Identifying New Genes in Mitochondrial Disease

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Abstract

Mendelian mitochondrial disease presents vast clinical and genetic heterogeneity, which provides challenges in attaining genetic diagnoses for affected patients. With approximately 1,200 proteins encoded by the nuclear genome that tightly coordinate mitochondrial function, targeted screening of a subset of genes provides a low diagnostic yield. Clinicians and researchers have increasingly turned to whole exome sequencing (WES), a targeted next-generation sequencing technology for the identification of all variants in the exons (coding regions) of all known genes for variant discovery and prioritisation, constituting approximately 1% of the human genome.

WES was utilised to provide genetic diagnoses in two patients cohorts with clinically well-defined, genetically undetermined mitochondrial disease: (i) adult-onset progressive external ophthalmoplegia (PEO) (n=19), a mitochondrial DNA (mtDNA) maintenance disorder characterised by extraocular paresis and skeletal muscle restricted multiple mtDNA deletions, with broad phenotypes ranging from indolent PEO and fatal multisystem PEO-plus; (ii) early-onset mitochondrial respiratory chain complex (RC) deficiency (n=20), typically affecting patients in the first decade of life and are associated with heterogeneous phenotypes. Pathogenic mtDNA mutations were previously excluded in all patients. Adult-onset PEO with multiple mtDNA deletions patients were recruited following negative genetic testing of commonly associated nuclear genes, while early-onset RC deficiency patients were negative genetic testing in a small selection of nuclear genes.

Using custom variant filtering strategies for each cohort, confirmed diagnoses were attained in 10% of adult-onset PEO with multiple mtDNA deletions patients and 35% of early-onset RC deficiency patients. This was comprised of known mitochondrial disease associated genes in both cohorts (*AARS2*, *EARS2*, *MRPS22*, *PDHA1*, *SCO1*, *TK2*, *TRMU*, *TWNK*), while novel candidate genes involved in mitochondrial translation, mtDNA replication, nucleotide metabolism and mitochondrial dynamics were also proposed. The pathological mechanism of a novel heterozygous *GMPR1* variant was characterised as a potential new candidate gene associated with late-onset PEO due to impaired nucleotide balance for mtDNA maintenance. *GTPBP3* was identified as a novel locus causing defective tRNA modification with multiple RC deficiency and the clinical, molecular and genetic features of *AARS2*, *EARS2* and *YARS2* mutations were expanded. Finally, this research highlights the potential significance of *de novo* and somatic mosaic variants in WES variant discovery and prioritisation.

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Author's Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. The research was conducted at the Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, under the supervision of Professor Robert W. Taylor and Dr Gráinne S. Gorman. All research is my own unless stated otherwise.

I certify that none of the material offered in this thesis has been previously submitted by me for a degree of any other qualification at this or any other university.

Publications

Material from this thesis has been published and presented as listed below.

Published Manuscripts

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Abbreviations

$\Delta\Psi_m$	Mitochondrial Membrane Potential
aaRS	Amino-Acyl tRNA Synthetase, Cytosolic
ACMG	American College of Medical Genetics and Genomics
AD	Allele Depth
ADOA	Autosomal Dominant Optic Atrophy
ADP	Adenosine Diphosphate
adPEO	Autosomal Dominant Progressive External Ophthalmoplegia
AFG3L2	AFG3-like Protein 2
AILF	Acute Infantile Liver Failure
AK2	Adenylate Kinase 2
AK3	Adenylate Kinase 3
AMP	Adenosine Monophosphate
ANS	Ataxia Neuropathy Spectrum
ANT1	Adenine Nucleotide Transporter 1
APE1	Apurinic/Apyrimidinic Endonuclease 1
APS	Ammonium Persulphate
APTX	Aprataxin
AR	Aspect Ratio
arPEO	Autosomal Recessive Progressive External Ophthalmoplegia
ATP	Adenosine Triphosphate
bp	Basepair
BER	Base Excision Repair
BLM	Bloom Syndrome Helicase
BSA	Bovine Serum Albumin
BWA	Barrow-Wheeler Alignment
CAGSSS	Cataracts, Growth Hormone Deficiency, Sensory Neuropathy, Sensorineural Hearing loss and Skeletal Dysplasia
CCDS	Consensus Coding Sequence
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridisation
COX	Cytochrome *c* Oxidase
CM	Cristae Membrane

CMT	Charcot-Marie-Tooth
CMTDIC	CMT Dominant Intermediate Type C
CMTRIB	Charcot-Marie-Tooth Recessive Intermediate Type B
CNM	Centronuclear Myopathy
CNS	Central Nervous System
CNV	Copy Number Variant
CODAS	Cerebral, Ocular, Dental, Auricular and Skeletal Abnormalities
CPAP	Continuous Positive Airway Pressure
CPEO	Chronic Progressive External Ophthalmoplegia
CSB	Conserved Sequence Block
CSF	Cerebrospinal Fluid
Ct	Cycle threshold
CytC	Cytochrome *c*
D-Loop	Displacement Loop
dA	Deoxyadenosine
DBS	Dialysed Bovine Serum
dC	Deoxycytidine
DD	Developmental Delay
ddNTP	Dideoxynucleotide Triphosphate
DFNB	Deafness, Autosomal Recessive
dG	Deoxyguanosine
dGDP	Deoxyguanosine Diphosphate
dGk/DGUOK	Deoxyguanosine Kinase
DMD	Duchene Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA2	DNA Replication ATP-Dependent Helicase/Nuclease DNA2
dNDP	Deoxynucleotide Diphosphate
DNM2	Dynamin 2
dNMP	Deoxynucleotide Monophosphate
dNTP	Deoxynucleotide Triphosphate
DP	Read Depth
DPBS	Dulbecco's Phosphate-Buffered Saline
DSBR	Double-Stranded Break Repair

dsDNA	Double-Stranded DNA
dT	Thymidine
DTT	Dithiothreitol
dU	Deoxyuridine
EIEE	Early Infantile-Onset Encephalopathy
ELAC2	Transfer Ribonuclease Z
EM	Electron Micrograph
EMG	Electromyography
ENT1	Equilibrative Nucleotide Transporter 1
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
ExAC	Exome Aggregation Consortium
FAD	Flavin Adenosine Nucleotide
FBS	Foetal Bovine Serum
FEN1	Flap Exonuclease 1
FF	Form Factor
FMN	Flavin Mononucleotide
FTT	Failure to Thrive
GATK	The Genome Analysis Toolkit
GDP	Guanosine Diphosphate
GMP	Guanosine Monophosphate
GMPR	Guanosine Monophosphate Reductase
GMPS	Guanosine Monophosphate Synthase
GO	Gene Ontology
GPx1	Glutathione Peroxidase 1
GQ	Genotype Quality
GRIDHH	Growth Retardation, Intellectual Developmental Disorder, Hypotonia and Hepatopathy
GTPBP3	GTP Binding Protein 3
GUK1	Guanylate Kinase 1
HBSL	Hypomyelination with Brainstem and Spinal Cord Involvement and Lactate Elevation
HCl	Hydrochloric Acid
HCM	Hypertrophic Cardiomyopathy
HGP	Human Genome Project

HLASA	Hydrops, Lactic Acidosis and Sideroblastic Anemia
HLD9	Hypomyelinating Leukodystrophy-9
HMN	Hereditary Motor Neuropathy
HNPP	Hereditary Neuropathy and Liability to Pressure Palsy
HRP	Horseradish Peroxidase
HSP	Hereditary Spastic Paraplegia
HUPRA	Hyperuricemia, Pulmonary Hypertension, Renal Failure and Alkalosis
HR2	Heptad Repeat Region
IBM	Inner Boundary Membrane
IFLS1	Infantile Liver Failure Syndrome 1
ILLD	Interstitial Lung and Liver Disease
IMM	Inner Mitochondrial Membrane
IMP	Inosine Monophosphate
IMPDH	Inosine Monophosphate Dehydrogenase
Indel	Insertion-Deletion
ISCU	Iron-Sulphur Cluster Assembly Enzyme
IUGR	Intrauterine Growth Restriction
kDa	Kilodalton
KSS	Kearns-Sayre Syndrome
LBSL	Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation
LETM1	Leucine Zipper-EF-Hand Containing Transmembrane Protein 1
LHON	Leber Hereditary Optic Neuropathy
LIG3	DNA Ligase III
LOF	Loss of Function
LONP1	Lon Peptidase 1
LTBL	Leukoencephalopathy with Thalamus and Brainstem Involvement and High Lactate
MAF	Minor Allele Frequency
Mb	Megabase
MCIA	Mitochondrial Complex I Assembly
MCU	Mitochondrial Ca^{2+} Uniporter
MDa	Megadalton
MELAS	Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-Like

	Episodes
MEM	Minimum Essential Medium
MEMSA	Myoclonic Epilepsy Myopathy Sensory Ataxia
MFF	Mitochondrial Fission Factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
$MgCl_2$	Magnesium Chloride
MGME1	Mitochondrial Genome Maintenance Exonuclease 1
MID	Mitochondrial Dynamics Protein
MIDD	Maternally Inherited Deafness and Diabetes
MIRAS	Mitochondrial Recessive Ataxia Syndrome
MLASA	Myopathy, Lactic Acidosis and Sideroblastic Anaemia
MLPA	Multi Ligation Probe Amplification
MNGIE	Mitochondrial Neurogastrointestinal Encephalopathy
MPC	Mitochondrial Pyruvate Carrier
MPV17	MPV17 Mitochondrial Inner Membrane Protein
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MRPL12	Mitochondrial Ribosomal Protein L12
MRPP	Mitochondrial Ribonuclease P
MRPS22	Mitochondrial Ribosomal Protein S22
MSA	Multiple Sequence Alignment
MSCCA	Microcephaly, Progressive Seizures and Cerebral and Cerebellar Atrophy
mt-aaRS	Mitochondrial Amino-Acyl tRNA Synthetase
mt-AlaRS	Mitochondrial Alanyl-tRNA Synthetase
mt-PheRS	Mitochondrial Phenyl-tRNA Synthetase
mt-GluRS	Mitochondrial Glutamyl-tRNA Synthetase
mt-TyrRS	Mitochondrial Tyrosyl-tRNA Synthetase
mtDNA	Mitochondrial DNA
MTERF1	Mitochondrial Transcription Termination Factor 1
mt-EFTu	Mitochondrial Elongation Factor Tu
mt-FMT	Methionyl-tRNA Formyltransferase
MTO1	Mitochondrial tRNA Translation Optimisation 1

MTPAP	Mitochondrial Poly(A) Polymerase
mtRF1a	Mitochondrial Release Factor 1a
MTS	Mitochondrial Targeting Sequence
mt-SSB	Mitochondrial Single-Stranded Binding Protein
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
MUTYH	MutY DNA Glycosylase
$NaCl_2$	Sodium Chloride
NdR	Deoxyribonucleotide
NEIL1	Nei-like DNA Glycosylase 1
NEIL2	Nei-like DNA Glycosylase 2
NFS1	Cysteine Desulfurase
NGS	Next-Generation Sequencing
NHLBI ESP	National Heart, Lung and Blood Institute Exome Sequencing Project
NIV	Non Invasive Ventilation
NS	Nervous System
NTE	N-Terminal Extension
O_H	Origin of Heavy Strand Replication
O_L	Origin of Light Strand Replication
OGG1	8-Oxoguanine-DNA Glycosylase 1
OMIM	Online Mendelian Inheritance in Man
OMM	Outer Mitochondrial Membrane
OPA1	Optic Atrophy Protein 1
OPMD	Oculopharyngeal Muscular Dystrophy
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PCH6	Pontocerebellar Hypoplasia-6
PCR	Polymerase Chain Reaction
PDA	Patent Ductus Arteriosus
PDHA1	Pyruvate Dehydrogenase Subunit E1-α
PDHc	Pyruvate Dehydrogenase Complex
PEG	Percutaneous Endoscopic Gastrostomy
PEO	Progressive External Ophthalmoplegia
PL	Genotype Phred-Likelihood
PMP22	Peripheral Myelin Protein 22

PMSF	Phenylmethylsulphonyl Fluoride
PNC1	Pyrimidine Nucleotide Carrier 1
PNC2	Pyrimidine Nucleotide Carrier 2
PNKP	Polynucleotide Kinase/Phosphatase
PNP	Purine Nucleoside Phosphorylase
POF	Premature Ovarian Failure
POLG	DNA Polymerase Gamma Subunit 1
POLG2	Polymerase (DNA) Gamma 2, Accessory Subunit
POLRMT	Mitochondrial RNA Polymerase
pRpp	Phosporibosyl Pyrophosphate
PTC	Peptidyl Transferase Centre
PTPIP51	Protein Tyrosine Phosphatase-Interacting Protein 51
PVDF	Polyvinylidene Fluoride
Q	Ubiquinone
QH_2	Ubiquinol
RC	Respiratory Chain
rCRS	Revised Cambridge Reference Sequence
REC	Research Ethics Committee
RIPA	Radioimmunoprecipitation Assay
RIRCD	Reversible Infantile Respiratory Chain Deficiency
RITOLS	Ribonucleotide Incorporated ThroughOut the Lagging Strand
rNDP	Ribonucleotide Diphosphate
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
RNASEH1	Ribonuclease H1
RNR	Ribonucleotide Reductase
ROS	Reactive Oxidative Species
RRM1	Ribonucleotide Reductase Catalytic Subunit M1
RRM2	Ribonucleotide Reductase Regulatory Subunit M2
RRM2B/p53R2	Ribonucleotide Reductase Subunit M2B
SAM	Sequence Alignment/Map
SAM Complex	Sorting and Assembly Machinery Complex
SAMHD1	SAM And HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1
SANDO	Sensory Ataxia Neuropathy Dysarthria and Ophthalmoplegia

SC	Supercomplex
SCAE	Spinocerebellar Ataxia and Epilepsy
SCO1	SCO1 Cytochrome *C* Oxidase Assembly Protein
SCO2	SCO2 Cytochrome *C* Oxidase Assembly Protein
SDH	Succinate Dehydrogenase
SDS	Sodium Dodecyl Sulphate
SEPT2	Septin 2
SLC25A4/ANT1	Solute Carrier Family 25 Member 4/ ADP/ATP Translocase 1
SMA	Spinal Muscular Atrophy
SMAN	Sensory Motor Axonal Neuropathy
SMGM	Skeletal Muscle Growth Medium
SNHL	Sensorineural Hearing Loss
SNP	Single Nucleotide Polymorphism
SOD1	Superoxide Dimutase 1
SOLiD	Sequencing by Oligo Ligation and Detection
SPG7	Paraplegin
SPG77	Spastic Paraplegia 77, Autosomal Recessive
SSBR	Single-Stranded Break Repair
TAE	Tris-Acetate-EDTA
TBST	Tris-Buffered Saline and Tween-20
TCA	Tricarboxylic Acid
TDP1	Tyrosyl-DNA-Phosphodiesterase 1
TEFM	Mitochondrial Transcription Elongation Factor
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
TFAM	Mitochondrial Transcription Factor A
TFB2M	Mitochondrial Transcription Factor B2
THF	N^{10}-formyl-TetraHydroFolate
TK1	Thymidine Kinase 1
TK2	Thymidine Kinase 2
TMRM	Tetramethylrhodamine Methyl Ester
TOM	Translocase of the Outer Membrane
TOP1mt	Mitochondrial Topoisomerase I
TOP3A	Topoisomerase III-α
Toprim	Topoisomerase-Primase
TRMU/MTU1	tRNA 5-Methylaminomethyl-2-Thiouridylate Methyltransferase

tRNA	Transfer RNA
TSAP	Thermosensitive Alkaline Phosphatase
TSFM	Mitochondrial Elongation Factor Ts
TYMP	Thymidine Phosphorylase
TWNK	Twinkle
UNG1	Uracil DNA Glycosylase 1
USH3B	Usher Syndrome Type 3B
V	Volts
VCF	Variant Call Format
VDAC	Voltage-Dependent Anion Channel
VSD	Ventricular Septic Defect
VUS	Variant of Unknown Significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
XMP	Xanthosine Monophosphate

Chapter 1. Introduction

1.1 Overview

Here, an overview of mitochondria functions and mitochondrial disease is discussed, which provides the backdrop to all studies in this thesis. Unless stated, this overview relates only to mammalian mitochondria. Additional pertinent background is also discussed in relevant chapters, which are indicated.

1.2 Mitochondria

Mitochondria are dynamic, intracellular double-membrane organelles found in almost all eukaryotic cells and are involved in a wide array of roles for maintaining cellular homeostasis. Derived from the Greek Latin '*mitos*' for 'thread' and '*chondrion*' meaning 'small grain', the major function of mitochondria is the production of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) (Hatefi, 1985). Nonetheless, mitochondria are also critical for regulating calcium (Ca^{2+}) homeostasis, apoptosis (Wang and Youle, 2009), iron-sulphur (Fe-S) cluster biogenesis (Rouault, 2012) and the tricarboxylic acid (TCA) cycle (Akram, 2014). Intriguingly, the mitochondrion is the only organelle other than the nucleus to possess its own genome (mtDNA), which encodes essential OXPHOS proteins, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) for mitochondrial function. However, the vast majority of mitochondrial proteins, approximately 1,200 (Lopez *et al.*, 2000; Calvo *et al.*, 2006), are now encoded by the nuclear genome with only 13 proteins encoded by mtDNA remaining (Anderson *et al.*, 1981).

1.2.1 Origins and Evolution of Mitochondria

Alternative theories have been proposed to explain the origins of mitochondria in eukaryotic cells (Martin *et al.*, 2015). The most widely accepted model is endosymbiotic theory in which mitochondria are believed to originate from free-living Eubacteria; the host cell received ATP and the Eubacterium benefited from a metabolite rich environment. Margulis (1971) was first to propose that mitochondria originated from Eubacteria incorporated into primitive eukaryotic cells in an independent endosymbiosis event that followed the formation of the nucleus from an Archaebacterium, with these two successive events referred to as the 'serial endosymbiosis' theory. Martin and Muller (1998) proposed an alternative hypothesis in which

both the nucleus and mitochondria were formed from the acquisition of a hydrogen-dependent Eubacteria by a hydrogen generating Archaebacterium, known as the 'hydrogen hypothesis'. Nonetheless, both theories propose that endosymbiosis took place and that the majority of genes were transferred from the Eubacterium to the host genome. These theories are supported by the shared characteristics between the mitochondrion and the primitive Eubacterium, particularly the evolutionary conservation between bacterial and mitochondrial respiratory chain (RC) complexes (Gray *et al.*, 1999).

1.2.2 Structure

Mitochondria are frequently depicted as autonomous ovoid or rod structures in textbooks and educational material. Indeed, the first electron micrograph (EM) images of the mitochondrion showed an ovoid structure measuring 1-2μm in length and 0.5-1μm in diameter, comprising a folded inner membrane with ridges, termed 'cristae', enclosed in an outer membrane (Palade, 1952) (**Figure 1.1A**).

The mitochondrion is comprised of five compartments; the outer mitochondrial membrane (OMM), the intermembrane space, the inner mitochondrial membrane (IMM), the matrix and cristae; highly folded invaginations of the IMM (**Figure 1.1B**).

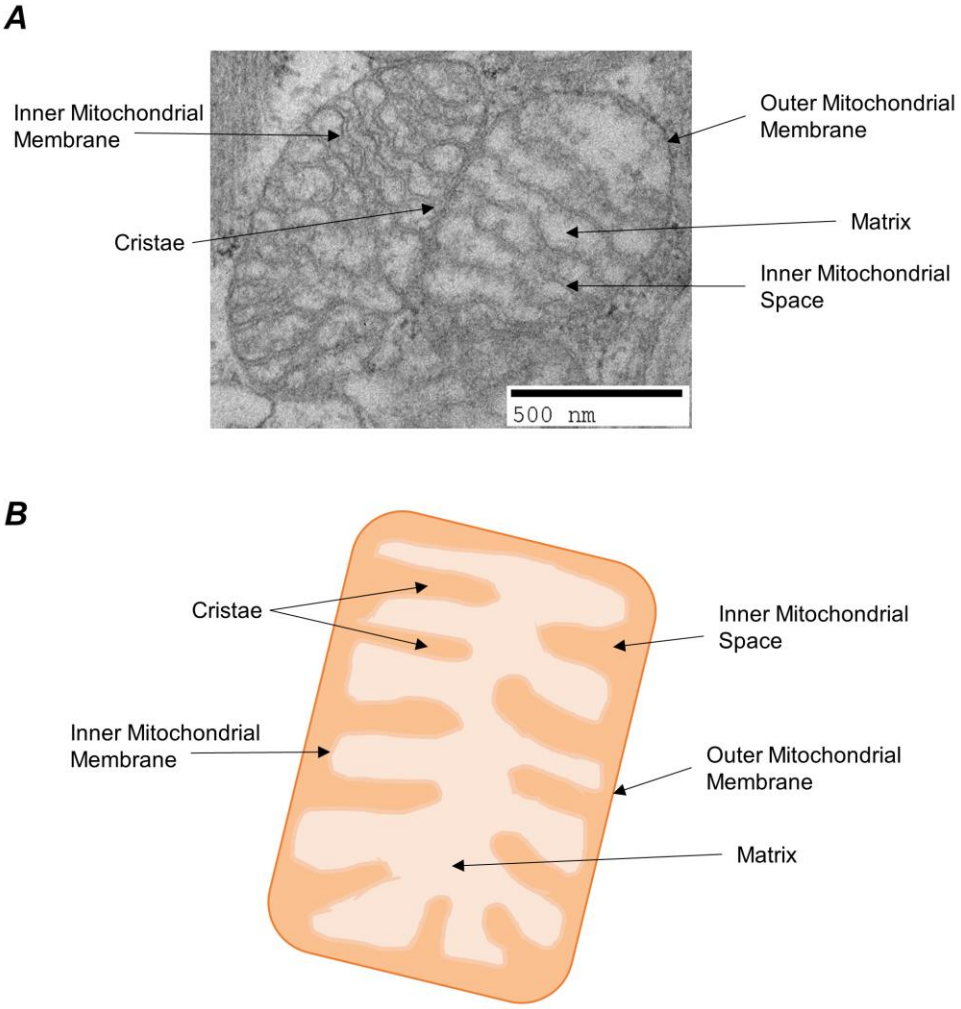


Figure 1.1 Mitochondrial Structure. (*A*) Electron micrograph (EM) image of two skeletal muscle mitochondria with aligned cristae. (*B*) Schematic of the mitochondrion showing key structures and compartments. Scale bar - 500 nanometers (nm). EM figure courtesy of Amy Vincent (unpublished).

The OMM is a highly porous membrane enclosing the organelle that contains membrane protein families that allow mitochondria to exchange molecules between the cytosol and communicate with the cellular environment. Voltage-dependent anion channels (VDACs) are highly abundant in the OMM with approximately 10,000 channels per mitochondrion, which allow small molecules up to 10 kilodaltons (kDa) to enter and exit the intermembrane space (Bayrhuber *et al.*, 2008; Shoshan-Barmatz *et al.*, 2010).

The IMM is composed of two distinct layers connected at cristae junctions by tubular structures; the inner boundary membrane (IBM) and the cristae membrane (CM) (Frey and Mannella, 2000). Due to the substantial surface area of folded cristae invaginations, the CM is enriched in the multi-subunit OXPHOS enzyme complexes for ATP generation. In contrast to the OMM, the IMM is permeable only to NH_3, H_2O, O_2 and CO_2 due to a protein rich lipid bilayer, thus separating the intermembrane space from the matrix. Hence, the import of small

molecules and proteins to the matrix requires transporter proteins. The translocase of the outer membrane (TOM) and sorting and assembly machinery (SAM) complexes allow the import of nuclear encoded mitochondrial proteins across the OMM (Hill *et al.*, 1998; Wiedemann *et al.*, 2003). Translocase of the inner membrane (TIM) forms a supercomplex with TOM to allow import of proteins with specific N-terminal mitochondrial targeting sequences (MTSs) (Bauer *et al.*, 2000), which are cleaved and processed further in the matrix (Hawlitschek *et al.*, 1988; Pfanner *et al.*, 1988). The IMM also contains additional transporter proteins for exchange of ATP, ADP and ions between the cytosol and matrix, including the abundant ADP/ATP carrier also referred to as adenosine nucleotide translocator 1 (ANT1) (Klingenberg, 2008).

The majority of mitochondrial processes occur in the mitochondrial matrix. It contains multiple copies of the mitochondrial genome and the essential machinery for mtDNA transcription, replication and translation of mitochondrial RC complex subunits, mt-tRNAs and rRNAs. The matrix is also the site of the TCA cycle, Fe-S cluster biogenesis and β-oxidation of fatty acids. Due to the alkali pH of 7.9 in the matrix, a transmembrane electrochemical gradient is generated that powers ATP synthesis (Llopis *et al.*, 1998).

1.2.3 Dynamics

Advances in imaging techniques have revealed that mitochondria form a dynamic network maintained by the continual processes of mitochondrial fusion, fission and turnover that regulate mitochondrial homeostasis, communication with the cytosol, morphology and content exchange between mitochondria (Chen and Chan, 2009) (**Figure 1.2**).

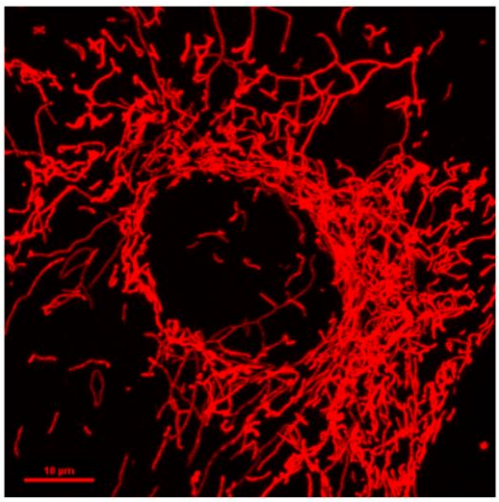


Figure 1.2 Mitochondrial Dynamic Network. Tetramethylrhodamine Methyl Ester (TMRM) staining and confocal microscopy imaging depicting the mitochondrial network in a single fibroblast cell (unpublished). Scale bar - 10µm.

1.2.3.1 Fusion

Mitochondrial fusion is dependent on the large dynamin-related GTPases mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy protein 1 (OPA1), allowing mixing of mitochondria content (**Figure 1.3**). MFN1/2 are surface exposed proteins in the OMM that interact with between mitofusins of adjacent mitochondria via a heptad repeat region (HR2), which acts as a tether to fuse together mitochondria (Chen *et al.*, 2003; Koshiba *et al.*, 2004). OPA1 is present in IMM on the side of the intermembrane space and in partnership with MFN1 (Cipolat *et al.*, 2004), coordinating fusion of the IMM (Olichon *et al.*, 2002; Ishihara *et al.*, 2006).

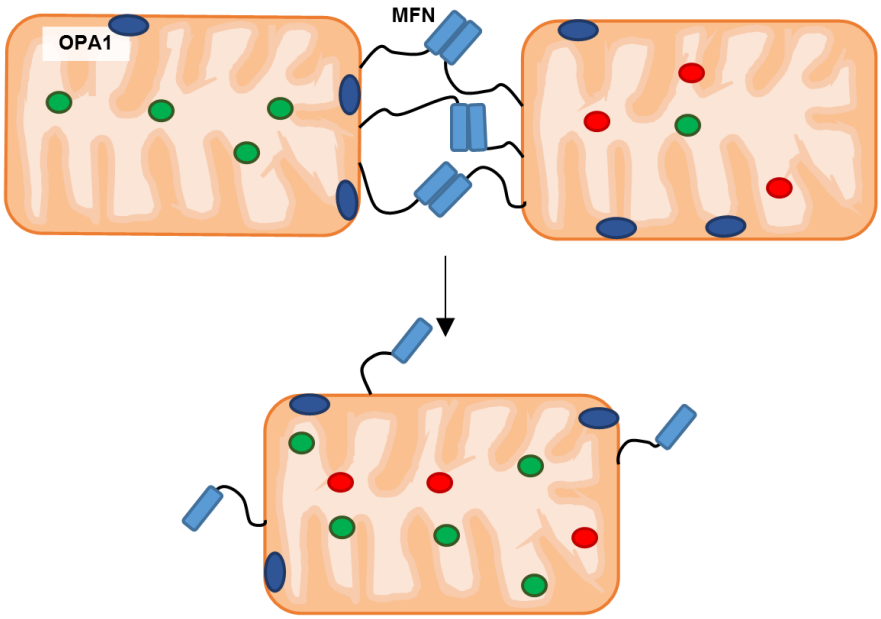


Figure 1.3 Mitochondrial Fusion. OPA1 coordinates fusion of the IMM. MFN1/2 interact with mitofusins of adjacent mitochondria via a HR2 as a tether for fusion. This allows mixing of mitochondrial contents (red and green).

1.2.3.2 Fission

Mitochondrial fission is dependent on dynamin-related protein 1 (Drp1), a predominantly cytosolic protein recruited to the OMM where it oligomerises into spirals around the mitochondrion, constricting and separating it into two organelles (**Figure 1.4**) (Ingerman *et al.*, 2005; Elgass *et al.*, 2013). Recruitment of Drp1 to the OMM is mediated by Fis1 in yeast (Okamoto and Shaw, 2005). However, knockout of Fis1 in mammalians prevents mitochondrial fission but does not alter recruitment of Drp1 to the OMM, suggesting that it has a minor role in mammalian mitochondrial fission (Lee *et al.*, 2004). Drp1 also interacts with mitochondrial fission factor (MFF), mitochondrial dynamics protein of 49 kDa (MID49)

and 51 kDa (MID51) (Loson *et al.*, 2013), which may be more essential for Drp1 recruitment in mammalians than in yeast. A recently characterised component of fission, septin 2 (SEPT2) (Pagliuso *et al.*, 2016), is discussed in **4.5.5.2**.

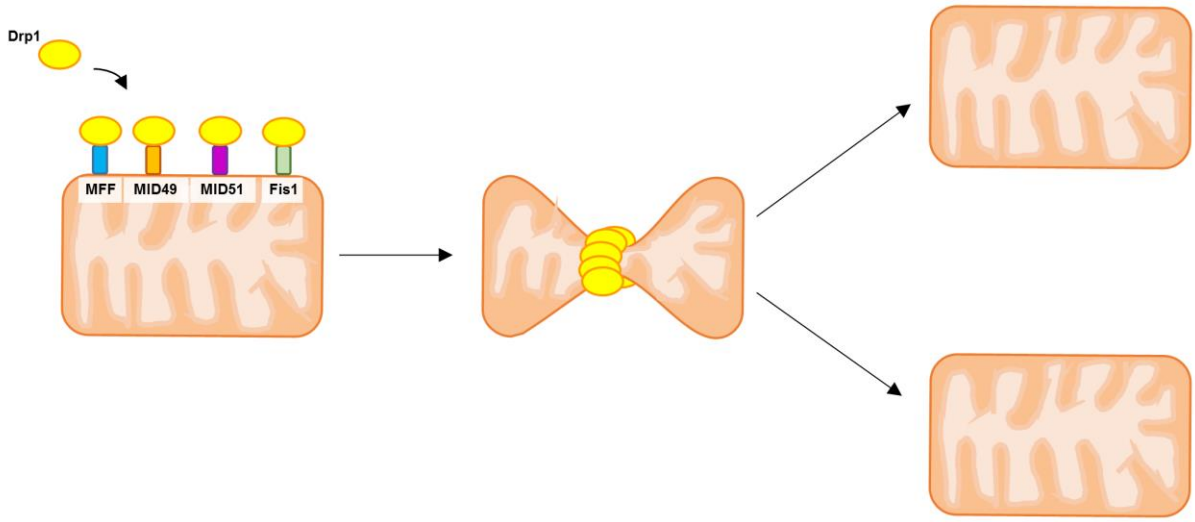


Figure 1.4 Mitochondrial Fission. Drp1 is recruited to the OMM where it oligomerises into spirals around the mitochondrion to constrict and separate it into two organelles. Recruitment is potentially mediated by MFF, MID49, MID51 or Fis1.

1.3 Mitochondrial Respiration and the Electron Transport Chain (ETC)

1.3.1 TCA Cycle and Initiation of ATP Production

The major role of mitochondria is the production of ATP via OXPHOS (Hatefi, 1985). This is initiated in the cytosol by the conversion of glucose to pyruvate in the final step of glycolysis. Glycolysis is summarised in the following equation:

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{P}_\text{i} \longrightarrow 2\text{Pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O}$$

Pyruvate is the main provider of carbon for the TCA cycle; a closed-loop cycle and key driver of cellular respiration. Pyruvate is transported from the intermembrane space to the matrix through the mitochondrial pyruvate carrier (MPC) (Bricker *et al.*, 2012). Next, pyruvate is irreversibly converted to acetyl Co-enzyme A (CoA), known as pyruvate decarboxylation, by the pyruvate dehydrogenase complex (PDHc) (Patel and Korotchkina, 2006). Pyruvate decarboxylation is summarised in the following equation:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{Acetyl CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

Acetyl CoA, NAD^+ and FAD are the major substrates for the TCA cycle (**Figure 1.5**) (Akram, 2014). Overall through eight intermediate redox reactions, acetyl CoA is oxidised to generate CoA, while NAD^+ and FAD are reduced to NADH and $FADH_2$. One cycle also generates one molecule of either ATP or guanosine triphosphate (GTP). The overall TCA cycle reaction is summarised in the following equation:

$$\text{Acetyl CoA} + 3NAD^+ + FAD + ADP + P_i + 2H_2O$$

$$\longrightarrow CoA + 3NADH + 2H^+ + FADH_2 + ATP + 2CO_2$$

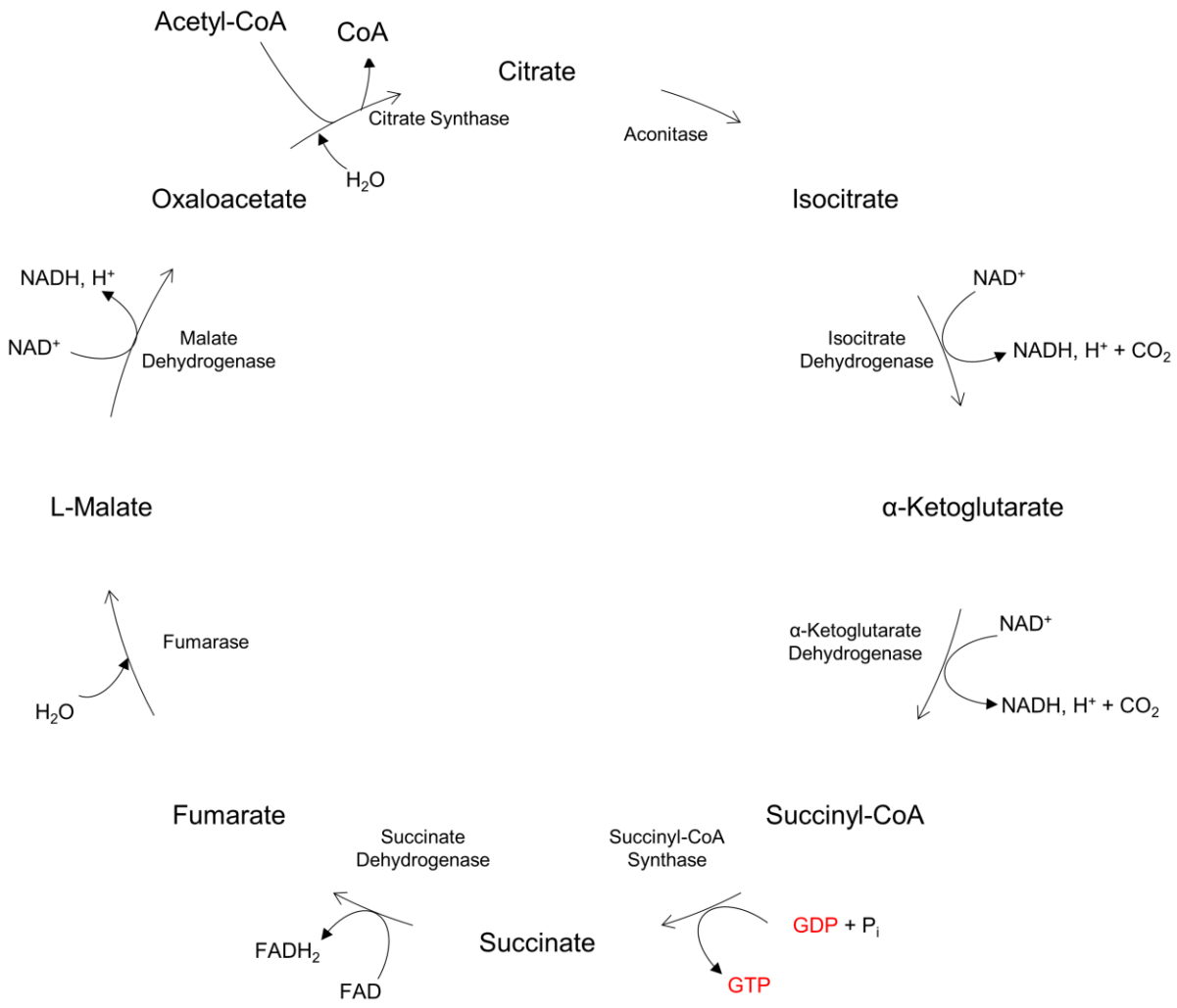


Figure 1.5 TCA Cycle. Schematic of the eight intermediate redox reactions for the conversion of Acetyl CoA to CoA. One molecule of GTP (red) or ATP is generated by transfer of an inorganic phosphate from succinyl-CoA to either GDP or ADP. Despite the generation of one GTP or ATP molecule, the NADH and $FADH_2$ molecules produced are essential electron carriers for the ETC.

The end stage of cellular respiration is OXPHOS for the generation of ATP, performed by four multi-subunit RC enzyme complexes (I-IV) and ATP synthase (V) that comprise the electron transport chain (ETC) (**Figure 1.11**). Despite producing only one ATP or GTP

molecule, the TCA cycle is also indirectly involved in ATP production via the ETC, since generated NADH and $FADH_2$ are critical electron carriers that donate electrons to complex I and II, which are then transferred to complex III and IV. Protons and H^+ released from the matrix into the intermembrane space create an electrochemical gradient, driving the conversion of ADP and P_i to ATP.

1.3.2 Complex I – NADH: Ubiquinone Reductase

Complex I (NADH: ubiquinone reductase) is the first and largest enzyme of the ETC at approximately 1.1 megadaltons (MDa), comprising 45 subunits; seven mtDNA encoded and 38 nuclear encoded (**Figure 1.6**) (Sazanov, 2015; Zhu *et al.*, 2016). Of these, 14 core subunits are evolutionarily conserved between bacteria and humans, which are considered essential yet sufficient for complex I enzyme activity (Hirst, 2011; Vinothkumar *et al.*, 2014), meaning that human mitochondria contain 31 supernumerary nuclear encoded structural subunits. The significance and characterisation of these 31 structural subunits was recently examined by Stroud *et al.* (2016), which demonstrated that 25 subunits were indispensable for assembly of functional complex I and one subunit, NDUFAB1, was required for cell viability. Complex I is also comprised of nine essential co-factors; eight Fe-S clusters and one flavin mononucleotide (FMN) for electron transport (Zhu *et al.*, 2016).

Complex I has an L-shaped conformation with a hydrophilic matrix arm and a hydrophobic membrane arm imbedded in the IMM, which are composed of three modules (N, Q, P) with independent functions. The N-module is located at the periphery of the matrix arm and is where NADH is oxidised to NAD^+, releasing two electrons that are donated to FMN. The Q-module is also located in the matrix arm, containing a chain of eight Fe-S clusters that pass one electron at a time to reduce ubiquinone (Q) to ubiquinol (QH_2), which is transferred to complex III. The P-module is located in the membrane arm and is required for the pumping of four hydrogen protons from the matrix across the IMM to the intermembrane space to create a membrane potential ($\Delta\Psi_m$) necessary to drive ATP synthesis by complex V. The complex I reaction is summarised in the following equation:

$$NADH + Q + 5H^+_{matrix} \longrightarrow NAD^+ + QH_2 + 4H^+_{ims}$$

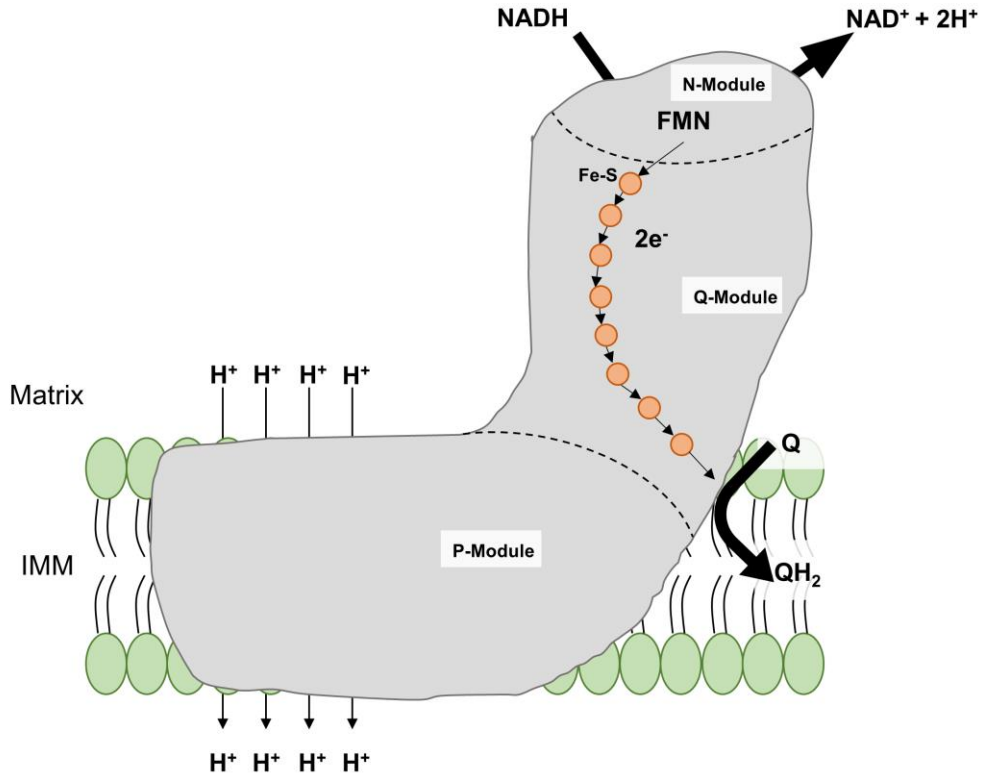


Figure 1.6 Complex I - NADH: Ubiquinone Reductase. Schematic of the complex I structure. This shows the passing of two electrons from the reduction of NADH donated to FMN, which are then passed along a chain of eight Fe-S clusters (brown circles) to reduce Q to QH_2. The N-, Q- and P-modules are distinguished by dashed-lines.

Assembly of complex I is a complex, phased process. Current models (Vogel *et al.*, 2007b; McKenzie and Ryan, 2010) suggest that intermediate modules of approximately 400-500 kDa are formed in the early-stages of assembly, composed of the core NDUFS2, NDUFS3 and the ND (mtDNA encoded) subunits of the P- and Q-modules. This is expanded further with the addition of the remaining structural subunits, with the N-module formed in the late-stages of assembly. Precise assembly of complex I is highly dependent on an increasing number of assembly factors (Heide *et al.*, 2012; Guarani *et al.*, 2014). This includes the mitochondrial complex I assembly (MCIA) complex, which is comprised of assembly factors NDUFAF1, ACAD9, ECSIT, TMEM126B and TIMMDC1 (Vogel *et al.*, 2005; Vogel *et al.*, 2007a; Nouws *et al.*, 2010; Heide *et al.*, 2012; Guarani *et al.*, 2014).

Complex I deficiency is one of the most frequent biochemical manifestations in mitochondrial disease, with isolated deficiency occurring due to mutations of mtDNA or nuclear encoded structural subunits, assembly factors or in combination with deficiency of other mitochondrial proteins (Loeffen *et al.*, 2000). Isolated complex I deficiency is particularly common in early-onset mitochondrial disease, affecting approximately 30% of patients (Kirby *et al.*, 1999). The clinically heterogeneous phenotypes are reflected in the increasing number of nuclear encoded

structural subunits and assembly factor defects that are now associated with the pathology of complex I deficiency. The most frequent presentation is Leigh syndrome, characterised by bilateral symmetrical hyperintensities of the brainstem and basal ganglia on brain MRI, whereby isolated complex I defects account for approximately 35% of all Leigh syndrome patients (Fassone and Rahman, 2012). Isolated complex I defects are also associated with mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (Liolitsa *et al.*, 2003), Leber hereditary optic neuropathy (LHON) (Yu-Wai-Man *et al.*, 2003), leukoencephalopathy (Ferreira *et al.*, 2011b) and neonatal lactic acidosis (Spiegel *et al.*, 2009). Multiple OXPHOS deficiencies that include complex I are predominantly due to nuclear gene defects of mtDNA maintenance, mitochondrial protein synthesis, cofactors of OXPHOS, dynamics and metabolism (Mayr *et al.*, 2015). However, isolated complex I defects have also been noted in patients with mutations of these additional nuclear encoded genes (Fassone and Rahman, 2012).

