), following the discovery of *PUS1* gene (Bykhovskaya *et al.*, 2004). In our centre, we initially identified two patients who harboured mutations in the *YARS2* gene: a Lebanese patient with typical MLASA syndrome and a Scottish patient with only myopathy and lactic acidosis. Through the review of case notes of a deceased Scottish patient who was originally referred to our centre in the early 1990s, I identified a third patient with MLASA syndrome, which in fact was the first case of MLASA syndrome originally described in the mid-1970s (Rawles and Weller, 1974).

The 4th case was most intriguing, as she was a 73 year old Scottish woman that presented with slowly progressive myopathy without lactic acidosis or sideroblastic anaemia. I compiled the case series, together with previously reported cases and showed that the triad of MLASA was only identified in approximately 70% of patients. Furthermore, the identification of three Scottish families highlights that the mutations in *YARS2* are not unique to ethnicities of the Middle East. I showed that severe cardio-respiratory involvement is evident in nearly 50% of cases that highlights the importance of active multi-specialist surveillance and treatment, in liaison with a cardiologist and respiratory physician.

Heteroplasmic mutations in the mtDNA are often associated with diverse clinical manifestations, as clearly illustrated in the case series of patients harbouring the m.13094T>C *MTND5* mutation. This mutation exhibits highly heterogeneous neurological manifestations including LS, MELAS/LS, spinocerebellar ataxia (SCA) with and without epileptic encephalopathy and LHON, and may mimic other neurological disorders such as demyelinating disease and SCA. Furthermore, useful pointers suggestive of a primary mitochondrial DNA disease such as maternal family history and abnormal muscle biopsy are often not identified in this uncommon mutation. Again, primary mtDNA disease can only be reliably excluded by performing whole mitochondrial genome sequencing in relevant clinical tissues given marked tissue segregation, as observed in the m.13094T>C mutation, may be evident.

The *RMND1* gene that encodes for Required for Meiotic Nuclear Division protein 1, has been linked to a mitochondrial translational defect and combined respiratory chain deficiencies (Janer *et al.*, 2012). RMND1 deficiency was initially described in severe, neonatal onset encephalomyopathy but subsequently, in patients with renal impairment and deafness (Janer *et al.*, 2015). To better delineate the clinical spectrum of RMND1 deficiency and inform clinical management, I led a multi-centre collaboration to collate clinical, biochemical and molecular genetics data (Ng *et al.*, 2016a). Whilst disease onset in all patients with mutations in *RMND1* gene was under two years (n=32), phenotypic expression and clinical outcomes were vastly heterogeneous. One of the striking findings was two-thirds of patients had evidence of kidney disease such as chronic renal impairment, secondary hypertension, dysplastic kidneys and renal tubular acidosis type 4. Another interesting observation was brady-arrhythmia only identified in patients of Pakistani origin with homozygous founder mutations. Patients with renal impairment had a better survival compared to those without renal impairment (the latter patients had CNS involvement primarily). The longer survival

may be attributable to the availability of active, supportive treatments such as correction of electrolytes, dialysis and renal transplant (n=4) (Parikh *et al.*, 2016) which have been instigated. Meticulous clinical phenotyping of rare mitochondrial disease remains crucial to guiding clinical management and surveillance for treatable complications, as exemplified by our findings in the case series of RMND1 deficiency.

Conclusions

The central theme of this thesis was to characterise the spectrum of clinical phenotypes further and identify complications that may be amenable to therapy with better clinical surveillance in patients with mitochondrial disease caused by mtDNA and nDNA mutations. I have demonstrated a connection between SADS and asymptomatic individuals with the m.3243A>G mutation, and highlighted that active family screening irrespective of disease status and proposed a simple risk stratification tool to tailor a strategy for cardiac surveillance and treatment accordingly. I have identified a high prevalence of IPO in m.3243AG- related mitochondrial disease and devised the Newcastle Gastrointestinal screening and management guidelines to streamline clinical practice based on these findings. Using the Mitocohort data, I have shown that dystonia and Parkinsonism are two common extrapyramidal movement disorders in patients, whose clinical management remains challenging. I have expanded the clinical features associated with *YARS2* mutation and highlighted that the typical MLASA syndrome was not essential to the clinical diagnosis. I have also identified that cardio-respiratory involvement is prominent and a common cause of death in patients with *YARS2* mutations, and suggested prompt recognition and supportive treatment may delay disease progression. Renal involvement is another example of a treatable complication that I have identified in a cohort of patients associated with RMND1 deficiency. Aggressive management is warranted, in such cases, as I have shown that a subgroup of patients with kidney involvement had a better long-term survival. These findings will serve to provide robust data on the delineation of natural history and prognostication in mitochondrial disease; and more importantly, will aid to set the standard for clinical care and help inform and develop nationally-endorsed clinical management guidelines for patients with mitochondrial disease whilst the hunt for a cure continues.