1.3.3 Complex II – Succinate Dehydrogenase

Uniquely, complex II (succinate dehydrogenase) is a heterotetrameric enzyme comprised of four nuclear encoded subunits only (**Figure 1.7**) (Cecchini, 2003). Complex II is also the only membrane-bound enzyme required for the TCA cycle, oxidising succinate to fumarate (**Figure 1.5**), in addition to participating in the ETC.

Of the four nuclear encoded subunits, hydrophilic 70kDa SDHA and 30kDa SDHB catalytic subunits are located in the matrix, while the hydrophobic SDHC and SDHD subunits ensure that complex II is attached to the IMM. SDHA contains a covalently attached FAD co-enzyme that is reduced to $FADH_2$ following the oxidation of succinate to fumarate. SDHB contains three Fe-S clusters (2Fe-2S, 3Fe-3S, 3Fe-4S), along which two electrons from flavin are transferred to reduce Q to QH_2. There is also a haem *b* group, although it is not clear if this participates in the ETC (Cecchini, 2003). Unlike the other ETC reactions, there is no transfer of protons across the IMM. The complex II reaction is summarised in the following equation:

$$\text{Succinate} + \text{Q} \longrightarrow \text{Fumarate} + \text{QH}_2$$

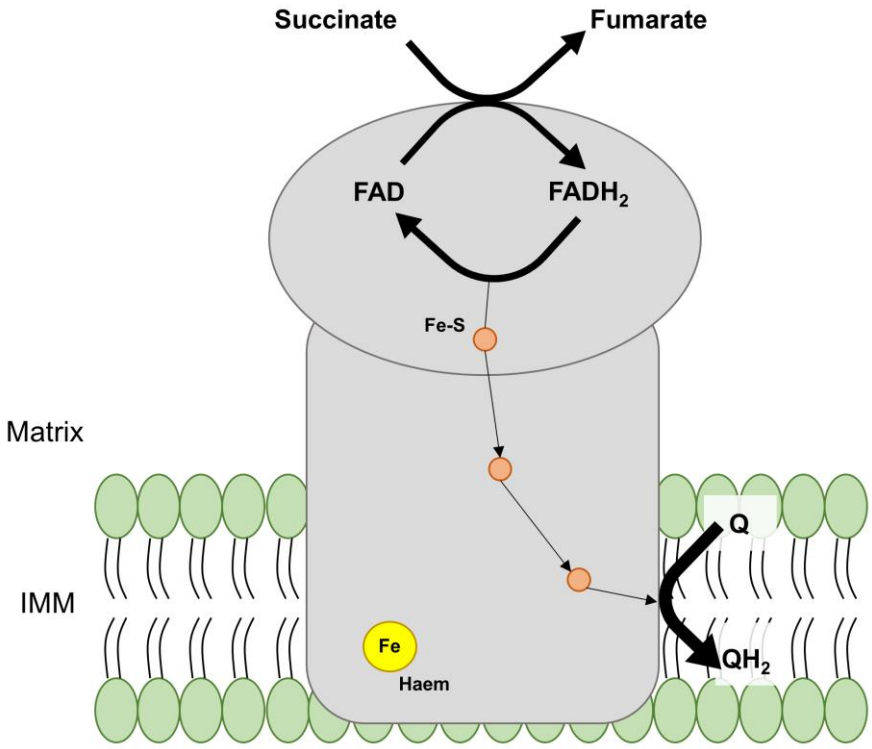


Figure 1.7 Complex II – Succinate Dehydrogenase. Schematic of complex II showing the passing of electrons from flavin along a chain of three Fe-S clusters (brown circles) for the reduction of Q to QH_2. Electrons are donated to FAD following oxidation of succinate to fumarate. However, it is unclear if the haem *b* group (yellow circle) participates in the ETC.

Although the role of the haem *b* group in the mammalian complex II is unclear, studies of *Escherichia coli* suggest a critical role in complex II assembly. In this bacterial model (Lenaz and Genova, 2010), haem *b* group binds to SdhD (SDHD in human nomenclature), which then links it to SdhC (SDHC) and finally provides a link to a complex II SdhA-SdhB (SDHA-SDHB) intermediate module. In humans, two nuclear encoded assembly factors (SDHAF1, SDHAF2) have been identified, but the precise roles in assembly are unclear (Ghezzi and Zeviani, 2012).

Mutations of the nuclear encoded complex II structural subunits and assembly factors have been predominantly associated with autosomal dominant hereditary paraganglioma–pheochromocytomas syndromes, which are tumours of the neuroendocrine tissues or adrenal medulla (Hoekstra and Bayley, 2013). Mitochondrial disease characterised biochemically by isolated complex II deficiency is rare (Hoekstra and Bayley, 2013), due to recessive mutations of *SDHA*, *SDHB*, *SDHD* and *SDHAF1* causing variable phenotypes including leukoencephalopathy and hypertrophic cardiomyopathy (HCM) (Ghezzi *et al.*, 2009; Alston *et al.*, 2012; Alston *et al.*, 2015). *SDHC* and *SDHAF2* are not currently associated with isolated complex II deficiency.

1.3.4 Complex III – Ubiquinol: Cytochrome *c* Reductase

Complex III (ubiquinol: cytochrome *c* reductase) is a homodimeric enzyme composed of 11 subunits; 10 nuclear encoded and one mtDNA encoded (cytochrome *b*) (**Figure 1.8**). It contains a catalytic core that comprises the cytochrome *b* subunit with two haem groups, cytochrome c_1 with one haem group and an Fe-S cluster referred to as the 'Rieske Fe-S cluster' (Xia *et al.*, 1997).

The process of electron transfer with complex III is referred to as the 'Q Cycle' (Mitchell, 1975). QH_2 from complex I binds to the Q_0 site to donate two electrons. One electron is passed through the Rieske Fe-S cluster and cytochrome c_1 where it next reduces one cytochrome *c* (CytC) molecule, which is released from complex III and two hydrogen protons are pumped from the matrix to the intermembrane space. Simultaneously, the second donated electron is cycled through the two haem groups of cytochrome *b*, where it reduces Q to a semi-ubiquinone (Q^-) at a Q_0 site. Binding of another QH_2 at the Q_1 site results in the release of two more electrons; one electron again reduces one CytC molecule that is released, while the second electron passes through the cytochrome *b* haem groups to reduce Q^- further to Q. Hence, two cycles of CytC reduction are necessary, which results in the release of four hydrogen protons from the intermembrane space to the matrix and the uptake of two protons

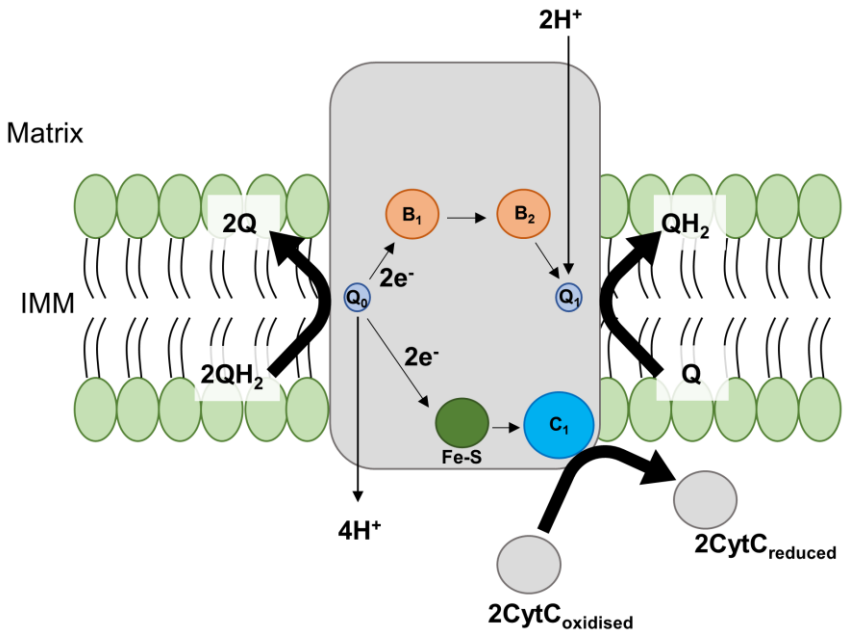


Figure 1.8 Complex III - Ubiquinol: Cytochrome c Reductase. Schematic of complex III showing the transfer of electrons in the process called the 'Q Cycle'. Overall, four electrons are required for the reduction of two cytochrome *c* (CytC) (grey circle) molecules and the pumping of four hydrogen protons from the matrix to the intermembrane space. The Rieske Fe-S cluster (green circle), cytochrome c_1 (blue circle), haem groups (orange circles) and Q_0 and Q_1 sites (small blue circles) are shown.

from the matrix. The complex III reaction is summarised in the following equation:

$$2QH_2 + Q + 2Cytc_{oxidised} + 2H^+_{matrix} \longrightarrow 2Q + QH_2 + 2Cytc_{reduced} + 4H^+_{ims}$$

Isolated complex III deficiency is a rare form of early-onset mitochondrial disease and has been associated with mutations of both structural subunits and assembly factors including *BCS1L* and *TTC19* (Bénit *et al.*, 2009; Tucker *et al.*, 2013; Ardissone *et al.*, 2015a; Dallabona *et al.*, 2016). Mutations of mtDNA encoded *MT-CYB* have been predominantly associated with exercise intolerance (Andreu *et al.*, 1998), but also multisystem disorders including encephalopathy, lactic acidosis, leukoencephalopathy and neurodegeneration (Bénit *et al.*, 2009; Tucker *et al.*, 2013; Ardissone *et al.*, 2015a; Dallabona *et al.*, 2016).

1.3.5 Complex IV – Cytochrome *c* Oxidase

Complex IV (cytochrome *c* oxidase, COX) is the final holoenzyme of the ETC and is a dimer composed of 14 subunits; three essential core mtDNA encoded (MT-COI, MT-COII, MT-COIII) and 11 nuclear encoded (**Figure 1.9**) (Tsukihara *et al.*, 1996). Initially thought to comprise 13 subunits, NDUFA4 was recently identified at the 14th subunit of human COX but not complex I (Balsa *et al.*, 2012), which has been supported by the identification of patients with *NDUFA4* mutations and isolated COX deficiency (Pitceathly *et al.*, 2013).

COX is comprised of two haem groups ($_{a}$ and $_{a3}$) and two copper binding sites (Cu_A and Cu_B). Together, three mtDNA encoded subunits constitute the catalytic core. MT-COI and MT-COII are catalytic subunits with highly conserved domains containing copper binding sites, whereas MT-COIII is the core structural subunit. MT-COI contains both haem groups and the copper binding site Cu_A, while MT-COII contains the copper binding site Cu_B. COX is essential for the reduction of O_2 to H_2O, requiring a total of eight hydrogen protons and four electrons donated from four reduced CytC molecules (Tsukihara *et al.*, 1996; Faxen *et al.*, 2005).

The formation of H_2O and the pumping of four hydrogen protons from the matrix to the intermembrane space occurs in two stages each requiring two electrons (Faxen *et al.*, 2005). Firstly, two electrons are passed sequentially across the electron acceptor Cu_A and haem$_a$ group from two molecules of reduced CytC, which are re-oxidised. At the catalytic binuclear site, one electron reduces haem$_{a3}$ and the second electron reduces Cu_B, establishing a peroxide bridge with O_2 between the haem$_{a3}$ and Cu_B sites. Secondly, a further two electrons are donated from two additional reduced CytC molecules, with the electrons passed through the

Cu_A site and $haem_a$ group to the catalytic binuclear site. Two hydrogen protons each bind to the Cu_B site and $haem_{a3}$ group, breaking the peroxide bridge and reducing the two oxygen atoms to release two H_2O molecules, while the Cu_B site and $haem_{a3}$ group return to their initial oxidised states. Simultaneously, four hydrogen protons are pumped across the IMM from the matrix to the intermembrane space. The complex IV reaction is summarised in the following equation:

$$4Cytc_{reduced} + 8H^{+} + O_2 \longrightarrow 4Cytc_{oxidised} + 2H_2O + 4H^{+}_{ims}$$

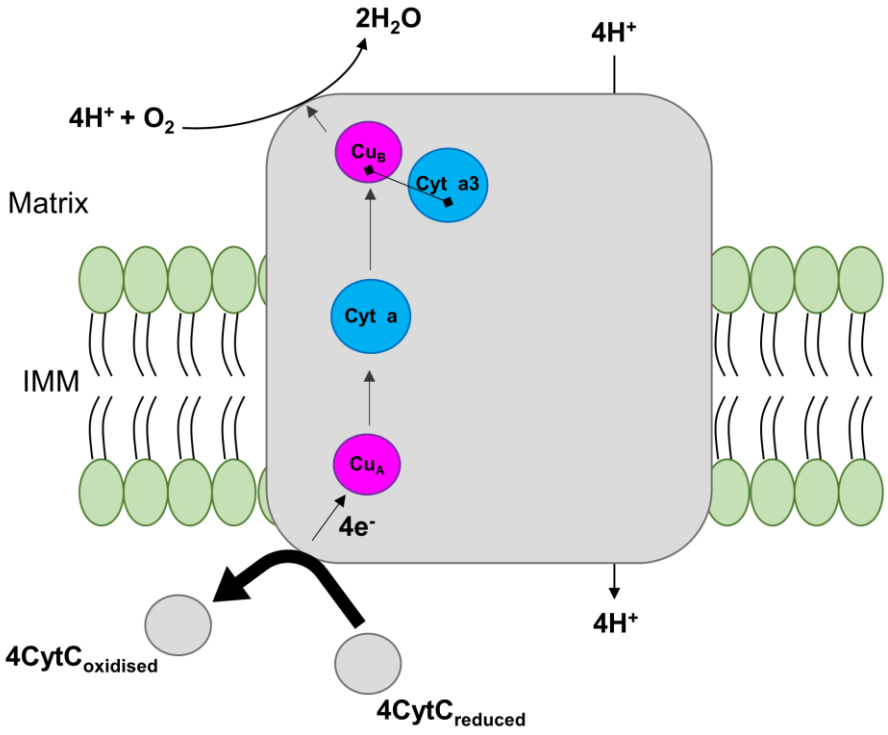


Figure 1.9 Complex IV – Cytochrome *c* Oxidase (COX). Schematic of COX showing the passing of four electrons through Cu_A and $haem_a$ group to the Cu_B-$haem_{a3}$ catalytic binuclear site. Together with four hydrogen protons pumped from the matrix to the intermembrane space and four hydrogen protons binding to the binuclear site, a total of two H_2O molecules are generated.

There are over 20 nuclear encoded ancillary factors currently known to be essential for COX structural assembly and biogenesis, which includes maturation of mtDNA-encoded subunits, and metalation of the copper and haem sites (Kadenbach and Hüttemann, 2015). Most recently, COA6 was identified as a copper chaperone for MT-COII maturation (Stroud *et al.*, 2015). COX assembly occurs in four sequential steps forming intermediate subcomplexes (Nijtmans *et al.*, 1998; Fontanesi *et al.*, 2006; Ghezzi and Zeviani, 2012). Synthesised MT-COI is imbedded in the IMM (S1), followed by incorporation of COX4 and COX5A (S2), MT-COIII, COX5b, COX8 and small nuclear encoded subunits (S3), and finally all remaining

subunits to complete assembly. Insertion of the haem groups and copper binding sites also occur during formation of the intermediate subcomplexes.

Isolated COX deficiency is a common form of mitochondrial disease that predominantly manifests in severe early-onset phenotypes, due to mutations of the three mtDNA encoded subunits or several of the nuclear encoded structural subunits or COX assembly factors (DiMauro *et al.*, 2012). The most common phenotype is Leigh Syndrome, which has been especially associated with recessive mutations of the COX biogenesis factor *SURF1* (Zhu *et al.*, 1998). Mutations of nuclear encoded COX assembly or biogenesis factors comprise a significant proportion of patients with isolated COX deficiency when compared to COX structural subunits, suggesting that mutations of structural subunits are predominantly incompatible with life (DiMauro *et al.*, 2012). Nonetheless, mutations of mtDNA-encoded COX subunits are rare, causing mild and broad late-onset phenotypes (Horváth *et al.*, 2005). Mutations of mt-tRNAs, nuclear encoded mitochondrial protein synthesis or other mitochondrial proteins have been associated with isolated COX deficiency, including *LRPPRC* (Oláhová *et al.*, 2015), *APOPT1* (Melchionda *et al.*, 2014) and mt-tRNA^{Glu} (Horvath *et al.*, 2009). Similar to complex I, COX deficiency with deficiencies other enzymes of the OXPHOS system is common due to nuclear encoded mutations, typically involving combined deficiencies of complexes I and IV, or complexes I, III and IV (Mayr *et al.*, 2015).

1.3.6 Complex V – ATP Synthase

Complex V (ATP synthase) is a ~600kDa dimeric enzyme composed of two regions; a soluble catalytic F_1 region located in the matrix and an F_0 region bound within the IMM (**Figure 1.10**) (Jonckheere *et al.*, 2012). The F_1 section comprises the central stalk and is composed of three copies each of α and β subunits forming a hexamer ring, plus one γ, δ and ε subunits. The F_0 section comprises a ring composed of approximately eight c subunits, plus one a, b, d, A6L and F_6 subunits, of which the b, d and F_6 subunits compose a peripheral stalk. Some of the structural subunits have multiple isoforms encoded by different nuclear genes, such as the α (*ATP5A1*, *ATP5A2*) and β (*ATPAF1*, *ATP5B*) subunits.

ATP synthase catalyses the reversible synthesis of ATP from ADP and inorganic phosphate (P_i) in the F_1 section, driven by the $\Delta\Psi_m$ generated by the ETC. A high $\Delta\Psi_m$ between 150 and 180 mV under normal conditions is favourable to ATP synthesis (Campanella *et al.*, 2009). The $\Delta\Psi_m$ provides energy that causes rotation of the c ring around the central stalk and rotation of the γ, δ and ε subunits in the F_1 section. Hydrogen protons are driven through the

F_0 section past the a subunit to the c ring of the F_0 section. It is the rotation of the γ subunit that permits the synthesis of ATP, in a mechanism known as 'binding change' (Boyer, 1975). Rotation causes alternating conformational changes to the nucleotide binding sites of the three catalytic β subunits, which are either 'open', 'loose' or 'tight' for ADP and P_i binding. Through rotation of the γ subunit and the conformation switches, the ADP and P_i molecules are brought 'tight' together to synthesise ATP.

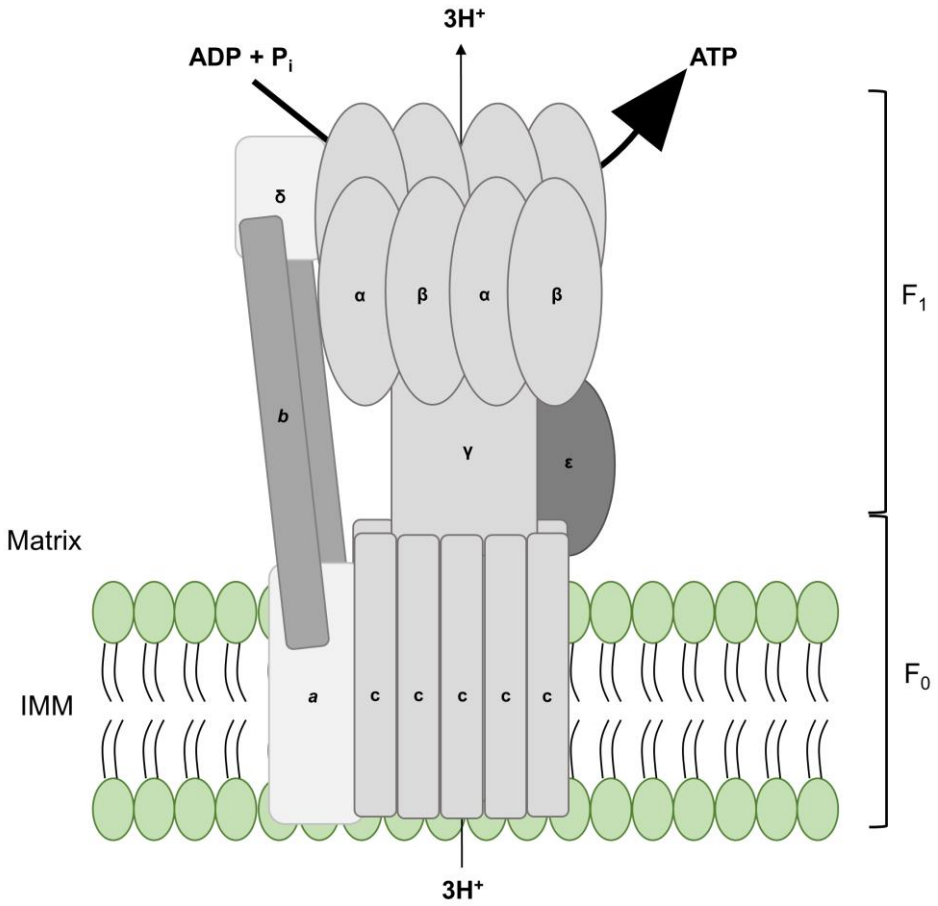
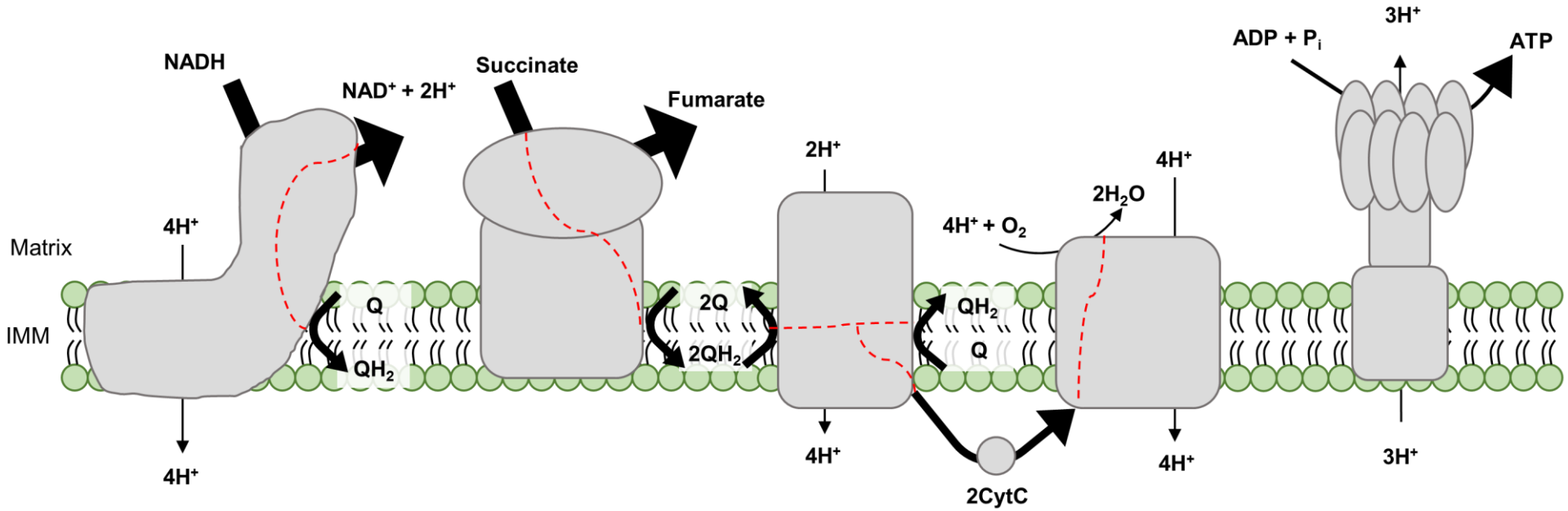


Figure 1.10 Complex V – ATP Synthase. Schematic of complex V showing the structural subunits that comprise the F_0 and F_1 sections. 'Rotary catalysis' of the γ subunit causes conformational changes of the β subunit – 'open', 'loose' and 'tight' – that permits the synthesis of ATP from ADP and P_i.

Assembly of the ATP synthase monomer has been well characterised in yeast (Wagner *et al.*, 2010; Rak *et al.*, 2011), although assembly is known to occur in step-wise stages in both yeast and mammalian mitochondria (Ruhle and Leister, 2015). The F_0 and F_1 sections are assembled independently followed by linkage, with the assistance of assembly factors ATP11 and ATP12 in mammalians. It is also known that a and A6L are the last two subunits to be incorporated to the assembled monomer (Wittig *et al.*, 2010).

Isolated complex V defects are a rare form of mitochondrial disease. Nonetheless, mutations of the mtDNA encoded subunit *MT-ATP6* are a common cause of Leigh syndrome (Lake *et al.*, 2016), which includes the recurrent m.8993T>G mutation (Santorelli *et al.*, 1993). Less common *MT-ATP6* phenotypes include motor neuron syndrome (Brum *et al.*, 2014) and adult-onset spinocerebellar ataxia (Pfeffer *et al.*, 2012). Mutations in the second mtDNA encoded structural subunit *MT-ATP8* have also been described (Jonckheere *et al.*, 2008), as have mutations in the nuclear encoded structural subunits α (*ATP5A1*) (Jonckheere *et al.*, 2013) and ε (*ATP5E*) (Mayr *et al.*, 2010), and two assembly factors (*ATP12*, *TMEM70*) (De Meirleir *et al.*, 2004; Cizkova *et al.*, 2008).



	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA Encoded	7	0	1	3	2
Nuclear Encoded	38	4	10	11	14

Figure 1.11 Electron Transport Chain (ETC) Multi-Subunit Complexes. The multi-subunit complexes that comprise the ETC are located within the IMM. The dashed red line denotes the pathways of electrons once entered the ETC. The numbers below each complex show the number of mtDNA and nuclear encoded subunits. CytC – cytochrome *c*; Q – ubiquinone; QH_2 – ubiquinol.

1.3.7 Supercomplexes (SC)

The ETC is frequently depicted as free-moving, isolated RC enzyme complexes that are linked together by the electrons carriers Q and CytC, known as the 'fluid model' (**Figure 1.11**) (Hackenbrock *et al.*, 1986). On the contrary, structural analyses suggest that the RC enzymes are dynamic, forming 'supercomplexes' (SCs) together with free-moving isolated RC enzymes in the IMM, known as the 'plasticity model' (Acin-Perez and Enriquez, 2014). This includes a so-called 'respirasome' (I+III+IV) and two SCs (I+III; III+IV), together with the free-moving isolated RC enzymes. Complex II does not form SCs with other RC enzymes, while active ATP synthase remains in a dimer structure. Evidence for SCs has been provided by blue-native polyacrylamide gel electrophoresis, emphasised by the identification of the respirasome comprising complex I, III and approximately four units of complex IV (Schagger and Pfeiffer, 2000). Electron cryo-microscopy has also been pivotal, used to establish the structure of the respirasome (Althoff *et al.*, 2011). In fact, complex I has been shown to be unstable in the absence of complex III or IV (Acin-Perez *et al.*, 2004; Diaz *et al.*, 2006; Vempati *et al.*, 2009). Loss of several complex I structural subunits also disrupts formation of complex I SCs (Stroud *et al.*, 2016). However, the precise roles of SCs have not been elucidated, although Lapuente-Brun *et al.* (2013) demonstrated the presence of two distinct pools of Q for electron supply in the I+III SC; one derived from the oxidation of NADH and the second from $FADH_2$. Furthermore, the assembly and binding of the individual complexes to form the SCs is not fully understood. Cardiolipins that compose a high proportion of the IMM membrane are thought to have a crucial role in SC assembly, as demonstrated for assembly of complexes III+IV in yeast (Bazan *et al.*, 2013). Most recently, *in silico* modelling of complex III+IV from bovine heart mitochondria suggested that cardiolipin uses binding sites on the surface of both RC enzymes to guide and assemble the complexes (Arnarez *et al.*, 2016).

1.4 Mitochondrial Functions

1.4.1 Generation of Reactive Oxidative Species (ROS)

Approximately 1-5% of O_2 consumed during OXPHOS is converted into reactive oxidative species (ROS), mainly at complexes I and III, that induce oxidative stress in mitochondria, including super oxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (Orrenius, 2007). Mitochondria have several antioxidant enzymes that catalyse the removal of ROS such as superoxide dimutase 1 (SOD1) (Barber *et al.*, 2006), glutathione peroxidase 1

(GPx1) (Handy *et al.*, 2009) and catalases (Chelikani *et al.*, 2004). Despite this, approximately 1% of ROS eludes enzymatic removal (Shoshan-Barmatz *et al.*, 2010). Mitochondria are highly susceptible to oxidative stress due to ROS, particularly mtDNA due to its close proximity to the ETC. ROS are also capable of damaging lipids of the mitochondrial membranes, oxidising macromolecules and releasing CytC plus additional pro-apoptotic proteins to the cytosol (Ott *et al.*, 2007). In spite of their toxicity, ROS are also involved in intracellular signalling pathways including apoptosis, differentiation and cell growth (Ott *et al.*, 2007).

1.4.2 Fe-S Cluster Biogenesis

Fe-S clusters are abundant inorganic co-factors that are essential for a wide range of cellular processes (Rouault, 2012). Fe-S clusters and the machinery for biogenesis are conserved in almost all organisms, highlighting their crucial role for even the most primitive of life. Mammalian Fe-S clusters are typically present in rhombic (Fe_2S_2) or cubic (Fe_4S_4) structures that are normally non-covalently bound to cysteine or less frequently to histidine protein residues (Beilschmidt and Puccio, 2014). Fe-S clusters are essential components of the OXPHOS system for accepting and donating electrons, particularly in complexes I, II and III (Stehling *et al.*, 2009). Indeed, electron transfer in complex I is dependent on a chain of eight Fe-S clusters (Sazanov, 2015). Fe-S clusters are also involved in the bindings of substrates for mitochondrial enzymatic reactions, notably to aconitase for the conversion of citrate to isocitrate, plus succinate dehydrogenase for the conversion of succinate to fumarate, with both reactions occurring in the TCA cycle (**Figure 1.5**) (Akram, 2014).

Fe-S biogenesis begins with the transport of reduced iron (Fe^{2+}) into the matrix by mitoferrins (Paradkar *et al.*, 2009). Cysteine desulfurase (NFS1) and its co-factor pyridoxal phosphate supply sulphur from cysteine, while iron-sulphur cluster assembly enzyme (ISCU) provides a backbone structure upon which the cluster can be synthesised from the reduced Fe^{2+} and inorganic sulphur (Lill, 2009; Rouault, 2012). Stability of NFS1 is also dependent upon on LYRM4 (ISD11) (Shi *et al.*, 2009). In bacteria, the basic machinery for Fe-S cluster biogenesis are encoded by a single *Isc* operon (Zheng *et al.*, 1998). However, following formation it is not yet fully understood how clusters are exported to the cytosol or how clusters are targeted and are incorporated into proteins (Rouault, 2012; Beilschmidt and Puccio, 2014).

1.4.3 Ca^{2+} Homeostasis

Mitochondria are important Ca^{2+} storage vessels essential for the intracellular homeostasis of Ca^{2+} (Newmeyer and Ferguson-Miller, 2003), channelling Ca^{2+} to and from the cytosol and endoplasmic reticulum (ER) (De Vos *et al.*, 2012), ATP production (Jouaville *et al.*, 1999) and apoptosis (Giorgi *et al.*, 2012). Ca^{2+} is also vital in the TCA cycle for activation of pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate (Denton, 2009). Thus, it is through control of the TCA cycle that Ca^{2+} regulates ATP production to meet the intracellular needs (Jouaville *et al.*, 1999).

Ca^{2+} is imported across the OMM into the intermembrane space via VDAC (Bayrhuber *et al.*, 2008; Shoshan-Barmatz *et al.*, 2010). Ca^{2+} is predominantly transported via a highly selective mitochondrial Ca^{2+} uniporter (MCU) located in the IMM, which is driven by the proton gradient but is independent of ATP synthesis or transport of other ions (Kirichok *et al.*, 2004). Additional IMM Ca^{2+} transporters for uptake and release have also been proposed, principally Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} exchange channel proteins, which may include leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) (Shao *et al.*, 2016).

1.4.4 Apoptosis

Apoptosis, also known as programmed cell death, is a controlled biochemical process essential for maintaining the cell population and the degradation of damaged cells. Apoptosis can be induced by damage from ROS, UV damage or starvation, which triggers CytC release from the matrix to the cytosol (Liu *et al.*, 1996; Ott *et al.*, 2007; Wang and Youle, 2009). Key to the release of CytC from mitochondria and activation of apoptosis pathways are the Bcl-2 proteins Bak and Bax (Cory and Adams, 2002). Under healthy conditions, Bax is localised to the cytosol, whereas Bak is associated with mitochondria. When cells are damaged, Bax translocates from the cytosol to the OMM (Hsu *et al.*, 1997; Wolter *et al.*, 1997), forming oligomers (Antonsson *et al.*, 2001) and interacts with another Bcl-2 protein, tBid, to induce permealisation of the membrane (Lovell *et al.*, 2008). Simultaneously, Bak continues to associate with mitochondria, but undergoes conformational changes to form oligomers (Griffiths *et al.*, 1999). The release of CytC from the matrix to the cytosol is critical to activation of the caspases, which are essential protease enzymes for controlled degradation of proteins during apoptosis (McIlwain *et al.*, 2013).

1.5 The Mitochondrial Genome

1.5.1 Structure and Organisation

Uniquely, the mitochondrion is the only organelle of eukaryotic cells except for the nucleus to have its own genome. Human mitochondrial DNA (mtDNA) is a compact, circular ~16,569 basepair (bp) double-stranded DNA molecule with 2-10 copies present in each mitochondrion (**Figure 1.12**), thus it is a multi-copy number genome in eukaryote cells. Initially sequenced by Anderson *et al.* (1981), the human mitochondrial genome sequence was revised by Andrews *et al.* (1999) and is referred to as the Revised Cambridge Reference Sequence (rCRS). The current GenBank accession number is NC_012920.

Throughout the evolution of mitochondria, almost all genes from the ancient Eubacterium have been lost or transferred to the nuclear genome. Hence, human mtDNA now has only 37 genes, encoding 13 proteins that comprise essential core subunits of complex I, III, IV and V. Also encoded are 22 mitochondrial transfer RNAs (mt-tRNAs) and two ribosomal RNAs

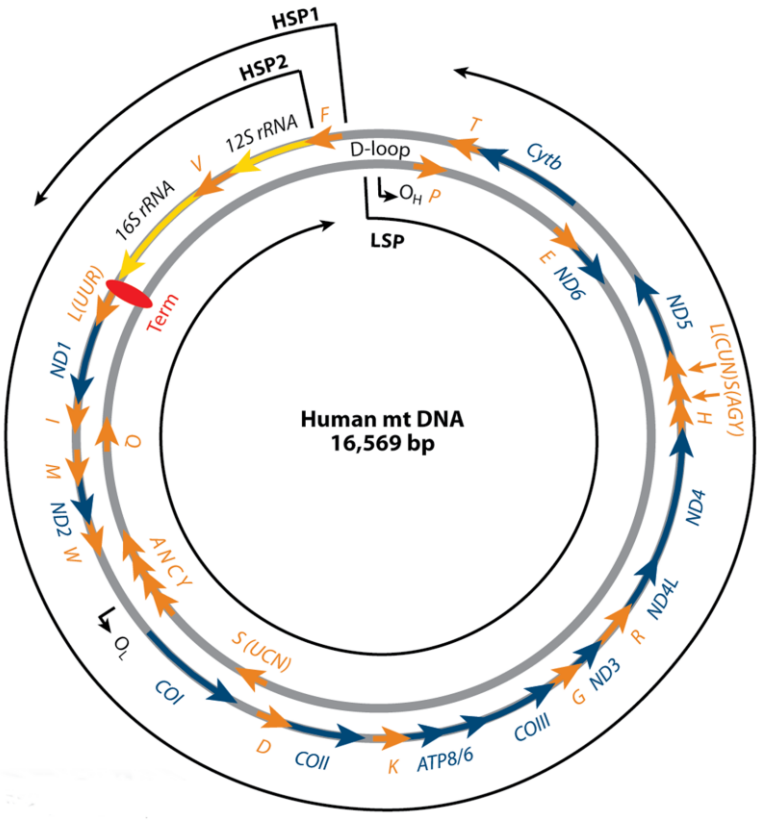


Figure 1.12 Human Mitochondrial Genome. Schematic representation of the human mitochondrial genome showing the H- and L-strands. Protein coding (blue), rRNA (yellow) and mt-tRNA (orange) genes are noted. 'Term' denotes the terminator of the short HSP1 transcript. Arrows show the direction of transcription. O_H and O_L denote the origins of H-strand and L-strand replication. Adopted from Suzuki *et al.* (2011).

(rRNAs) (12S and 16S) for mitochondrial protein synthesis. Since all remaining mitochondrial proteins are encoded in the nucleus and imported, mitochondria are therefore dependent upon the close genetic coordination between the nuclear and mitochondrial genomes.

The double-stranded mitochondrial genome comprises an outer guanine-rich heavy (H-) strand and a cysteine-rich inner light- (L-) strand (Anderson *et al.*, 1981). Unlike the nuclear genome, there are no introns within mtDNA genes, nor are there intergenic regions between each of the encoded genes. The major exception of the human mtDNA is a 1.1kb triple-stranded displacement loop (D-loop) (Kasamatsu *et al.*, 1971). This contains the origin of H-strand replication (O_H), one H-strand promoter (HSP1), the L-strand promoter (LSP), binding sites for mitochondrial transcription factor A (TFAM) and three conserved sequence blocks (CSB1, CSB2, CSB3). A second H-strand promoter (HSP2) is located near the 5' end of the *MT-RNR1* gene, while the origin of L-strand replication (O_L) is located between the *MT-TC* and *MT-TN* genes (Montoya *et al.*, 1982).

Unlike the nuclear genome which is organised in regular nucleosomal array structures (Kornberg, 1974), mtDNA is packaged throughout the mitochondrial network in compact, spherical nucleoprotein complexes called the 'nucleoids' containing one or more copies of mtDNA (Garrido *et al.*, 2003; Bogenhagen, 2012). There are a number of proteins associating with the nucleoids that are involved in mitochondrial transcription, replication and translation. A central component of mammalian nucleoids is TFAM (Garrido *et al.*, 2003; Wang and Bogenhagen, 2006; Kaufman *et al.*, 2007). Also co-localising with the nucleoids are Lon protease 1 (LONP1) (Lu *et al.*, 2007), DNA polymerase gamma subunit 1 (POLG1, POLγA) (Bogenhagen *et al.*, 2008), the mitochondrial RNA polymerase (POLRMT) (Bogenhagen *et al.*, 2008), the twinkle helicase (TWNK) (Garrido *et al.*, 2003; Rajala *et al.*, 2014) and mitochondrial single-stranded binding protein (mt-SSB) (Wang and Bogenhagen, 2006; Bogenhagen *et al.*, 2008; Rajala *et al.*, 2014).

1.5.2 Maternal Inheritance

In contrast to nuclear DNA, the mammalian mitochondrial genome is strictly inherited through the maternal lineage (Hutchison *et al.*, 1974; Giles *et al.*, 1980). However, there is one apparent instance, a male patient who harboured a pathogenic 2-bp deletion in *MT-ND2*, where mtDNA was apparently inherited paternally (Schwartz and Vissing, 2002). The mechanisms for elimination of the paternal mtDNA in mammalians are not fully understood

(Sato and Sato, 2013). Currently, the prevailing hypothesis is that mammalian paternal mitochondria are tagged for ubiquitin-mediated degradation and are eliminated before the four-cell stage (Sutovsky *et al.*, 1999).

1.5.3 mtDNA Haplogroups

The mitochondrial genome has a mutation rate approximately 10- to 17-fold faster than the nuclear genome (Brown *et al.*, 1979), due to the close proximity of the nucleoids to the ROS generated from ETC. As a consequence, a significant proportion of neutral or benign human mtDNA variants that have occurred sporadically. Since mtDNA is maternally inherited, these polymorphisms can be grouped and subject to phylogenetic analysis, allowing the tracking of human migration out of Sub-Saharan Africa (Kivisild, 2015). Thus, these mtDNA 'haplogroups' have been assigned alphabetical labels to denote different geographical populations (Torroni *et al.*, 1993; Torroni *et al.*, 1994).

1.5.4 Mitochondrial Transcription

Transcription of the mitochondrial genome occurs bi-directionally and is initiated at the three promoter regions, two H-strand promoters (HSP1, HSP2) and one L-strand promoter (LSP). Transcription from the HSP2 and LSP promoters produce near-genome length polycistronic transcripts, covering almost all of the coding regions on the L-strand and H-strand. On the other hand, the HSP1 promoter generates a short polycistronic transcript that encompasses the two rRNAs (12S and 16S) and two mt-tRNA (mt-tRNA^{Phe} and mt-tRNA^{Val}).

In vitro studies have shown that three proteins are essential for the initiation of transcription, forming the mitochondrial transcription initiation complex; POLRMT, TFAM and mitochondrial transcription factor B2 (TFB2M) (Falkenberg *et al.*, 2002; Shi *et al.*, 2012; Yakubovskaya *et al.*, 2014). A second transcription factor, TFB1M, was also initially thought to participate in transcription (Falkenberg *et al.*, 2002; McCulloch and Shadel, 2003), but this is no longer the case (Metodiev *et al.*, 2009). Transcription of mtDNA is performed by a single-subunit of POLRMT, which is unable to transcribe nuclear DNA (Kuhl *et al.*, 2014). POLRMT contains an N-terminal extension (NTE) with an unclear role in transcription, although it could act as a transcription repressor (Gustafsson *et al.*, 2016).

Initiation of transcription (**Figure 1.13**) begins with the binding of TFAM approximately 10-15bp upstream of the promoter site, which introduces a 180^{O}C bend in mtDNA (Gaspari *et al.*, 2004; Shi *et al.*, 2012). This conformational change allows TFAM to coordinate the

recruitment of POLRMT, which binds to the promoter region, upstream bases and TFAM. POLRMT itself undergoes a change in conformation, which allows TFB2M binding to the pre-initiation complex, thus generating the fully assembled initiation complex that encompasses the promoter region (Yakubovskaya *et al.*, 2014). An additional protein, mitochondrial transcription elongation factor (TEFM) has been shown to interact with POLRMT for transcribing longer sections of RNA and is indispensable for transcription (Minczuk *et al.*, 2011). However, it is not clear whether TEFM is a putative second subunit of POLRMT or if it is an elongation accessory subunit (Gustafsson *et al.*, 2016).

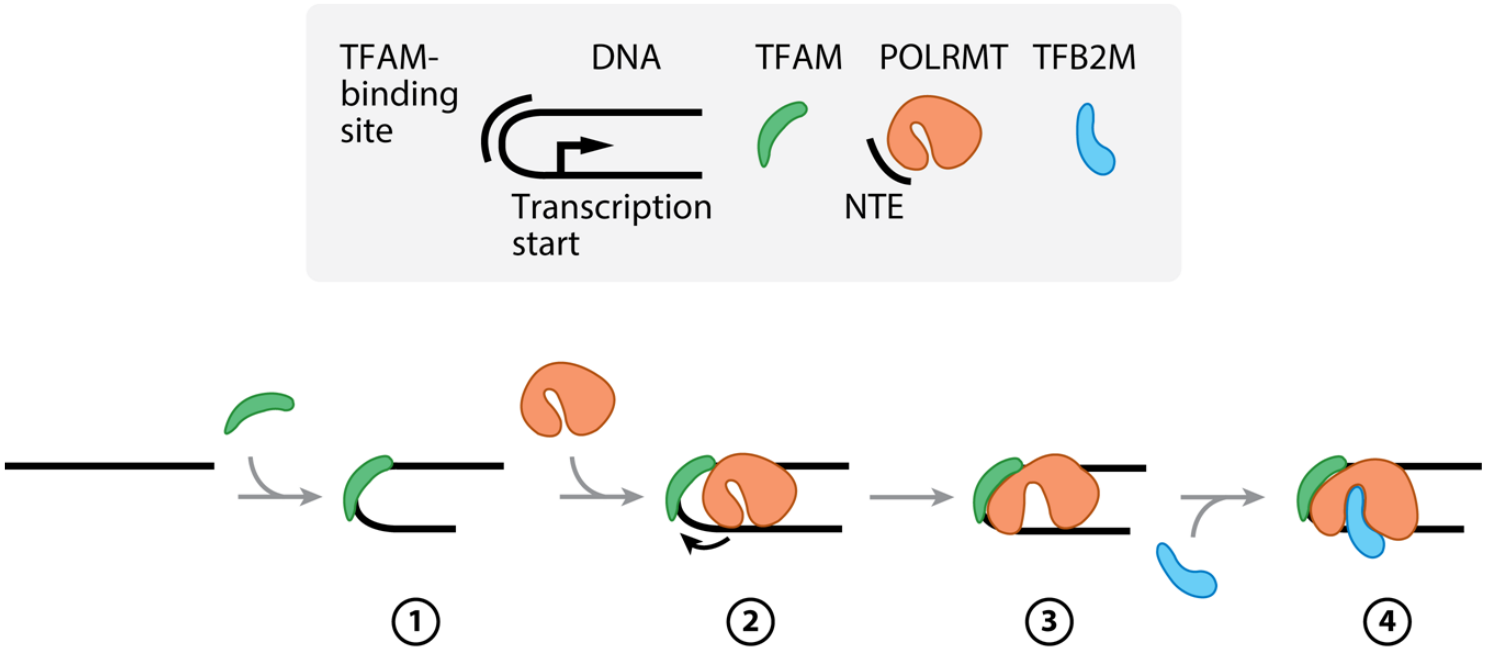


Figure 1.13 Mitochondrial Transcription Initiation. Transcription is initiated by binding of TFAM upstream of the promoter region, which induces a 180^{O} bend in mtDNA (1). This facilitates binding of POLRMT to mtDNA and TFAM (2). POLRMT undergoes a conformational change (3) to allow binding of TFB2M to generate the fully assembled transcription initiation complex. Adopted from Gustafsson *et al.* (2016).

Mitochondrial transcription termination factor 1 (MTERF1) is the responsible protein for termination from all three transcription promoters (Kruse *et al.*, 1989; Fernandez-Silva *et al.*, 1997). However, only the HSP1 termination site has been functionally characterised; MTERF1 binds to 28bp region that is located downstream of the 16S rRNA gene (Kruse *et al.*, 1989). However, knockout of *Mterf1* in mice did not show a phenotype or altered rRNA or messenger RNA (mRNA) levels, suggesting that it is dispensable for transcription of rRNA through the HSP1 promoter region (Terzioglu *et al.*, 2013).

Two additional MTERF proteins have also been identified in mammalians; MTERF2 and MTERF3 (Roberti *et al.*, 2009). MTERF2 is poorly characterised but has been shown to co-localise with the nucleoids, which suggests a role in mtDNA expression (Pellegrini *et al.*, 2009). On the other hand, MTERF3 has been demonstrated as a repressor of transcription, since heart-specific knockout in mice caused significant up-regulation in transcription (Park *et al.*, 2007).

1.5.5 Mitochondrial Genome Replication

1.5.5.1 Replication Machinery

Unlike the nuclear genome, the mammalian mitochondrial genome is not coupled to the cell cycle and hence, there is constant replication and turnover of mtDNA (Bogenhagen and Clayton, 1977). Mitochondrial genome replication is dependent on the replisome, comprising POLG1 (POLγA) and POLG2 (POLγB) that together compose the only known replicative mitochondrial DNA polymerase (POLG), TWNK for unwinding of the dsDNA and mt-SSB for stabilisation of ssDNA, plus POLRMT that likely synthesises the RNA primers needed for replication (**Figure 1.14**) (Falkenberg *et al.*, 2007; Wanrooij *et al.*, 2008; Fuste *et al.*, 2010; Gustafsson *et al.*, 2016).

1.5.5.2 Models of Replication

Despite characterisation of mtDNA replication machinery, the precise mechanism(s) are still debated (**Figure 1.15**). Currently, there are two prevailing models proposed; strand-displacement (asynchronous) and strand-coupled (synchronous) replication (Holt and Reyes, 2012; McKinney and Oliveira, 2013).

In the strand-displacement model, replication is initiated at the O_H (leading-strand) and continues 'unidirectionally' until the O_L (lagging-strand) is exposed by the underwinding of

DNA by TWNK, which initiates replication of the lagging-strand (Kasamatsu and Vinograd, 1973; Tapper and Clayton, 1981; Clayton, 1982).

In the strand-coupled model, replication is 'bi-directional' with initiated at the O_H and at multiple initiation sites for lagging-strand synthesis, revealed during unwinding of the DNA, until replication is terminated at the O_H (Holt *et al.*, 2000). This model proposes the existence of Okazaki fragments as replication intermediates, but its presence in mitochondria have not yet been proved (Wanrooij and Falkenberg, 2010; McKinney and Oliveira, 2013).

A third controversial model similar to the strand-coupled hypothesis has been proposed, referred to as the Ribonucleotide Incorporated ThroughOut the Lagging Strand (RITOLS) model (Yang *et al.*, 2002; Yasukawa *et al.*, 2006). This model proposes that leading- and lagging-strand synthesis occur simultaneously but in the presence of ribonucleotides that are incorporated into the synthesised lagging-strand and mature into DNA probably following exposure of the O_L.

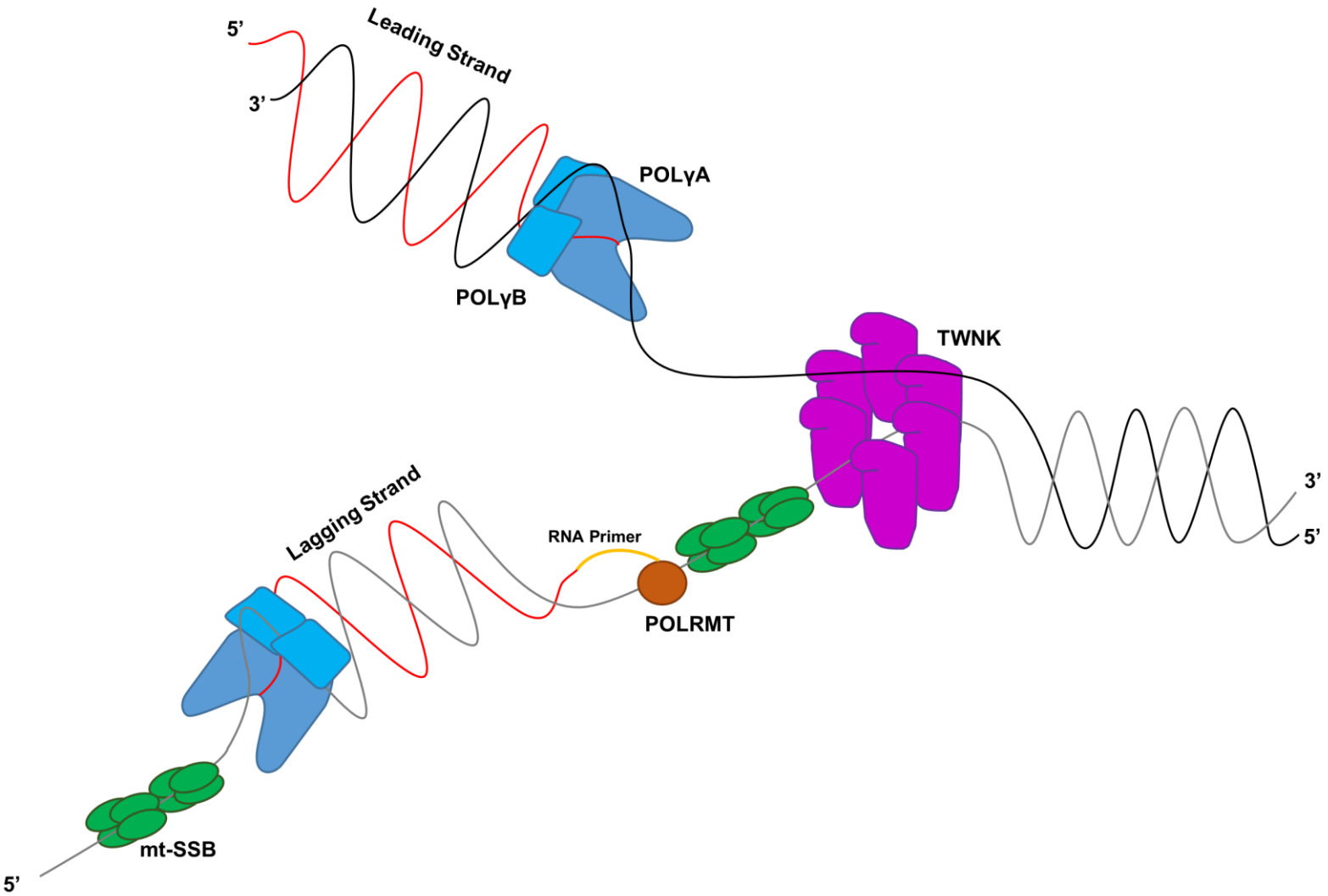


Figure 1.14 Mitochondrial Replication Machinery. Mitochondrial genome replication is shown in a 5’ to 3’ direction. Replication is dependent on the replisome comprising POLγA (dark blue), POLγB (light blue), twinkle (purple), mt-SSB (green) and POLRMT (orange). Based on Wanrooij and Falkenberg (2010).

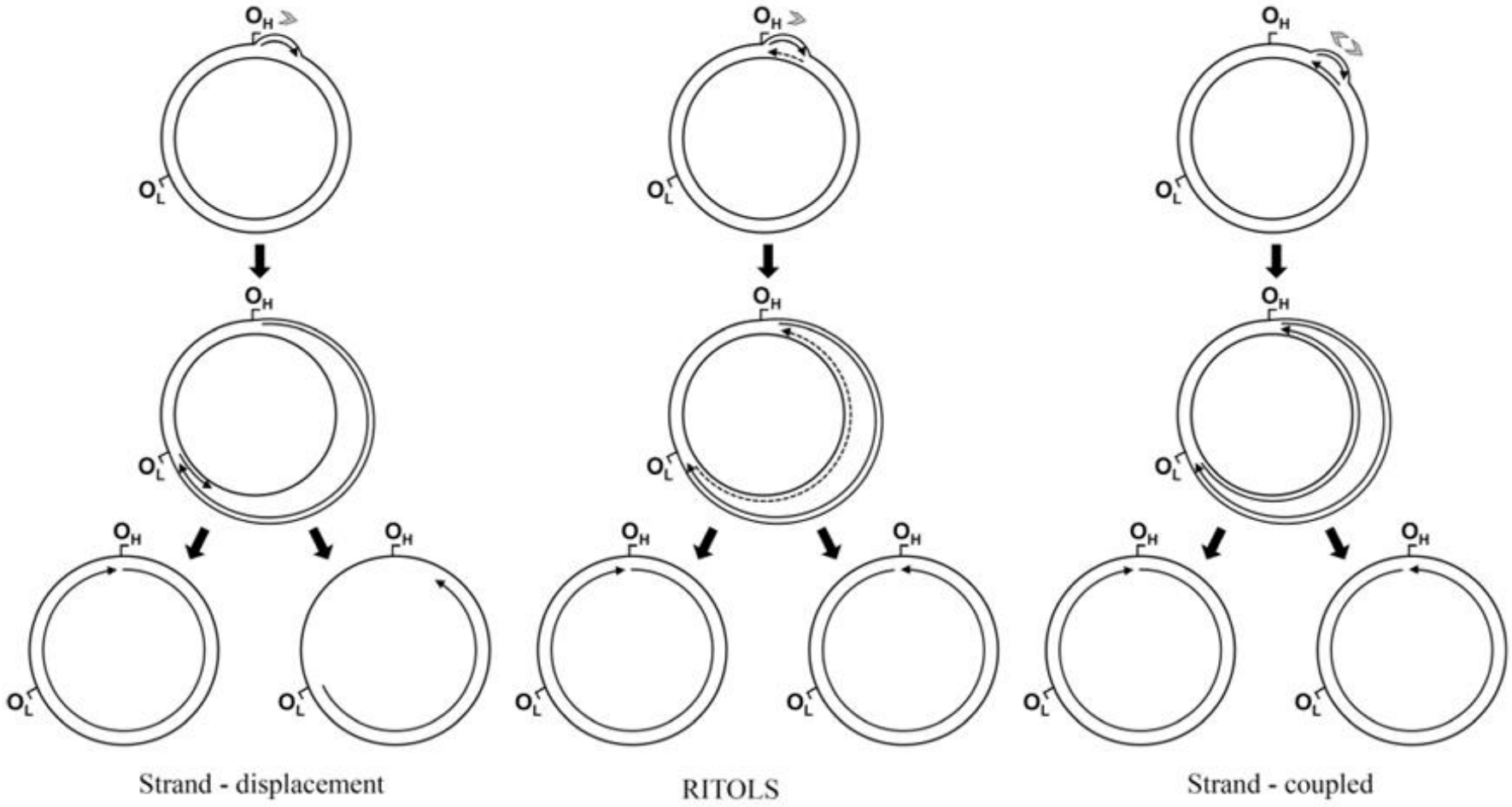


Figure 1.15 Models of Mitochondrial Genome Replication. The current models of mitochondrial genome replication; strand-displacement (asynchronous), RITOLS and strand-coupled (synchronous). Adopted from McKinney and Oliveira (2013).

1.5.6 mtDNA Maintenance and Repair

Initially thought to have a reduced capacity for DNA repair (Clayton *et al.*, 1974; Miyaki *et al.*, 1977), mitochondria are now known to possess several complex mechanisms for mtDNA maintenance, involving the coordination of a wide array of repair enzymes that are often shared with the nuclear genome and distinguished by a MTS (Kazak *et al.*, 2012; Alexeyev *et al.*, 2013). Repair mechanisms of mtDNA are extensively reviewed by Kazak *et al.* (2012) and Alexeyev *et al.* (2013).

Base excision repair (BER) is the most well-characterised and perhaps the main repair mechanism in mitochondria (Kazak *et al.*, 2012), which involves repair of damaged nucleotide base(s) (Svilar *et al.*, 2011). Since mitochondrial BER proteins are localised at the IMM, BER only occurs when mtDNA is associated with the membrane (Stuart *et al.*, 2005). This first requires recognition and removal of damaged base(s) by either monofunctional (uracil DNA glycosylase 1 – UNG1; MutY DNA glycosylase – MUTYH) or bifunctional (8-oxoguanine-DNA glycosylase 1 – OGG1; Nth-like DNA glycosylase 1 – NTHL1; Nei-like DNA glycosylase 1 and 2 – NEIL1, NEIL2) DNA glycosylases. This generates an abasic site that is typically removed by apurinic/apyrimidinic endonuclease 1 (APE1) (Chattopadhyay *et al.*, 2006). However, gaps introduced by the bifunctional glycosylases NEIL1/2 are processed by polynucleotide kinase/phosphatase (PNKP) (Jilani *et al.*, 1999). Single-strand breaks are then repaired by either long-patch or short-batch BER. Short-batch BER occurs when a 3'-OH group is generated at one end of the break, with single nucleotide insertion and synthesis performed by POLG (Van Goethem *et al.*, 2001), together with a 5'-phosphate. On the other hand, when 5'-ends are cannot be ligated, long-batch BER occurs. POLG mediated-displacement synthesis with two or more nucleotides creates a single-strand DNA flap. Short flaps of eight nucleotides or less are cleaved by flap exonuclease 1 (FEN1) (Kalifa *et al.*, 2009). On the other hand, the ATP dependent helicase/nuclease DNA2 is required to generate a 'stub' on the end of longer flaps (Duxin *et al.*, 2009), which can then be cleaved by FEN1. Following both short- and long-batch BER, the ends are ligated by the only known mammalian mitochondrial DNA ligase, LIG3 (Simsek *et al.*, 2011).

Mitochondria also possess single-strand break repair (SSBR) for when mitochondrial topoisomerase I (TOP1mt) fails to re-join DNA ends during replication (Zhang *et al.*, 2001), ROS or from BER. SSBR involves the several of the same steps and enzymes as BER and is often considered a subpathway of BER (Kazak *et al.*, 2012). Two important enzymes involved in mitochondrial SSBR are aprataxin (APTX) and tyrosyl-DNA-phosphodiesterase 1

(TDP1) (Meagher and Lightowlers, 2014). APTX cleaves a 5' adenosine monophosphate (AMP) from a lesion created from abortive ligation, so a further attempt at ligation can proceed (Sykora *et al.*, 2011). When TOP1mt collides with DNA and RNA polymerases, it can remain covalently attached to DNA (Zhang *et al.*, 2001). Following degradation of TOP1mt, a 3' phosphotyrosine bond that remains is removed by TDP1 to leave a 3' phosphate that can undergo further processing for ligation (Das *et al.*, 2010).

It is critical that the nuclear genome possesses double-strand break repair (DSBR) mechanisms, but since mtDNA is a multi-copy genome it is not as equally crucial to repair (Kazak *et al.*, 2012). Two key pathways of DSBR are homologous recombination - requiring a template sequence to exchange nucleotide sequences to repair end breaks- and non-homologous end joining that simply requires the ligation of breaks. There is some evidence that mammalian mtDNA undergoes homologous recombination and non-homologous end joining to repair double-strand breaks (Coffey *et al.*, 1999; D'Aurelio *et al.*, 2004; Bacman *et al.*, 2009). However, this evidence suggests that DSBR is an uncommon or minor repair mechanism in mammalian mitochondria (Alexeyev *et al.*, 2013).

Mitochondria could also possess a mismatch repair mechanism to prevent misincorporation and proofreading errors of nucleotides by POLG (Alexeyev *et al.*, 2013). Although mismatch repair mechanisms of the nuclear genome are well characterised (Li, 2008), it is not yet clear in mitochondria although Y-box binding protein 1 (YBX1) has been shown to recgonise and have base mismatch repair activity in mitochondria (de Souza-Pinto *et al.*, 2009).

Mitochondrial genome replication is also dependent upon a balanced supply of all four deoxynucleotide triphosphates (dNTPs), derived from cytosolic and mitochondrial salvage pathways (Pica-Mattoccia and Attardi, 1972; Saada, 2009). Incorporation of damaged dNTPs represent a significant source of mismatch errors (Pursell *et al.*, 2008). Supply of dNTPs for mtDNA maintenance is detailed in **Chapter 5**.

1.5.7 mt-tRNA and mRNA Processing

Following transcription, synthesised polycistronic transcripts undergo processing based largely upon the position and folding of the mt-tRNAs. These are excised by tRNA ribonuclease (RNase) Z (ELAC2) that has 3'-end cleavage activity (Brzezniak *et al.*, 2011) and mitochondrial ribonuclease P (RNase P) comprising three subunits that has 5'-end cleavage activity (MRPP1, MRPP2, MRPP3) (Holzmann *et al.*, 2008). This releases the mRNA and mt-tRNA transcripts for additional processing and modification.

Following mt-tRNA modification, a –CAA sequence is added to the end of the 3'-acceptor stem (Nagaike *et al.*, 2001), which allows aminoacylation (charging) of the cognate mt-tRNAs with their specific amino acid, catalysed by the corresponding mitochondrial amino-acyl tRNA synthetase (mt-aaRS). Aminoacylation of the mt-tRNAs by the mt-aaRS is outlined in detail throughout **Chapter 7**.

At the same time or after cleavage of the mt-tRNAs, all rRNA and mRNA transcripts are polyadenylated by the mitochondrial poly(A) polymerase (MTPAP) to introduce a poly(A) or oligo(A) extension (Slomovic *et al.*, 2005; Bratic *et al.*, 2016). This completes the UAA stop codon in seven mRNA transcripts and may stabilise some mRNAs (Ojala *et al.*, 1981; Smits *et al.*, 2010). In mammalian mitochondria, UAA or UAG are the two conventional stop codons for mRNA transcripts (Suzuki *et al.*, 2011). However, human MT-COI and MT-ND6 possess AGA and AGR stop codons, which requires the mitoribosome to promote a -1 frameshift at these codons to allow termination with the conventional UAG codon (Temperley *et al.*, 2010).

1.5.8 Post-Transcriptional mt-tRNA Modification

Post-transcriptional modification of mt-tRNAs at the anti-codon stem wobble-base, the first base of the anti-codon, is essential for the decoding, stability, folding and interaction between mt-tRNA and mRNA since mammalian mitochondria have a different decoding system to the universal code (Suzuki *et al.*, 2011). Hence, nucleoside modification at the wobble-base is critical for decoding the mRNA.

Elucidating the number of mt-tRNA modifications has been historically challenging due to the difficulties in isolating and mass spectrometry analysis of individual mt-tRNAs. Furthermore, not all modification enzymes have been characterised to date, although they are evolutionarily inferred. Most recently, Van Haute *et al.* (2016) demonstrated for the first time that NSUN3 was required for methylation (m^5C) and formylation (f^5C) in position 5 of the U34 wobble-base in mt-$tRNA^{Met}$. The f^5C modification of mt-$tRNA^{Met}$ had been predicted in humans since it is evolutionary conserved (Moriya *et al.*, 1994; Bilbille *et al.*, 2011), whereas the m^5C modification was previously unknown. Nonetheless, compared with cytosolic tRNAs, mammalian mt-tRNAs have fewer modifications at the wobble-base (Suzuki *et al.*, 2011).

Modification of the U34 wobble-base and three factors involved (MTU1, GTPBP3, MTO1) are discussed in **6.5.1.2**.

1.5.9 Mitochondrial Translation

After post-transcriptional processing, there are nine monocistronic and two dicistronic mRNA transcripts that are translated into proteins via three phases; initiation, elongation and termination (**Figure 1.16**). This is outlined in detail by Mai *et al.* (2016) and (Ott *et al.*, 2016).

1.5.9.1 Initiation

First, the mitoribosome is dissociated into its two subunits; the large 39S subunit (comprising 50 subunits and 16S rRNA) and the small 28S subunit (comprising 30 subunits and 12S rRNA) (Amunts *et al.*, 2015; Greber *et al.*, 2015). mRNA is recruited to the 28S subunit, which is bound to initiation factor $IF3_{mt}$ to prevent re-association of the mitoribosome. Met-$tRNA^{Met}$ is formylated by mitochondrial methionyl-tRNA formyltransferase (mt-FMT) to generate an fMet-$tRNA^{Met}$ species (Takeuchi *et al.*, 2001). Since mtDNA encodes only one $tRNA^{Met}$, fMet-$tRNA^{Met}$ is used for translation initiation and non-formylated Met-$tRNA^{Met}$ is required for elongation. fMet-$tRNA^{Met}$ is recruited by GTP-bound $IF2_{mt}$ to the P-site of the 28S subunit. Correct codon:anti-codon binding causes $IF3_{mt}$ to be released and hydrolysis of the GTP-bound $IF2_{mt}$, allowing re-association of the mitoribosome subunits.

1.5.9.2 Elongation

Elongation of the polypeptide requires the delivery of complex comprising an aminoacylated mt-tRNA by the mitochondrial elongation factor Tu (mt-EFTu) and GTP. Correct codon:anti-codon binding causes GTP hydrolysis and the release of mt-ETFu. The mt-EFTu:GTP complex is restored by mitochondrial translation factor Ts (TSFM) (Schwartzbach and Spremulli, 1989). After the release of mt-EFTu, a peptide bond is formed at the peptidyl transferase centre (PTC) of the 39S subunit. This leaves an uncharged mt-tRNA occupying the P-site and a dipeptidyl mt-tRNA at the A-site. A third mitochondrial elongation factor, G1 (mt-EFG1), initiates a conformational change in the mitoribosome structure that causes translocation of the uncharged mt-tRNA to the E-site and its release from the mitoribosome, then translocation of the dipeptidyl mt-tRNA across three nucleotides from the A-site to the P-site.

1.5.9.3 Termination

Elongation of the polypeptide continues until a stop codon – either UAA or UAG - at the A-site is reached, for which a mt-tRNA is unable to bind. The stop-codon is recognised by mitochondrial release factor 1a (mt-RF1a), which hydrolyses the ester bond between the mt-

tRNA in the P-site and the last amino acid of the synthesised polypeptide, thus releasing the polypeptide from 39S subunit (Soleimanpour-Lichaei *et al.*, 2007). Following release of the polypeptide, dissociation of the mitoribosome and release of the uncharged mt-tRNA and mRNA occurs through two mitoribosome recycling factors; mitochondrial release factor 1 (mt-RRF1) and mitochondrial elongation factor G2 (mt-EFG2) (Rorbach *et al.*, 2008). Despite the nomenclature, the function of mt-EFG2 is restricted to mitoribosome recycling and not elongation (Tsuboi *et al.*, 2009).

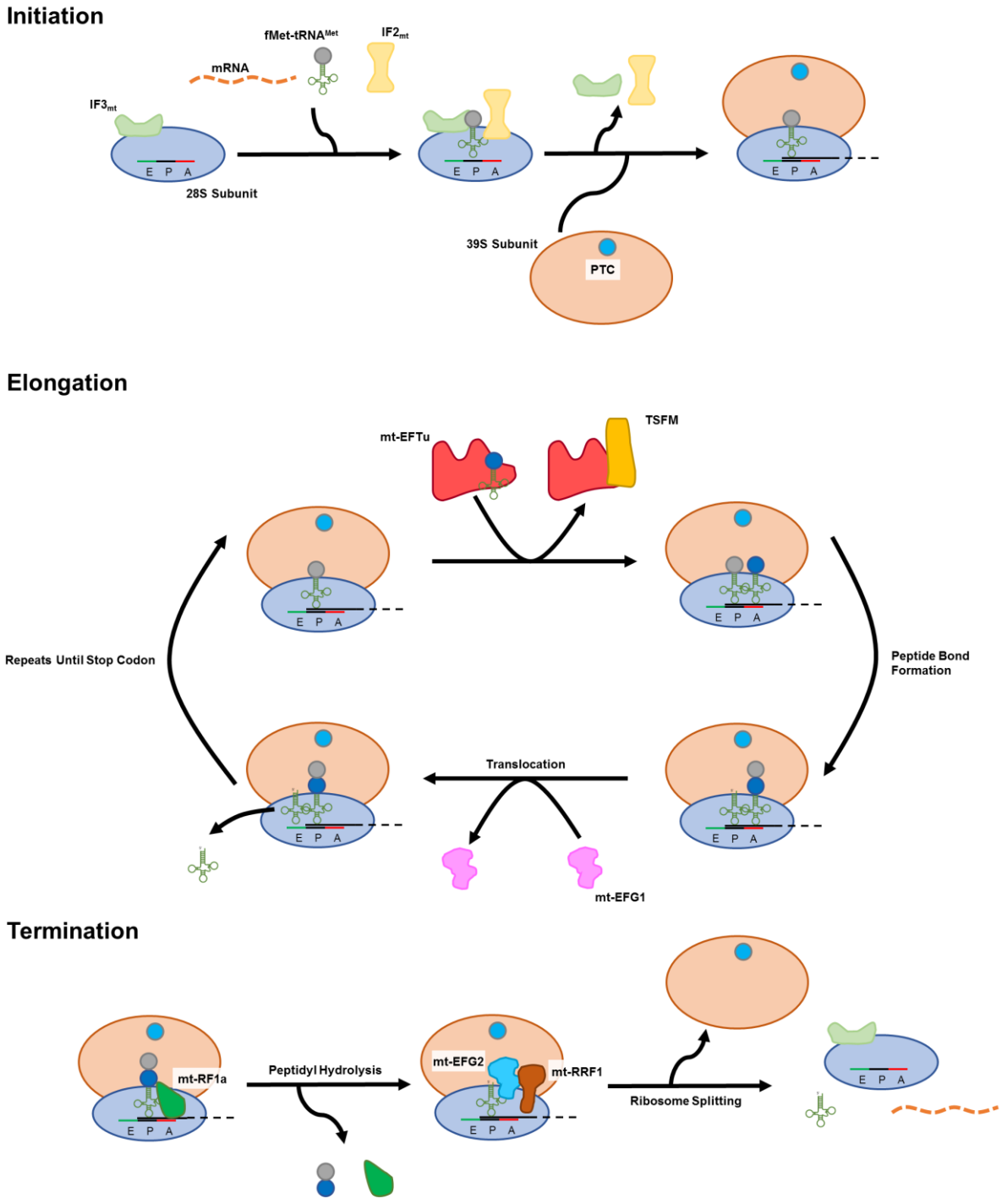


Figure 1.16 Mitochondrial Translation. Schematic showing the initiation, elongation and termination stages of mitochondrial translation. mt-EFG1 – mitochondrial elongation factor G1; mt-EFG2 – mitochondrial elongation factor G2; mt-EFTu – mitochondrial elongation factor Tu; mt-RF1a – mitochondrial release factor 1a; mt-RRF1 – mitochondrial ribosomal recycling factor 1; PTC – peptidyl transferase centre; TSFM – mitochondrial elongation factor Ts. Based on Mai *et al.* (2016) and Ott *et al.* (2016).

1.6 Mitochondrial Disease

Mitochondrial disease encompasses a common group of clinically heterogeneous metabolic disorders, defined as dysfunction of the OXPHOS system due to mitochondrial genome or nuclear gene defects (Lightowlers *et al.*, 2015; Alston *et al.*, 2017). Mitochondrial abnormalities have also been associated with the normal process of mammalian ageing, which is largely based around the 'free radical theory' in which ROS causes mtDNA mutations (Harman, 1956). However, the role of mitochondria in ageing is still debated and is likely due to multiple processes (Payne and Chinnery, 2015). Mitochondrial dysfunction has also been associated with other Mendelian and age-related disorders that include Alzheimer's disease (Coskun *et al.*, 2012), Parkinson's disease (McCoy and Cookson, 2012), amyotrophic lateral sclerosis (ALS) (Cozzolino and Carrì, 2012), myofibrillar myopathy (Vincent *et al.*, 2016) and cardiac disease (Rosca and Hoppel, 2013).

1.6.1 Primary mtDNA Disease

Primary mitochondrial DNA diseases arise predominantly due to mtDNA mutations or single, large-scale mtDNA deletions. It is estimated that 1 in 200 individuals harbour a heteroplasmic pathogenic mtDNA point mutation (Chinnery *et al.*, 2012), while the minimum point prevalence of adults manifesting primary mitochondrial disease is estimated at 1 in 5000, with 1 in 9,346 individuals estimated to be at risk of developing mtDNA disease (Gorman *et al.*, 2015b).

1.6.1.1 Point Mutations and Indels

mtDNA point mutations including small insertions or deletions (indels) are predominantly inherited through the maternal lineage, while the prevalence of *de novo* mtDNA mutations, usually manifesting in severe early-onset mitochondrial disease has been estimated at 24.6% (Sallevelt *et al.*, 2016). Pathogenic mutations of all mtDNA genes have been reported and are curated in the human mitochondrial genome database MITOMAP (Lott *et al.*, 2013; Lott, 2016).

LHON is the most common mtDNA disease (Gorman *et al.*, 2015b), with m.3460G>A (*MT-ND1*), m.11778G>A (*MT-ND4*) and m.14484T>C (*MT-ND6*) mutations in complex I subunit genes comprising over 90% of LHON patients (Yu-Wai-Man *et al.*, 2003). These mutations principally manifest as visual failure in young adults due to degeneration of the retinal ganglion cell layer and typically sporadic or homoplasmic (Yu-Wai-Man *et al.*, 2003).

However, there is incomplete penetrance of LHON mutations since only 40% of males and 10% of females are manifest disease symptoms despite being homoplasmic for a mutation.

Despite comprising a small proportion of the mitochondrial genome, point mutations of the mt-tRNAs have emerged as an important cause of mitochondrial disease (Yarham *et al.*, 2010). This includes the most common mtDNA mutation, m.3243A>G mutation occurring in *MT-TL1* (mt-tRNA$^{Leu(UUR)}$). Initially associated with MELAS (Goto *et al.*, 1990b), the m.3243A>G mutation is associated with variable, overlapping syndromes that also include maternally inherited deafness and diabetes (MIDD) and progressive external ophthalmoplegia (PEO) (Nesbitt *et al.*, 2013).

1.6.1.2 Single Large-scale mtDNA Deletions

Single large-scale mtDNA deletions are the most common primary mtDNA re-arrangement associated with mitochondrial disease. They are typically sporadic in comparison to mtDNA point mutations (Chinnery *et al.*, 2004), with an estimated minimum prevalence of 1.5 in 100,000 (Gorman *et al.*, 2015b). The clinical manifestations of a large-scale deletion are categorised into three classic syndromes, though these are now recognised as an overlapping continuum of syndromes (Mancuso *et al.*, 2015); Kearn-Sayre syndrome (KSS) (Kearns and Sayre, 1958; Kearns, 1965), Pearson's syndrome (Pearson *et al.*, 1979; McShane *et al.*, 1991) and PEO to PEO-plus phenotypes (Moraes *et al.*, 1989). PEO is a frequent manifestation in all three classic syndromes due to single large-scale mtDNA deletions and is discussed further in **Chapter 3**.

Deletion size is variable but approximately one-third of patients harbour a 'common deletion' of 4,977kb (Schon *et al.*, 1989). However, it is debated whether the size or location of the mtDNA deletion correlates with the clinical phenotypes (Aure *et al.*, 2007; Yamashita *et al.*, 2008; Lopez-Gallardo *et al.*, 2009).

1.6.1.3 Secondary multiple mtDNA deletions

Secondary multiple mtDNA deletions of varying size and position occur predominantly due to mutations of nuclear gene encoding mtDNA transcription, replication and maintenance machinery (Kaukonen *et al.*, 2000; Spelbrink *et al.*, 2001; Van Goethem *et al.*, 2001). mtDNA deletions have been also been shown to accumulate in post-mitotic tissue during the process of healthy ageing (Kraytsberg *et al.*, 2006; Yu-Wai-Man *et al.*, 2010b). Disorders of mtDNA maintenance are discussed in **1.6.2.1**.

1.6.1.4 Heteroplasmy and the Threshold Effect

Since mtDNA is a multi-copy genome in eukaryotes, two or more populations of mtDNA harbouring a single causative mutation or large-scale rearrangements can arise. The phenomenon of mixed mtDNA populations is referred to as heteroplasmy (Stewart and Chinnery, 2015). Conversely, homoplasmy refers to a population of mtDNA that are identical.

Some rare causative mtDNA mutations are homoplasmic, but most causative mutations are heteroplasmic (Lightowlers *et al.*, 2015). Most heteroplasmic mutations occur at low levels and do not manifest mitochondrial disease. Thus, there is a critical threshold in which a minimum proportion of mutant mtDNA is necessary before biochemical and clinical features are exhibited, referred to as the 'threshold effect' (**Figure 1.17**) (Rossignol *et al.*, 2003; Stewart and Chinnery, 2015). This threshold is variable depending on the mtDNA mutation, the type of mutation and the tissues harbouring mutant mtDNA.

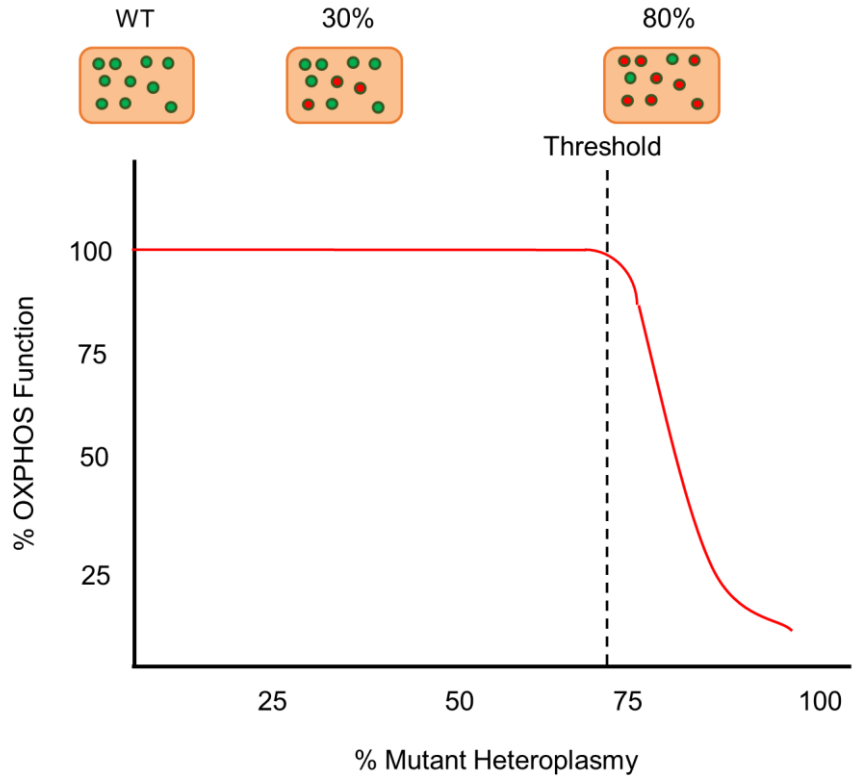


Figure 1.17 Heteroplasmy and the Threshold Effect. Schematic demonstrating a critical threshold of approximately 70%. The minimum proportion of mutant mtDNA must reach this threshold before biochemical and clinical features manifest. Wild-type mtDNA are shown in green. Mutant mtDNA are shown in red.

1.6.1.5 Clonal Expansion

The accumulation of somatic mtDNA mutations or mtDNA deletions over time in post-mitotic tissue is known as ‘clonal expansion’. The mechanism continues to be debated, although there are currently three principal hypotheses.

The first hypothesis, termed ‘survival of the smallest’, proposes a replicative advantage for mtDNA with larger deletions (Wallace, 1989). This hypothesis suggests that smaller mtDNA replicate faster than wild-type mtDNA, leading to an accumulation of deleted molecules over time. Although this hypothesis has been studied extensively (Diaz *et al.*, 2002; Fukui and Moraes, 2009), it fails to provide an explanation for the accumulation of point mutations since these do not confer a replicative advantage. More recently, Campbell *et al.* (2014a) demonstrated no replicative advantage for larger deleted mtDNA molecules in skeletal muscle compared to wild-type mtDNA.

The second hypothesis, termed ‘survival of the slowest’, proposes that pathogenic mtDNA mutations reduces the respiratory capacity of the mitochondrion, leading to lower levels of ROS, slower replication and slow accumulation of mtDNA damage (De Grey, 1997). Wild-type mtDNA respire normally, which generates higher levels ROS and faster accumulation of mtDNA damage. Therefore, there is a selective advantage for slow replicating mtDNA molecules. However, this hypothesis only provides an explanation for when respiratory capacity has been already affected. Furthermore, it also suggests that mitotic tissue should accumulate a greater proportion of mtDNA deletions than post-mitotic tissue, given that replicative turnover is higher.

The third hypothesis, termed ‘random genetic drift’, was developed through mathematical modelling and proposes that relaxed replication alone without any replicative advantage can lead to clonal expansion of mtDNA deletions throughout human life (Elson *et al.*, 2001). Further modelling showed that this could apply to point mutations in normal human ageing and tumours (Coller *et al.*, 2001; Poovathingal *et al.*, 2009). However, the predicted levels of clonal expansion were not observed in aged human substantia nigra neurons (Bender *et al.*, 2006; Reeve *et al.*, 2008).

1.6.2 Mendelian Mitochondrial Disease

The nuclear genome is estimated to encode for 1,500 proteins that are required for the coordination of mitochondrial processes (Lopez *et al.*, 2000; Calvo *et al.*, 2006). Therefore, a mutation of any one of these nuclear genes could cause mitochondrial disease. The minimum

point prevalence of nuclear mutations in diagnosed adults with mitochondrial disease is 2.9 in 100,000, which is approximately one-third the prevalence mtDNA mutations (Gorman *et al.*, 2015b). Thus, a nuclear aetiology accounts for a significant proportion of mitochondrial disease patients.

Nuclear mitochondrial disease obeys Mendelian inheritance, thus causative mutations can be dominant (heterozygous), recessive (compound heterozygous or homozygous), X-linked (hemizygous) or occur *de novo*. Reflecting the vast mitochondrial proteome and pathways involved, Mendelian mitochondrial disease manifests in broad phenotypic and genetic diversity. There are approximately 245 nuclear genes currently associated with Mendelian mitochondrial disease (**Figure 1.18**) (Mayr *et al.*, 2015). However, this catalogue of nuclear defects continues to expand due to advances in DNA sequencing technologies.

This thesis focuses on two Mendelian mitochondrial disorders characterised by extreme clinical and genetic heterogeneity; (i) adult-onset Mendelian mitochondrial PEO with mtDNA instability and (ii) early-onset mitochondrial RC deficiency. These Mendelian disorders are outlined in detail throughout **Chapter 3**, **Chapter 4** and **Chapter 6**.

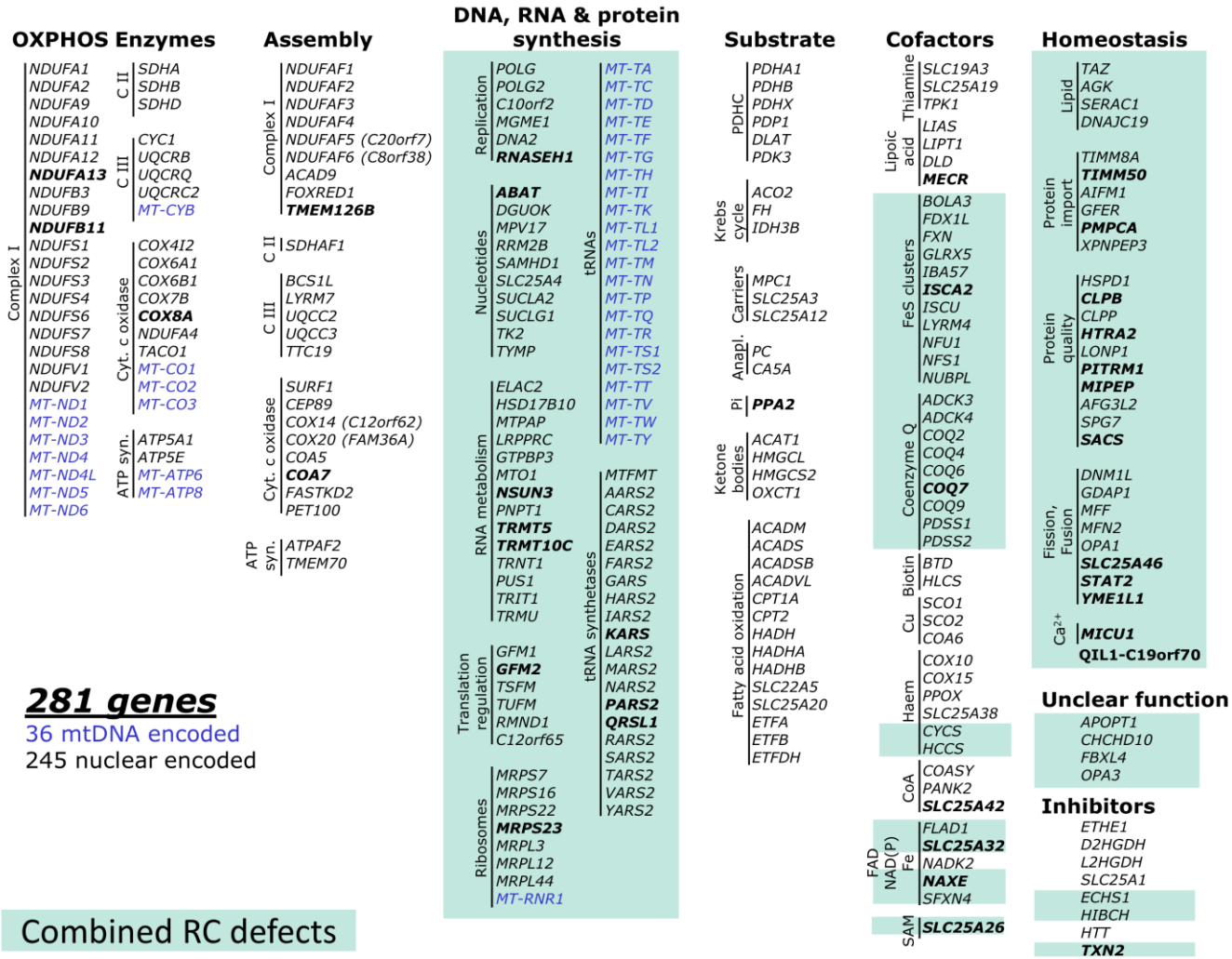


Figure 1.18 Genes Associated with Mitochondrial OXPHOS Defects. All mtDNA and nuclear genes associated with mitochondrial disease (n=281). Genes in green are associated with multiple RC deficiencies. Bold genes indicate those reported in human disease since publication of the Mayr *et al.* (2015) review. Figure courtesy of Charlotte Alston and amended from (Mayr *et al.*, 2015).

1.6.2.1 Disorders of mtDNA Maintenance

Quantitative and qualitative defects of mtDNA maintenance have been recognised as an important syndromes in early- and adult-onset Mendelian mitochondrial disease (El-Hattab and Scaglia, 2013; Ahmed *et al.*, 2015). Disorders of mtDNA maintenance can be classified into two broad groups with overlapping features; (i) autosomal recessive mtDNA depletion syndrome, characterised by a severe reduction in mtDNA copy number and (ii) late-onset mtDNA instability, characterised by multiple mtDNA deletions in post-mitotic tissues due to dominant or recessive mutations. These disorders are overwhelming associated with mutations in nuclear genes encoding mtDNA transcription, replication or maintenance machinery, nucleotide metabolism enzymes and nucleotide transporters (**Figure 1.19**) (Sommerville *et al.*, 2014).

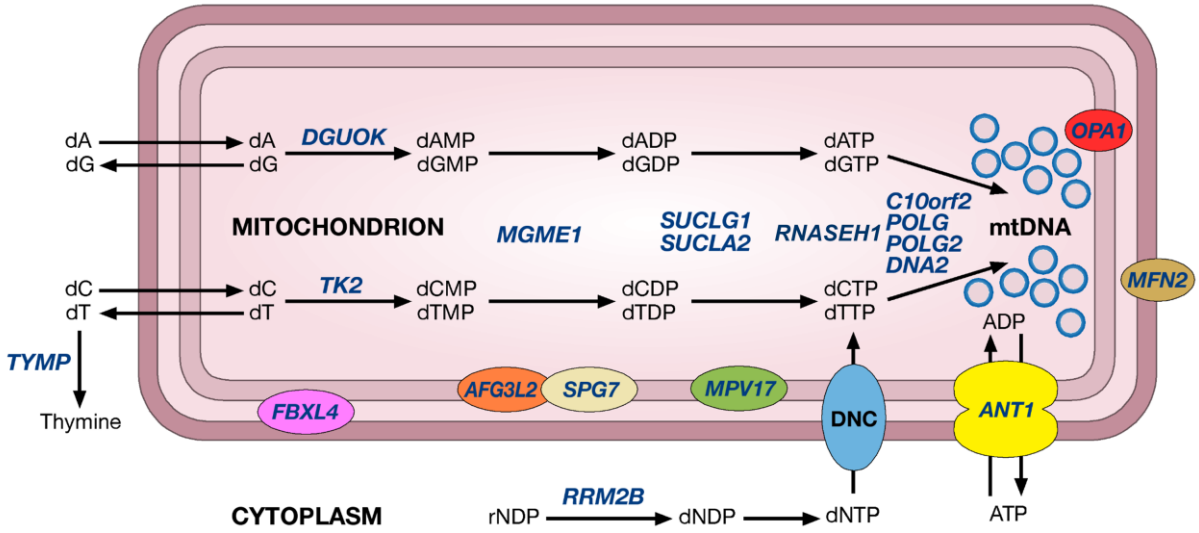


Figure 1.19 Defects of mtDNA Maintenance. Schematic showing the major proteins associated with mtDNA maintenance disorders. Adopted and amended from Sommerville *et al.* (2014).