Future work

(1) Potential for future research and preventative and early treatments in intestinal pseudo-obstruction

Unpublished data from the MRC Mitochondrial Disease Patient Cohort (Newcastle) show that over 70% of patients with the m.3243A>G mutation have variable degrees of GI symptoms, corroborated with the previous reports (Kaufmann *et al.*, 2011; de Laat *et al.*, 2015). Intestinal pseudo-obstruction is at the end of the spectrum of gut dysmotility, and it is intuitive to hypothesise that early and aggressive treatment of chronic constipation may reduce the risk or prevent the development of IPO. Current evidence suggests a role for smooth muscle involvement (high mutant heteroplasmy levels and COX deficiency) in the development of gut dysmotility among patients with the m.3243A>G mutation (Betts *et al.*, 2008). However, the role of the enteric nervous system has not been investigated extensively. It is possible that bacterial overgrowth plays an important part in the development of recurrent pseudo-obstruction given that gas filled dilated loops of bowel are frequently observed on imaging. Interrogation of the gut microbiome in those individuals affected by IPO and stroke-like episodes and those who are unaffected might further elucidate the mechanism of 'neuro-gastrointestinal crisis' given that at least 50% of patients presented with acute metabolic stroke also developed IPO concurrently. In addition, systematic evaluation of dietary intervention such as low residue diet and probiotics, and antimicrobial therapy for bacterial overgrowth in these patients are warranted.

(2) Future research on neuro-radiological techniques for specific genetic defects

Neuroimaging is an essential tool for the diagnosis of mitochondrial disease with predominant CNS involvement, but it remains under-utilized in clinical research. Several studies showed that MR spectroscopy appeared to be a useful biomarker on identifying individuals with the m.3243A>G mutation who were at risk of developing stroke-like episodes (Abe *et al.*, 2004; Weiduschat *et al.*, 2014). However, such findings need to be replicated in larger, prospective studies before translating into routine clinical practice and other mtDNA mutations such as *MTND5* should also be investigated. Stroke-like episodes with a preferential involvement of posterior cerebral cortex are enigmatic in primary mtDNA disease and *POLG*-related disorders. Functional MR and positron emission tomography (PET) scans would be useful on interrogating the cellular metabolism in different brain regions of patients with mitochondrial

disease *in vivo*. Tractography, a 3D-modelling MRI technique, can be applied to investigate the roles of white matter and brain circuits in seizure propagation, given the spreading of stroke-like lesions is believed to be driven primarily by the neuronal hyper-excitability (Iizuka *et al.*, 2002; Iizuka *et al.*, 2003).

Cognitive impairment and ataxia are clinical features commonly found in patients at their later stage of the mitochondrial disease. The imaging findings of cerebral and cerebellar atrophy together with low brain weight and evidence of neuronal loss in post-mortem studies strongly suggest an active neurodegenerative process in mitochondrial disease, irrespective of genotype. Brain volumetric study can provide quantitative data on investigating the extent and rate of atrophy, which may serve as an important biomarker and outcome measure in future drug trials.

(3) Explore genotype/phenotype correlation for identifying more accurate prognostic markers.

A large patient number is essential for exploring genotype-phenotype correlation, identifying and validating putative prognostic factors that are relevant in clinical practice. The establishment of the national patient registry in the UK, Italy, Germany, United States and Australia is a significant development towards a better understanding of the natural history of mitochondrial disease caused by different genetic defects over the last decade. Several studies derived from these national cohort data have further defined the clinical phenotypes of prevalent mutations such as m.3243A>G (Mancuso *et al.*, 2013a; Nesbitt *et al.*, 2013), m.8344A>G (Mancuso *et al.*, 2013b; Altmann *et al.*, 2016) and single, large scale mtDNA deletion (Grady *et al.*, 2014; Mancuso *et al.*, 2015), and provided a more objective and in-depth evaluation of the spectrum of disease burden in these patients when compared to early case reports and small case series. Standardisation of clinical assessment and harmonisation of data collection such as using human phenotype ontology terms between these national registries would allow pooling clinical data for robust analysis. More importantly, international collaboration should help to establish consensus guidance, especially on surveillance for complications and treatment, prioritise areas of clinical research and recruit patients to future drug trials.

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