Adult-onset Mendelian PEO with secondary, clonally expanded skeletal-muscle restricted multiple mtDNA deletions fits into this latter category and is discussed in **Chapter 3**.

1.6.2.2 Early-Onset Mitochondrial RC Disease

With an estimated minimum prevalence of 1 in 5000 live births (Skladal *et al.*, 2003), early-onset RC disorders are amongst the most common metabolic disorders; this is discussed further in **Chapter 6**.

1.6.3 Diagnostic Algorithm in Mitochondrial Disease

The diagnosis of mitochondrial disease requires a multidisciplinary approach that combines clinical characteristics, histopathological, biochemical and genetic investigations, for which a diagnostic algorithm has been devised (**Figure 1.20**) (McFarland *et al.*, 2010).

Despite the vast clinical heterogeneity of mitochondrial diseases, precedent exists for which targeted mtDNA or nuclear gene screening can be performed based on clinical features alone. For example, visual failure in a young adult may prompt targeted sequencing of the three LHON mtDNA mutations (Yu-Wai-Man *et al.*, 2003), while the classic MELAS phenotype evokes screening for the common m.3243A>G mutation (Nesbitt *et al.*, 2013).

Nonetheless, paramount to the diagnosis of mitochondrial disease for almost all patients is the requirement for clinically affected tissue samples, which is often skeletal muscle but can also include liver, brain or cardiac tissue if available. Available tissue can be subject to histopathological and biochemical analyses to direct the future diagnostic actions.

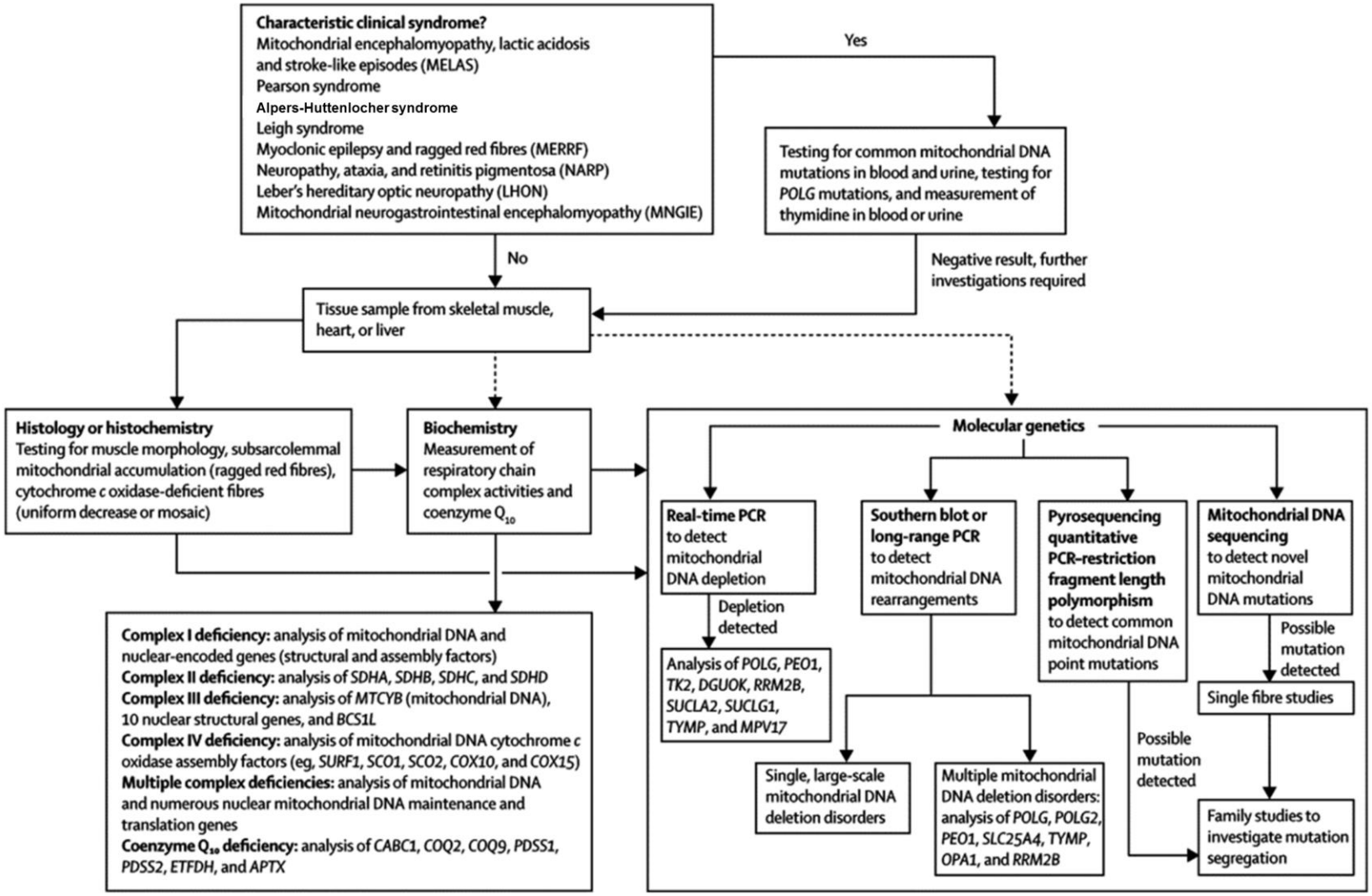


Figure 1.20 Current Diagnostic Algorithm for Investigating Mitochondrial Disease. Despite the increasing number of mtDNA and nuclear gene mutations associated with mitochondrial disease and advances in investigative tools and techniques, the diagnostic algorithm for the investigation of mitochondrial disease published in 2010 still holds true. Adopted from McFarland *et al.* (2010).

1.6.3.1 Histopathology

Two common stains used are haematoxylin and eosin (H&E) and modified Gomori trichrome. H&E is a general stain for observing muscle fibre morphological abnormalities (**Figure 1.21A**). Modified Gomori trichrome stain is used to detect the presence of 'ragged red fibres' (Gomori, 1950; Engel and Cunningham, 1963), characterised by the abnormal subsarcolemmal accumulation of mitochondria, which indicates mitochondrial proliferation.

The standard histochemical assay used is sequential cytochrome *c* oxidase/succinate dehydrogenase (COX-SDH) histochemistry (**Figure 1.21A**) (Old and Johnson, 1989; Sciacco and Bonilla, 1996), which can visualise RC activities for complex IV and II. Global or mosaic patterns of COX-deficiency can be visualised by the presence of blue fibres, which can occur due to mtDNA defects or nuclear mutations. A mosaic pattern of COX-deficiency is typically associated with mtDNA mutations, often due to heteroplasmy and the multi-copy nature of mtDNA. However, COX-deficient fibres are also noted in other neuromuscular diseases and can be observed as part of the normal ageing process (Greaves *et al.*, 2011; Vincent *et al.*, 2016).

The disadvantage of sequential COX-SDH histochemistry is that it does accommodate activities of the other RC complexes, particularly complex I which is the one of the most commonly affected enzymes of the OXPHOS system (Loeffen *et al.*, 2000). Recently, a novel quadruple immunofluorescence assay has been developed for the quantification of complex I and IV abundance in individual skeletal muscle fibres using monoclonal antibodies for NDUFB8 and MT-COI, together with VDAC1/porin as a mitochondrial mass marker and laminin as a marker of myofibre boundaries (**Figure 1.21B**) (Rocha *et al.*, 2015). It has been demonstrated as accurate and reproducible in a large proportion of muscle fibres from range mitochondrial disease patients with mtDNA and nuclear genetic defects, thus showing promise in aiding the diagnostic process.

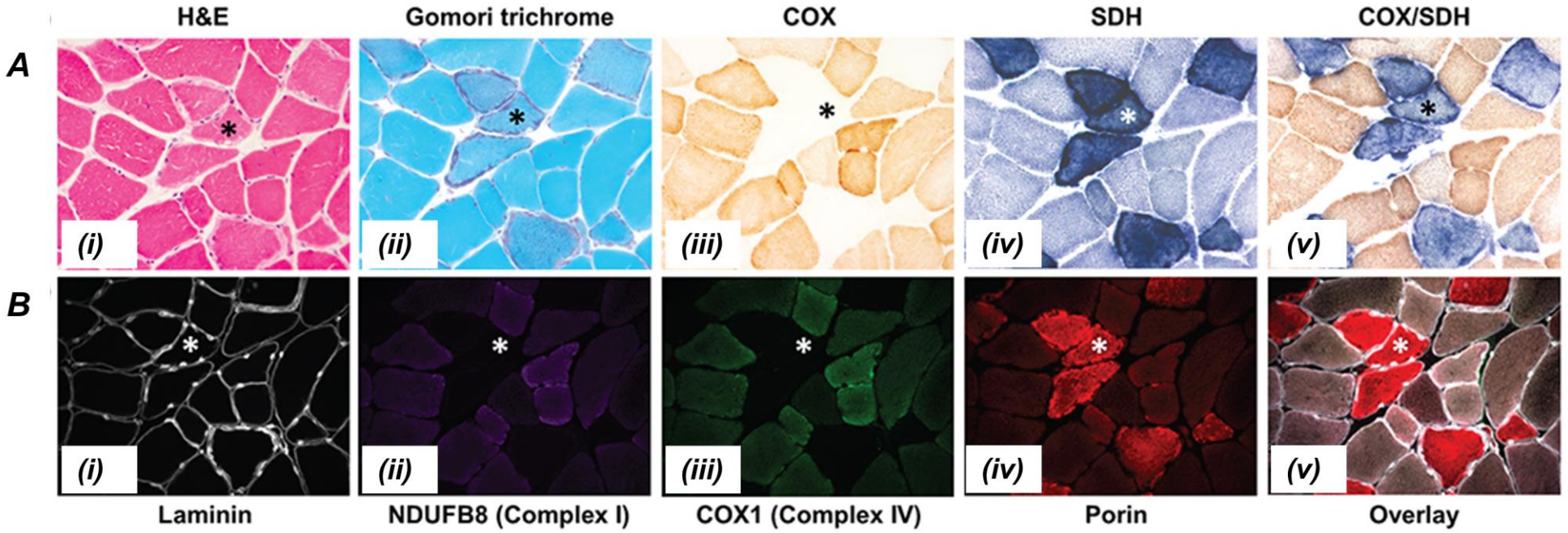


Figure 1.21 Histopathological Techniques in the Diagnosis of Mitochondrial Disease. Panel demonstrating sections of (*A*) current standard histopathology techniques in skeletal muscle from a patient harbouring a single large-scale mtDNA deletion, which show ragged red fibres and COX-deficient fibres; (*i*) H&E and (*ii*) modified Gomori trichrome stain, (*iii*) COX, (*iv*) SDH and (*v*) sequential (merged) COX-SDH reactions. Panel demonstrating sections from the same patient subjected to the novel quadruple immunofluorescence assay for the quantification of complex I and IV protein abundance; (*i*) laminin is used as a marker of myofibre boundaries, quantification of (*ii*) NDUFB8 (complex I), (*iii*) MT-COI (complex IV) and (*iv*) VDAC1/porin, plus (*v*) a merged image. Adopted and amended from Alston *et al.* (2017).

1.6.3.2 Biochemistry

In vitro spectrophotometric biochemical assays for measuring the activity of each individual RC complex in muscle relative to activity of the matrix enzyme citrate synthase in frozen muscle is an essential diagnostic method, particularly in suspected early-onset mitochondrial disorders (Kirby *et al.*, 2007). However, assays require a significant quantity of muscle (>50mg) and complex V cannot be reliably measured in frozen muscle.

1.6.3.3 Genetic and Molecular Studies

Genetic investigations and the order of sequenced mutations or genes is dependent upon the clinical, histochemical and biochemical findings. Rapid whole mitochondrial genome sequencing using next generation sequencing (NGS) that can also accurately measure heteroplasmy may be considered in a significant proportion of patients following exclusion of common mtDNA mutations (Tang *et al.*, 2013). Targeted Sanger sequencing of a subset of known causative nuclear genes is also routinely performed following exclusion of mtDNA mutations. This includes mutational screening of nuclear genes involved mtDNA maintenance or mitochondrial translation in patients with multiple RC deficiency in affected tissues, or RC enzyme complex subunits and assembly factors for patients with isolated RC deficiency. Segregation studies are also integral to confirming maternal inheritance of a causative mtDNA mutation and the carrier status of unaffected and affected relatives of the proband. This is not always feasible however, especially in late-onset patients where access to DNA from parents or relatives is frequently a challenge.

Regarding mtDNA maintenance disorders, quantitative real-time PCR of muscle DNA is used to determine relative mtDNA copy number (Dimmock *et al.*, 2010; Venegas and Halberg, 2012). Single large-scale mtDNA deletion and multiple mtDNA deletions are typically detected by long range PCR or occasionally be Southern blotting (**Figure 3.1C and D**). The presence of multiple mtDNA deletions, depletion or both would allow a mutational screening of a subset of known causative nuclear genes associated with mtDNA maintenance disorders. (El-Hattab and Scaglia, 2013; Ahmed *et al.*, 2015).

1.7 Applications of DNA Sequencing and the Emergence of Next Generation Sequencing (NGS) Technologies

The advance of human society has been greatly enriched by the ability to sequence the genetic code, comprising of just four dNTPs, that dictates the vast diversity of all life on Earth. The

power of DNA sequencing has led to landmark discoveries and outcomes, including the discovery of non-coding RNAs (Eddy, 2001), estimation of evolutionary trees (Felsenstein, 1981) and of course, sequencing of the mitochondrial genome (Anderson *et al.*, 1981).

1.7.1 First-Generation Sequencing

Many of the first seminal discoveries to date have relied upon 'first-generation' DNA sequencing methodologies. The earliest such methodologies were largely chemical based, including Maxam and Gilbert sequencing, which used four chemical cleavage reactions (Maxam and Gilbert, 1977). However Sanger sequencing, also known as chain-termination sequencing, was adopted over the Maxam and Gilbert methodology and continues to be used today. Developed by the Nobel Laureate Frederick Sanger almost four decades ago (Sanger *et al.*, 1977b), the initial Sanger sequencing protocol was essentially a modified PCR reaction that relied upon a DNA polymerase, DNA primers, dNTPs and modified di-dNTPs (ddNTPs) for termination of DNA synthesis and a single-stranded DNA (ssDNA) template. The ssDNA was fragmented into short single-stranded DNA (ssDNA) of varying lengths and added to four reaction mixtures comprising each of the dNTPs and ddNTPs. These short ssDNA fragments were amplified and terminated with radio-labelled ddNTPs, with each of the four reaction mixtures then separated by length through a polyacrylamide radiograph gel. This gel was then visualised by x-ray, with dark bands in the gel representing each base. Thus, the nucleotide sequence was manually read from the top to bottom. Sanger sequencing was enhanced by the development of automation technologies, which instead relied upon fluorescently labelled ddNTPs that were excited with a laser and the emitted colour recorded, corresponding to one of the four bases. Using this chain-termination chemistry the first full genome, bacteriophage phi X174, was sequenced (Sanger *et al.*, 1977a).

Sequencing continued to be enhanced over the following decades thanks to the development of PCR for amplification of short DNA fragments, thermal cyclers and discoveries of heat-resistant polymerases including Taq from *Thermus aquaticus* (Saiki *et al.*, 1985; Smith *et al.*, 1986; Saiki *et al.*, 1988). This also included development of the first automated sequencer, the ABI 370 by Applied Biosystems, allowing all four necessary reactions to be contained within a single mixture (Prober *et al.*, 1987). Computer processing power and storage capabilities also increased dramatically throughout the 1980s and 90s. For the curation of millions of DNA sequences generated, the GenBank database was established in 1982 as an open-access, publically available source of nucleotide and amino acid sequences (Benson *et al.*, 2013).

Updated every 2 months, GenBank grows exponentially with each release (Benson *et al.*, 2013).

Despite continued improvements, Sanger sequencing was only able to sequence short DNA reads. For sequencing of larger DNA regions such as whole chromosomes or genomes, high-throughput methods including shotgun *de novo* sequencing was developed. This initially involved randomly fragmenting DNA into short reads by sonification, cloning the fragments into a plasmid vector and transformation into a host organism including *E. coli*, which then incorporated Sanger sequencing of the short reads (Shendure and Ji, 2008). The first genome to be sequenced using this strategy was the cauliflower mosaic virus (Gardner *et al.*, 1981). Following the advent of PCR (Saiki *et al.*, 1985; Saiki *et al.*, 1988), fragmented DNA could be amplified with paired primers flanking the target. This became known as pairwise-end reading and allowed for more accurate alignment with the DNA fragment. This approach was utilised to sequence the first free-living organism, *Haemophilus influenza* (Fleischmann *et al.*, 1995).

Shotgun sequencing was also the strategy of choice for sequencing of the human genome throughout the 1990s and early 2000s. Initiated by the international collaborative Human Genome Project (HGP) in 1990, this kick-started a race between the publically-funded HGP and private-funded Celera Corporation founded by Craig Venter. The outcome was the publication of the draft human genome in 2001 (Lander *et al.*, 2001; Venter *et al.*, 2001), followed by the declaration of completion in 2003 and publication in 2004 (Human Genome Sequencing, 2004; Schmutz *et al.*, 2004). Key findings were that the human genome contained far less protein-coding genes than anticipated, ranging from 19,000 to 23,000, and the prevalence of large segmental duplications that constituted the remaining gaps. Expected to herald a new era in precision medicine, over a decade on from publication of the draft sequence the legacy of the first human genome sequencing efforts continue to be debated (Lander, 2011).

1.7.2 Second-Generation Sequencing

Although the first complete human genome was sequenced ahead of time and under-budget, the sequencing cost in 2001 was estimated at approximately $100 million (**Figure 1.22**) (Wetterstrand, 2016). It became clear that novel technologies were essential for rapid high-throughput sequencing with significant reductions in production costs. While revolutionary, Shotgun sequencing was not fit for purpose, but served as the precursor for all next-generation

sequencing (NGS) technologies used today. These new technologies were termed ‘second-generation’ DNA methodologies (Shendure and Ji, 2008). Although it is necessary for DNA fragments to be shorter in length (50-150bp) than for Sanger sequencing, single or paired-end reads can be rapidly quantified compared with the previous shotgun approach.

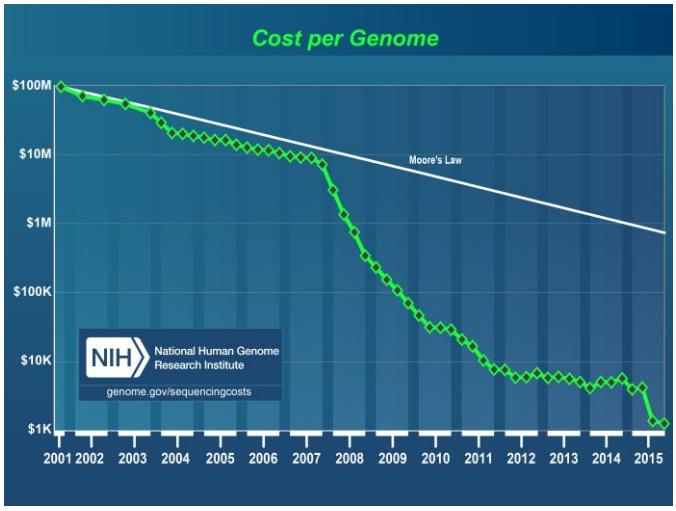


Figure 1.22 Cost Per Genome Since 2001. The cost of sequencing one human genome has fallen dramatically since 2001 and does not follow Moore’s Law. Adopted from Wetterstrand (2016).

The first commercially available NGS platform was 454 Pyrosequencing from Life Sciences (now Roche) (Roche, 2016), also known as ‘sequencing by synthesis’ (Rothberg and Leamon, 2008). 454 Pyrosequencing was based on emulsion PCR, a common genomic library preparation tool still used today, in which denatured ssDNA are compartmentalised in water droplets on beads in a water-in-oil emulsion (Kanagal-Shamanna, 2016). Performed on a microtiter plate (Margulies *et al.*, 2005), incorporation of one complementary dNTP at a time released pyrophosphate that emitted a unique wavelength detectable by the sequencer, allowing automated reading of a wavelength trace generated (Rothberg and Leamon, 2008). This platform was used to sequence the second human genome, of James Watson, more rapidly and at a much reduced cost compared to Shotgun sequencing (Wheeler *et al.*, 2008).

This ‘sequence-by-synthesis’ approach is similar to that of Illumina NGS platforms (also referred to as Solexa platforms) used today. In contrast to 454 Pyrosequencing, Illumina platforms use ‘bridge amplification’ for library preparation (Adessi *et al.*, 2000; Fedurco *et al.*, 2006). DNA fragments are flanked by adaptors ligated to each end and denature to form ssDNA. These are added to a ‘flow cell’ with corresponding fixed adaptors that bind to one end of the ssDNA fragments. Fragments are elongated by PCR with the terminus adaptors ‘bridging’ (binding) to their corresponding fixed adaptor. Strands are denatured and further

cycles proceed to generate clusters of DNA for sequencing. Fragments undergo single-base extension with chemically-cleavable fluorescent-labelled dNTPs with distinct emissions that can be imaged (Turcatti *et al.*, 2008). Cleavage of the modified dNTPs allows further single-base extensions and imaging to occur to determine the nucleotide sequences. Currently, the most popular Illumina NGS genetic analysers used are the HiSeq and MiSeq systems (Illumina, 2016).

ThermoFisher Scientific and Applied Biosystems market a third NGS platform, referred to as 'Sequencing by Oligo Ligation and Detection' (SOLiD) (Life Technologies, 2016). Library preparation previously used emulsion PCR, similar to that of 454 Pyrosequencing. More recently, the SOLiD platform switched to 'Wildfire technology' with amplification using a flowchip with fixed adaptors for hybridisation of DNA fragments and pair-wise amplification (Ma *et al.*, 2013). Moreover, the SOLiD platform uses a DNA ligase instead of a DNA polymerase for amplification, with the fluorescence emitted from repeated cycles of ligation and extension that can be read to determine the fragment sequences (Shendure and Ji, 2008).

The advent of second-generation sequencing has seen the cost of sequencing one human genome fall dramatically over the past 15 years, with the current cost estimated at just over $1000 (Wetterstrand, 2016). Moore's Law states that the number of transistors per square inch on an integrated circuit doubles approximately every 2 years and hence, computing power is increased (Moore, 1998). Therefore the technological costs decrease, although there is expected to be a physical limitation to Moore's Law that could soon be reached. The cost of genome sequencing has significantly outpaced the cost predicted by Moore's Law since early 2008 when second-generation sequencing began to reach the fore-front of genomics (**Figure 1.22**). Nonetheless, the sequencing cost per human genome is still economically unviable for many public-funded research institutes and groups, who seek other cost-effective alternatives.

1.7.3 Whole Exome Sequencing (WES)

One of the NGS platforms developed in the mid-2000s provided such opportunities as a cost-effective, targeted tool of coding variation only, termed 'whole exome sequencing' (WES).

Since it has been estimated that protein-coding changes in the coding regions (exons) of the account for 85% for human Mendelian disorders, the potential use of WES in the diagnosis of Mendelian disease has been widely recognised (Choi *et al.*, 2009; Ng *et al.*, 2010; Bamshad *et al.*, 2011). The first diagnoses attained by WES were a novel homozygous *SLC26A3* missense mutation in a patient with a differential diagnosis of Bartter Syndrome (Choi *et al.*, 2009) and

an X-linked *XIAP* missense mutation in a young patient who presented with inflammatory bowel disease (Worthey *et al.*, 2011). Identification of the causative *XIAP* mutation was seminal in guiding therapeutic strategies, in this case an allogeneic hematopoietic progenitor cell transplant that greatly improved quality of life for the patient (Worthey *et al.*, 2011).

Aside from costs, WES has been an attractive tool in Mendelian disease diagnosis compared with whole genome sequencing (WGS) or virtual targeted gene panel approaches (**Figure 1.23**).

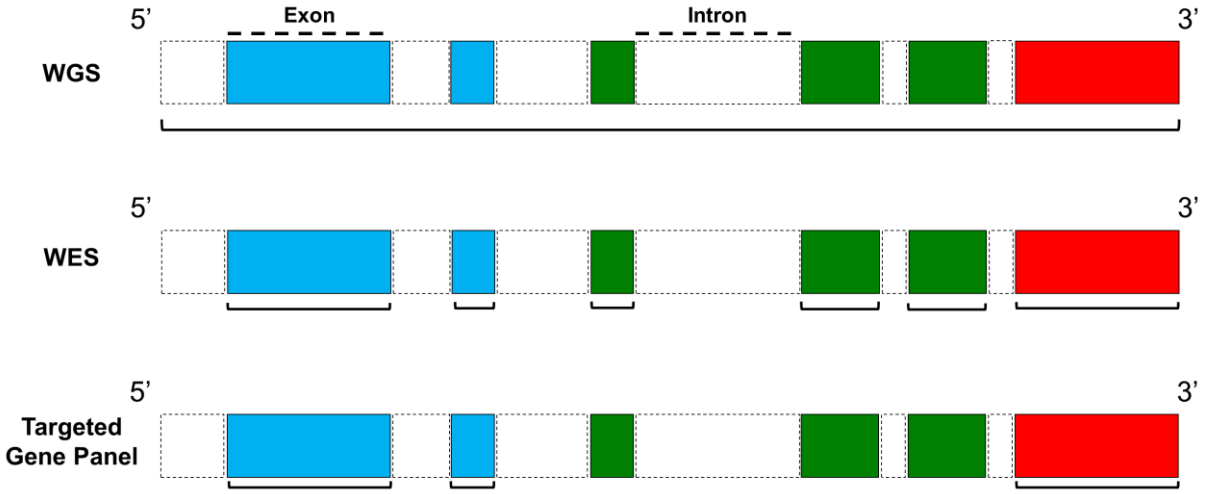


Figure 1.23 Comparison of WGS, WES and Targeted Gene Panel Approaches. A schematic comparing WGS, WES and targeted gene panel approaches to the diagnosis of Mendelian disease. Individual boxes represent one exon. Blue, green and red exon(s) represent distinct genes. Grey and dashed boxes represent introns.

The basic workflow for WES is described by Bamshad *et al.* (2011) and is performed using hybridisation exome-capture kits, which include the TruSeq and Nextera Rapid Exome Capture kits used in this thesis (Illumina, 2016). Genomic DNA is fragmented to create a shotgun library. Fragments are flanked with adapters and subjected to aqueous-phase hybridisation capture, in which fragments enriched with exons are hybridised to biotinylated DNA baits in the presence of blocking oligonucleotides. Hybridised fragments are recovered by streptavidin-based pulldown. These fragments are then amplified using bridge amplification for library preparation and 'sequencing by synthesis' using the Illumina HiSeq or MiSeq genetic analysers. These hybridisation-based exome capture kits require a relatively substantial quantity of genomic DNA for exome sequencing, approximately 2-3µg (Rykalina *et al.*, 2014).

Since it has been estimated that protein-coding changes in the coding regions (exons) of the genome account for 85% for human Mendelian disorders, the potential use of WES in the diagnosis of Mendelian disease has been widely recognised (Choi *et al.*, 2009; Ng *et al.*, 2010; Bamshad *et al.*, 2011). The first diagnoses attained were a novel homozygous *SLC26A3* missense mutation in a patient with a differential diagnosis of Bartter Syndrome (Choi *et al.*, 2009) and an X-linked *XIAP* missense mutation in a young patient who presented with inflammatory bowel disease (Worthey *et al.*, 2011). Identification of the causative *XIAP* mutation was seminal in guiding therapeutic strategies, in this case an allogeneic hematopoietic progenitor cell transplant that greatly improved quality of life for the patient (Worthey *et al.*, 2011).

Although WES has been demonstrated as an effective diagnostic and research tool, there has been continued challenges regarding the assessment of identified variants. The identification of 'variants of unknown significance' (VUS), defined as a non-informative variants that could be associated with human pathology but lack additional evidence, such as segregation studies or functional validation, to confirm pathogenicity. Similarly, the identification of all protein-coding changes raises ethical concerns regarding 'incidental' or 'secondary' findings. Such findings are typically unrelated to the primary genetic investigations but could be variants associated with increased risk of developing certain cancers, including in *BRCA1* or *BRCA2* genes (Levy-Lahad and Friedman, 2007), or drug metabolism (Yu *et al.*, 2014). Guidance and opinion on the return of genetic findings to patients including secondary findings continue to be fiercely debated (Bennette *et al.*, 2013; Yu *et al.*, 2014; Roche and Berg, 2015), including whether such findings are clinically relevant to the primary investigations, medically actionable or if additional family members could also be at risk. Ultimately, the definition of secondary findings and the actions taken by the clinicians are likely to be determined on a 'case-by-case' basis.

WES also provides some technical challenges in identifying potentially pathogenic mutations. WES is dependent on PCR-amplification steps to create a shotgun library from fragmented DNA that aims to provide adequate coverage of the genome (Bamshad *et al.*, 2011). However, this can often lead to poor or uneven coverage, especially in GC-rich regions and because read lengths are typically short (Kozarewa *et al.*, 2009; Veal *et al.*, 2012). Short read lengths and poor or uneven coverage can also prevent the detection of large genomic rearrangements, expanded or triplet repeats, which are often implicated in neurological diseases (Orr *et al.*, 1993; Campuzano *et al.*, 1996; Walker, 2007).

Nonetheless, WES has emerged as a cost-effective targeted NGS tool with the potential to transform the diagnostic algorithm for mitochondrial disorders. The use of WES in the diagnosis of adult-onset mtDNA maintenance disorders and early-onset RC disease is discussed in **Chapter 4** and **Chapter 6**.

1.8 Aims and Objectives

Over the past decade, NGS sequencing technologies have rapidly become part of the clinicians and researchers toolkit in the diagnosis and investigation of human disease. Mendelian mitochondrial disease is no exception, providing diagnoses for a significant proportion of patients while also identifying novel genes associated with this complex disease. Owing to the broad phenotypic and genetic heterogeneity of adult-onset PEO with multiple mtDNA deletions and early-onset RC disease, many patients remain without a genetic diagnosis following routine diagnostic investigations. Hence, NGS technologies including WES offer new opportunities to elucidate the genetic aetiology. This thesis seeks to take advantage of WES in a diagnostic and research orientated approach to mitochondrial diseases, specific to adult-onset Mendelian PEO with multiple mtDNA deletions and early-onset RC deficiency.

First, the clinical, molecular and genetic characteristic of all published patients with adult-onset Mendelian PEO and mtDNA instability will be systematically reviewed. The phenotypic spectrum of this mitochondrial disorder is poorly characterised and has not been previously reviewed. Such a systematic review of all known nuclear gene defects aims delineate the reported phenotypes and to identify genotype-phenotype correlations, where possible.

Secondly, the findings from this systematic review will be used to develop a custom variant filtering strategy for WES analysis and will be applied to a cohort of patients with clinically well-defined, genetically undetermined Mendelian PEO with multiple mtDNA deletions. The systematic review findings will help direct the category of gene variants that will be filtered for and will assist in the prioritisation of candidates.

Simultaneously, a second custom WES filtering strategy for patients with early-onset RC deficiency will be developed and applied to a cohort of undiagnosed patients. It is anticipated that this filtering strategy will differ from that used for the first cohort, but should nonetheless be effective in prioritising the causative variants.

For all causative or putative causative variants, Sanger sequencing will be performed to provide confirmation of variants identified. Critically, segregation studies of unaffected and affected family members will also be performed, where possible, to confirm carrier status. This will be especially pertinent for parents and families with affected children, for which confirmation of the causative variants could inform future reproductive options.

Finally, known and novel candidate gene variants identified from both patient cohorts will be characterised, depending on the availability of patient tissue for study. This includes skeletal muscle, fibroblasts (skin cells) using techniques to investigate the effect on mitochondrial physiology and function. Where possible, *in vitro* and *in silico* tools plus models will be incorporated to complement laboratory studies.

Overall, this thesis aims to demonstrate the use of WES in the diagnosis and investigation of mitochondrial diseases with extreme clinical and genetic heterogeneity, with the use of appropriate filtering and prioritisation strategies.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Equipment

ABI 3130*xl* Genetic Analyser	Applied Biosystems
Analogue Tube Roller, SRT6	Stuart
Autoclave	Astell
Balance, AR3130	Ohaus
Benchtop Centrifuge 5417R (Refrigerated)	Eppendorf
Benchtop Centrifuge 5418	Eppendorf
Benchtop Centrifuge 5804	Eppendorf
Benchtop Centrifuge U-32	Boeco
BH-EN-2004 Class II Microbiological Safety Cabinet	Faster
Biopipette 8 Channel Pipette, 1-10µl	Labnet
Cellometer Auto 1000	Nexcelom Bioscience
ChemiDoc MP Imaging System	Bio-Rad
CO_2 Cell Culture Incubator, MCO-18AIC	Panasonic
Dry Heat Block	Techne
GeneAmp PCR System 9700	Applied Biosystems
Leica DM IL LED Microscope	Leica
Magnetic Stirrer, RCT Basic	IKA
Magnetic Stirring Bars	Camlab
Microcentrifuge, Technicomini	Griffin Education
Mini Electrophoresis Blotting System	Hoefer
Mini-Protean Tetra Cell System	Bio-Rad
Mini Trans-Blot Cell System	Bio-Rad
NanoDrop Spectrophotometer, ND-1000	Thermo Scientific
NANOpureII Water Purification System	Barnstead
Nikon A1R Resonant Scanning Confocal System	Nikon Instruments
Orbital Shaker SSL1	Stuart
PCR UV Cabinet	Bioair Instruments
PerfectBlue Maxi Gel System M	PEQLAB

Pestle and Mortar	CoorsTek
pH Meter 3510	Jenway
Pipette Controller	StarLab
Power Supply, 250 Volts	Cleaver Scientific
SpectraMax M5e Multimode Plate Reader	Molecular Devices
StepOnePlus™ Real-Time PCR System	Applied Biosystems
Stirrer, Ceramic Plate U151	Stuart
Ultra-Turrax Homogeniser, T25	IKA
Ultra-Turrax Homogeniser, T8	IKA
Vacusafe Aspiration System	Integra
Veriti 96 Well Thermal Cycler	Applied Biosystems
Vortex Genie 2	Scientific Industries
Vortex IR	StarLab

2.1.2 Consumables

µ-Dish, 35mm, High, ibiTreat	iBidi
24-Well PCR Plate, Non-Skirted, Elevated Wells	StarLab
8-Strip PCR Caps, Domed	StarLab
90mm Petri Dish, Sterile	Thermo Scientific
96-Well PCR Plate, Semi-Skirted, Clear	4titutde
96-Well PCR Plate, Semi-Skirted with Raised Rim	StarLab
Advanced Polyolefin StarSeal, X-Clear	StarLab
Aluminium Foil	Terinex
Aluminium StarSeal	StarLab
Autoclave tape	Fisher Scientific
Cellstar Falcon Tubes (15ml, 50ml)	Greiner Bio-One
Cellstar Aspirating Pipette, 2ml	Greiner Bio-One
Cellstar Disposable Pipettes (5ml, 10ml, 25ml)	Greiner Bio-One
Cellstar Tissue Culture Flasks ($25cm^2$, $75cm^2$)	Greiner Bio-One
Chromatography Paper, 3mm	Whatman
CryoPure Tubes	Sarstedt
DNeasy Blood and Tissue Kit	Qiagen
FrameStar Break-A-Way 96 wells, Clear Tubes,	4titutde

Blue Frame	
Gloves	StarLab
Immun-Blot PVDF Membrane	Bio-Rad
MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1ml	Applied Biosystems
Microfuge Tubes (0.6ml, 1.5ml, 2ml)	StarLab
Nunc Cryotube Vials	Thermo-Scientific
Parafilm, Pre-Cut	M
Pasteur Pipette, 3ml	StarLab
Performa® DTR V3 96-Well Short Plate Kit	EdgeBio
Sarogold Pro Food Wrap	Sarogold
Scalpel	Swan-Morton
Stericup-GP, 0.22µm, Polyethersulfone, 500ml, Radio Sterilised	Merick-Millipore
Syringe Filter Unit, 0.22µm, Polyethersulfone, 33mm, Gamma Sterilised	Millex
Syringes Without Filters, 1ml	BD Plastipak

2.1.3 Solutions

Ammonium Persulphate (APS), (w/v) 10%	1g APS Dissolve in 10ml ddH_2O
Bromophenol Blue, 1% (w/v)	100mg Bromophenol Blue Dissolve in 10ml ddH_2O
Cell Lysate Buffer	500µl 1M Tris-HCl, pH 7.5 600µl 2M $NaCl_2$ 20µl 1M $MgCl_2$ 100µl Triton X-100 1X EDTA protease inhibitor cocktail tablet Top-up to 10ml with ddH_2O Aliquot and store at -20^{O}C Before use, add 1µl 100mM PMSF per 100µl cell lysate buffer
Dithiothreitol (DTT), 1M	0.308g DTT

	Dissolve in 2ml ddH_2O
DNA Loading Buffer	0.25% (w/v) Bromophenol Blue
	0.25% (w/v) Xylene Cyanol
	30% (v/v) Glycerol
Electrophoresis Buffer	900ml ddH_2O
	100ml TAE, 1X
Laemmli Buffer, 4X	4ml 50% (v/v) Glycerol
	0.8g SDS
	5ml 0.5M Tris-HCl, pH 6.8
	0.25% (w/v) Bromophenol Blue
	0.25% (w/v) Xylene Cyanol
	Make up to 10ml
	Aliquot and store at room temperature
	Before use, add 1µl 1M DTT per
	100µl Laemmli buffer (4X)
Laemmli Buffer, 5X	4ml 1.5M Tris-HCl, pH 6.8
	1ml 1% Bromophenol Blue
	10ml Glycerol
	2.0g SDS
	5ml β-Mercaptoethanol
	Aliquot and store at -20^{O}C
Magnesium Chloride ($MgCl_2$), 1M	0.203g $MgCl_2$
	Dissolve in 1L ddH_2O
Phenylmethylsulphonyl Fluoride (PMSF), 100mM	192mg PMSF
	Dissolve in 10ml Isopropanol
Phosphate Buffered Saline (PBS), 1X, pH 7.4	1X PBS tablet
	Dissolve in 200ml
Ponceau S Staining Solution	1g Ponceau S
	50ml Acetic Acid
	Make up to 1L with ddH_2O
Protease Buffer	10ml RIPA Buffer
	1X EDTA protease inhibitor cocktail tablet
	100µl PMSF
Radioimmunoprecipitation Assay (RIPA) Buffer	250µl 10% SDS

	0.125g Sodium Deoxycholate 250µl 100% Igepal 375µl Triton-X 100 16.47µl β-Mercaptoethanol Make up to 25ml in 1X PBS
Running Buffer (Western Blot), 10X	30g Trisma Base 144g Glycine 10g SDS Make up to 1L with ddH_2O
Sodium Dodecyl Sulphate (SDS), (w/v) 10 %	1g SDS Make up to 10ml with ddH_2O
Tris-Acetate-EDTA (TAE) Buffer, 10X	48.4g Trisma Base 11.42ml Glacial Acetic Acid 20ml 500mM EDTA pH 8.0 Make up to 1L with ddH_2O
Tris-Acetate-EDTA (TAE) Buffer, 1X	100ml 10X TAE Make up to 1L with ddH_2O
Tris-Buffered Saline and Tween-20 (TBST), 5X, pH 7.6	60.5g Trisma Base 45g NaCl 2.5ml Tween-20 Make up to 800ml with ddH_2O Adjust to pH 7.6 with concentrated HCl Top-up to 1L
Transfer Buffer (Western Blot), 10X	30.3g Trisma Base 144g Glycine Dissolve in 1L ddH_2O
Tris-HCl, 0.5M, pH 6.8	6.06g Trisma Base Make up to 100ml with ddH_2O Adjust to pH 6.8 with concentrated HCl
Tris-HCl, 1M, pH 7.5	12.13g Trisma Base Make up to 100ml with ddH_2O Adjust to pH 7.5 with concentrated HCl

Tris-HCl, 1.5M, pH 6.8	18.18g Trisma Base Make up to 100ml with ddH_2O Adjust to pH 6.8 with concentrated HCl
Tris-HCl, 3.75M, pH 8.5	45.45g Trisma Base Make up to 100ml with ddH_2O Adjust to pH 8.5 with concentrated HCl

2.1.4 Chemicals and Reagents

Ethanol, 100%	Fisher Chemical
Liquid Nitrogen	BOC
Methanol, 100%	Fisher Chemical

2.1.5 Polymerase Chain Reaction (PCR) Reagents

Colorless GoTaq Flexi Buffer, 5X	Promega
Colorless GoTaq Reaction Buffer, 5X	Promega
Deoxyribonucleotide Triphosphate (dNTP) Mix, 2mM	Bioline
dNTP Mixture, 2.5mM	TaKaRa
GoTaq G2 DNA Polymerase, 5U μl^{-1}	Promega
GoTaq G2 Hot Start Polymerase, 5U μl^{-1}	Promega
$MgCl_2$ Solution, 25mM	Promega
PrimeSTAR GXL DNA Polymerase, 1.25U μl^{-1}	TaKaRa
PrimeSTAR GXL Buffer, 5X	TaKaRa

2.1.6 Sanger Sequencing Reagents

BigDye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Exonuclease I, 20U μl^{-1}	Thermo Scientific
Thermosensitive Alkaline Phosphatase (TSAP)	Promega

2.1.7 Tissue Culture Reagents

Dialysed Foetal Bovine Serum (DBS)	Sigma-Aldrich

Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Dulbecco's Phosphate-Buffered Saline (DPBS)	Gibco
Ethidium Bromide, 10mg ml^{-1}	Sigma-Aldrich
Foetal Bovine Serum (FBS)	Gibco
Gentamicin, 50mg ml^{-1}	Gibco
GlutaMAX Supplement, 100X	Gibco
L-Glutamine, 2mM	Gibco
Minimum Essential Medium (MEM)	Gibco
MEM Non-essential Amino Acid Solution (100X)	Sigma-Aldrich
MEM Vitamin Solution, 100X	Gibco
Penicillin and Streptomycin Solution	Gibco
PBS, 1X	Gibco
Picogreen	Life Technologies
Skeletal Muscle Cell Growth Medium (SMGM)	PromoCell
Sodium Pyruvate, 100mM	Gibco
Tetramethylrhodamine Methyl Ester (TMRM), 25mg	Invitrogen
TrypLE Express Enzyme (1X), Phenol Red	Gibco
Uridine	Sigma-Aldrich

2.1.8 Gel Electrophoresis Reagents

Agarose (Molecular Grade)	Bioline
1Kb DNA Ladder, 0.1µg $µl^{-1}$	Promega
SYBR Safe DNA Gel Stain, 10,000X	Invitrogen

2.1.9 SDS-Page and Western Blot Reagents

N,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Acrylamide/Bis-acrylamide, 30%, 29:1	NBS Biologicals
Benzonase Nuclease, Purity >90%	Merck Millipore
Clarity Western ECL Substrate	Bio-Rad
Glycine	Sigma-Aldrich
Marvel Dried Skimmed Milk Powder	Marvel
Polysorbate 20 (Tween 20)	Acros Organics
Ponceau S	Sigma-Aldrich

SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific
Trisma Base	Sigma-Aldrich

2.1.10 Molecular Reagents

Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Pierce Bovine Serum Albumin Standard, 2mg ml^{-1}	Thermo Scientific
Protein Assay Dye Reagent Concentrate	Bio-Rad
Spectra Multicolor Broad Range Protein Ladder	Thermo Scientific

2.1.11 Software

Alamut	Interactive Biosoftware
Discovery Studio Visualiser (Version 4.1)	Accelrys Software Incorporated
FinchTV (Version 1.4.0)	Geospiza Incorporated
Foundation Data Collection (Version 3.0)	Applied Biosystems
IBS: Illustrator for Biological Sequences (Version 1.01)	The CUCKOO Workgroup
ImageJ (Version 1.48)	Open Source
Image Lab (Version 4.0.1)	Bio-Rad
Mutation Surveyor	SoftGenetics
NIS-Elements Viewer (Version 4.2)	Nikon
Prism 5 (Version 5.02)	GraphPad Software Incorporated
SeaView (Version 4.5.3)	Manolo Gouy Laboratoire de Biometrie et Biologie Evolutive CNRS / Universite Lyon I
StepOne Sofware (Version 2.1)	Applied Biosystems

2.2 Patients

2.2.1 Recruitment of Patients

Patients were recruited via the NHS Highly Specialised Service for Rare Mitochondrial Disorders and the NHS Highly Specialised Mitochondrial Diagnostic Service in Newcastle upon Tyne (NHS Highly Specialised Service for Rare Mitochondrial Disorders, 2016). The research undertaken was approved by the NHS Research Ethics Committee (REC) under references 16NE/0267 and 8061. Patient and control tissue samples were obtained from the Diagnostic laboratory via a tissue and DNA request form.

2.2.2 Ethical Guidelines

All procedures were approved and performed under the ethical guidelines of the NHS Highly Specialised Mitochondrial Diagnostic Service and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent had been previously obtained from patient and/or guardians for research purposes.

2.2.3 Diagnostic Investigations

Unless stated, all recruited patients underwent clinical, histochemical, molecular and genetic investigations performed by the NHS Highly Specialised Mitochondrial Diagnostic Service. Whole mitochondrial genome sequencing was performed to exclude mtDNA mutations. MtDNA rearrangements were determined in skeletal muscle DNA by one or more of long-range PCR, quantitative real time PCR or Southern blot assays. Analysis of the muscle mitochondrial respiratory chain complex activities were performed for early-onset patients (Kirby *et al.*, 2007). Muscle histochemical studies performed were sequential COX-SDH and Gomori trichrome staining (Taylor *et al.*, 2004), if tissue was available. Targeted nuclear-encoded gene screening was performed in several patients to exclude variants commonly associated with Mendelian mitochondrial disease.

2.2.4 Control Tissue

Appropriate age-matched control fibroblast, myoblast cell lines and muscle tissue were made available from the NHS Highly Specialised Mitochondrial Diagnostic Service for research purposes. This comprised of four paediatric and three adult fibroblast cell lines, two adult myoblast cell lines, two paediatric muscle and two adult muscle blocks.

2.3 Whole Exome Sequencing

2.3.1 Exome Capture and Enrichment

Forty patients with clinically well-defined, undiagnosed mitochondrial disease who underwent WES between July 2011 and April 2016 were analysed for this project. All genomic DNA for WES was prepared by the Highly Specialised Newcastle upon Tyne Mitochondrial Diagnostic Laboratory. Patient blood or muscle genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen). Patients 1-34 were sequenced externally by AROS Applied Biotechnology A/S (AROS, 2016) and patients 35-40 were sequenced in-house. For each patient, 2-3µg of genomic DNA was prepared and sent externally or internally for WES. Genomic DNA was fragmented and exome enriched by either Illumina TruSeq 62Mb (15 patients), Illumina TruSeq Rapid Exome 45Mb (six patients), Nextera Rapid Exome Capture 37Mb (14 patients) or Agilent Sure Select Human All Exon V5 50Mb (five patients) capture kits and sequenced using either the Illumina HiSeq 2000 or Illumina GAIIx in 100 base pair (bp) reads. The final outputs were raw sequence reads in FASTQ format, comprising the nucleotide sequence and the quality score (Cock *et al.*, 2010).

2.3.2 In-House WES Analysis Pipeline

The WES analysis pipeline was developed and run in-house by bioinformaticians (Helen Griffin, Wei Wei, Yaobo Xu, Konstantinos Douroudis) based at the Centre for Life, Institute of Genetic Medicine, Newcastle University. The in-house pipeline was run using Linux command line with a series of Bash Shell scripts that automated necessary tools or software commands. This was able to run simultaneous exomes using one initial Shell script with the file names and corresponding sample identification numbers. Samples were run together in batches with approximately 2 days to complete the analyses. FastUniq was used to remove duplicate reads (Xu *et al.*, 2012), which identified duplicates by comparing adjacent paired reads and by sequence comparison. Raw sequence reads were aligned to the human reference genome sequence (UCSC hg19) using the Burrows-Wheeler Alignment (BWA) tool (Li and Durbin, 2009) and alignments were generated in Sequence Alignment/Map (SAM) format.

The Genome Analysis Toolkit (GATK) was used for variant annotation of single nucleotide variants (SNVs), short insertions or deletions (INDELs) and genotyping (Van der Auwera *et al.*, 2013). SAM files were converted to the equivalent Binary Alignment/Map (BAM) format. Variant annotation generated a Variant Call Format (VCF) file for each patient comprising of

fixed fields; chromosome, (genomic) position, identifier, reference base(s), alternate base(s), quality score, filter status and additional information. Missing values were expressed as '.'.

The quality score was based on a phred-scaled likelihood that the alternate base(s) was called correctly (Ewing and Green, 1998; Ewing *et al.*, 1998). Quality scores (Q) for each base were logarithmically related to the probability (p) that the base(s) were called incorrectly:

$$Q = -10 \times \log_{10}(p)$$

For example, a quality score of 40 is equivalent to a 1 in 10,000 probability that a base(s) was called incorrectly and hence, the base call accuracy would be 99.99%.

The filter status was expressed as 'PASS' if the variant had passed all variant quality score filters or as a semi-colon separated list of all failed filters. For purposes of identifying variants associated with Mendelian disease, variants that did not pass the quality filters were excluded from analysis. For each exome in each genomic position the additional information field comprised of genotype fields in the following format; genotype (GT), unfiltered allele depth (AD), filtered read depth (DP), genotype quality (GQ) and likelihood of the called genotype (PL). GQ and PL values were based upon the Phred-scaled quality scores for each base. The most likely GT was expressed as two allele values separated by '/'. The first allele value represented the human genome sequence and the second allele variant represented the alternative (patient) sequence at that position. Since human cells are diploid, heterozygous variants were called as '0/1' and homozygous as '1/1'. AD was expressed as the total number of unfiltered reads at each position that supported the reported allele value. For example, '34,37' represented 34 reads with the reference position and 37 reads with the alternate position. On the other hand, DP expressed the total number of reads that passed quality score filtering to support the reported allele value. PL was represented by three values, which expressed the normalised phred-scaled likelihoods of each possible genotype at a given position; 0/0 (variant absent), 0/1 and 1/1. The most likely genotype was assigned a normalised PL value of 0 on the phred scale. GQ expressed the genotype quality as a phred-scaled confidence score based upon the PL values. This was calculated as the difference between the second most likely genotype PL value and the most likely PL value. For differences between PL values that were greater than 99, then the GQ value was capped at 99. This indicated the highest quality genotype value for the most likely genotype as given by the PL field.

The full specifications for the SAM/BAM and VCF data formats are available at Samtools at GitHub (samtools, 2016).

Functional annotation of called variants stored VCF files was performed using Annovar (Wang *et al.*, 2010). Gene annotation determined whether called variants occurred in genes, if these were affecting coding or non-coding regions, the affected exons and the specific amino acids affected. Reference systems used for gene annotation were RefSeq (O'Leary *et al.*, 2016), UCSC Genes (Rosenbloom *et al.*, 2015) and Ensembl Genes, using the latest system versions from Annovar. Next, called variants were annotated with allele frequency data from external exome databases and protein effect prediction scores from several *in silico* tool databases for SNVs. Allele frequency data assigned to called variants were derived from Exome Aggregation Consortium (ExAC) (Lek *et al.*, 2016), National Heart, Lung and Blood Institute Exome Sequencing Project (NHLBI ESP), 1000 Genomes Project (February 2012 data release) (The Genomes Project, 2015) and Complete Genomics (Drmanac *et al.*, 2010). The *in silico* protein prediction tool databases assigned to called SNVs were SIFT, PolyPhen 2, LRT, Mutation Taster, Mutation Assessor, FATHMM, RadialSVM, LR, GERP++, PhyloP and SiPhy.

The final output was a Microsoft Excel Open XML format spreadsheet (.xlsx) file generated by a Perl script that concatenated the fields from variant and functional annotation into single columns each, with called variants each represented by one row. Called variants were also annotated with additional allele frequency data from up to 378 in-house exomes that had previously been analysed by the WES pipeline. Both Microsoft Excel .xlsx and VCF files for each patient were made available for purposes of variant analysis.

2.3.3 WES Variant Analysis for Mendelian Mitochondrial Disease

VCF and .xlsx files generated by the in-house WES analysis pipeline required additional, extensive investigations for the prioritisation of potentially pathogenic variants associated with Mendelian mitochondrial disease. Analysis of variants discovered and annotated by the in-house WES pipeline was performed by filtering of the fixed fields provided in the .xlsx file. On-target variants with a minor allele frequency (MAF) greater than or equal to 0.01, corresponding to a frequency of 1% or greater in external databases were excluded. The external databases used were Exome Aggregation Consortium (ExAC), National Heart, Lung and Blood Institute Exome Sequencing Project (NHLBI ESP), 1000 Genomes Project (The Genomes Project, 2015), Complete Genomics and in-house exomes. Variants were restricted to exonic (coding) or splice-site variants. Although functional annotation of called variants by Annovar assigned several *in silico* protein effect predictions tool databases, only PolyPhen 2 (Adzhubei *et al.*, 2013), SIFT (Sim *et al.*, 2012) and Align-GVGD (Tavtigian *et al.*, 2006)

were considered in the analysis of variants of interest. Insertions, deletions, splice-site and truncating variants were immediately considered as damaging to either protein function or structure. Possible effects on pre-mRNA splicing were predicted using the Human Splicing Finder v.3.0 (Desmet *et al.*, 2009). Variants of interest were analysed using the mutation analysis software Alamut (Interactive Biosoftware), which incorporated gene transcripts, MAFs, *in silico* protein effect prediction tools and amino acid conservation across species.

The filtering and analysis strategy of variants differed between the adult-onset PEO with multiple mtDNA deletions cohort and the early-onset mitochondrial respiratory chain complex deficiency cohort, by taking the possible mode of inheritance and GO-Terms of nuclear-encoded genes into consideration. Briefly, the filtering of variants for early-onset respiratory chain complex deficiency patients prioritised the identification of recessive (homozygous or compound heterozygous) variants in nuclear-encoded genes encoding mitochondrial-targeted proteins, with additional prioritisation of genes associated with mitochondrial translation. On the other hand, the strategy for the adult-onset PEO with multiple mtDNA deletions cohort prioritised nuclear-encoded genes associated with DNA maintenance, repair and replication with both dominant (heterozygous) and recessive (homozygous or compound heterozygous) inheritance considered. Full outlines of the WES analysis and filtering strategies employed are described in **Chapter 4** and **Chapter 6**.

2.3.4 Copy Number Variants (CNVs)

Copy number variations (CNVs) were predicted using ExomeDepth (Plagnol *et al.*, 2012), which uses an algorithm to compare the ratios of each exon read depth counts of a patient between approximately 10 control samples as a reference, while reducing technical variability between samples. Deletions and duplications were determined by quantifying the read depth ratio between the patient sample (reads observed) and the control samples (reads expected). For example, a hemizygous deletion gives a ratio of 1:2 (patient:controls) to equal 0.5. Conversely, a duplication gives a ratio of 3:2 to equal 1.5.

CNV outputs were generated using a Perl script that provided the start and end genomic positions of the CNVs, the type of CNV (deletion or duplication), the number of exons affected, the CNV length (bp), chromosome, reads expected, reads observed, gene abbreviation(s) and exon(s), full gene name(s) and Online Mendelian Inheritance in Man (OMIM) gene-phenotype relationship if applicable. The Bayes Factor for each CNV was also given, which computed the likelihood of the CNV presence by assessing read depth,

chromosome, genomic coordinates and the type of CNV. The minimum, maximum and average observations of each CNV from in-house exomes were also given. Common CNVs detected by Conrad *et al.* (2010) were annotated. Known, pathogenic CNVs from the ClinVar archive were also annotated (Landrum *et al.*, 2016). CNVs were constrained to those observed in a minimum of zero to three in-house exomes. Hence, only novel, rare or known pathogenic CNVs were considered. Filtering and prioritisation of CNVs employed the same GO-Terms that were applied to the respective WES patient cohorts.

2.3.5 Primers

Primers used for molecular and genetic studies were made available from the Mitochondrial Diagnostic Service, colleagues from the Wellcome Trust Centre for Mitochondrial Research or were custom-designed. All full list of all primers used is provided in **Appendix A**.

2.3.6 Custom Primer Design

Whole exons or exonic regions harbouring putative pathogenic variants were selected and flanked by 200 bps upstream (5') and downstream (3'). Custom-designed forward and reverse primers were designed using Primer3 (Resnick, 1996). SNPCheck3 (NGRL, 2016) was used to identify single nucleotide polymorphisms (SNPs) in the primer binding sites. Final designs were flanked by universal M13-derived forward (5'-TGTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGACC-3') primers and ordered from Integrated DNA Technologies. Unless stated, all primers were aliquoted in 10µM stocks containing both the forward and reverse primers.

2.3.7 Polymerase Chain Reaction (PCR)

PCRs were all prepared in ice on the laboratory bench. Amplification was performed using either the GeneAmp PCR System 9700 or the Veriti 96 Well Thermal Cycler. For each run a no template control was also performed.

Standard PCR amplification was performed in a total of 25µl comprising 13.8µl NANOpure water, 5.0µl 5X Colourless GoTaq G2 Reaction Buffer, 2.5µl 2mM dNTPs, 0.2µl GoTaq G2 DNA Polymerase, 2.5µl 10µM (forward and reverse) primers and 1µl DNA at a concentration at least 5ng μl^{-1}. Unless stated, the standard PCR protocol was one cycle of $94^{O}C$ for 1 minute then 30 cycles of $94^{O}C$ for 1 minute, $60^{O}C$ for 1 minute and $72^{O}C$ for 1 minute. The final extension step was $72^{O}C$ for 10 minutes.

Hot Start PCR amplification was performed in a total of 25µl comprising 10.0µl nuclease-free water, 5.0µl 5X Colorless GoTaq Flexi Buffer, 2.5µl 2mM dNTPs, 2.0µl 25mM $MgCl_2$, 0.5µl GoTaq G2 Hot Start Polymerase, 4.0µl 10µM (forward and reverse) primers and 1.0µl DNA. Unless stated the Hot Start PCR protocol was one cycle of $94^{O}C$ for 4 minutes, then 30 cycles of $94^{O}C$ for 45 seconds, $60^{O}C$ for 45 seconds and $72^{O}C$ for 1 minute. The final extension step was $72^{O}C$ for 10 minutes.

2.3.8 Gradient PCR for Primer Optimisation

For all forward and reverse primers, $60^{O}C$ was the standard annealing temperature for standard PCR and Hot Start PCR amplification. For primers where amplification failed to occur at $60^{O}C$, a gradient PCR was performed using the Veriti 96 Well Thermal Cycler with annealing temperatures tested at $56^{O}C$, $58^{O}C$, $60^{O}C$, $62^{O}C$ and $64^{O}C$. Additionally, PCRs were tested at all temperatures with and without the addition of 2.0µl DMSO to each reaction.

2.3.9 Long Range PCR

Long range PCR primers were designed by Georgia Campbell, with optimisation also done by Georgia Campbell and Amy Vincent. Amplification was performed to investigate the presence of single large-scale mtDNA deletions or multiple mtDNA deletions. Diagnostic long range PCR was also performed using extracted muscle DNA. Extracted DNA samples were run in triplicate using the PrimeSTAR GXL DNA Polymerase kit (TaKaRa). Two pairs of forward and reverse primers for amplification of the whole mitochondrial genome were used on all samples (**Table 2.1**), together with control blood homogenate DNA, two additional control DNAs and a no template control. PCRs were prepared in ice on the laboratory bench. Each amplification was performed in a total of 50µl comprising 32µl nuclease-free water, 10µl 5X PrimeSTAR GXL buffer, 4.0µl 2.5µM dNTPs, 1.0µl forward primer, 1.0µl reverse primer, 1.0µl PrimeSTAR GXL DNA polymerase and 1.0µl stock DNA. The PCR block was pre-heated to $94^{O}C$. The long range PCR protocol was 35 cycles of $98^{O}C$ for 10 seconds and $68^{O}C$ for 11 minutes. Products were visualised using a 0.7% agarose gel.

Primer	Sequence	Fragment Size (bp)	Amplified Region
719/16422	F-gttcaccctctaaatcaccacgatcaaaag R-tattgatttcacggaggatggtggtcaag	15704	ChrM:719-16422
122/16309	F-cagtatctgtctttgattcctgcctcatc R-ctatgtactgttaagggtgggtaggtttg	16188	ChrM:122-16309

Table 2.1 Long Range PCR Primers. Forward and reverse primers for long range PCR amplification of mtDNA.

2.3.10 Quantitative Real Time PCR for mtDNA Quantification

The quantitative real time PCR protocol and primers were optimised by Ilaria Dalla Rosa for mtDNA copy number quantification. Fibroblast DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol and quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). All tested samples were run in triplicate on 96-well reaction plates, with each plate also run in triplicate. Master mixes and samples were prepared in ice on the laboratory bench and plates prepared in the PCR UV cabinet. Each amplification was performed in a total of 25.0µl comprising 12.5µl Power SYBR Green PCR Master Mix (Applied Biosystems), 1.25µl forward primer, 1.25µl reverse primer, 5.0µl nuclease-free water and 5.0µl DNA at a concentration of 5.0ng μl^{-1}, loaded in MicroAmp® Fast Optical 96-Well Reaction Plates and centrifuged at 1600rpm for 2 minutes. The real time PCR programme was $50^{O}C$ for 2 minutes, $95^{O}C$ for 5 minutes, then 40 cycles of $95^{O}C$ for 5 seconds and $60^{O}C$ for 1 minute. MtDNA was amplified with forward and reverse primers specific for human *MT-COII* gene as the target gene, with forward and reverse primers for human *APP* encoding amyloid-β precursor protein used as a nuclear-encoded reference gene (**Table 2.2**). *MT-COII* primer stocks were prepared at a concentration of 10µM, whereas *APP* primer stocks were at a concentration of 30µM.

Primer	Sequence	Amplified Region
MT-COII	F-cgtctgaactatcctgcccg R-tggtaagggagggatcgttg	ChrM:7774-7874
APP	F-tttttgtgtgctctcccaggtct R-tggtcactggttggttggc	Chr21:27462334-27462407

Table 2.2 Quantitative Real Time PCR Primers. Human *MT-COII* and *APP* forward and reverse primers for mtDNA quantification by real time PCR.

Quantification of mtDNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). As this was a comparative methodology for mtDNA quantification, a control sample

was always used alongside the untested patient samples. Cycle threshold (ct) values for *APP* and *MT-COII* used were the average of the three triplicates. This reflected the number of cycles required for exponential amplification and for fluorescent signal to cross the threshold value to exceed background levels. The average ct values were used in the following calculations:

$$\Delta\mathrm{Ct}(\mathrm{control}) = \mathrm{Ct}(\textit{MT-COII}) - \mathrm{Ct}(\textit{APP})$$

$$\Delta\mathrm{Ct}(\mathrm{untested}) = \mathrm{Ct}(\textit{MT-COII}) - \mathrm{Ct}(\textit{APP})$$

$$\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct}\ (\mathrm{control}) - \Delta\mathrm{Ct}\ (\mathrm{untested})$$

Therefore, the relative expression fold change was calculated as $2^{-\Delta\Delta Ct}$. Ct values for *MT-COII* were higher when mtDNA was depleted because more amplification is required to reach exponentiation. Relative mtDNA quantities were expressed as fold changes relative to one control. Student's t test was performed for statistical comparison between controls and patient samples.

2.3.11 Gel Electrophoresis

Unless stated, PCR products were visualised using a 1% agarose gel. Gels were prepared by heating 1.0g agarose and 100ml 1X TAE in a microwave until the agarose had fully dissolved. After cooling, 3.0µl of SYBR Safe DNA Gel Stain was swirled into the gel mixture. The gel mixture was poured and left to set for 30 minutes. For each PCR product, 5.0µl was mixed with 1.0µl DNA loading buffer and loaded into the agarose gel. In each row used, 3.0µl of 1Kb DNA ladder was loaded. Unless specified, products were run using a benchtop power supply at 120 volts (v) for 40 minutes. Products were visualised using the ChemiDoc MP Imaging System using the SYBR Safe protocol, according to the manufacturer guidelines.

2.3.12 Sanger Sequencing

Prior to Sanger sequencing, the PCR products were cleaned-up. On ice, 5µl of PCR product and 1.5µl ExoSAP-IT was added to a 96-well PCR plate in duplicate for sequencing with forward and reverse universal primers, capped with a rubber seal and mixed by pulse centrifuge. The ExoSAP-IT PCR clean-up programme was 37^{O}C for 15 minutes and 80^{O}C for 15 minutes. ExoSAP-IT enzyme removes unbound dNTPs and residual PCR primers that may interfere with sequencing.

For 20µl of cycling, a reaction mixture comprising 7µl nuclease-free water, 3µl 5X BigDye v.3.1 sequencing buffer, 2µl BigDye v.3.1. ready reaction mix and 1µl universal forward or reverse primer was added to each PCR-ExoSAP-IT mixture, capped with a rubber seal and mixed by pulse centrifuge. The cycle sequencing programme was 96^{O}C for 1 minute, then 35 cycles of 96^{O}C for 10 seconds, 50^{O}C for 5 seconds and 60^{O}C for 4 minutes.

The final step of PCR product clean-up was filtration through the Performa DTR V3 96-well plate, a gel filtration plate for the removal of unincorporated BigDye v.3.1. terminator dye, dNTPs and additional low molecular weight material. The Performa DTR 96-well plate was placed onto a clean 96-well PCR plate and centrifuged at 2,300rpm for 3 minutes to remove excess eluate. The total volume of PCR products for sequencing were added to the centre of each well in the Performa DTR 96-well plate, which was then placed on top of a clean 96-well PCR plate and centrifuged at 2,300rpm for 5 minutes. The eluted products in the 96-well PCR were retained and sequenced using the ABI 3130*xl* Genetic Analyser. Sequencing programmes were setup using the Foundation Data Collection (Applied Biosystems) software.

Completed DNA sequencing from the ABI 3130*xl* Genetic Analyser was generated into .ab1 files for each amplification, comprising of the raw DNA base sequence and electropherogram. Mutation Surveyor (SoftGenetics) or FinchTV (Geospiza) software was used to analyse the sequencing files and figure preparation.

2.4 Tissue Culture

2.4.1 Cell Culturing

All cells were incubated in a CO_2 Cell Culture Incubator at 37^{O}C with 5% CO_2. Cell culturing techniques were performed in a Class II Microbiological Safety Cabinet (Faster) using aseptic technique and sterile or autoclaved equipment. Unless stated, all cells were grown in T75 tissue culture flasks (Greiner Bio-One).

Cultured fibroblasts were grown in MEM with 10% FBS, 1% MEM vitamins, 1% non-essential amino acids, 50U ml^{-1} penicillin, 50µg ml^{-1} streptomycin, 1mM sodium pyruvate solution, 25mg ml^{-1} uridine solution and 2mM L-glutamine. Cultured myoblasts were grown in SMGM with 10% FBS, 1% GlutaMAX supplement and 50µg ml^{-1} liquid gentamycin.

2.4.2 Subculturing of Fibroblasts

Fibroblast cells were passaged at approximately 90% confluency. Fibroblast MEM was aspirated since this inhibits activity of the trypsin, rinsed with 2ml TrypLE Express Enzyme (Gibco) to ensure full coverage of cells and incubated at 37^{O}C for 5 minutes. Cells were quenched with 4ml fresh MEM, transferred to a 50ml falcon tube, centrifuged at 1,200rpm for 4 minutes to obtain a pellet and the supernatant aspirated. Pellets were suspended in 1ml fresh MEM, which was divided equally into T75 tissue culture flasks with 15ml fresh MEM and incubated.

2.4.3 Subculturing of Myoblasts

Myoblasts were passaged at approximately 60% confluency to prevent differentiation in myotubes. SMGM was aspirated, myoblasts were washed with 5ml sterile PBS then aspirated, rinsed with 2ml TrypLE Express Enzyme (Gibco) and incubated at 37^{O}C for 6 minutes. Cells were quenched with 4ml fresh SMGM, divided equally into T75 tissue culture flasks with 15ml fresh SMGM and incubated.

2.4.4 Harvesting of Cells

Both cultured fibroblasts and myoblasts were prepared for harvesting using the steps described in **2.4.2**, until a pellet was obtained in a 50ml falcon tube. Pellets were re-suspended in 1ml MEM or SMGM and transferred to a clean 1.5ml eppendorf tube. After further centrifugation at 1,200rpm for 4-5 minutes the supernatant was aspirated and the pellet rapidly frozen in liquid nitrogen for storage at -80^{O}C.

2.4.5 Freezing of Cells

Both cultured fibroblasts and myoblasts were prepared for freezing using the steps described in **2.4.2**, until a pellet was obtained in a 50ml falcon tube. Fibroblast pellets were carefully resuspended dropwise in 500µl fibroblast freezing medium containing 90% FBS and 10% DMSO. Myoblast pellets were carefully resuspended dropwise in 1ml myoblast freezing medium containing 70% DMEM, 20% FBS and 10% DMSO. Resuspended cells were stored for 24 hours at -80^{O}C, then transferred for long term storage in liquid nitrogen stores.

2.4.6 Generation and Harvesting of Quiescent Fibroblasts

Quiescent (non-dividing) fibroblasts were generated to repress the cytosolic nucleotide salvage pathway and constrain mitochondria dNTP supply to the mitochondrial nucleotide salvage pathway (Dalla Rosa *et al.*, 2016). Once fibroblast cells were confluent in T75 tissue culture flasks, MEM was aspirated and replaced with MEM that contained 0.1% DBS, instead of 10% FBS as described in **2.4.1**. Fibroblasts were incubated in quiescent MEM for between 10 and 14 days, with quiescent MEM replenished every 3-4 days.

Quiescent fibroblasts were harvested by aspirating the quiescent MEM, washing the cells with 4ml PBS, rinsing the cells with 2ml TrypLE Express Enzyme and incubating for 5 minutes. Cells were quenched with 500µl FBS, 4ml PBS, transferred to a 50ml falcon tube, centrifuged at 1,200rpm for 5 minutes to obtain a pellet and the supernatant aspirated. Pellets were resuspsended in 500µl PBS and transferred to a clean 1.5ml eppendorf tube. After further centrifugation at 1,200rpm for 5 minutes the supernatant was aspirated and the pellet rapidly frozen in liquid nitrogen for storage at -80^{O}C.

2.5 Western Blotting

2.5.1 Whole Cell Lysis

Lysis of fibroblast or myoblast pellets was performed on ice. The cell lysate buffer comprised 50mM Tris-HCl, 130mM NaCl, 2mM $MgCl_2$, 1% NP-40 (v/v) and 1X EDTA-protease inhibitor cocktail tablet. Cell lysate buffer was prepared in 10ml stocks and stored at $^{-}$20^{O}C in 200µl aliquots. Immediately before use, 1mM PMSF was added to the lysate buffer. Dependent on the pellet size, 50-100µl cell lysate buffer was added to each pellet and mixed by pipetting. All samples were vortexed thoroughly for 30 seconds, then centrifuged at 4^{O}C, 2,300rpm for 2 minutes. After centrifuging, the supernatant was transferred to a clean 1.5ml eppendorf tube then rapidly frozen in liquid nitrogen for storage at -80^{o}C.

2.5.2 Muscle Homogenisation

Frozen skeletal muscle was homogenised for western blot studies using a protease buffer prepared from RIPA buffer. Liquid nitrogen was added to a pestle and mortar for rapid cooling, then 10-20mg frozen control or patient muscle was added to the mortar. Using the pestle, the muscle was crushed to obtain a powder with further liquid nitrogen added to cool the muscle, pestle and mortar. Once a fine powder was attained, 0.5-1ml protease buffer was added to the tissue. Once frozen, the tissue continued to be crushed until the protease buffer

and tissue were thawed in a homogenate, then pipetted into a clean 1.5ml eppendorf tube. The homogenate was vortexed for 15 seconds then placed on ice for 5 seconds. This was repeated a further four times, then placed on ice for 45 minutes. Next, the homogenate was homogenised using the either the T25 or T8 Ultra-Turrax homogeniser (IKA) or for 5 seconds, placed on ice for 15 seconds, then repeated. The homogenate was returned to a clean 1.5ml eppendorf tube and centrifuged in a chilled centrifuge at 4^{O}C, 11,500rpm for 10 minutes. After centrifuging, the supernatant was transferred to a clean 1.5ml eppendorf tube and rapidly frozen in liquid nitrogen for storage at -80^{O}C.

2.5.3 Bradford Assay

Protein quantification was performed using a Bradford assay with bovine serum albumin (BSA) to produce a standard curve. BSA stocks were prepared at 1mg ml^{-1} and stored at -20^{O}C. The standard curve was prepared in duplicate with BSA calibration points at 0, 2, 5, 10, 15 and 20mg ml^{-1}. Protein samples of unknown concentrations were prepared at 1µl and 2µl dilutions; no duplicates were made. All tubes were vortexed after addition of all the reagents and left at room temperature for 5 minutes. Once the BSA calibration points and protein samples for quantification were prepared, 200µl were aliquoted using reverse pipetting into a 96-well plate. Each protein sample for quantification was aliquoted twice. Plates were then read at 595nm using the SpectraMax M5e Multimode Plate Reader (Molecular Devices).

2.5.4 SDS-Page Sample Preparation

Fibroblast, myoblast and muscle lysates were prepared for western blot loading using 4x or 5x laemmli sample buffer. Hence, the ratio of lysate to sample buffer was 3:1 or 4:1. Prepared samples were heated at 37^{O}C on a dry heat block for 30 minutes. After heating, 0.5µl benzonase nuclease (Merck Millipore) was added to each sample to degrade residual nucleic acid. Samples were flicked to mix and kept on the laboratory bench at room temperature for a further 15 minutes.

2.5.5 SDS-Page

Polyacrylamide gels were handcast and electrophoresis performed using the Mini-Protean Tetra Cell system (BioRad) or the Mini Electrophoresis Blotting System (Hoefer). Either 10% or 12% polyacrylamide gels were used, depending on the molecular weight of the proteins of interest (**Table 2.3**). Once the resolving gel was poured, isopropanol was added on top to

produce an even edge until polymerisation. After the isopropanol was washed away, the 3.75% stacking gel was poured on top.

	10% Resolving Gel	12% Resolving Gel	3.75% Stacking Gel
Acrylamide/Bis-acrylamide, 30%, 29:1	1.667ml	2ml	0.625ml
3.75M Tris-HCl, pH 8.5	0.5ml	0.5ml	/
0.5M Tris-HCl, pH 6.8	/	/	1.25ml
dH_2O	2.726ml	2.395ml	3.02ml
10% SDS	50µl	50µl	50µl
TEMED	5µl	5µl	5µl
10% APS	50µl	50µl	50µl

Table 2.3 Polyacrylamide Gel Casting Reagents and Volumes. List of reagents and volumes for casting a 10% or 12% resolving gel and the 3.75% stacking gel.

SDS-page electrophoresis was performed in 1X running buffer (25mM Tris, 192mM glycine, 0.1% SDS) with 25-50µg protein per sample as prepared in 2.5.4, plus 10µl Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). Electrophoresis was run at a constant 150V using the Bio-Rad Mini-Protean Tetra Cell System or 120V using the Hoefer Mini Electrophoresis Blotting System until the sample buffer dye reached the bottom of the polyacrylamide gel.

2.5.6 Wet Electroblotting Transfer and Blocking

The transfer of proteins from the SDS-page gel to a polyvinylidine fluoride (PVDF) membrane (Bio-Rad) was performed using wet electroblotting transfer with the Mini Trans-Blot Cell System (Bio-Rad). The SDS-page gel was removed from the gel running system and placed in 1X transfer buffer (25mM Tris, 192mM glycine). The PVDF membrane (8.5x5.5cm) was activated in 100% methanol for 30 seconds and rinsed in distilled water. A cassette was assembled with the gel and membrane between four cut-pieces (8.5x5.5cm) 3mm thick chromatography paper (Whatman) and two sponges. This was placed in the transfer tank filled with 1X transfer buffer, a magnetic stirring bar and an ice pack. Wet transfer was performed at 100V for 60 minutes, at $4^{O}C$ on a magnetic stirrer to keep the transfer buffer cool. Following transfer, membranes were blocked in 5% powdered milk (Marvel) in 1X TBST for 1 hour at room temperature to prevent non-specific binding of antibodies.

2.5.7 Ponceau S Staining

Ponceau S staining solution was used for qualitative visualisation of protein loading on PVDF membranes. Following wet electroblotting transfer, membranes were incubated with Ponceau S solution for 2 minutes at room temperature and rinsed in dH_2O until background staining was removed. After visualisation, membranes were further rinsed in dH_2O until all staining solution was removed.

2.5.8 Primary and Secondary Antibodies

Membranes were probed with primary antibodies diluted in 5% powdered milk in 1X TBST (**Table 2.4**); either overnight at $4^{O}C$ or for 2 hours at room temperature. Anti-alpha Tubulin, anti-β-actin, anti-GAPDH and anti-Vinculin were used as loading controls.

Antibody	Dilution	Predicted (Observed) Size, kDa	Host Species	Clonality
Anti-AK2 (ab37594, Abcam)	1:1000	26	Rabbit	Polyclonal
Anti-AK3 (ab119058, Abcam)	1:1000	26	Mouse	Monoclonal
Anti-alpha Tubulin (ab7291, Abcam)	1:10000	50	Mouse	Monoclonal
Anti-ATP5B (ab14730, Abcam)	1:2000	52	Mouse	Monoclonal
Anti-beta Actin (A5316, Sigma)	1:10000	42	Mouse	Monoclonal
Anti-GAPDH (ab8245, Abcam)	1:10000	40 (36)	Mouse	Monoclonal
Anti-GMPR1 (ab118751, Abcam)	1:1000	37	Rabbit	Polyclonal
Anti-GMPR1 (SAB1101144, Sigma)	1:1000	37 (40-42)	Rabbit	Polyclonal
Anti-LONP1 (15440-1-AP, Proteintech)	1:1000	100	Rabbit	Polyclonal
Anti-MRPL12 (Affinity purified in Lightowler laboratory)	1:500	19	Rabbit	
Anti-MTCOI (ab14705, Abcam)	1:1000	57 (40)	Mouse	Monoclonal
Anti-MTCOII (ab110258, Abcam)	1:1000	26	Mouse	Monoclonal
Anti-mtAlaRS/AARS2 (ab197367, Abcam)	1:1000	107	Rabbit	Polyclonal
Anti-mtGluRS/EARS2 (HPA043633, Sigma)	1:1000	60	Rabbit	Polyclonal
Anti-mtTyrRS/YARS2 (AP7838a, Abgent)	1:500	60	Rabbit	Polyclonal
Anti-mtRNA Polymerase/POLRMT (ab32988, Abcam)	1:250	139 (140)	Rabbit	Polyclonal

Antibody	Dilution	Predicted (Observed) Size, kDa	Host Species	Clonality
Anti-MT-TFA/TFAM (ab119684, Abcam)	1:1000	29 (25)	Mouse	Monoclonal
Anti-NDUFB8 (ab110242, Abcam)	1:1000	22	Mouse	Monoclonal
Anti-p53R2 (ab8105, Abcam)	1:5000	41 (39)	Rabbit	Polyclonal
Anti-R1 (sc-22786, Santa Cruz)	1:1000	94	Rabbit	Polyclonal
Anti-R2 (sc-10846, Santa Cruz)	1:1000	45	Goat	Polyclonal
Anti-RMDN3/PTPIP51 (HPA009975, Sigma)	1:500	62	Rabbit	Polyclonal
Anti-SDHA (ab14715, Abcam)	1:1000	70	Mouse	Monoclonal
Anti-SLC25A33/PNC1 (ab97820, Abcam)	1:1000	35	Rabbit	Polyclonal
Anti-SLC25A36/PNC2 (ab154559, Abcam)	1:1000	34	Rabbit	Polyclonal
Anti-SLC29A1/ENT1 (LS-C96639, Lifespan Biosciences)	1:1000	50	Rabbit	Polyclonal
Anti-TK1 (H00007083-M02, Abnova)	1:500	26	Mouse	Monoclonal
Anti-TK2 (Liya Wang)	1:1000	26	Rabbit	Polyclonal
Anti-UQCRC2 (ab14745, Abcam)	1:1000	48	Mouse	Monoclonal
Anti-VDAC1/Porin (ab14734, Abcam)	1:10000	39 (37)	Mouse	Monoclonal
Anti-Vinculin/VCL (ab18058, Abcam)	1:2000	130	Mouse	Monoclonal

Table 2.4 Primary Antibodies. List of primary antibodies used for western blotting.

Membranes incubated overnight at 4^{O}C were transferred to room temperature for a further 20 minutes of incubation to enhance the signal. Next, membranes were washed five times at 5 minutes in 1X TBST, then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako) diluted in 5% powdered milk in 1X TBST (**Table 2.5**) for 1 hour at room temperature.

Antibody	Dilution	Host Species	Clonality
Anti-Goat Secondary (P0449, Dako)	1:3000	Rabbit	Polyclonal
Anti-Mouse Secondary (P0260, Dako)	1:2000	Rabbit	Polyclonal
Anti-Rabbit Secondary (P0399, Dako)	1:3000	Swine	Polyclonal

Table 2.5 Secondary Antibodies. List of HRP-conjugated secondary antibodies used for immunodetection.

After secondary antibody incubation, membranes were washed a further five times for 5 minutes in 1X TBST. Detection of secondary antibodies was performed using either Clarity Western ECL Substrate (Bio-Rad) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). A 1:1 ratio of the luminol/enhancer reagent and stable peroxide solution were mixed, added to the membrane surface with the protein side up and incubated in darkness for 5 minutes. Fluorescence was measured using the ChemiDoc MP Imaging System (Bio-Rad) and analysed using the Image Lab (Bio-Rad) software.

2.6 Live Cell Imaging

2.6.1 Fluorescent Dyes

TMRM (Invitrogen) sequestered by mitochondria was used for visualisation of mitochondrial network morphology. PicoGreen (Life Technologies) was used to stain double-stranded DNA (dsDNA) for the visualisation of mitochondrial nucleoid morphology.

2.6.2 Seeding of Fibroblasts

Passage matched fibroblasts were seeded approximately 24 hours before live cell imaging studies. At least two controls and patient fibroblasts were seeded at 50,000 cells ml^{-1} in 35mm glass bottom imaging dishes (iBidi) with 2ml MEM and incubated overnight at $37^{O}C$. Approximately 50 minutes prior to imaging, the MEM was aspirated and replaced with fresh MEM containing 5nmol TMRM or 3µl ml^{-1} PicoGreen dye.

2.6.3 Confocal Microscopy

Live cell imaging of fibroblast cells was performed at The Bio-Imaging Unit (Central Campus, Newcastle University) using the Nikon A1R Invert point scanning confocal microscope. Imaging was done in an enclosed environmental chamber at $37^{O}C$ with 5% CO_2 using resonant scanning for fast scanning and an increased frame rate with a 63X oil

immersion objective. At least 10 cells from each fibroblast line were captured as a series of Z-stacks for volume analysis.

2.6.4 Mitochondrial Network Morphology Analysis

ImageJ (Open Source) was used to process all live cell images. Z-stacks were processed into a single image using a maximum projection method. A kernel filter was applied using deconvolution to smooth the image signal. Next, images were binarised to allow the automated quantification of mitochondrial morphological features, demonstrated as aspect ratio (AR) and form factor (FF). AR reflected the ratio between the major and minor axis, or the length-to-width ratio of mitochondria. FF reflected the degree of branching, which was calculated using mitochondria perimeter and area as follows:

$$FF = \frac{Perimeter^2}{4\pi \times Area}$$

Average AR and FF for each replicate and fibroblast line were calculated. Student's t test and Dunn's Multiple Comparison test were performed to compare the median AR and FF between control and patient fibroblasts.

2.7 Bioinformatics Analysis of Protein Sequences and Structures

2.7.1 Protein Amino Acid Sequencing and Secondary Structure Retrieval

Full length primary amino acid sequences encoded by genes of interest were retrieved from the UCSC Genome Browser in FASTA format (Genome Bioinformatics Group, 2009; Rosenbloom *et al.*, 2015).

Secondary protein structures were retrieved from the RCSB Protein Data Bank in .pdb format (Berman *et al.*, 2000; The Protein Data Bank (PDB), 2016).

2.7.2 Identification and Visualisation of Protein Domains

Full length amino acid sequences were submitted to InterProScan 5 for identification of protein domains (Jones *et al.*, 2014; InterPro, 2016). Graphical representation of proteins domains was generated using IBS: Illustrator for Biological Sequences (Liu *et al.*, 2015).

2.7.3 Multiple Sequence Alignment (MSA)

Protein sequences of interest were aligned to infer the evolutionary relationship between species or between orthologs. Full length amino acid sequences were arranged in FASTA files with a single line description line for each sequence denoted by the greater-than sign (‘>’) and one or more lines of the protein sequence. FASTA files were read by SeaView (Manolo Gouy, Laboratoire de Biometrie et Biologie Evolutive, CNRS/Universite Lyon I) (Gouy *et al.*, 2010) and aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004b; Edgar, 2004a).

2.7.4 Secondary Structure Analysis

Secondary protein structures were analysed using Discovery Studio Visualiser (Accelrys Software Incorporated), allowing investigation of intramolecular bonds and mutational analysis. To facilitate analysis, water molecules were removed.

Chapter 3. A Comprehensive Systematic Review of Adult-Onset Mendelian Mitochondrial PEO and mtDNA Instability

This review was published (Sommerville *et al.*, 2014) in *Journal of Neuromuscular Diseases* and has been updated to include newly described genes and patients, plus material from Garone *et al.* (submitted manuscript).

3.1 Introduction

Progressive external ophthalmoplegia (PEO) is an adult-onset mitochondrial disorder characterised by paresis of the extraocular muscles and drooping of the eyelids (ptosis) (**Figure 3.1A**). Historically, PEO associated with mitochondrial dysfunction has been widely described in the literature (Iannaccone *et al.*, 1974; Muller Hocker *et al.*, 1983; Marolda *et al.*, 1984; Byrne *et al.*, 1985; Turnbull *et al.*, 1985; Cooles *et al.*, 1988; Federico *et al.*, 1988; Yamamoto and Nonaka, 1988). Mitochondrial PEO is typically associated with single, large-scale mtDNA deletions or mtDNA point mutations (Zeviani *et al.*, 1988; Moraes *et al.*, 1989). Maternally-inherited or sporadic single, large scale mtDNA deletions can cause Kearns-Sayre syndrome (Zeviani *et al.*, 1988; Holt *et al.*, 1989), a mitochondrial cytopathy associated with PEO, retinitis pigmentosa, cardiomyopathy and ataxia (Kearns and Sayre, 1958; Kearns, 1965) or indolent PEO phenotypes (Moraes *et al.*, 1989; Goto *et al.*, 1990a). Single mtDNA point mutations of several mtDNA genes with varying adult-onset PEO phenotypes include $tRNA^{Ala}$ (Spagnolo *et al.*, 2001; Pinos *et al.*, 2011; Filosto *et al.*, 2016), $tRNA^{Leu(UUR)}$ including the common m.3243A>G mutation (Moraes *et al.*, 1993; Verma *et al.*, 1996; Koga *et al.*, 2000; Campos *et al.*, 2001; Nesbitt *et al.*, 2013), $tRNA^{Leu(CUN)}$ (Fu *et al.*, 1996; Cardaioli *et al.*, 2008), $tRNA^{Lys}$ (Tiranti *et al.*, 1999), $tRNA^{Tyr}$ (Raffelsberger *et al.*, 2001; Sahashi *et al.*, 2001), $tRNA^{Glu}$ (Alston *et al.*, 2010), $tRNA^{Ile}$ (Franceschina *et al.*, 1998; Corona *et al.*, 2002; Berardo *et al.*, 2010; Schaller *et al.*, 2011), $tRNA^{Pro}$ (Hardy *et al.*, 2016), $tRNA^{Asn}$ (Seibel *et al.*, 1994; Ronchi *et al.*, 2012d), $tRNA^{Val}$ (Yan *et al.*, 2010) and $tRNA^{Ser(UCN)}$ (Cardaioli *et al.*, 2007; Souilem *et al.*, 2010).

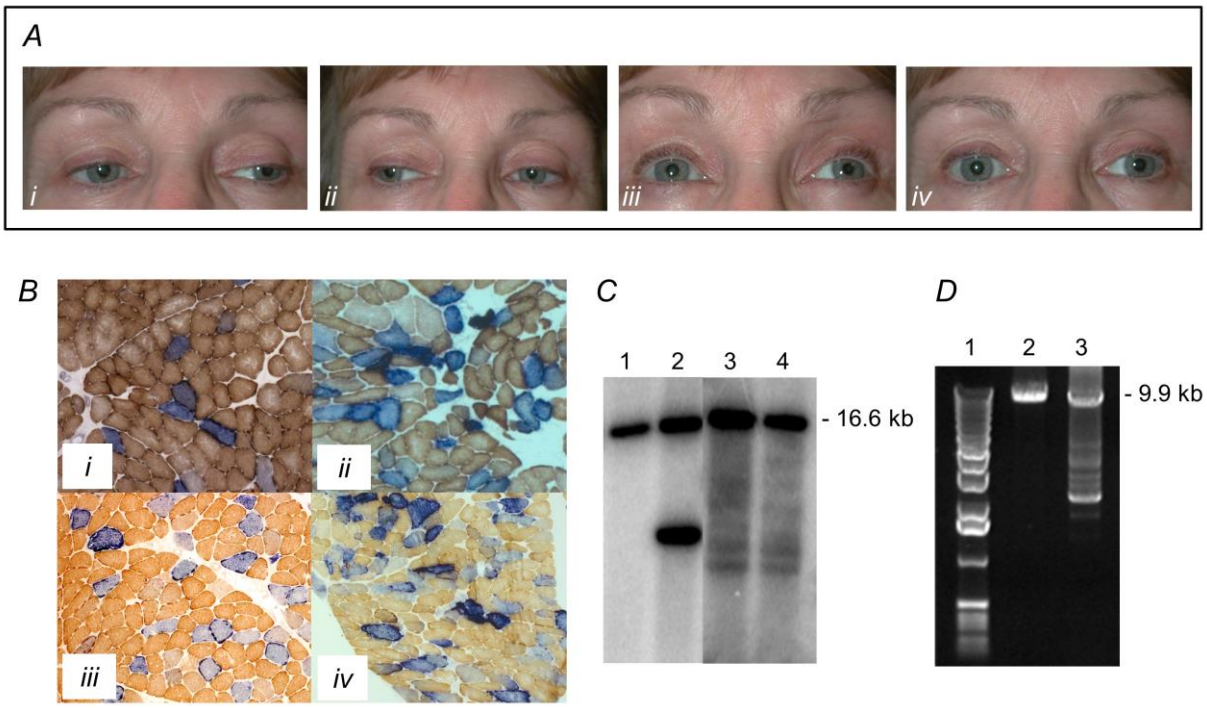


Figure 3.1 Clinical, Histochemical and Molecular Features of Adult-Onset Mendelian PEO and mtDNA Instability (*A*) Paresis of the extraocular muscles is demonstrated by a patient asked to look (*i*) left, (*ii*) right, (*iii*) up and (iv) down. (*B*) Sequential COX-SDH histochemical studies from patients with (*i*) a homozygous p.Ala467Thr *POLG* variant; (*ii*) a heterozygous p.Gln458His *TWNK* variant; (*iii*) compound heterozygous p.Arg186Gly and p.Thr218Ile *RRM2B* variants; (*iv*) compound heterozygous p.Asn288* and p.Lys558* *SPG7* variants. (*C*) Southern blotting of muscle DNA demonstrating typical banding pattern associated with multiple mtDNA deletions (lanes 3 and 4), a large scale single deletion (lane 2) and wild-type (lane 1). (*D*) Long range PCR of muscle DNA also shows a banding pattern typical of multiple mtDNA deletions (lane 3), together with wild-type mtDNA (lane 2) and a size marker (lane 1). Adopted from Sommerville *et al.* (2014).

Mutations of nuclear encoded genes essential for maintenance and repair of mtDNA have been increasingly recognised as important causes of adult-onset PEO associated with secondary, clonally expanded skeletal muscle restricted multiple mtDNA deletions (Zeviani *et al.*, 1989). However the phenotypic spectrum is broad, ranging from indolent or isolated PEO to fatal multisystem disorders. The most widely recognised nuclear encoded gene associated with adult-onset Mendelian PEO is *POLG*, encoding the catalytic subunit of the only known mitochondrial DNA polymerase (Van Goethem *et al.*, 2001). Over the past two decades, additional mutated genes have emerged as important causes of mtDNA maintenance disorders, including *TWNK* (formerly known as *C10orf2* or Twinkle) (Spelbrink *et al.*, 2001), *SLC25A4* (also known as *ANT1*) (Kaukonen *et al.*, 2000) and *RRM2B* (Tyynismaa *et al.*, 2009b). Sequential COX-SDH histochemistry of patient skeletal muscle characteristically show COX-deficient fibres (**Figure 3.1B**), while modified Gomori trichrome staining also often demonstrates ragged-red fibres. Similar to patients with single large-scale mtDNA

deletions, multiple mtDNA deletions are typically detected by long range PCR or occasionally by Southern blotting of muscle DNA, which show a typical multiple banding pattern (**Figure 3.1C and D**).

Despite the identification of several associated nuclear encoded genes, there remains a poor correlation between phenotypes and genotypes. Genetic diagnosis of patients has been enhanced by WES and NGS technologies (Garone *et al.*, 2012; Ronchi *et al.*, 2012b; Tyynismaa *et al.*, 2012; Kornblum *et al.*, 2013; Ronchi *et al.*, 2013; Pfeffer *et al.*, 2014; Wedding *et al.*, 2014; Gorman *et al.*, 2015a; Reyes *et al.*, 2015). Unlike early-onset mitochondrial disorders that are overwhelmingly recessively inherited, the prioritisation of variants identified by NGS is challenging since both dominant and recessive variants are associated with adult-onset Mendelian PEO. Since these disorders are predominantly late-onset, for identified candidate heterozygous variants it is often impossible to perform segregation studies with parental DNA samples; it is unknown whether candidate variants were indeed inherited or occurred *de novo*. Therefore, NGS studies of undiagnosed mitochondrial disease has principally focused on early-onset mitochondrial disorders that are considered more likely to lead to genetic diagnoses (Taylor *et al.*, 2014; Wortmann *et al.*, 2015; Pronicka *et al.*, 2016). Clinically, some patients with overlapping phenotypes may have different genetic aetiologies, while other patients with different phenotypes may have a similar genetic aetiology. Furthermore, muscle histopathology and molecular studies also demonstrate variability between patients, sometimes providing inconclusive evidence of mitochondrial dysfunction or mtDNA rearrangements. Therefore, approximately half of patients with clinically well-defined adult-onset Mendelian PEO remain without a confirmed genetic diagnosis following exclusion of known associated nuclear encoded genes.

3.2 Aims

This chapter reviews the genotypic and phenotypic heterogeneity of all patients from the literature with adult-onset Mendelian PEO and mtDNA instability, classifying all currently known nuclear encoded genes.

3.3 Methods

3.3.1 Patient Selection

Published patients were identified using the following inclusion criteria; (1) adult patients, defined as 16 years old or older; (2) presenting PEO, which included the use of additional

descriptors such as ‘CPEO’, ‘ophthalmoparesis’, ‘ophthalmoplegia’, paresis or restricted movement of the extraocular muscles; (3) have evidence of skeletal muscle restricted multiple mtDNA deletions, mtDNA depletion or both; (4) have a confirmed genetic diagnosis of nuclear aetiology.

3.3.2 Literature Search Strategy

Electronic searches were performed for articles published between 1st January 1970 and 8th November using three online literature database; Scopus, PubMed and Genetics Abstracts, with additional searches performed with UniProt (**Appendices B-E)**. This wide date range was selected to allow historic patients who received a genetic diagnosis in later publications to be included. Only English-language articles were included and duplicate articles retrieved from combined electronic searches were excluded. Patients reported in two or more published articles were included once only, with careful consideration taken when analysing articles reporting large, undefined patient cohorts. The following keywords were used for searches: ‘PEO’, ‘CPEO’, ‘ophthalmoplegia’, ‘ophthalmoparesis’, ‘adult’ and ‘adult-onset’. Additional electronic searches were performed after 8th November 2013 to expand the initial remit of the systematic review.

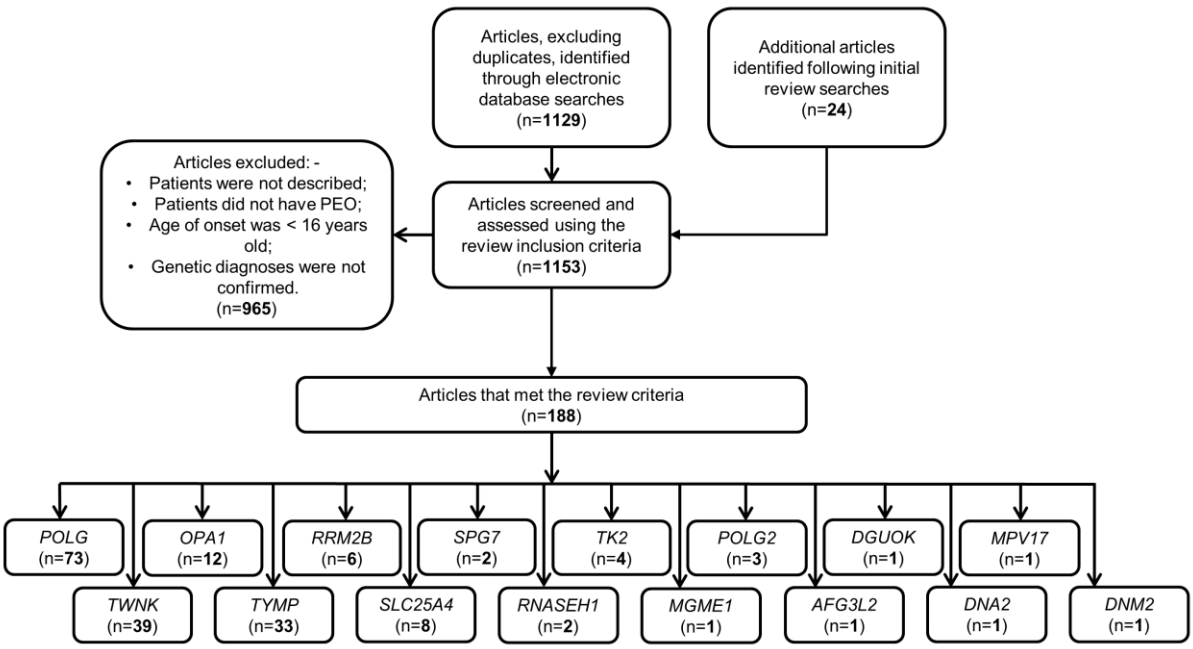


Figure 3.2 Flow of Articles Identified Systematically and Manually. Assessment of articles identified systematically (1st January 1970 and 8th November 2013) and from additional searches. Figure adopted and amended from Sommerville *et al.* (2014).

3.3.3 Data Extraction and Analysis

Articles were arranged by genes in the order of discovery with regards to involvement in adult-onset Mendelian PEO and mtDNA instability. Statistical and meta-analyses were not performed due to the vast numbers of patients and the variation in the quality of articles.

3.4 Results

3.4.1 Identification and Assessment of Adult-Onset PEO and mtDNA Instability

A total of 1129 articles were retrieved from the electronic searches using the set search terms. Of this, 164 articles described 594 patients who met the inclusion criteria and harboured variants of 12 nuclear encoded genes. A large number of patients initially identified were excluded, due to symptoms presenting before 16 years of age, an undetermined genetic aetiology or a non-mitochondrial aetiology. Additional manual searches after 8th November 2013 identified a further 73 patients in 24 articles (including one unpublished article) with *SPG7*, *AFG3L2*, *DNM2* and *RNASEH1* variants, plus additional patients with variants of known genes (**Figure 3.2**). Five patients were also reported with digenic inheritance of two heterozygous mutations associated with PEO. Hence, 16 nuclear encoded genes were identified where variants cause adult-onset PEO and mtDNA instability in 667 patients (**Table 3.1**). All mutations are listed in **Appendices F, G, I and K**.

Gene	Genotype	Effect on mtDNA	# Patients	# Articles
POLG	AD and AR	Multiple deletions and depletion	280	73
TWNK	AD	Multiple deletions and depletion	161	39
OPA1	AD	Multiple deletions	53	12
TYMP	AR	Multiple deletions and depletion	51	33
RRM2B	AD and AR	Multiple deletions and depletion	42	6
SLC25A4	AD	Multiple deletions	39	8
SPG7	AD and AR	Multiple deletions	12	2
RNASEH1	AR	Multiple deletions	12	2
TK2	AR	Multiple deletions	8	4
MGME1	AR	Multiple deletions	3	1
POLG2	AD	Multiple deletions	3	3
AFG3L2	AD	Multiple deletions	2	1
DGUOK	AR	Multiple deletions	2	1
DNA2	AD	Multiple deletions	1	1
MPV17	AR	Multiple deletions	1	1
DNM2	AD	Multiple deletions	1	1

Table 3.1 Nuclear Genes Associated with Adult-onset PEO and mtDNA Instability. All 16 genes currently associated with adult-onset Mendelian PEO and mtDNA instability and the number of affected patients reported. Four patients had digenic variants (variants of two genes). AD – autosomal dominant. AR- autosomal recessive.

3.4.2 *TYMP* – Thymidine Phosphorylase

TYMP encodes thymidine phosphorylase, which is essential for the nucleotide salvage pathway, converting thymidine (dT) to thymine and deoxyuridine (dU) to uracil. *TYMP* variants cause the multisystem disorder mitochondrial neurogastrointestinal encephalopathy (MNGIE), of which PEO is a prominent feature. There were 51/667 (7.6%) patients from 33 articles that met the review criteria. All patients had recessive variants; either homozygous or compound heterozygous. Splice variants were particularly prevalent in comparison to variants of other genes associated with PEO and mtDNA instability (**Appendix G**), which were generally missense changes. Hence, patients had depleted TYMP activity that was typically below 5% compared to controls. Most patients had mtDNA depletion in the skeletal muscle where such analyses were performed, although a small number had multiple mtDNA deletions or both (Nishino *et al.*, 1999a; Nishino *et al.*, 2000).

The most prominent feature of MNGIE was gastrointestinal dysmotility, which varied in severity between patients. Some of the common associated symptoms included borborygmi,

abdominal pain, diarrhoea, vomiting and early satiety. MNGIE-like phenotypes were described in several patients with pathogenic variants of different PEO-associated nuclear genes, including a patient with recessive *RRM2B* variants (Shaibani *et al.*, 2009) and some patients with *POLG* variants (Filosto *et al.*, 2003; Bostan *et al.*, 2012; Tang *et al.*, 2012; Woodbridge *et al.*, 2013; Horga *et al.*, 2014), but whom all had normal TYMP activity. Additionally, leukoencephalopathy typical of *TYMP* patients was also absent in these additional patients (Van Goethem *et al.*, 2003a). Almost all patients had myopathy, which included muscle weakness and atrophy. Patients also frequently had peripheral or sensory neuropathies, including absence of deep tendon reflexes. Retinopathy, hearing loss or both were also frequently described (Hirano *et al.*, 1994; Hamano *et al.*, 1997; Carrozzo *et al.*, 1998; Hirano *et al.*, 1998; Nishino *et al.*, 2000; Blazquez *et al.*, 2005; Blondon *et al.*, 2005a; Marti *et al.*, 2005; Amiot *et al.*, 2009; Laforce *et al.*, 2009; Massa *et al.*, 2009; Filosto *et al.*, 2011; Etienne *et al.*, 2012). Three patients also apparently had no gastrointestinal dysmotility (Gamez *et al.*, 2002; Giordano *et al.*, 2006; Filosto *et al.*, 2011). However, the distinct and progressive MNGIE phenotype observed in all other patients suggest that this may yet to present.

MNGIE is an early-onset mitochondrial disorder with a distinct, progressive phenotype with poor prognosis. Typically, patients presented with in the first decade of life or early teens and therefore, several were excluded from the review. Onset of symptoms after 30 years of age was rare. Despite the characteristic features of MNGIE, there was no correlation between *TYMP* variants and phenotype.

3.4.3 *SLC25A4* – ADP/ATP Translocase 1

SLC25A4, also known as *ANT1*, encodes ADP/ATP translocase 1 and is required for the exchange of ADP from the cytoplasm with ATP generated by mitochondria (Stepien *et al.*, 1992). There were 39/667 (5.8%) patients with dominant *SLC25A4* variants in eight articles who presented indolent or mild PEO phenotypes, with or without ptosis (Kaukonen *et al.*, 2000; Napoli *et al.*, 2001; Komaki *et al.*, 2002; Deschauer *et al.*, 2005). The majority of patients had a heterozygous c.340G>C (p.Ala114Pro) variant. Reduced hearing ability was described in several members of an Italian family, while generalised myopathy was described in some members of another Italian family (Kaukonen *et al.*, 2000).

Four patients had striking psychiatric symptoms (Siciliano *et al.*, 2003; Deschauer *et al.*, 2005; Galassi *et al.*, 2008). This included a late-onset patient with severe depression,

insomnia and nocturnal panic attacks, bradykinesia, rigidity, slurred speech, dysphagia, dysmetria, bilateral postural-action tremor and absent deep-knee tendon reflexes. This patient was found to have heterozygous *SLC25A4* c.865G>A (p.Val289Met) and *POLG* c.1399G>A (p.Ala467Thr) variants that may explain the severe phenotype in comparison to other *SLC25A4* patients. Schizoaffective disorder was described in a female with bilateral ptosis, mild limb weakness and retinopathy but who had two affected siblings without psychiatric features (Deschauer *et al.*, 2005). Bipolar affective disorder was also described in a mother and daughter; the mother had bilateral facial bradykinesia, temporal wasting, ataxic gait, peripheral neuropathy and hearing loss, while her daughter had a milder phenotype of exercise intolerance (Siciliano *et al.*, 2003). Both shared a heterozygous p.Leu98Pro variant that was also found in Italian families with mild PEO phenotypes but without psychiatric symptoms.

Dominant *SLC25A4* variants manifest mild but often varied adult-onset PEO phenotypes, even in patients harbouring the same variant. A family history of PEO was reported in all cases except for one apparently *de novo* case. Multiple mtDNA deletions were also found in muscle from all analysed patients. Recently, the genotype-phenotype correlation has been further complicated by the identification of neonatal-onset patients with severe mtDNA depletion due to *de novo* heterozygous *SLC25A4* variants (Thompson *et al.*, 2016). Recessive *SLC25A4* variants have also been associated with mitochondrial myopathy, hypertrophic cardiomyopathy (HCM) and mtDNA instability, but without PEO or muscle weakness (Palmieri *et al.*, 2005; Echaniz-Laguna *et al.*, 2012; Korver-Keularts *et al.*, 2015).

SLC25A4 variants continue to be a rare cause of adult-onset PEO and mtDNA instability but should be considered in all suspect patients with mild or indolent PEO, particularly those with facial weakness.

3.4.4 *POLG* – DNA Polymerase Gamma Subunit 1

POLG encodes the catalytic subunit of the only known DNA polymerase of mitochondria, required for the binding and processing of newly synthesised DNA. There were 280/667 (42.0%) patients from 73 articles that met the review criteria harbouring predominantly dominantly inherited, heterozygous *POLG* variants and a smaller number harbouring recessive variants.

POLG variants were the most frequently reported cause of adult-onset Mendelian PEO and mtDNA instability in the literature, presenting a heterogeneous, broad spectrum of

overlapping features (**Appendix H**). Patients typically presented systemic or complex phenotypes; indolent PEO with or without ptosis was uncommon. Due to significant numbers of patients identified, adult-onset PEO associated with mtDNA instability and *POLG* variants have been categorised by clinical features to assist in diagnosis (Cohen *et al.*, 1993).

- Autosomal dominant PEO (adPEO) – Patients have phenotypes ranging from indolent PEO to PEO-plus, which also frequently include non-systemic features.
- Autosomal recessive PEO (arPEO) – Similar to adPEO, some patients present non-systemic features. However, ataxia and MNGIE-like manifestations have also been described (Van Goethem *et al.*, 2003a; Tang *et al.*, 2012).
- Myoclonic epilepsy myopathy and sensory ataxia (MEMSA) – This includes patients who present with ataxia usually as the first symptom, epilepsy, proximal or distal myopathies, but who do not have ophthalmoparesis. MEMSA was previously classified as Spinocerebellar Ataxia with Epilepsy (SCAE).
- Ataxia Neuropathy Spectrum (ANS) – Approximately 90% of patients within this spectrum present with ataxia and neuropathy, while muscle weakness is uncommon. This encompasses the previous classifications of Sensory Ataxia Neuropathy Dysarthria and Ophthalmoparesis (SANDO) and Mitochondrial Recessive Ataxia Syndrome (MIRAS).

Sensory or cerebellar ataxia were common features due to *POLG* variants and were often associated with peripheral sensory neuronopathy. However, the true extent of affected patients reported in the literature was unclear due to incomplete patient descriptions or non-specific terms for neuropathies. *POLG*-associated muscle weakness was predominantly proximal or manifested as exercise intolerance, although a significant number of patients presented distal weakness; a rare feature due to variants of other adult-onset PEO associated nuclear-genes.

Additional, non-syndromic features reported included diabetes mellitus, dysphagia, dysarthria and dysphonia. Liver involvement was also uncommon in adults with *POLG* variants (Neeve *et al.*, 2012). Interestingly several female patients had premature ovarian failure (POF) (Pagnamenta *et al.*, 2006; Hudson *et al.*, 2007; Blok *et al.*, 2009; Baruffini *et al.*, 2011), which appeared to be confined to *POLG* variants with the exception of one female with a pathogenic *TWNK* variant (Virgilio *et al.*, 2008). Extrapyramidal features were also frequently reported. There were 46 patients who had Parkinsonism (Del Bo *et al.*, 2003; Luoma *et al.*, 2004; Mancuso *et al.*, 2004b; Pagnamenta *et al.*, 2006; Invernizzi *et al.*, 2008; Remes *et al.*, 2008; Betts-Henderson *et al.*, 2009; Synofzik *et al.*, 2010a; Ferreira *et al.*, 2011a; Milone *et al.*, 2011; Gurgel-Giannetti *et al.*, 2012; Dolhun *et al.*, 2013; Mukai *et al.*, 2013; Horga *et al.*,

2014; Miguel *et al.*, 2014; Delgado-Alvarado *et al.*, 2015; Martikainen *et al.*, 2016; Mongin *et al.*, 2016). Dystonia was less common but may also be suggestive of a *POLG* associated disorder (Paus *et al.*, 2008; Remes *et al.*, 2008; Horga *et al.*, 2014; Rajakulendran *et al.*, 2016). Dementia was also a common manifestation (Van Goethem *et al.*, 2004; Horvath *et al.*, 2006b; Komulainen *et al.*, 2010; Martikainen *et al.*, 2010; Tang *et al.*, 2011; Dolhun *et al.*, 2013; Roos *et al.*, 2013a), as was depression or avoidant personalities (Lamantea *et al.*, 2002; Van Goethem *et al.*, 2003a; Luoma *et al.*, 2004; Mancuso *et al.*, 2004a; Kollberg *et al.*, 2005; Hudson *et al.*, 2007; Galassi *et al.*, 2008; Schulte *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Synofzik *et al.*, 2010a; Gurgel-Giannetti *et al.*, 2012; Palin *et al.*, 2012; Dolhun *et al.*, 2013).

There were 91 *POLG* variants identified that were associated with adult-onset PEO (**Appendix I**). This comprised of 78 missense changes, six nonsense variants, three frameshift variants, two splice-site variants, one in-frame deletion and one stop-loss variant. The most commonly occurring variants were c.1399G>A, p.Ala467Thr; c.2243G>C, p.Trp748Ser, c.1760C>T, p.Pro587Leu and c.752C>T, p.Thr251Ile, which were associated with recessive inheritance. Nonetheless, most variants were reported in small numbers or in single patients and were chiefly pathogenic dominant variants. However, there was no correlation between variants and phenotypes. Patients with overlapping features who harbour known and novel *POLG* variants continue to be reported, often using previous classifications of SANDO, SCAE and MIRAS for diagnosis. Taken together, both dominant and recessive pathogenic *POLG* variants should be screened for in undiagnosed adult-onset PEO patients with suspected Mendelian aetiology.

3.4.5 *TWNK* – Twinkle

TWNK (*C10orf2* or Twinkle) encodes a DNA helicase that catalyses the ATP-dependent unwinding of mtDNA in the 5’ to 3’ direction, co-localising with the mtDNA in the nucleoids (Spelbrink *et al.*, 2001; Korhonen *et al.*, 2003). Autosomal dominant *TWNK* variants comprised the second largest group of patients, with 161/667 (24.1%) patients described in 39 articles. *TWNK* variants manifested in a broad phenotypic spectrum ranging from indolent PEO to a fatal multisystem disorder (**Appendices J** and **K**). Features that were particularly common included proximal muscle weakness, gait weakness, exercise intolerance and fatigue. Additional features included diabetes mellitus, visual loss or impairment and ataxia. One patient had also POF (Virgilio *et al.*, 2008), a feature more frequently observed in females with pathogenic *POLG* variants but also in Perrault syndrome due to recessive *TWNK* variants

without ophthalmoparesis or mtDNA instability (Morino *et al.*, 2014; Demain *et al.*, 2016; Lerat *et al.*, 2016).

Three patients were reported harbouring digenic variants that included one pathogenic *TWNK* variant. The first had heterozygous *TWNK* c.1001G>A (p.Arg334Gln) and *POLG* c.2542G>A (p.Gly848Ser) variants who presented PEO, ptosis, depression and severe dysphagia that led to cachexia, dysarthria, generalised myopathy and pyramidal signs (Van Goethem *et al.*, 2003a). The second patient had heterozygous *TWNK* c.1142T>C (p.Leu381Pro) and *POLG* c.2831A>G (p.Glu944Gly) variants and presented late-onset PEO, bilateral ptosis, had a short stature, dysphagia and reduced deep tendon reflexes (Da Pozzo *et al.*, 2015). The third patient had heterozygous *TWNK* c.1121G>A (p.Arg374Gln) and *TYMP* c.317G>C (p.Glu106Gly) variants leading to a complex, late-onset MNGIE-like phenotype comprising PEO, bilateral ptosis, irregular abdominal pain, gastrointestinal dysmotility, fatigue and prominent myopathy, without evidence of mtDNA instability (Nakhro *et al.*, 2011). The identification of patients with co-existing variants causing adult-onset PEO suggests that digenic inheritance may be a rare but important contributor to the pathogenesis.

The prevalence of psychiatric symptoms was higher in patients with *TWNK* variants than in other adult-onset PEO associated genes. Depression and avoidant personalities were described in a cohort of patients (Spelbrink *et al.*, 2001). The prevalence of dementia and Alzheimer's disease was also higher than would be by chance (Spelbrink *et al.*, 2001; Hudson *et al.*, 2005; Virgilio *et al.*, 2008; Echaniz-Laguna *et al.*, 2010a). Parkinsonism was described in seven patients, including three affected members of the same family (Baloh *et al.*, 2007). A deceased patient was also found to have scattered white matter lesions, but did not apparently present psychiatric symptoms, Alzheimer's disease, Parkinsonism or dementia (Martin-Negrier *et al.*, 2011).

HCM was described in several patients. This included a patient with right bundle branch block (Lewis *et al.*, 2002), a cohort with left ventricular hypertrophy (Fratter *et al.*, 2010) and a Chinese family with familial heart disease (Hong *et al.*, 2010). An Arabian family with double nucleotide changes (c.1078C>G and c.1079T>G) leading to a heterozygous p.Leu360Gly missense change, had congestive cardiac failure together with severe hepatopathy and acute encephalopathy (Bohlega *et al.*, 2009).

Although present in all tested patients, multiple mtDNA deletions were not always prominent in the skeletal muscle. One patient also had mtDNA depletion in addition to mtDNA deletions (Jeppesen *et al.*, 2008). Although less frequently described, *TWNK* variants present a broad

phenotypic spectrum similar to that of *POLG* variants, though multisystem disorders are less common. With variable and heterogeneous phenotypes, *TWNK* variants should be considered in all suspect adult-onset PEO patients, particularly those with psychiatric symptoms, cardiomyopathies or both.

3.4.6 *OPA1* – Optic Atrophy Protein 1

OPA1 encodes the mitochondrial dynamin-like 120 kDa protein, which is required for regulating mitochondrial fusion and morphology (Olichon *et al.*, 2002; Ishihara *et al.*, 2006).

Reviewing the clinical presentations of patients with pathogenic *OPA1* variants revealed a distinctive, progressive phenotype that required amendment of the review criteria. Typically, *OPA1* variants caused optic neuropathy, sometimes with additional visual abnormalities, within the first decade of life that progressed to PEO and other mitochondrial disease associated symptoms in adult life. Therefore, the review criteria were relaxed to include patients that presented before 16 years old. With this amended criteria, 53/667 (7.9%) patients in 12 articles with pathogenic heterozygous *OPA1* variants were identified.

Sensorineural hearing loss (SNHL) was the most prominent feature, present in almost all patients. Other frequently described features included ataxia, axonal neuropathy, ptosis, proximal myopathy and exercise intolerance. A small number of patients also had one or more of migraines, diabetes, Parkinsonism, dementia or epilepsy (Yu-Wai-Man *et al.*, 2010a; Carelli *et al.*, 2015). One patient who also presented with hypogonadism and macrocytic anemia was initially diagnosed with a heterozygous *POLG2* variant but was later found to have a pathogenic *OPA1* variant, which was presumed the most likely genetic aetiology (Ferraris *et al.*, 2008). Multiple mtDNA deletions were detected in all patients where such analyses were performed.

OPA1 variants were first described in patients presenting non-syndromic autosomal dominant optic atrophy (ADOA), with mitochondrial disease associated features described later (Payne *et al.*, 2004), referred to as ADOA-plus. However, the phenotype had been historically described in the literature (Treft *et al.*, 1984; Meire *et al.*, 1985). Delineating the genotype-phenotype correlation between ADOA and ADOA-plus is not currently possible since these overlapping phenotypes can be caused by the same variant. For example, the c.1334G>A, p.Arg455His variant has been described in 12 patients with optic atrophy, PEO and multiple mtDNA deletions, but also in patients with pure ADOA (Shimizu *et al.*, 2003).

Phenotypically, patients with *OPA1* variants are similar to those with Leber Hereditary Optic Neuropathy (LHON) (Yu-Wai-Man *et al.*, 2003).

OPA1 variants have been extensively studied and described in patients with ADOA-plus, which include features such as ophthalmoparesis and hearing loss or with pure ADOA. Therefore, targeted *OPA1* screening should be performed in all patients that present optic atrophy within the first decade of life or adolescence, independent of additional syndromic features.

3.4.7 *POLG2* – DNA Polymerase Gamma Subunit 2

POLG2 encodes the mitochondrial DNA polymerase gamma accessory subunit, which binds to single-stranded DNA and is required for mtDNA replication and embryogenesis, as evident from mouse models demonstrating a single copy of *POLG2* is sufficient to sustain life (Humble *et al.*, 2013).

There were 3/667 (0.4%) patients with heterozygous *POLG2* variants reported in three articles. The first was a 19 year old female with a heterozygous p.Arg369Gly variant presented a multisystem disorder comprising PEO and ptosis with gastrointestinal reflux, delayed gastric emptying, apnoea and respiratory insufficiency (Young *et al.*, 2011). She had Duchene Muscular Dystrophy (DMD)-like muscle weakness, was easily fatigued and suffered from exercise-induced cramps. The second patient was a 55 year old male who also had the same *POLG2* variant (Craig *et al.*, 2012), for whom a family history of PEO and ptosis was reported. He developed ptosis at 30 years of age, followed by the gradual progression of ophthalmoparesis, cerebellar ataxia, exercise intolerance, proximal myopathy, absent knee jerk response and the inability to walk from heel to toe. The third patient was a 60 year old female with a heterozygous c.1352G>A (p.Gly451Glu) variant (Longley *et al.*, 2006), presenting progressive PEO and ptosis that was preceded by exercise intolerance and muscle pain from the age of 40. Mild facial and limb muscle weakness, glucose intolerance and a cardiac conduction defect were also noted. A family history of PEO and ptosis was also reported including her similarly affected mother.

Classification of adult-onset PEO patients with heterozygous *POLG2* variants is difficult due to small number identified, the broad range of symptoms and the variation in clinical progression. This is further complicated by the identification of patients with juvenile-onset phenotypes (Young *et al.*, 2011) and the identification of a patient with early-onset mtDNA hepatocerebral depletion syndrome associated with a homozygous *POLG2* variant (Varma *et*

al., 2016). Nevertheless, proximal muscle was a common feature in all three adult PEO patients. Although rare, *POLG2* variants should be considered in all adult-onset PEO patients, especially those with multisystem phenotypes.

3.4.8 *RRM2B* – Ribonucleotide Reductase Subunit M2B

RRM2B encoding ribonucleoside-diphosphate reductase subunit M2 B (p53R2) is required for the supply of deoxynucleotide triphosphates (dNTPs) for nuclear and mtDNA replication (Bourdon *et al.*, 2007).

There were 42/667 (6.3%) patients identified in six articles with pathogenic *RRM2B* variants, resulting in broad phenotypes comparable to those caused by *POLG* and *TWNK* variants. In total, there were 16 pathogenic *RRM2B* variants reported. The most common pathogenic variant was the c.979C>T (p.Arg327*) nonsense change harboured by 19 patients. This was described in a four generation family, including some members who were unaware that they were affected with mild PEO, myopathy and exercise intolerance, plus a Hungarian family with the proband also presenting with mild gait ataxia and decreased reflexes (Tyynismaa *et al.*, 2009b). Among all *RRM2B* patients, the most frequent features were hearing loss, muscle atrophy and exercise intolerance (Fratter *et al.*, 2011; Kato *et al.*, 2011; Takata *et al.*, 2011).

Almost all patients had dominant *RRM2B* variants, but two adult patients with recessive variants were also reported. The first patient had a homozygous p.Pro33Ser variant and presented with PEO, ptosis, hearing loss and had a depressive mood and anxiety (Takata *et al.*, 2011). The second patient had compound heterozygous (c.329G>A, p.Arg110His and c.362G>A, p.Arg121His) variants and presented PEO, ptosis, nausea, gastrointestinal dysmotility, cachexia, dysarthria and hearing loss (Shaibani *et al.*, 2009), which closely resembled MNGIE and early-onset *RRM2B* patient phenotypes (Bourdon *et al.*, 2007). Furthermore, this was the only adult-onset *RRM2B* patient to have mtDNA depletion; at 12% compared to age-matched controls. Multiple mtDNA deletions were described in all other tested patients.

Interestingly, a synonymous c.48G>A variant was also identified in three patients (Pitceathly *et al.*, 2012). This c.48G>A change was found to occur in the last nucleotide of exon 1 and predicted to result in aberrant splicing, which was demonstrated by RNA analysis showing the abolition of normal exon 1 and 2 splicing with partial intron retention leading to premature translation (Pitceathly *et al.*, 2012). This is currently the only known pathogenic synonymous variant in any of the 16 known adult-onset PEO associated genes.

RRM2B variants are the fifth most described cause of adult-onset PEO and present a broad phenotypic spectrum. Although there was a single patient with a MNGIE-like phenotype, multisystem disorders arising from *RRM2B* variants are considered rare. Targeted *RRM2B* screening is recommended in all adult-onset PEO patients, especially where other systemic features such as hearing loss, bulbar involvement and gastrointestinal dysmotility present.

3.4.9 *TK2* – Thymidine Kinase 2

TK2 encodes thymidine kinase 2, an essential component of the mitochondrial nucleotide salvage pathway that generate dNTPs for mtDNA replication. There were 8/667 (1.2%) patients identified with recessive *TK2* variants; four patients from the literature (Tyynismaa *et al.*, 2012; Alston *et al.*, 2013; Camara *et al.*, 2015) and four unpublished patients (Garone *et al.*, submitted). TK2-deficiency has been more widely described in early-onset patients with mtDNA depletion syndrome, who present a severe phenotype leading to premature death (Saada *et al.*, 2001).

The four published adult patients comprised two affected sisters and two unrelated female patients. The affected first sister developed progressive bilateral ptosis at 47 years of age and received blepharoplasty at 52. Skin sarcoidosis, progressive leg muscle weakness and mild atrophy, scapular winging and difficulty in rising from a squatted position were noted at 49; PEO did not develop until 52 years old. Although able to walk unaided at 64, she developed dysarthria, dysphagia and died of pancreatitis, associated pneumonia and respiratory insufficiency. Her affected sister developed ptosis in her 30s or 40s, which was operated on three times (41, 46 and 48 years old). During her 40s she also developed progressive proximal arm and leg muscle weakness, but did not seek medical attention until 54 years old. At this time PEO and walking difficulties were also noted, but at 59 years old normal eye movements and only mild ptosis were seen. She also had scapular winging on both sides, dysphagia without facial muscle weakness and respiratory insufficiency. Both sisters were found to have compound heterozygous *TK2* variants (c.547C>T, p.Arg183Trp and c.562A>G, p.Thr188Ala) (Tyynismaa *et al.*, 2012). The third patient was a 74 year old female with hearing loss, mild ptosis, subtle PEO, facial weakness, proximal muscle atrophy including scapular winging and of the sternocleidomastoids and weak neck flexion and extension. She carried compound heterozygous *TK2* variants (c.103C>T, p.Gln35* and c.582G>T, p.Lys194Asn) (Alston *et al.*, 2013). The fourth patient presented with mild PEO, severe ptosis, limb girdle muscle weakness, dysphagia, dysarthria and facial diplegia. This patient harboured a homozygous in-frame c.604_606del (p.Lys202del) deletion (Camara *et al.*, 2015). All four unpublished

patients (Garone *et al.* submitted) presented initially with progressive myopathy, PEO, ptosis and with multiple mtDNA deletions. Three patients had dysphagia. Respiratory insufficiency also manifested in three patients, of which two required ventilatory support. An unpublished patient corresponds to patient 4 (**4.4.6**), who harboured a homozygous c.323C>T (p.Thr108Met) *TK2* missense variant identified by WES.

All eight adult-onset PEO patients with TK2-deficiency presented with a late-onset and slowly progressive myopathy, with PEO and ptosis of varying severity while often developing scapular winging and respiratory involvement. Although rare, targeted *TK2* gene screening should be considered in all PEO patients with progressive myopathies and reduced respiratory capacity.

3.4.10 *DGUOK* – Deoxyguanosine Kinase

DGUOK encodes deoxyguanosine kinase and is essential for dNTP generation via the mitochondrial nucleotide salvage pathway for mtDNA replication (Jullig and Eriksson, 2000), similar to TK2. There were only 2/667 (0.3%) patients described with recessive *DGUOK* variants in one article (Ronchi *et al.*, 2012b).

The first adult patient was a 69 year old female who presented indolent PEO and ptosis at 58 years old, although a sibling was noted to have exercise intolerance. The second patient was a 72 year old female who presented with PEO and bilateral ptosis for over 20 years, occasional dysphagia for liquids and complained of upper and lower limb girdle muscle weakness with leg cramps in later life. Both these patients shared a heterozygous c.462T>A (p.Asn154Lys) *DGUOK* variant. However, the 69 year old female also had a c.605_606del (p.Arg202Tyrfs*) frameshift variant, while the 72 year old female had an additional c.130G>A (p.Glu44Lys) missense change. These variants had also been previously described in early-onset *DGUOK* patients (Dimmock *et al.*, 2008). Unlike the mtDNA depletion syndrome patients, liver function in both adults was normal.

To date, pathogenic *DGUOK* variants are predominantly associated with early-onset hepatocerebal mtDNA depletion syndrome (Mandel *et al.*, 2001; Dimmock *et al.*, 2008). Therefore, *DGUOK* variants remain a very rare cause of adult-onset PEO.

3.4.11 *MPV17* – MPV17 Mitochondrial Inner Membrane Protein

MPV17 encodes a mitochondrial inner membrane protein that has yet to be fully characterised, although it is believed to be associated with the mitochondrial nucleotide salvage pathway and dNTP balance for mtDNA replication (Spinazzola *et al.*, 2006; Dalla Rosa *et al.*, 2016). Currently, only 1/667 (0.1%) patient with adult-onset PEO, multiple mtDNA deletions who had recessive *MPV17* variants has been reported in one article (Garone *et al.*, 2012). *MPV17* variants are frequently associated with early-onset hepatocerebal mtDNA depletion syndrome (Spinazzola *et al.*, 2006; El-Hattab *et al.*, 2010), which is comparable to that caused by *DGUOK* variants.

The patient was a 67 year old male born to non-consanguineous parents who developed distal limb muscle weakness and numbness at 34 years old, leading to an initial diagnosis of Charcot-Marie-Tooth disease following nerve conduction and EMG studies. This progressed to proximal limb muscle weakness, exercise intolerance, diabetes, ptosis, ophthalmoparesis, hearing loss, severe constipation due to gastrointestinal dysmotility and depression in his 40s. At 65 years old, he had Parkinsonism with bradykinesia, bilateral resting tremor and mild rigidity. He was also found to have a fatty liver following ultrasonography of the abdomen. His mother reportedly had ptosis, while his sister died of unexplained liver failure at 39 years old. WES identified three heterozygous *MPV17* variants (c.263A>T, p.Lys88Met; c.265A>T, p.Met89Leu; and c.428T>G, p.Leu143*). The p.Lys88Met missense change had been previously described in patients with early-onset hepatocerebal mtDNA depletion syndrome (El-Hattab *et al.*, 2010).

Ophthalmoparesis and axonal sensory-motor neuropathy preceding liver dysfunction in the adult patient was similar to that of some early-onset patients with *MPV17* variants, whereas Parkinsonism and gastrointestinal dysmotility were additional features. Comparable to recessive *DGUOK* variants, *MPV17* variants represent an extremely rare cause of adult-onset mitochondrial disease.

3.4.12 *MGME1* – Mitochondrial Genome Maintenance Exonuclease 1

MGME1 encodes mitochondrial genome maintenance exonuclease 1, which has been demonstrated to be the cleavage of single-stranded DNA, processing DNA flap substrates and maintaining 7S DNA levels (Kornblum *et al.*, 2013; Szczesny *et al.*, 2013; Uhler *et al.*, 2016). Only 3/667 (0.4%) patients from one article harbouring homozygous *MGME1* variants were included in this review (Kornblum *et al.*, 2013); two Italian brothers with a homozygous

c.456G>A (p.Trp152*) nonsense variant and a German female with a homozygous c.698A>G (p.Tyr233Cys) missense change.

The Italian brothers, aged 53 and 42, were born to consanguineous parents and presented with mild bilateral ptosis in the third decade of life that progressed to insidious PEO, proximal myopathy, generalised muscle wasting and mild kyphosis that developed over time. Both were profoundly emaciated and suffered from respiratory insufficiency. The younger sibling developed sleep apnoeas and became dependent on continuous positive airway pressure (CPAP) at night, followed shortly afterward by the elder sibling who also required CPAP occasionally during the day. The German female presented with bilateral ptosis, generalised muscle weakness and fatigue at the age of 57. Further examination at 65 years old revealed PEO, diplopia, decreased deep tendon reflexes and dyspnea. Similarly to the Italian siblings, she developed respiratory insufficiency at 66 years old and began non-invasive ventilation with oxygen therapy at 67. Furthermore, she also suffered from chronic renal failure, cardiac arrhythmias, depressive episodes and memory lapses.

Early-onset patients with *MGME1* variants were also described, including three siblings who shared the same homozygous p.Trp152* variant as the two Italian brothers, with similar clinical presentations. Although the phenotype appears to be distinct and progressive, *MGME1* variants remain extremely rare.

3.4.13 *DNA2* – DNA Replication ATP-Dependent Helicase/Nuclease DNA2

DNA2 encodes a dual nuclear-mitochondrial helicase required for Okazaki fragment processing, nuclear and mtDNA repair, and telomere maintenance (Duxin *et al.*, 2009). Only 1/667 (0.1%) female patient, who presented at 35 years old, from one article has been described with adult-onset PEO, multiple mtDNA deletions and a single heterozygous c.2167G>A (p.Val723Ile) *DNA2* variant (Ronchi *et al.*, 2013). Clinical examination at 55 years old showed that she had lower limb weakness, ophthalmoparesis, diplopia and myalgia. Three other adult patients presenting progressive myopathy, multiple mtDNA deletions and with heterozygous *DNA2* variants have also been reported, but did not have extra ocular features. Nonetheless, *DNA2* variants are considered a rare cause of adult-onset mitochondrial disease.

3.4.14 *SPG7* – Paraplegin

SPG7 encodes paraplegin, a mitochondrial inner membrane metalloprotease forming an m-AAA protease holo-oligmer with AFG-3 like protein 2 (Casari *et al.*, 1998). Following the initial systematic searches for adult-onset PEO and multiple mtDNA deletion patients, Wedding *et al.* (2014) and Pfeffer *et al.* (2014) described 12/667 (1.8%) patients identified by WES and targeted sequencing, who met the review criteria with dominant and recessive *SPG7* variants. There were 11 patients identified from a cohort of 68 patients with undiagnosed adult-onset PEO, indicating that *SPG7* variants may be a common genetic cause. Eight patients had recessive variants and four had heterozygous variants, including the commonly described c.1529C>T (p.Ala510Val) variant. Of these, nine presented with the classical spastic paraplegia that had been previously associated with recessive *SPG7* variants (Casari *et al.*, 1998). Brain MRI revealed cerebellar atrophy in six patients and ataxia was described in 10 patients, of which one patient was spastic. Only one patient presented isolated PEO.

Typically, patients with *SPG7* variants presented PEO and spastic ataxia or a progressive ataxia disorder. With a high frequency of patients reported with spastic paraplegia or progressive ataxia, this may direct targeted gene screening of *SPG7* in undiagnosed patients.

3.4.15 *AFG3L2* – AFG3-like Protein 2

AFG3L2 encodes AFG-like protein 2 and is known to associate with paraplegin in the mitochondrial inner membrane (Banfi *et al.*, 1999; Di Bella *et al.*, 2010). Pathogenic heterozygous *AFG3L2* variants have been typically associated with adult-onset spinocerebellar ataxia 28 (SCA28). Only 2/667 (0.3%) patients from one article were identified following WES and targeted *AFG3L2* screening (Gorman *et al.*, 2015a). The first patient had an indolent gait and limb ataxia that had begun as a teenager. On clinical examination, this had progressed to a broad-ataxic gait with dysmetria and dysarthria. There was also a family history of ataxia. The second patient presented with slowly progressive ataxia and limb spasticity. Brain MRI revealed marked cerebellar atrophy. However, there was no prior family history. Nonetheless, both patients presented late-onset, slowly progressive PEO with slurred speech and limb muscle weakness. The first patient had a heterozygous c.2011G>T (p.Gly671Trp) variant, while the second patient had a heterozygous c.2065T>C (p.Tyr689His) variant. Both variants occurred in the hot-spot for pathogenic *AFG3L2* variants, lending support to pathogenicity.

With only two patients identified, this suggests that pathogenic heterozygous *AFG3L2* variants are a very rare cause of late-onset PEO and multiple mtDNA deletions. Hence, targeted *AFG3L2* gene screening may be considered for undiagnosed patients presenting late-onset PEO with ataxia, spasticity or both.

3.4.16 *DNM2* – Dynamin 2

DNM2 encodes dynamin-2, a widely expressed dynamin belonging to a subfamily of GTP-binding proteins and is required for a variety of functions including membrane trafficking, endocytosis, neuron morphology and growth (Tinelli *et al.*, 2013). Autosomal dominant *DNM2* variants had been previously been reported as a cause of centronuclear myopathy (CNM) (Bitoun *et al.*, 2005), CMT (Zuchner *et al.*, 2005), hereditary spastic paraplegia (HSP) (Sambuughin *et al.*, 2015) and a homozygous variant associated with lethal congenital contracture syndrome (Koutsopoulos *et al.*, 2013). Mitochondrial abnormalities have also been described but are uncommon (Zanoteli *et al.*, 2009; Catteruccia *et al.*, 2013; Gal *et al.*, 2015).

To date, 1/667 (0.1%) patient from one article, a 47 year old Hungarian female from a single article has been reported with PEO and multiple mtDNA deletions due to a heterozygous c.1105C>T (p.Arg369Trp) *DNM2* variant (Gal *et al.*, 2015). The p.Arg369Trp variant had been previously associated with centronuclear myopathy (Bitoun *et al.*, 2005). The patient presented at 32 years old with difficulty tiptoeing, stair climbing, standing from a seated position and had frequent ankle sprains, weakness in the hands and generalised muscle weakness. On clinical examination at 42 years, she had a long myopathic face, ophthalmoparesis mostly affecting the left side, bilateral ptosis, diplopia, moderate atrophy of the hand and feet muscles, hammer toe and mild, predominantly distal muscle weakness. She also had cardiomyopathy, axonal neuropathy and mild depression. Her mother was also affected by ophthalmoparesis and severe cardiac failure but genetic investigation was not possible.

Targeted *DNM2* gene screening may be considered in adult patients with mtDNA deletions presenting PEO with axonal neuropathy and muscle weakness. However, the presence of central nuclei and low-level COX-deficiency in skeletal muscle together with clinical investigations, suggest that muscle histochemical studies are more beneficial in leading to the diagnosis of *DNM2* patients.

3.4.17 *RNASEH1* – Ribonuclease H1

RNASEH1 encodes ribonuclease H1, a dual nuclear and mitochondrial endonuclease required for the degradation of RNA-DNA hybrids (Cerritelli and Crouch, 1998). Recessive *RNASEH1* variants are the most recent to be associated with adult-onset PEO and multiple mtDNA deletions. Currently, 12/667 (1.8%) patients from two articles have been reported (Reyes *et al.*, 2015; Akman *et al.*, 2016).

Most patients presented initially with ptosis and progressive PEO during their twenties, although one patient presented at 45 years old, progressing to gait difficulties, ataxia and muscle weakness. One patient became wheelchair dependent late in life. Sensorimotor neuropathies manifested in several patients, while one presented with a mild motor myelinating neuropathy. In late life, three of four affected siblings had respiratory impairment. A fourth unrelated patient also had reduced respiratory capacity leading to an acute episode requiring a tracheotomy, but only mild respiratory deficit thereafter. One patient had HCM, though a second patient also died after a sudden cardiac event. Multiple mtDNA deletions were also detected in the skeletal muscle of all tested patients. Furthermore, all patients harboured a c.424G>A (p.Val142Ile) variant either homozygous (10 patients) or in compound (two patients) with a second variant (c.469C>T, p.Arg157* or c.554C>T, p.Ala185Val).

RNASEH1 variants have only been recently described and therefore only a small number of patients are currently known. Nonetheless, all reported patients presented initially with adult-onset ptosis and progressive PEO, which progresses to a multisystem disorder with respiratory involvement.

3.5 Discussion

Adult-onset mitochondrial PEO remains predominantly caused by mtDNA point mutations or a single, large scale deletion. Sixteen nuclear-encoded genes were identified that are associated with adult-onset PEO and mtDNA instability, leading to a broad spectrum of clinical features affecting multiple body systems (**Table 3.2**). There were 667 patients described in 188 published articles with confirmed genetic diagnoses (**Table 3.1**). Twelve genes were identified systematically; *SPG7, AFG3L2, DNM2* and *RNASEH1* variants were identified with additional searches, using the initial search terms employed.

Gene	Muscle	NS	Psychiatric	Cardiovascular	Endocrine	Digestive	Respiratory	Reproductive	Urinary
POLG	++	++	+	+	+	+	-	+	-
TWNK	++	+	++	+	+	+	-	+	-
OPA1	++	++	+	-	-	+	-	-	-
TYMP	++	++	+	-	-	++	-	-	-
RRM2B	++	++	+	+	-	+	+	-	+
SLC25A4	++	+	+	-	+	-	-	-	-
SPG7	++	++	+	-	-	-	-	-	+
RNASEH1	++	++	-	+	-	-	++	-	-
TK2	++	+	-	-	-	-	+	-	-
MGME1	++	-	+	+	-	-	++	-	+
POLG2	++	++	-	-	-	+	+	-	-
AFG3L2	++	++	-	-	-	-	-	-	-
DGUOK	++	-	-	-	-	++	-	-	-
MPV17	++	++	++	-	++	++	-	-	-
DNA2	++	-	-	-	-	-	-	-	-
DNM2	++	++	++	++	-	-	-	-	-

Table 3.2 Body Systems Affected in Adult-Onset PEO and mtDNA Instability Disorders. Summary of the body systems affected in adult-onset PEO and mtDNA instability due to nuclear-gene defects. NS – nervous system. '++' - frequently involved. '+' - rarely involved. '-' – not involved. Adopted and amended from Sommerville *et al.* (2014).

3.5.1 Influence of Publications on Systematic Review Quality

Systematic reviews are greatly influenced by the quality of the papers describing the case studies. With 188 papers reporting patients from a period greater than 10 years, there was vast variation in the quality of reporting. Several patients identified from the literature were initially described without a confirmed genetic diagnosis, particularly before 1999, with molecular and genetic analyses of the same patients reported in later articles. For the purpose of this review, articles that described patients in the greatest detail were considered of a high quality. This included a full case report for each patient; containing the age of onset, age at clinical examination, a complete description of the phenotype, and full molecular and genetic analyses. This was notable in recent articles, but earlier papers and those describing large patient cohorts were poor. Age of onset was a key detail for this review, but was not described

in some earlier papers. It was challenging to determine if some patients should be included in the review. Ultimately, such patients were included if a dominant aetiology with multiple mtDNA deletions were confirmed, which was more likely to suggest onset of symptoms in adult-life. Articles reporting 'juvenile' patients were excluded, while 'young adults' were included. Therefore, it was not feasible to perform meta-analyses or statistical comparisons.

3.5.2 Broad Phenotypic Spectrum of Adult-Onset PEO with Multiple mtDNA Deletions

Patients with adult-onset PEO and mtDNA instability presented a broad spectrum of clinical features ranging from indolent or isolated PEO to fatal multisystem phenotypes. The broadest features were observed in patients harbouring *POLG* and *TWNK* variants, comprising over 65% of all described patients in the literature who met the review criteria. It should be noted though that the prevalence of mutations in each gene identified from the literature may not be representative of the wider population (Gorman *et al.*, 2015b). It was also not uncommon for the same pathogenic heterozygous variants of either *POLG* or *TWNK* to cause divergent phenotypes between patients. Psychiatric features such as depression or psychosis were particularly prevalent in patients with *POLG* and *TWNK* variants compared to other associated genes. POF was also confined to patients with either *POLG* or *TWNK* variants only. *RRM2B* variants also showed a diverse phenotypic spectrum, but were uncommon compared to *POLG* and *TWNK* variants. Nonetheless *TWNK*, *RRM2B* and *SLC25A4* variants were more frequently associated with indolent or mild PEO phenotypes. *POLG2* mutations were also associated with mild PEO and ptosis, with all patients also presenting proximal muscle weakness. Multisystem phenotypes were commonly associated with dominant or recessive *POLG* variants. Sensory ataxia, neuronopathy and Parkinsonism were also indicative of *POLG* variants.

Though defining the genotype-phenotype correlations remains challenging, it was possible to delineate distinct phenotypes due to pathogenic *OPA1*, *TYMP* and *SPG7* variants. Dominant, heterozygous *OPA1* mutations caused optic atrophy with varying severity of visual loss in childhood, followed by ophthalmoparesis and mitochondrial myopathy in adult-life (Payne *et al.*, 2004). Hence, it was vital to provide an exemption of the review criteria for *OPA1* to allow inclusion this review. Similarly, recessive *TYMP* variants cause MNGIE with patients typically presenting gastrointestinal dysmotility of varying severity, encephalopathy, PEO and sensorimotor neuropathy (Nishino *et al.*, 1999a). Unlike other associated nuclear-encoded genes, *TYMP* variants were frequently associated with mtDNA depletion in the skeletal muscle; multiple mtDNA deletions were uncommon. For patients with dominant and

recessive *SPG7* variants, spastic ataxia was the prominent feature, in addition to PEO (Pfeffer *et al.*, 2014; Wedding *et al.*, 2014).

Delineating the genotype-phenotype correlation for the remaining associated nuclear-encoded genes was not possible. This was due the inherent lack of patients who met the review criteria or because few patients were reported. Nonetheless, discrete features may direct targeted gene screening in undiagnosed patients. Respiratory insufficiency and myopathy were prominent in patients with recessive *TK2*, *MGME1* or *RNASEH1* variants. Also, patients with *TK2* and *MGME1* variants had generalised muscle wasting. However, onset in *TK2* patients was typically later than those with *MGME1* or *RNASEH1* variants. Liver involvement in adult-onset PEO was unmistakably rare, except for adults homozygous for the *POLG* p.Ala467Thr variant (Neeve *et al.*, 2012), one adult patient with recessive *DGUOK* variants (Ronchi *et al.*, 2012b) and one adult patient with recessive *MPV17* variants (Garone *et al.*, 2012).

Mendelian adult-onset, mitochondrial PEO was predominantly associated with multiple mtDNA deletions in the skeletal muscle. The exception was *TYMP*, which was generally association with mtDNA depletion. *POLG* variants were also associated with mtDNA depletion in adults harbouring recessive variants, particularly the frequently occurring p.Ala467Thr, p.Thr251Ile, p.Pro587Leu and p.Trp748Ser variants. Additional patients identified with depletion included one with a heterozygous *TWNK* variant (Jeppesen *et al.*, 2008) and one with compound heterozygous *RRM2B* variants that phenocopied MNGIE (Shaibani *et al.*, 2009). Furthermore, disturbed mtDNA maintenance was not confirmed in a significant number of patients, which may be due to unavailable skeletal muscle for analysis.

Inheritance of adult-onset Mendelian PEO was overwhelmingly due to autosomal dominant, heterozygous variants that were inherited or occurred *de novo*. *POLG*, *RRM2B*, and *SPG7* were associated with both dominant and recessively inherited PEO. *TYMP*, *RNASEH1*, *TK2*, *MGME1*, *DGUOK* and *MPV17* variants were also associated with recessively inherited PEO only. Although rare, digenic inheritance of variants in nuclear-encoded genes associated with adult-onset PEO and mtDNA maintenance occurred more frequently than would perhaps be expected (Van Goethem *et al.*, 2003a; Galassi *et al.*, 2008; Nakhro *et al.*, 2011; Da Pozzo *et al.*, 2015), suggesting that it may be an under-recognised pathological mechanism.

3.5.3 Concluding Remarks

PEO is the most prominent feature of Mendelian mtDNA maintenance disorders, due to defects of nuclear-encoded genes essential for mtDNA integrity. Adult-onset Mendelian PEO

with multiple mtDNA deletions has been historically described in the literature (Zeviani *et al.*, 1989), though it was not until the discovery of *TYMP* variants using linkage analysis that the underlying genetic aetiology began to be deciphered (Nishino *et al.*, 1999a). Although there are overlapping clinical features shared between mutations of different nuclear genes, there are some discrete features that can direct targeted gene sequencing of undiagnosed patients. Nonetheless, the phenotypic spectrum is broad and for several genes there are currently few patients reported to allow a complete delineation of phenotypes and progression. As further patients are reported in the literature, continued revision of the clinical spectrum of adult-onset Mendelian PEO and mtDNA instability with further serve both clinicians and patients, providing crucial guidance on disease progression, treatment options and the development of therapeutic strategies.

Chapter 4. WES of Adult-Onset PEO with Multiple mtDNA Deletions

4.1 Introduction

This chapter contains material from Garone *et al.* (submitted manuscript). Additional clinical, diagnostic and research support are provided from colleagues and external collaborators who are appropriately acknowledged.

4.1.1 Adult-Onset PEO with Multiple mtDNA Deletions

As discussed in **Chapter 3**, adult-onset Mendelian PEO with multiple mtDNA deletions presents a broad phenotypic spectrum ranging from indolent or isolated PEO to fatal multisystem PEO-plus syndromes (Sommerville *et al.*, 2014). However, approximately 50% of patients are currently without a diagnosis. Overlapping phenotypes and variable muscle biopsy findings are often insufficient to indicate specific genes for targeted mutational screening. Also, family history is often negative or there is reduced penetrance between any additional affected members. Thus, determining autosomal dominant or recessive inheritance has been historically difficult. This is further complicated by patients presenting late in life, which limits segregation studies. Consequently, it has been challenging to provide a genetic diagnosis for these patients. This is reflected by the identification of only 14 known nuclear genes, expanded to 16 nuclear genes during this study (**Chapter 3**), that have been associated with this disorder to date, with roles in mtDNA replication, maintenance and repair.

4.1.2 Identification of Novel mtDNA Maintenance Disorders using WES and WGS

Although large-scale studies have primarily focused on early-onset mitochondrial respiratory chain deficiency (Lieber *et al.*, 2013; Taylor *et al.*, 2014; Wortmann *et al.*, 2015; Kohda *et al.*, 2016; Pronicka *et al.*, 2016), WES and WGS have successfully elucidated the genetic aetiology of some adult-onset PEO with multiple mtDNA deletions patients due to *RRM2B* (Tyynismaa *et al.*, 2009a), *TK2* (Tyynismaa *et al.*, 2012), *DGUOK* (Ronchi *et al.*, 2012a), *MPV17* (Garone *et al.*, 2012), *MGME1* (Kornblum *et al.*, 2013), *DNA2* (Ronchi *et al.*, 2013), *SPG7* (Pfeffer *et al.*, 2014), *AFG3L2* (Gorman *et al.*, 2015a) and *RNASEH1* (Reyes *et al.*, 2015) mutations. Mutations of *RRM2B*, *TK2*, *DGUOK* and *MPV17* were previously associated with early-onset mtDNA depletion syndrome (Mandel *et al.*, 2001; Saada *et al.*, 2001; Spinazzola *et al.*, 2006; Bourdon *et al.*, 2007), hence rare or novel variants identified in

these genes would have been highly prioritised as potentially causing a late-onset mtDNA maintenance disorder. Autosomal recessive mutations of *SPG7* encoding the mitochondrial-AAA metalloprotease paraplegin, were first described in patients presenting with spastic paraplegia with additional neurological features that included ophthalmoparesis and ptosis with evidence of mitochondrial dysfunction (Casari *et al.*, 1998). Autosomal dominant *AFG3L2* mutations, encoding the catalytic subunit of the mitochondrial-AAA protease that interacts with paraplegia, were first reported in patients with spinocerebellar ataxia 28 (SCA28) including some patients who additionally had ophthalmoparesis and ptosis (Cagnoli *et al.*, 2006; Di Bella *et al.*, 2010). Since (i) both *AFG3L2* and *SPG7* mutations were known to cause ophthalmoparesis, (ii) AFG-like protein 2 and paraplegin interact in the mitochondrial membrane and (iii) mitochondrial dysfunction had been noted in muscle biopsies of some *SPG7* patients (Casari *et al.*, 1998), the identified mutations in these genes from patients with adult-onset PEO and multiple mtDNA deletions would have been prioritised (Pfeffer *et al.*, 2014; Wedding *et al.*, 2014; Gorman *et al.*, 2015a). *MGME1*, *DNA2* and *RNASEH1* were the only genes that had not been associated with Mendelian human disease, prior to the identification of adults with mtDNA instability.

4.1.3 Challenges of WES Variant Filtering and Prioritisation

Almost all nuclear genes associated with adult-onset mtDNA maintenance disorders are directly involved with mtDNA replication, maintenance, repair or the synthesis and balance of nucleotide pools. However, current strategies employed for the filtering and prioritisation of mitochondrial disease candidate gene variants are incompatible for adult-onset PEO with multiple mtDNA deletions. Unless an autosomal dominant aetiology was anticipated, current strategies have focused on early-onset Mendelian mitochondrial disease with expected autosomal recessive (compound heterozygous and homozygous) or X-linked inheritance (Vasta *et al.*, 2009; Calvo *et al.*, 2012; Vasta *et al.*, 2012; DaRe *et al.*, 2013; Lieber *et al.*, 2013; Neveling *et al.*, 2013; Taylor *et al.*, 2014; Wortmann *et al.*, 2015; Kohda *et al.*, 2016; Legati *et al.*, 2016; Pronicka *et al.*, 2016). As stated in **Chapter 3**, adult-onset Mendelian PEO with multiple mtDNA deletions is predominantly associated with dominant, heterozygous variants and less frequently with autosomal recessively inherited variants. Secondly, several nuclear genes encode proteins that are not localised to mitochondria. Cytosolic enzymes are essential for the synthesis and balance of nucleotides that are imported to mitochondria for mtDNA replication, which includes *TYMP* encoding thymidine phosphorylase (Nishino *et al.*, 1999b) and *RRM2B* encoding p53R2 (Bourdon *et al.*, 2007). Although seemingly uncommon, filtering for nuclear genes encoding mitochondrial proteins

would exclude genes encoding cytosolic-targeted enzymes. Additionally, *DNM2* encodes a dynamin-GTPase with its role in mitochondrial function yet to be fully understood. Finally, the complete mitochondrial proteome and mtDNA maintenance pathways have yet to be fully elucidated. MGME1 is the most prominent example of an essential enzyme for mtDNA maintenance that was uncharacterised prior to the identification of patients with multiple mtDNA deletions (Kornblum *et al.*, 2013). Previously referred to as *C20orf72*, Kornblum *et al.* (2013) demonstrated that *MGME1* encoded a mitochondrial exonuclease required for the cleavage of single-stranded DNA and the processing of DNA flap-substrates. The identification of patients allowed additional characterisation into the role of MGME1 in mtDNA replication. Nicholls *et al.* (2014) used *MGME1* patient muscle and fibroblasts to demonstrate that MGME1 played a role in 5' end processing of mtDNA, with unusually large mtDNA rearrangements in affected patients and that it interacted with POLG at the nucleoids. Further efforts to identify mtDNA maintenance genes included a genome-wide RNA interference (RNAi) screen in *Drosophila melanogaster* cells (Fukuoh *et al.*, 2014). However, the study of model organisms such as *D. melanogaster* and *Saccharomyces cerevisiae* for identifying human mtDNA maintenance orthologs is not always appropriate due to evolutionary divergent pathways and mechanisms. It is also possible that genes encoding known nuclear genome maintenance proteins are dual-localised to nuclear DNA and mtDNA, such as *DNA2* (Duxin *et al.*, 2009), but to date have not been associated with mtDNA maintenance.

Therefore, there is a need to devise a clear, concise strategy for the discovery and filtering of candidate gene variants identified by WES or NGS of adult-onset mtDNA maintenance disorders. This filtering strategy would be required to incorporate (i) autosomal dominant (heterozygous) and recessive (compound heterozygous or homozygous) inheritance, as patient family history may dictate; (ii) the prioritisation of genes encoding mitochondrial, cytosolic and nuclear proteins, which (iii) have known or putative roles in the transcription, replication or repair of DNA. In this chapter, a custom filtering strategy for the prioritisation of candidate gene variants is designed and applied to patients with clinically well-defined, genetically undetermined adult-onset PEO with multiple mtDNA deletions.

4.2 Aims

This chapter identifies candidate gene variants using a custom filtering strategy for the prioritisation of genes and variants in patients with clinically well-defined, genetically

undetermined adult-onset PEO with multiple mtDNA deletions, ranging from indolent PEO to fatal, PEO-plus multisystem phenotypes.

4.3 Methods

4.3.1 Recruitment of Patients

Patients were clinically examined at the NHS Highly Specialised Service for Rare Mitochondrial Disorders, with diagnostic testing performed by the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory in Newcastle upon Tyne. There were 67 patients (35 male, 31 female) identified with genetically undetermined, clinically well-defined adult-onset PEO and multiple mtDNA deletions. Diagnostic investigations were performed as described in **2.2.3**.

4.3.2 Targeted *RNASEH1* Sanger Sequencing

In addition to diagnostic genetic analyses, custom forward and reverse primer designs for all eight exons of *RNASEH1* were designed to exclude pathogenic or likely pathogenic mutations in all 67 patients. Sanger sequencing confirmation and analysis were performed as described in **2.3**.

4.3.3 WES Selection Criteria

Of the 67 clinically well-defined, genetically undetermined adult-onset PEO with multiple mtDNA deletions patients, 20 unrelated patients (14 male, 6 female) were selected for WES (**Table 4.2**). Patients were selected due to significant clinical, biochemical and molecular evidence suggestive of a disorder of mtDNA maintenance. Hence, it was considered highly likely that candidate variants in genes required or implicated in mtDNA maintenance would be identified. Criteria for selection were: -

- Clinically well-defined adult-onset PEO;
- Presence of multiple mtDNA deletions in skeletal muscle detected by at least one diagnostic assay (long-range PCR, Southern blotting or quantitative real time PCR assays);
- COX-SDH histochemistry demonstrating ≥5% COX-deficient fibres;
- Negative results in diagnostic whole mitochondrial genome sequencing;

- Negative results in diagnostically targeted nuclear gene screening of common PEO and multiple mtDNA deletions associated genes.

4.3.4 Diagnostic Genetic Analysis of Nuclear Genes

Diagnostic targeted gene screening of *POLG* (NM_002693), *RRM2B* (NM_015713), *SLC25A4* (NM_001151), *TWNK* (NM_021830) and *POLG2* (NM_007215.3) was performed in all 20 patients recruited for WES. *TK2* (NM_004614.4) was sequenced for all patients except patients 3, 4 and 12. *PABPN1* (NM_004643.3) GCN repeat analysis of exon 1 to exclude polyalanine tracts was performed for patient 14.

4.3.5 WES Filtering and Analysis

Called variants that passed quality score filtering were restricted to exonic (coding) or splice-site variants, with a minor allele frequency (MAF) equal to or less than 0.01 (1%) of in-house exomes or external exome databases. The effect of missense variants on protein function were predicted using PolyPhen2, Align-GVGD and SIFT. However, *in silico* predictions were purely advisory and were not used to directly exclude candidates. Splice-site or nonsense loss-of-function (LOF) variants were also considered.

To confirm diagnostically targeted genes and *RNASEH1* screening results, any filtered variants identified of the genes listed in **4.3.4** were examined. Next, any filtered variants identified in all other published nuclear genes associated with adult-onset PEO and multiple mtDNA deletions were examined; *TYMP* (NM_001257989), *OPA1* (NM_130837), *TK2* (NM_004614), *MPV17* (NM_002437), *DGUOK* (NM_080916), *DNA2* (NM_001080449), *SPG7* (NM_003119) and *AFG3L2* (NM_006796). Filtered variants occurring in additional nuclear genes associated with mtDNA maintenance disorders were also examined; *ABAT* (NM_020686), *DDHD1* (NM_030637), *GFER* (NM_005262), *FBXL4* (NM_001278716), *MFN2* (NM_001127660), *SUCLA2* (NM_003850), *SUCLG1* (NM_003849), *TFAM* (NM_003201) and *WFS1* (NM_006005).

Following the exclusion of known nuclear genes associated with mtDNA maintenance disorders, variants were filtered using GO-Terms associated with mitochondrial localisation, DNA replication and repair. GO-Terms employed allowed the inclusion of nuclear genes encoding proteins involved in DNA replication and repair, but that had not necessarily been associated with mtDNA maintenance to date. Therefore, nuclear and mtDNA maintenance genes were considered. GO-Terms used were the wildcard term ‘mitochondr*’, ‘DNA repair’,

‘replication’, ‘transcription’, ‘nucleotide’, ‘purine’, ‘pyrimidine’, ‘exonuclease’, ‘polymerase’, ‘topoisomerase’, ‘ligase’, ‘helicase’ and ‘nucleoside’. CNVs were also analysed using the same employed GO-Terms.

Lists of genes and called variants were categorised according to gene role and function, expected inheritance and association with human disease (if known). Autosomal dominant (heterozygous) variants were prioritised for patients with a known family history extending at least one generation. For patients with no known family history, both dominant and autosomal recessive (homozygous or compound heterozygous) variants were prioritised.

To assess the potential pathogenicity of called variants, a simplified scoring system was used (**Table 4.1**). Since trios comprising the proband and parental WES datasets were not sequenced, segregation of variants was not included in variant prioritisation. The effect of missense on protein function were predicted using PolyPhen2, Align-GVGD and SIFT. However, *in silico* predictions were purely advisory and were not used to directly excluded candidates using the scoring system. Splice-site or nonsense loss-of-function (LOF) variants were also considered but not scored. Due to both autosomal dominant and recessive mutations in Mendelian mitochondrial PEO, the scoring system was also purely advisory.

Mutation(s)	**Genotype**	**GO-Terms**		**Mitochondrial Disease Gene?**			**Score**
		Mitochondr*	***Replication/Maintenance/Repair Terms***	**Y/N**	**Allele-1**	**Allele-2**	
c.?, p.?							

Table 4.1 Basic WES Variant Scoring System for Adult-Onset PEO with Multiple mtDNA deletions Patients. Genotype – 1 point for a heterozygous variant, 2 points for a recessive (homozygous, compound heterozygous) or hemizygous variant(s). Since mitochondrial disease was confirmed in biochemical and histopathological studies, 2 points were given for mitochondr* hits. ‘*Replication/Maintenance/Repair Terms*’ denotes any of the GO-Terms used for DNA replication and maintenance, with 1 point awarded for each matched term. 1 point was given if they gene was previously associated with mitochondrial disease. 1 point was given to each known pathogenic variant (compound heterozygous) or 2 points for a known pathogenic homozygous or hemizygous variant. CNVs were also included in analysis. For this patient cohort, the scoring system was purely advisory in guiding prioritisation.

Owing to the scores attained, variants could be classified into categories. Category-1 comprised the highest level candidates. These variants met the following criteria:

- Autosomal dominant (1 point) **or** autosomal recessive (homozygous, compound heterozygous) or hemizygous (2 points), dependent on a known family history.
- GO-Terms matched ‘mitochondr*’ (2 points) **or** one or more DNA replication/maintenance/repair terms (1 point each).
- Known causative gene associated with adult-onset Mendelian PEO and multiple mtDNA deletions (1 point).
- One or more previously reported causative mutation(s) (1-2 points).

Category-2 was comprised of medium prioritised candidates. These variants met the following criteria:

- Autosomal dominant (1 point) **or** autosomal recessive (homozygous, compound heterozygous) or hemizygous (2 points), dependent on a known family history.
- GO-Terms matched ‘mitochondr*’ (2 points) **or** one or more DNA replication/maintenance/repair terms (1 point each).
- Known causative gene associated with adult-onset Mendelian PEO and multiple mtDNA deletions (1 point) **or** a different mtDNA maintenance disorder (½ point).
- One or more previously reported causative mutation(s) (1-2 points).

Category-3 variants were considered low priority, since any role in mtDNA replication or maintenance would be unclear. These variants met the following criteria:

- Autosomal dominant (1 point) **or** autosomal recessive (homozygous, compound heterozygous) or hemizygous (2 point), dependent on a known family history.
- GO-Terms matched ‘mitochondr*’ (2 points) **or** one or more DNA replication/maintenance/repair terms (1 point each).
- No known association with a mtDNA maintenance disorder (0 points).

Finally, the last category were considered variants of unknown significance (VUS). In this instance, no appropriate candidate variants in nuclear genes encoding mitochondrial localised proteins were identified. Upon this failure, *all* dominant VUS were evaluated individually, since this was considered the most likely aetiology. All listed VUS in unsolved cases were reviewed at least every 2-3 months to ensure up-to-date findings from the literature were reflected.

- Autosomal dominant (1 point).
- No GO-Terms matched (0 points).

- No known association with a mtDNA maintenance disorder or other known human pathology (0 points).

In comparison, the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation use a set of devised terminology to assess sequence variants (Richards *et al.*, 2015). The ACMG standards and guidelines proposed two sets of criteria; one for classifying pathogenic or likely pathogenic variants and the second for classifying benign or likely benign variants. The criteria using this system were weighted upon the evidence available, including *in silico* scores, segregation of candidate variants, loss-of-function, allele frequency and if the variant or gene were previously associated with human disease. To contrast with this study, the authors of the ACMG standards and guidelines did not opt to use a point scoring system, since as quantitative system was viewed as insufficient to account for the complexity of human genetic disorders.

Custom forward and reverse primer design for candidate variants, PCRs, Sanger sequencing confirmation and analyses were performed as described in **2.3**. If available, primers designed within the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory were used.

A schematic of WES filtering for adult-onset PEO with multiple mtDNA deletions is given in **Figure 4.1.**

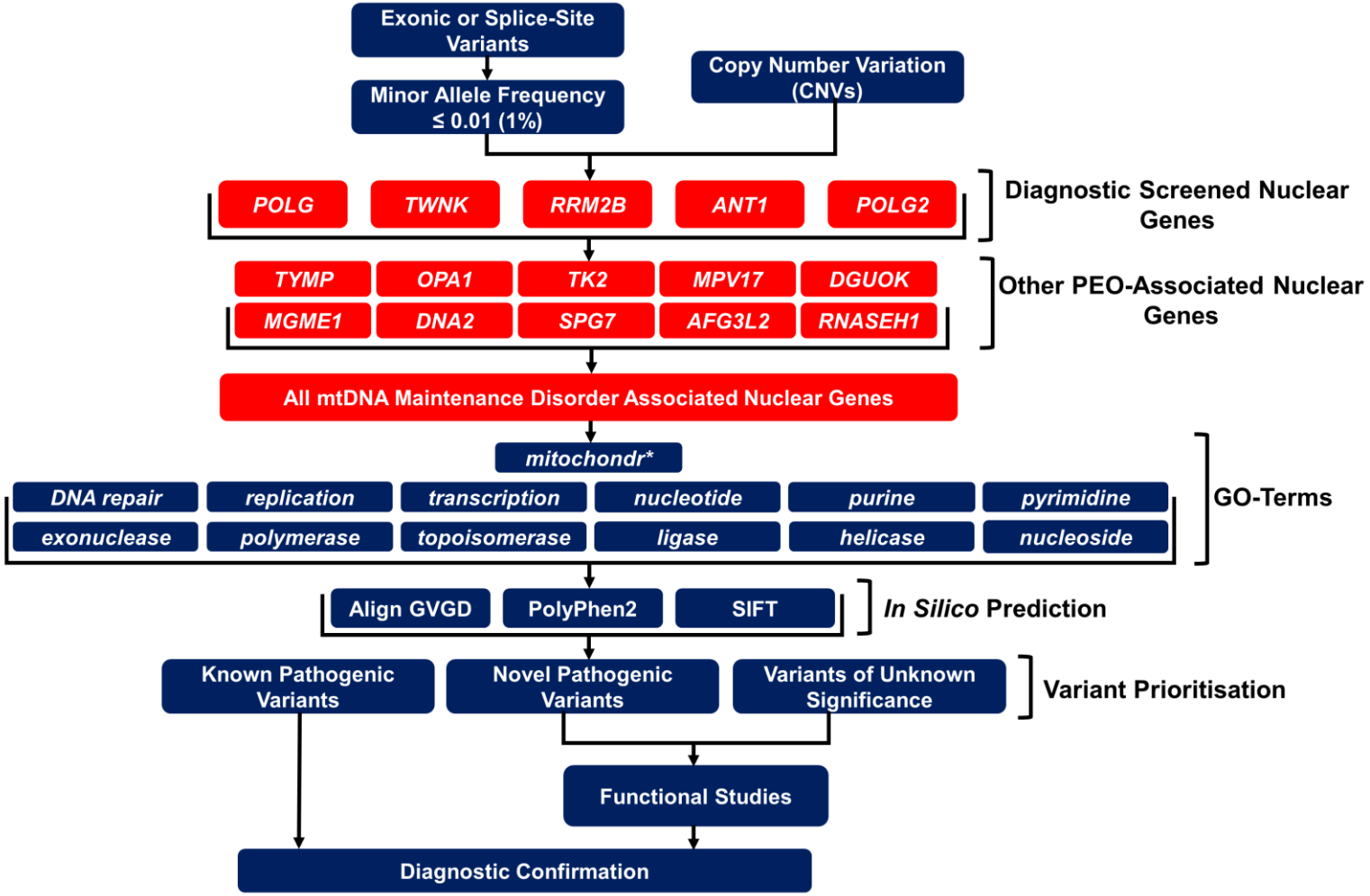


Figure 4.1 Adult-Onset PEO with Multiple mtDNA Deletions WES Filtering Strategy.

4.3.6 Cell Culture

Cultured fibroblasts were grown for patient 14 and two appropriate age-matched controls. Subculturing, freezing, harvesting and generation of quiescent fibroblasts were performed as described in **2.4**.

4.3.7 Western Blotting

Patient 14 and control fibroblast lysates were prepared and subjected to 12% SDS-page, probed with primary and secondary antibodies (**Table 2.4**, **Table 2.5**) then detected as outlined in **2.5**. Membranes were incubated with primary antibodies specific to POLRMT, MRPL12 and SDHA. β-Actin was used as a loading control.

4.3.8 Quantitative Real Time PCR for mtDNA Quantification

DNA was extracted from proliferating and quiescent fibroblasts of patient 14 and two controls. Quantitative real time PCR was performed as outlined in **2.3.10**.

4.3.9 Long Range PCR of Quiescent Fibroblast DNA

Long-range PCR of quiescent patient 14 and two control fibroblast DNA plus 1:10 control blood DNA homogenate was performed as outlined in **2.3.9**.

4.4 Results

4.4.1 Targeted *RNASEH1* Screening

In anticipation of WES, Sanger sequencing of all eight coding exons of *RNASEH1* was performed in all referred undiagnosed adult-onset PEO and multiple mtDNA deletion patients (n=67) (**Appendix L**). No non-synonymous variants were identified. A common synonymous c.498A>G (p.Pro166Pro) (rs10186193) variant of exon 4 was found in 38 patients, either heterozygous or homozygous. Two additional rare synonymous changes were also identified; c.474A>G (p.Ala158Ala) (rs61738918) of exon 4 in one patient and c.702A>G (p.Ala234Ala) (rs143510329) of exon 7 in two patients. Common intronic variants identified were c.409+34C>A (rs75663960) in 40 patients (heterozygous or homozygous), c.245-118_245-115het_delAGAG (rs151141402) in 20 patients (heterozygous or homozygous), c.564+16C>T (rs375447388) in two patients, c.649+13A>G (rs75789502) in two patients and c.774+132A>T (rs116083909) in two patients. Two novel deep intronic variants were also

identified, c.128+44G>A and c.509+44C>G, in patients 4 and 10 respectively, which were confirmed by WES. However in the absence of coding or non-coding variants and with an alternative diagnosis reach for patient 4, these were not deemed pathogenic. Therefore, no patients with pathogenic or likely pathogenic *RNASEH1* variants were identified.

4.4.2 Clinical and Molecular Features

After WES was performed and analysis had commenced, further details obtained externally for patient 13 showed no clinical, biochemical or molecular evidence of adult-onset PEO with multiple mtDNA deletions. Instead, this patient presented hearing-loss only. Therefore, patient 13 was omitted from the study and the total number of patients analysed was 19.

The clinical and molecular features of patients recruited for WES from the undiagnosed cohort are summarised in **Table 4.2**. Almost all patients had PEO (18/19, 94.7%) and all had ptosis (19/19, 100%). Over half of patients had myopathy, fatigue or exercise intolerance (12/19, 63.2%). Less common features were sensorineural hearing loss (SNHL) (7/19, 36.8%), dysphagia (6/19, 31.6%), neuropathy (6/19, 31.6%), diplopia (4/19, 21.1%), ataxia (4/19, 21.1%), cataracts (3/19, 15.8%), dysarthria (2/19, 10.5%), HCM (2/19, 10.5%), respiratory insufficiency (2/19, 10.5%), retinitis pigmentosa (2/19, 10.5%), liver involvement (2/19, 10.5%) and diabetes (2/19, 10.5%). Rare features noted in single cases were scapular winging, tremor, muscle pain, myalgia and optic neuropathy. Posterior fossa vascular malformation and cystinuria were features considered unrelated to the PEO phenotypes. One patient presented a MNGIE-like phenotype. Oculopharyngeal-muscular dystrophy (OPMD)-like phenotype was noted in 3/19 (15.8%) patients, typically associated with GCN-repeat tracts or mutations of *PABPN1* encoding polyadenylate-binding nuclear protein 1 (Banerjee *et al.*, 2013). All patients had skeletal muscle restricted multiple mtDNA deletions confirmed by long range PCR. Southern blotting (3/19, 15.8%) and quantitative real time PCR (2/19, 10.5%) were also used diagnostically. A family history of PEO was reported in only 8/19 (42.1%) of cases and 3/19 (15.8%) patients were deceased.

Patient	Sex	Age of Onset	Family History	Clinical Features	Muscle Histopathology	Evidence of Multiple mtDNA Deletions
1	M	n.d.	-	PEO, bilateral ptosis, mild axonal neuropathy, SHNL	COX-deficient fibres	+ (long-range PCR)
2†	M	40s	-	PEO, areflexic peripheral neuropathy, emaciated, MNGIE-like	20% COX-deficient fibres 9% Ragged-red fibres	+ (long-range PCR)
3	M	40s	+ (AD)	PEO, ptosis, OPMD-like, SHNL, diplopia, dysphagia	COX deficient fibres Ragged-red fibres	+ (long-range PCR, Southern blot)
4	M	Mid 50s	-	PEO, bilateral ptosis, myopathy, dysarthria, dysphagia, respiratory insufficiency	25% COX-deficient fibres 6% Ragged-red fibres	+ (long-range PCR)
5	M	n.d.	+ (AD)	PEO, bilateral ptosis, cataracts, diplopia, proximal muscle (neck) weakness, tremor, muscle pain in arms, shoulder and neck, SNHL	20% COX-deficient fibres 7% Ragged-red fibres	+ (long-range PCR)
6	M	n.d.	+ (AD)	PEO, bilateral ptosis, OPMD-like, diplopia, dysphagia, facial muscle weakness and wasting, SHNL	35% COX-deficient fibres 15% Ragged-red fibres	+ (long-range PCR)
7	M	Late 40s	n.d.	PEO, bilateral ptosis, OPMD-like, facial muscle weakness, bulbar involvement	35% COX-deficient fibres 13% Ragged-red fibres	+ (long-range PCR)
8	F	Mid 40s	+ (AD/AR)	PEO, ptosis, ataxia, axonal neuropathy, HCM (cardiac conduction defect), dysphagia, SHNL, proximal muscle weakness	10% COX-deficient fibres 10% Ragged-red fibres	+ (long-range PCR, Southern blot)
9†	M	n.d.	+ (AD/AR)	Ptosis, proximal muscle weakness, SNHL, diabetes, endocrinopathy	13% COX-deficient fibres	+ (long-range PCR)
10	F	n.d.	-	PEO, ptosis	30% COX-deficient fibres 10% Ragged-red fibres	+ (long-range PCR)
11	F	n.d.	-	PEO, bilateral ptosis, diplopia	15% COX-deficient fibres 3% Ragged-red fibres	+ (long-range PCR)

Patient	Sex	Age of Onset	Family History	Clinical Features	Muscle Histopathology	Evidence of Multiple mtDNA Deletions
12†	F	Mid 40s	-	PEO, ptosis, sensory ataxia, SMAN, bulbar involvement, cataracts, dysphagia, proximal and facial muscle weakness, scapular winging, myalgia, optic neuropathy, respiratory insufficiency, retinitis pigmentosa	20% COX-deficient fibres 8% Ragged-red fibres	+ (long-range PCR, Southern blot)
14	M	n.d.	-	PEO, ptosis, ataxia, HCM, cataracts, dysarthria, exercise intolerance, facial muscle weakness, myopathy, SMAN, neuronopathy	20% COX-deficient fibres Ragged-red fibres	+ (long-range PCR)
15	M	20s	+ (AD)	PEO, ptosis	COX-deficient fibres Ragged-red fibres	+ (long-range PCR, real time PCR)
16	F	n.d.	-	PEO	5% COX-deficient fibres	+ (long-range PCR)
17	M	n.d.	+ (AD)	PEO, ptosis, mild dysphagia	30% COX-deficient fibres	+ (long-range PCR)
18	F	n.d.	-	PEO, ptosis, small posterior fossa vascular malformation	20% COX-deficient fibres	+ (long-range PCR)
19	M	n.d.	-	PEO, ptosis, myopathy, fatigue, cystinuria	18% COX-deficient fibres 3% Ragged-red fibres	+ (long-range PCR)
20	M	n.d.	-	PEO, ptosis, ataxia, diabetes, subtle facial muscle weakness, liver cirrhosis, proximal muscle weakness, retinitis pigmentosa	COX-deficient fibres	+ (long-range PCR , real time PCR)

Table 4.2 Clinical and Molecular Features of the Adult-Onset PEO with Multiple mtDNA Deletions Cohort for WES. Patients with adult-onset PEO and multiple mtDNA deletions selected for WES. AD – autosomal dominant; AR – autosomal recessive; HCM – hypertrophic cardiomyopathy; MNGIE – mitochondrial neurogastrointestinal encephalopathy; OPMD – oculopharyngeal muscular dystrophy; SMAN – sensory motor axonal neuropathy; SNHL – sensorineural hearing loss. † - deceased. '+' – yes; '-' – no. Patient 11 underwent corrective squint surgery at 60 years old, but no additional details are available for onset of PEO (see **5.4.1**). Patient 13 presented SHNL only and was omitted from the study. 'n.a.' – not applicable; 'n.d.' – not determined. AD/AR denotes cases with possible AD or AR family, such as an affected sibling but with no details on a possible affected parent for example.

4.4.3 WES Read Coverage and Depth Statistics

WES read coverage and depth statistics of the adult-onset PEO and multiple mtDNA deletions cohort was calculated for 32,947,520 exome consensus coding sequence (CCDS) bases (bp) (**Appendix M**). The mean depth per CCDS bases was 59-fold. The mean percentage of CCDS bases at 20-fold coverage was 77.83%.

4.4.4 WES Analysis

Using the simplified scoring system devised to advise the prioritisation of candidates, causative or likely causative variants and VUS are listed in **Table 4.3** with MAF data and *in silico* predictions. Causative (2/19, 10.5%) and likely causative variants associating with adult-onset PEO and multiple mtDNA deletions (4/19, 21.1%) were identified in six patients, providing a diagnostic yield of 31.6%.

Causative mutations were identified in two nuclear genes previously associated adult-onset mtDNA instability disorders for two patients (4 and 18); somatic *TWNK* and homozygous *TK2* missense variants. Both variants were previously reported as pathogenic and were confirmed by Sanger sequencing using diagnostic forward and reverse primers. After evaluation and confirmation, variants were diagnostically reported.

Likely causative variants were identified in four patients (3, 5, 11 and 19) in *RRM1* (NM_001033.3), *VDAC1* (NM_003374.2), *GMPR1* (NM_006877.3) and *SEPT2* (NM_006155). All variants require additional validation including segregation and molecular studies to confirm pathogenicity.

Two patients had digenic inheritance of known pathogenic mutations not associated with a mtDNA maintenance disorder, but were nonetheless actionable variants. Patient 1 had a known pathogenic heterozygous 1.4Mb deletion in chromosome 17 (chr17:14095307-15477497) associated with neuropathy. Patient 19 had two known pathogenic mutations of *SLC3A1* associated with isolated cystinuria.

In spite of no PEO and multiple mtDNA deletions for patient 13, analysis of known genes associated with SNHL revealed a likely causative *MYH14* (NM_001145809) mutation.

VUS were identified in five patients (1, 6, 8, 15 and 17) in *ABAT* (NM_000663.4), *RRM2B*, *TOP3A* (NM_004618.3), *POLRMT* (NM_005035.3) and *MGME1*. Candidate genes were

either not currently associated with human disease or occurred at low MAFs in external databases.

Analysis of 8 patients did not identify causative or likely causative variants (2, 7, 9, 10, 12, 14, 16 and 20). VUS were listed for these patients (**Appendix N**) but it was not possible to determine the significance due to a lack of information on the function of encoded proteins.

To attain genetic diagnoses, patient 6, 8, 12 and 15 genomic DNA were prepared and sent for WGS to the Human Genome Sequencing Center at the Baylor College of Medicine in Houston, Texas. At the time of writing, an *RRM2B* (rs72554098) missense change and *POLRMT* splice-site variant remain the top prioritised candidates for patients 6 and 15.

Patient	Clinical Features	Gene	Mutations		Minor Allele Frequency			*In Silico* Predictions		
			cDNA Change	Amino Acid Change	ExAC	NHLBI ESP	1000G	PolyPhen 2	Align-GVGD	SIFT
Causative Variants										
1	PEO, bilateral ptosis, mild axonal neuropathy, SHNL	1.4Mb Chromosome 17 Deletion including *PMP22* (chr17:14095307-15477497), Heterozygous								
4	PEO, bilateral ptosis, myopathy, dysarthria, dysphagia, respiratory insufficiency	*TK2*	Homozygous c.323C>T	p.Thr108Met (rs137854431), Homozygous	0.0000248	Ø	Ø	1.000	Class 15	0.03
18	PEO, ptosis, small posterior fossa vascular malformation	*TWNK*	c.1121G>A/=	p.Arg374Gln, Heterozygous (Somatic Mosaic)	Ø	Ø	Ø	1.000	Class 35	0.26
19	PEO, ptosis, myopathy, fatigue, cystinuria	*SLC3A1*	c.1400C>T/=	p.Met476Thr (rs121912691), Heterozygous	0.002713	0.002076	0.0003993	0.823	Class 65	0.05
			Exon 5-9, 13.98kb Duplication (chr2:44527111-44541090), Heterozygous							
Likely Causative Variants										
3	PEO, ptosis, OPMD-like, SHNL, diplopia, dysphagia	*RRM1*	c.1281C>A/=	p.Asn427Lys, Heterozygous	Ø	Ø	Ø	1.000	Class 65	0.00
5	PEO, bilateral ptosis, cataracts, diplopia, proximal muscle (neck) weakness, tremor,	*VDAC1*	c.239C>T/=	p.Thr80Ile, Heterozygous	Ø	Ø	Ø	0.997	Class 15	0.12

	muscle pain in arms, shoulder and neck, SNHL									
11	PEO, bilateral ptosis, diplopia	*GMPR1*	c.547G>C/=	p.Gly183Arg, Heterozygous	Ø	Ø	Ø	1.000	Class 65	0.00
13	SNHL	*MYH14*	c.5185C>T/=	p.Arg1729Trp, Heterozygous	0.0000859	Ø	Ø	1.000	Class 65	0.00
19	PEO, ptosis, myopathy, fatigue, cystinuria	*SEPT2*	c.988C>T/=	p.Arg330Cys, Heterozygous	Ø	Ø	Ø	0.990	Class 65	0.00
Variants of Unknown Significant (VUS)										
1	PEO, bilateral ptosis, mild axonal neuropathy, SHNL	*ABAT*	c.1157C>T/=	p.Pro386Leu, Heterozygous	0.0000165	Ø	Ø	1.000	Class 65	0.00
6	PEO, bilateral ptosis, OPMD-like, diplopia, dysphagia, facial muscle weakness and wasting, SHNL	*RRM2B*	c.482C>T/=	p.Thr161Ile (rs72554098), Heterozygous	0.0000082	0.0000769	Ø	0.508	Class 0	0.00
8	PEO, ptosis, ataxia, axonal neuropathy, HCM (cardiac conduction defect), dysphagia, SHNL, proximal muscle weakness	*TOP3A*	c.298A>G c.403C>T	p.Met100Val p.Arg135*	0.0000580 0.0001983	0.0000769 0.0000769	Ø Ø	0.001 n.a.	Class 0 n.a.	0.68 n.a.
15	PEO, ptosis	*POLRMT*	c.2641-1G>C/=	Splice-Acceptor Site, Heterozygous	0.0000163	Ø	Ø	n.a.	n.a.	n.a.
17	PEO, ptosis, mild dysphagia	*MGME1*	c.706C>G/=	p.Leu236Val, Heterozygous	Ø	Ø	Ø	0.978	Class 15	0.13

Table 4.3 Pathogenic variants, potential pathogenic variants and VUS identified with possible associations with adult-onset PEO and multiple mtDNA deletions. 'Ø' denotes that a variant absent from external databases. HCM – hypertrophic cardiomyopathy; OPMD – oculopharyngeal muscular dystrophy; SNHL – sensorineural hearing loss. n.a. – not applicable.

4.4.5 *TK2* – Thymidine Kinase 2

Patient 4 was a 64 year old male who initially presented with bilateral ptosis in his mid-50s. This progressed to PEO, myopathy, dysarthria, dysphagia and respiratory failure requiring ventilatory support. Diagnostic long-range PCR confirmed the presence of multiple mtDNA deletions in the skeletal muscle. Muscle histopathology revealed 25% COX-deficient fibres and 6% ragged-red fibres (**Figure 4.2A**). Family history was negative for mitochondrial myopathy.

WES analysis identified a homozygous c.323C>T (p.Thr108Met) (rs137854431) variant in *TK2* (16q21), matching GO-Terms 'mitochondr*', 'pyrimidine' and 'replication'. Sanger sequencing using diagnostic forward and reverse primers for *TK2* exon 5 confirmed the variant (**Figure 4.2B**). The p.Thr108Met variant affected a highly conserved residue in a highly conserved region of TK2 (**Figure 4.2C**) and had been previously identified either homozygous or was compound heterozygous with an additional variant in patients with TK2-deficiency. The p.Thr108Met variant was present in 3/121070 alleles (MAF=0.00002478) in ExAC; two non-Finnish Europeans and one Latino, all in heterozygous state. It was absent from in-house exomes, NHLBI ESP and 1000 Genomes Project. The parents were not known to be consanguineous.

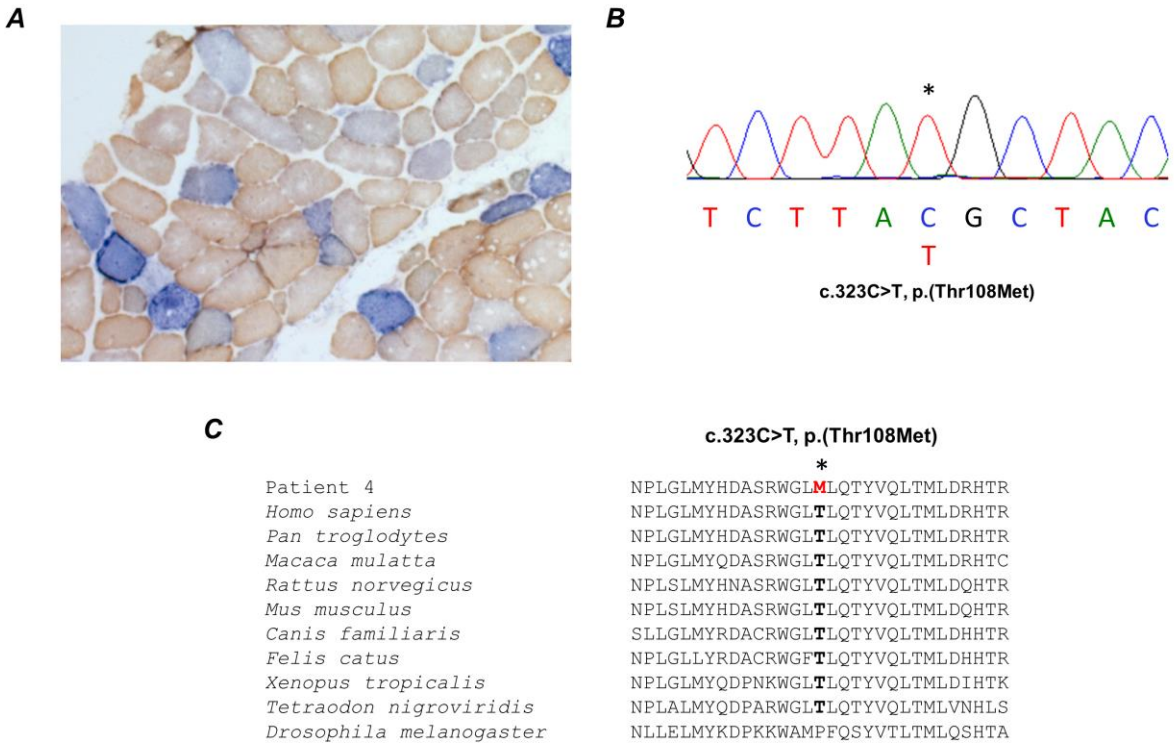


Figure 4.2 Histochemical and Genetic Features of Patient 4. (*A*) Sequential COX-SDH histochemistry of patient 4 skeletal muscle demonstrating COX-deficient fibres. (*B*) Sanger sequencing confirmation of the homozygous p.Thr108Met *TK2* missense variant. (*C*) Multiple sequence alignment (MSA) of the TK2 Thr108 residue.

4.4.6 *TWNK* - Twinkle

Patient 18 was a female who presented late-onset PEO, ptosis and had small posterior fossa vascular malformation. Muscle histopathology showed 20% COX-deficient fibres. Long-range PCR of skeletal muscle detected multiple mtDNA deletions. Family history was negative for extraocular paresis.

WES analysis identified a heterozygous c.1121G>A (p.Arg374Gln) variant in *TWNK* (10q24.31), matching GO-Terms ‘mitochondr*’, ‘replication’, ‘transcription’ and ‘helicase’. Suspicions were raised for the identified p.Arg374Gln *TWNK* variant due to its known pathogenicity (Spelbrink *et al.*, 2001) and since all coding exons and intronic regions of *TWNK* had been diagnostically sequenced. Examination of WES coverage at the c.1121 (Chr10:102749088) site showed 22 reads that passed quality score filtering, with an unfiltered AD of 16 reads at the reference (c.1121G) position and 6 reads (27.3%) with the alternate (c.1121G>A) base. The PL of the heterozygous alternate base was 0, indicating that heterozygosity was the most likely genotype. GQ was also 99, the highest possible phred-scaled quality score. Examination of the diagnostic electropherogram for *TWNK* exon 1c with

blood DNA showed that the variant was apparently absent except for a small peak at the c.1121 site. Repeated Sanger sequencing with blood (**Figure 4.3A**) and muscle (**Figure 4.3B**) genomic DNA also showed a small peak at the c.1121 site. Unfortunately, available urine DNA failed to amplify. Segregation studies were not possible.

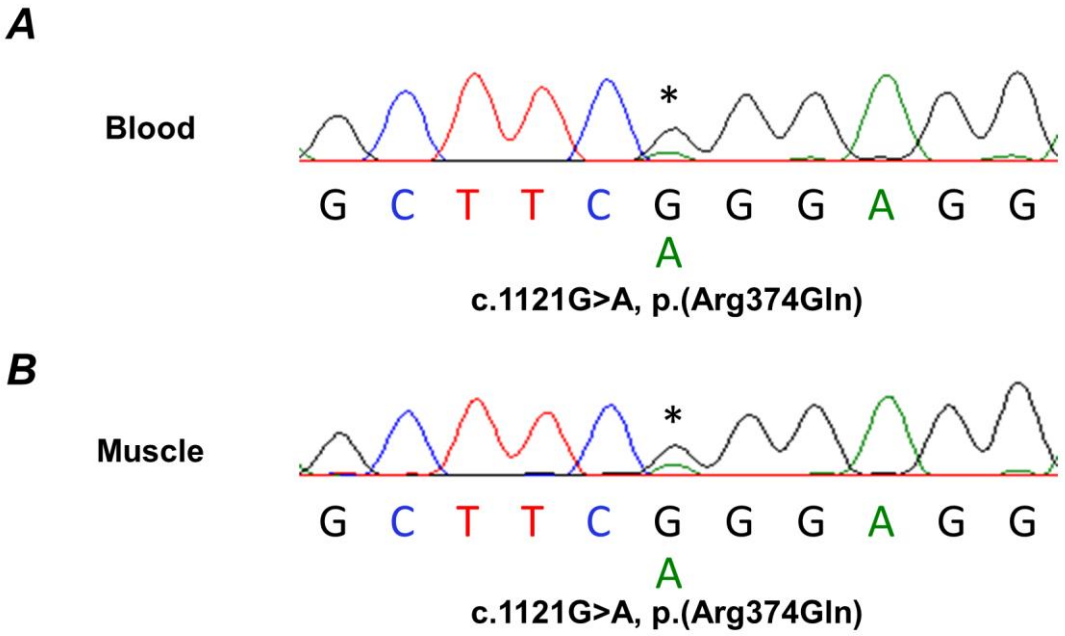


Figure 4.3 Electropherograms of the Somatic Mosaic p.Arg374Gln Variant in Patient 18 Blood and Muscle DNA. Both electropherograms of patient 18 (*A*) blood and (*B*) muscle genomic DNA show a small peak at the *TWNK* c.1121 site for the G>A nucleotide substitution.

4.4.7 *RRM1* - Ribonucleotide Reductase Subunit M1

Patient 3 was a 66 year old male who presented an OPMD-like phenotype with PEO, ptosis, hearing loss, diplopia and dysphagia. Muscle histopathology showed COX-deficient and ragged-red fibres. Multiple mtDNA deletions were confirmed by long-range PCR and Southern blotting. Family history was significant; his mother also presented with the same phenotype.

WES analysis for heterozygous variants identified a c.1281C>A (p.Asn427Lys) missense change in *RRM1* encoding ribonucleotide reductase subunit M1 (R1). *RRM1* (11p15.4) matched GO-Terms ‘replication’, ‘purine’ and ‘nucleotide’. Sanger sequencing using custom forward and reverse primers for *RRM1* exon 12 confirmed the variant (**Figure 4.4A**). Segregation studies including of the affected mother were not possible. Asn427 was

conserved from humans to *S. cerevisiae* (**Figure 4.4B**). The Asn427Lys variant was absent from in-house exomes and external databases.

Independent of this WES study, two apparently unrelated patients with childhood-onset PEO and multiple mtDNA deletions were identified harbouring a homozygous c.1142G>A (p.Arg381His) *RRM1* missense change. The p.Arg381His missense change was absent from all external exome databases and affected a highly conserved residue in all tested species except *Danio rerio*, *D. melanogaster* and *S. cerevisiae* (**Figure 4.4B**).

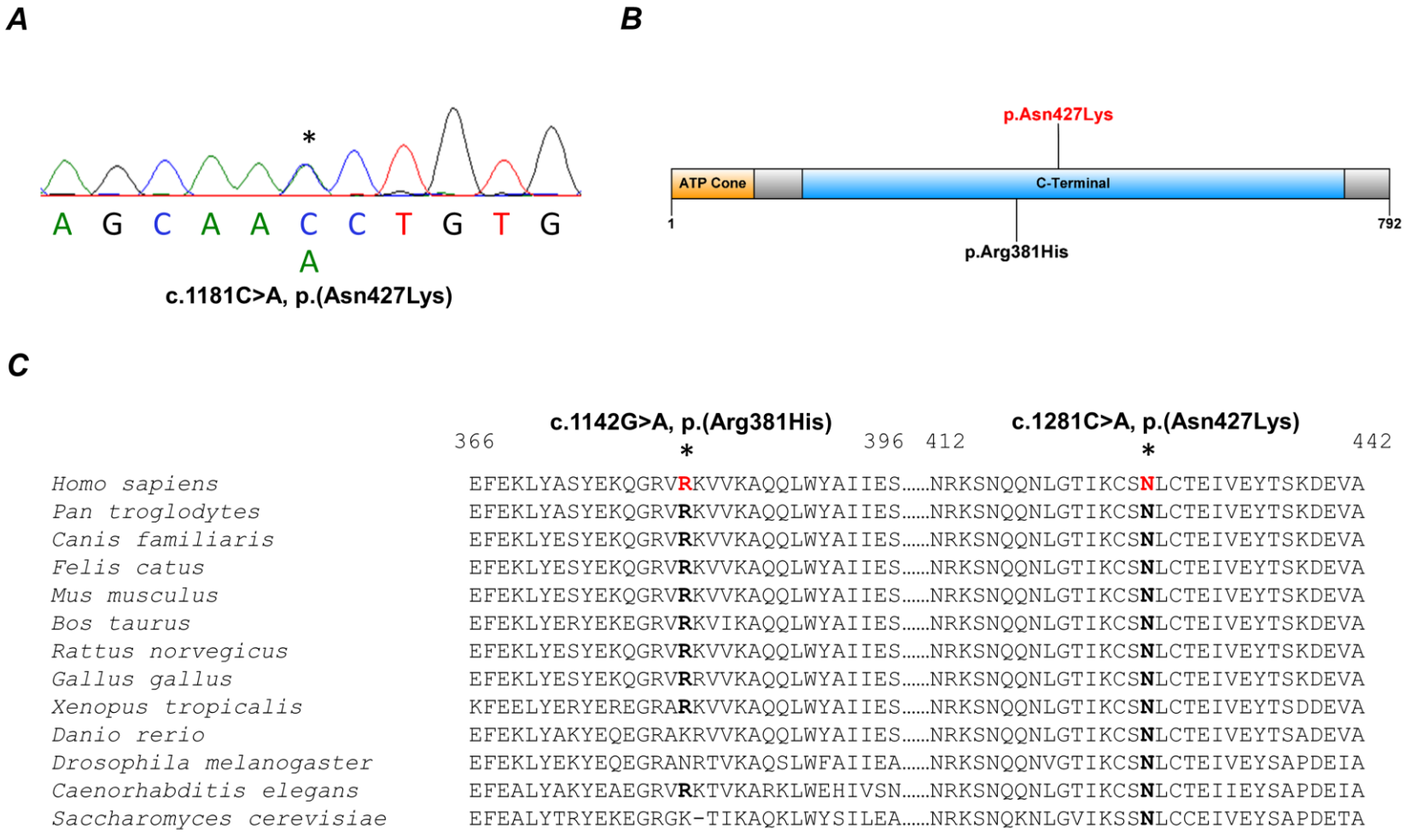


Figure 4.4 *RRM1* Sanger Sequencing Confirmation, Domain Architecture and MSA. (*A*) Sanger sequencing confirmation of novel p.Asn427Lys *RRM1* missense variant in patient 3. (*B*) RRM1 domain architecture showing location of the dominant heterozygous p.Asn427Lys (red) and the reported homozygous p.Arg381His missense variant (black). (*C*) MSA of RRM1 Arg381 and Asn427 residues. '.....' denotes the space between the residue where the residues are located.

4.4.8 *VDAC1* – Voltage Dependent Anion Channel 1

Patient 5 was a 64 year old male with a history of asthma and migraine since childhood. Upon clinical examination, the patient had PEO, ptosis, neck muscle weakness, a tremor, cataracts and SNHL. He had features of irritable bowel syndrome and complained of muscle pain in his arms, neck and shoulders as well as double vision. Muscle histopathology disclosed 20% COX-deficient fibres and 7% ragged-red fibres. Multiple mtDNA deletions were also confirmed by diagnostic long-range PCR. His mother, grandmother and sister all had ptosis, while his mother also had hearing loss.

WES analysis of heterozygous variants identified a c.239C>T (p.Thr80Ile) missense change in *VDAC1* encoding voltage dependent anion channel 1, also known as porin. *VDAC1* (5q31.1) matched the GO-Term 'mitochondr*'. Sanger sequencing confirmed the variant with custom forward and reverse primers for *VDAC1* exon 3 **(Figure 4.5A**). Segregation studies of affected and unaffected family members were not possible. The p.Thr80Ile variant was absent from in-house exomes and all external exome databases. Analysis of the human VDAC1 secondary protein structure (PDB ID 2JK4) (Bayrhuber *et al.*, 2008) showed that the Thr80 residue was located at the edge of a β-strand fold (**Figure 4.5B**). Thr80 was conserved in all tested species except *S. cerevisiae*, occurring in a highly conserved region of the protein (**Figure 4.5C**).

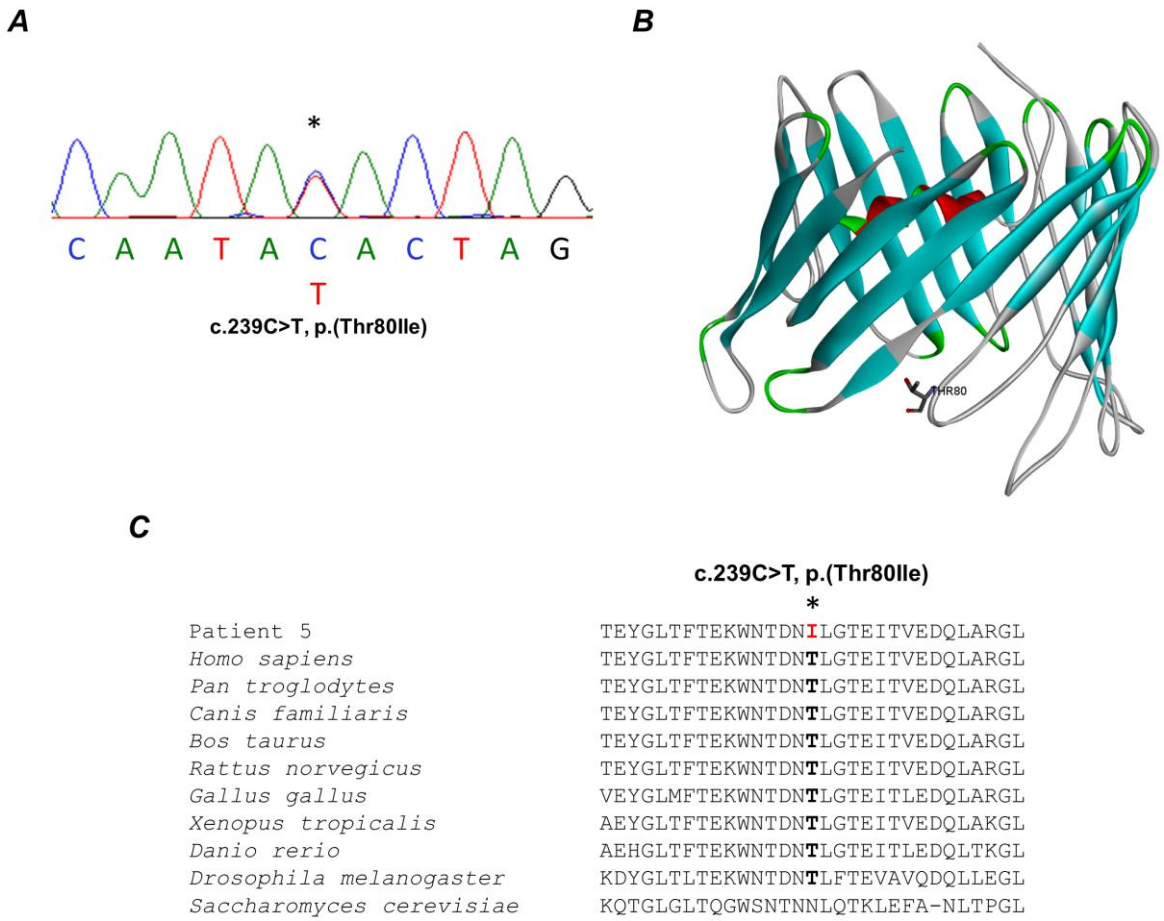


Figure 4.5 Sanger Sequencing Confirmation, VDAC1 Secondary Structure and MSA. (*A*) Sanger sequencing confirmation of the novel p.Thr80Ile *VDAC1* missense change. (*B*) Secondary structure of VDAC1 demonstrating the location of the Thr80 residue. (*C*) MSA of the VDAC1 Thr80 residue. VDAC1 secondary structure adopted and amended from PDB ID 2JK4.

4.4.9 *GMPR1* – Guanosine Monophosphate Reductase 1

Application of the WES strategy for patient 11 identified a novel heterozygous *GMPR1* c.547G>C (p.Gly183Arg) missense change in *GMPR1* encoding guanosine monophosphate reductase 1. This is investigated in **Chapter 5** as a likely novel causative gene associated with late-onset PEO and multiple mtDNA deletions.

4.4.10 *SEPT2* – Septin 2

Patient 19 was a 50 year old male who first presented cystinuria, a disease characterised by the build-up of cysteine in urine leading to the formation of stones, which required a partial nephrectomy and treatment with penicillamine. This was followed by what was initially presumed to be penicillamine-induced myasthenia gravis, manifesting as PEO, ptosis and

fatigue. Serum lactate was also mildly elevated (3.1mmol/L; normal <2.5mmol/L). However, withdrawal of penicillamine did not resolve the symptoms and the patient was investigated for a possible mitochondrial myopathy. Muscle histopathology disclosed 18% COX-deficient fibres and 3% ragged-red fibres. Multiple mtDNA deletions were detected by long-range PCR. Family history was negative for ophthalmoparesis or mitochondrial myopathy.

Application of the WES filtering strategy did not reveal any causative or likely causative variants and hence, rare dominant and recessive variants were listed.

Recently, Pagliuso *et al.* (2016) demonstrated that septin 2, a cytoskeleton component encoded by *SEPT2*, was essential for Drp1-mediated mitochondrial fission. A cohort-wide search for rare or novel *SEPT2* variants identified a heterozygous c.988C>T (p.Arg330Cys) missense change harboured by patient 19, confirmed with custom forward and reverse primers for *SEPT2* exon 12 (**Figure 4.6A**). The GO-Terms for *SEPT2* did not match those of the WES filtering strategy devised for adult-onset PEO and multiple mtDNA deletions patients. Arg330 was fully conserved from humans to *D. melanogaster*, occurring in a highly conserved region (**Figure 4.6C**). The variant was absent from in-house exomes and all external databases. Segregation studies were not possible and therefore, the p.Arg330Cys variant was presumed *de novo* or parental germline mosaic.

Due to the cystinuria presented by this patient, the co-existence of two or more Mendelian disorders was considered. To date, isolated cystinuria has been associated with autosomal recessive mutations of *SLC3A1* (type A) (Calonge *et al.*, 1994) and *SLC7A9* (type B) (Feliubadalo *et al.*, 1999). WES analysis of *SLC3A1* and *SLC7A9* genes identified a known pathogenic heterozygous c.1400T>C (p.Met467Thr) (rs121912691) *SLC3A1* missense variant, confirmed with custom forward and reverse primers for *SLC3A1* exon 8 (**Figure 4.6B**). No pathogenic or likely pathogenic *SLC7A9* variants were identified. Analysis of CNVs identified an additional known pathogenic 13.98kb *SLC3A1* exon 5-9 duplication (chr2:44527111-44541090). Unfortunately there was insufficient DNA to perform quantitative real time PCR to confirm the presence of the duplication. Therefore, this resolved the genetic aetiology of the cystinuria and likely the elevated serum lactate readings.

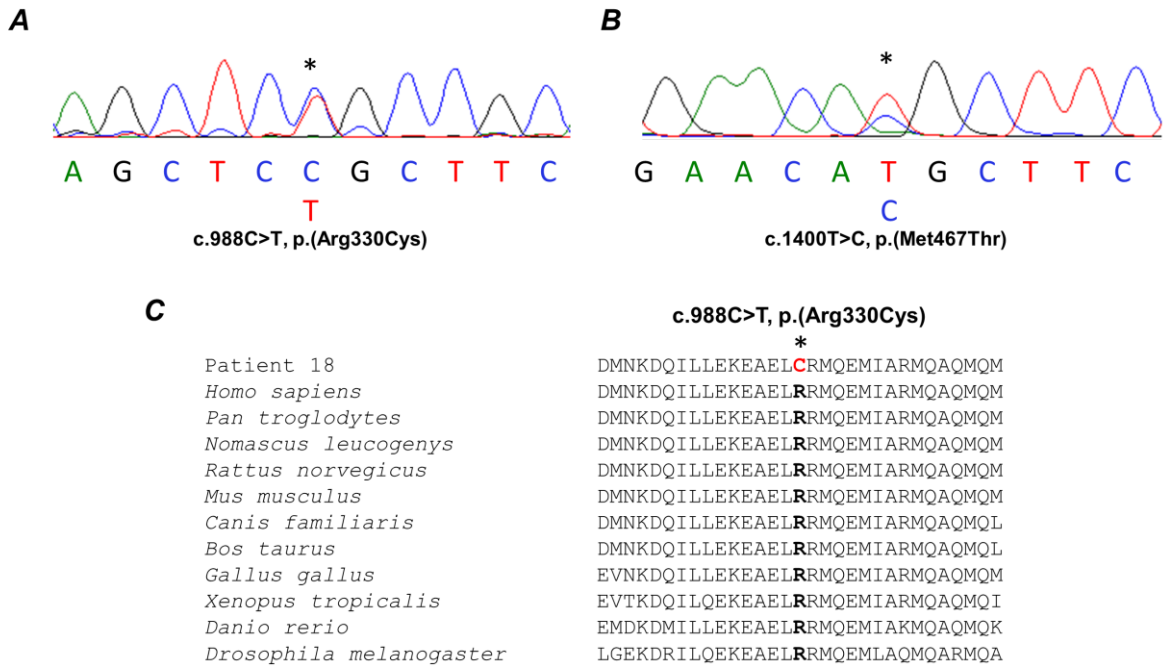


Figure 4.6 *SEPT2* and *SLC3A1* Sanger Sequencing Confirmation and SEPT2 MSA. Sanger sequencing confirmation of (*A*) the novel *SEPT2* p.Arg330Cys missense change and (*B*) the known pathogenic heterozygous *SLC3A1* p.Met467Thr missense change. (*C*) MSA of the SEPT2 Arg330 residue.

4.4.11 *ABAT* (VUS) – GABA Transaminase

Patient 1 was a 72 year old male with gradual progressive bilateral ptosis since his late 40s, PEO, a mild axonal neuropathy, mild hearing loss and intermittent difficulty swallowing. Muscle histopathology disclosed COX-deficient and ragged-red fibres. Multiple mtDNA deletions were detected by diagnostic long-range PCR. Family history was negative.

WES analysis identified a heterozygous c.1157C>T (p.Pro386Leu) missense variant of *ABAT* encoding the GABA transaminase. *ABAT* (16p13.2) matched the GO-Term ‘mitochondr*’. Sanger sequencing using custom forward and reverse primers for *ABAT* exon 13 confirmed the variant (**Figure 4.7A**). The missense change was absent from in-house exomes. However, it was reported in 2/121064 heterozygous non-Finnish European alleles (MAF=0.00001652) in ExAC. Pro386 was fully conserved in all tested species and occurred in a moderately conserved region of the protein (**Figure 4.7B**).

Analysis of CNVs for patient 1 revealed a known pathogenic 1.4Mb deletion in chromosome 17 (chr17:14095307-15477497, 17p12). Within this deletion were all coding exons of *PMP22* (peripheral myelin protein 22) and *COX10* (cytochrome *c* oxidase assembly factor 10) exon 6. GO-Terms matched were ‘mitochondr*’, ‘polymerase’ and ‘transcription’. Unfortunately

there was insufficient DNA to confirm the presence of the deletion either by real time PCR or array comparative genomic hybridisation (CGH) assays.

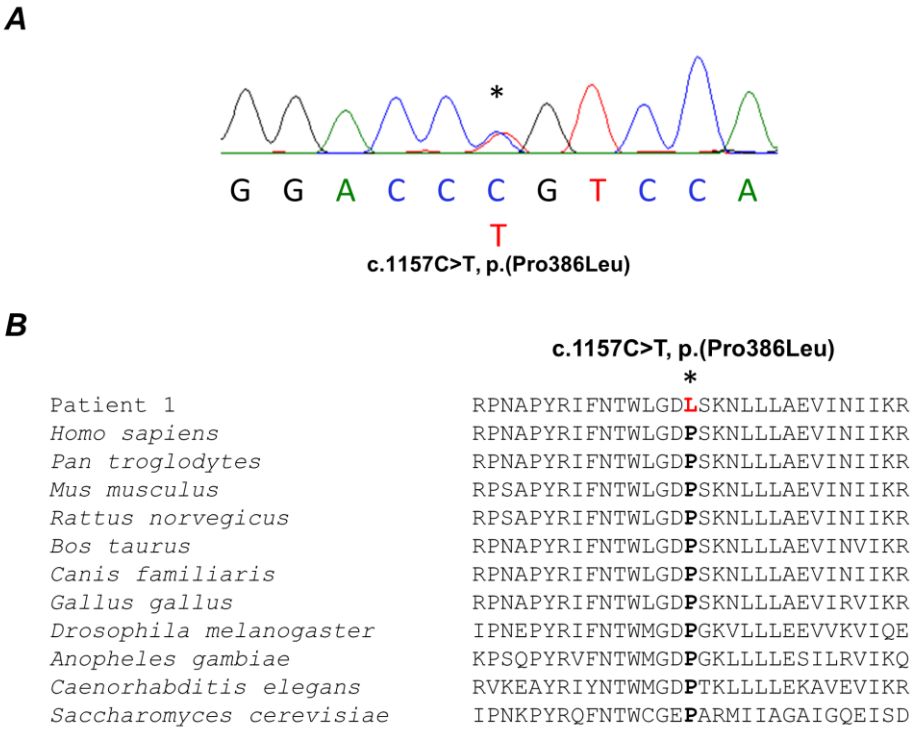


Figure 4.7 *ABAT* Sanger Sequencing Confirmation and ABAT MSA. (*A*) Sanger sequencing confirmation of the heterozygous p.Pro386Leu *ABAT* missense change. (*B*) MSA of the ABAT Pro386 residue.

4.4.12 *RRM2B* (VUS) – Ribonucleotide Reductase Subunit M2B

Patient 6 was a 76 year old male presenting an OPMD-like phenotype with PEO, bilateral ptosis, facial muscle weakness with mild wasting of the facial muscles, dysphagia, intermittent diplopia and SNHL. Muscle histopathology showed 35% COX-deficient fibres and 15% ragged-red fibres. Diagnostic long-range PCR demonstrated the presence of multiple mtDNA deletions. There was a family history of hearing loss but extraocular paresis was confined to patient 6.

WES analysis revealed a heterozygous *RRM2B* missense change, c.482C>T (p.Thr161Ile), that had previously been catalogued (rs72554098) and had been excluded diagnostically as a causative variant. *RRM2B* (8q22.3) matched GO-Terms ‘DNA repair’, ‘nucleoside’ and ‘replication’. The missense change was absent from in-house exomes and the 1000 Genomes Project, but was reported in 1/121346 non-Finnish European allele (MAF=0.000008241) in ExAC and in 1/13006 (MAF=0.00007689) in the NHLBI ESP, both in heterozygous state. The Thr161 residue was fully conserved, occurring in a highly conserved region of the protein

(**Figure 4.8A**). In the absence of additional candidate variants, p.Thr161Ile was classified as a VUS.

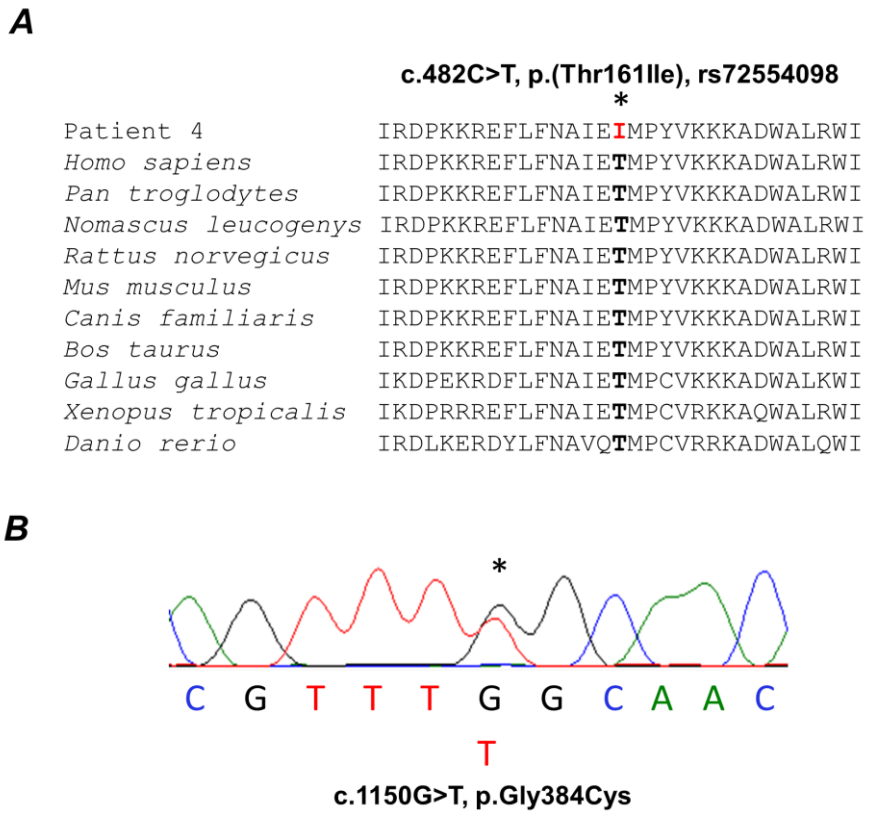


Figure 4.8 RRM2B MSA and *MYH14* p.Gly384Cys Sanger Sequencing Confirmation. (*A*) MSA of the RRM2B Thr161 residue. (*B*) Sanger sequencing confirmation of the heterozygous p.Gly384Cys *MYH14* missense change.

Further analysis of rare annotated disease-causing variants identified a heterozygous c.1150G>T (p.Gly384Cys) (rs119103280) missense change of *MYH14*, encoding non-conventional non-muscle myosin heavy-chain 14, which had been previously categorised as pathogenic (Donaudy *et al.*, 2004). The p.Gly384Cys variant was confirmed by Sanger sequencing using custom forward and reverse primers for *MYH14* exon 11 (**Figure 4.8B**).

4.4.13 *TOP3A* (VUS) – Topoisomerase III-α

Patient 8 was a 67 year old female presenting with a complex PEO-plus phenotype with onset of ptosis at 44 years old. She also manifested with PEO, proximal muscle weakness, ataxia, axonal neuropathy, dysphagia, decreased visual acuity and hearing loss. A pacemaker was fitted due to a cardiac conduction defect. Interestingly, the patient also had telangiectasia; a condition with red lines or other patterns on the skin due to dilated blood vessels near the surface of the skin. Muscle histopathology disclosed >10% COX-deficient fibres and >10%

ragged red fibres. Multiple mtDNA deletions were confirmed by diagnostic long-range PCR and Southern blotting. Family history was significant for a female sibling who died during her late 20s, apparently due to lupus although there were no further details available.

WES analysis identified compound heterozygous variants in *TOP3A* encoding topoisomerase III-α; a c.298A>G (p.Met100Val) missense change and a c.403C>T (p.Arg135*) nonsense variant. *TOP3A* (17p11.2) matched the GO-Term 'topoisomerase'. The variants were confirmed by Sanger sequencing with custom forward and reverse primers for *TOP3A* exons 3 and 5 (**Figure 4.9A**). Both variants were absent from in-house exomes. The p.Met100Val missense change was reported in 7/120600 alleles (MAF=0.00005804) in ExAC comprising five non-Finnish Europeans, one Finn and one Latino, all in heterozygous state. It was also reported in 1/13006 heterozygous allele (MAF=0.00007689) in the NHLBI ESP. The p.Arg135* nonsense change was reported in 24/121054 alleles (MAF=0.0001983) in ExAC comprising 20 non-Finnish Europeans, three Finns and one undisclosed individual, all in heterozygous state. It was also reported in 1/13006 heterozygous allele (MAF=0.00007689) in the NHLBI ESP. Both variants occurred in the Toprim domain of TOP3A (**Figure 4.9B**). The Met100 residue was poorly conserved; it was a valine residue in *D. melanogaster* (**Figure 4.9C**). Segregation studies performed by Charlotte Alston (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne) confirmed that the mother and her daughter were heterozygous carriers of the p.Arg135* nonsense mutation.

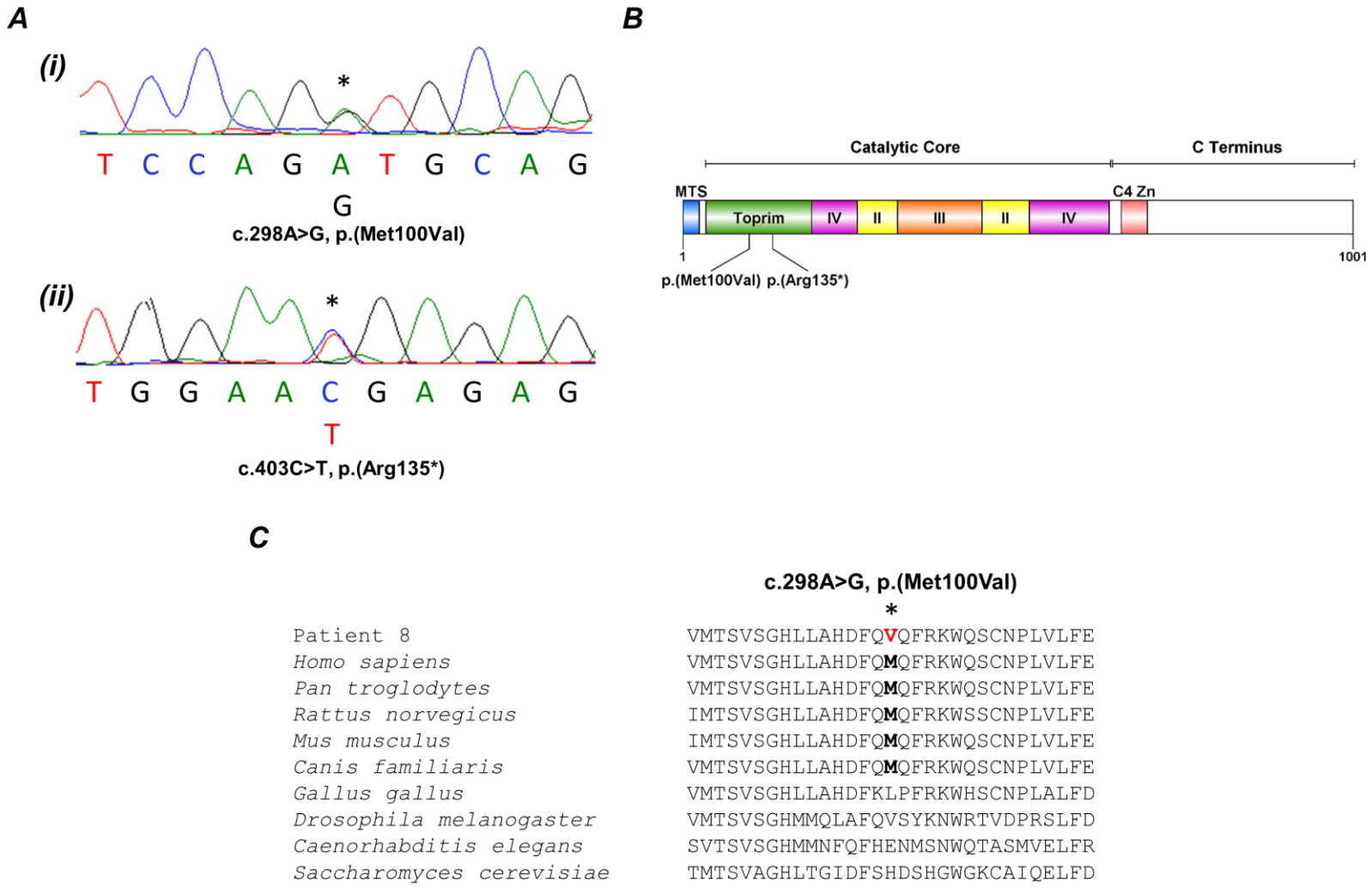


Figure 4.9 *TOP3A* Sanger Sequencing Confirmation, Domain Architecture and MSA. (*A*) Sanger sequencing confirmation of the *TOP3A* p.Met100Val and p.Arg135* variants harboured by patient 8. (*B*) TOP3A domain architecture demonstrating the locations of the identified *TOP3A* variants. (*C*) MSA of the TOP3A Met100 residue.

4.4.14 *POLRMT* (VUS) – Mitochondrial RNA Polymerase

Patient 15 was a 56 year old male presenting with an indolent PEO phenotype. Ophthalmoplegia was noted incidentally on clinical examination at 28 years old, while ptosis predated this for an unknown duration. Brain MRI was normal and there had been no disease progression since initial examination. Muscle histopathology showed COX-deficient and ragged-red fibres (**Figure 4.10A and B**). Multiple mtDNA deletions were detected by diagnostic long-range PCR and quantitative real time PCR assays. The patient had an uncle who was also thought to have PEO but this was not clinically confirmed.

WES filtering identified a heterozygous c.2641-1G>C splice acceptor site variant in intron 10 of *POLRMT* encoding the mitochondrial RNA polymerase. The Human Splicing Finder predicted that this would lead to a broken splice-acceptor site, with possible skipping of exon 11. *POLRMT* (19p13.3) the GO-Terms 'mitochondr*', 'transcription' and 'polymerase'. The splice-site variant was absent from in-house exomes, the 1000 Genomes Project and NHBLI Exome Variant Server databases, but was present in 1/61428 heterozygous allele (MAF=0.00001628) in ExAC. The splice-site variant was confirmed by Sanger sequencing using custom forward and reverse primers for *POLRMT* exon 11 (**Figure 4.10C**). Fibroblast RNA was extracted from patient 14 fibroblasts and one control, then cDNA was synthesised for sequencing. However, four sets of custom forward and reverse primers failed to amplify the cDNA for sequencing due to a high GC-content of the flanking exons. Segregation studies were also not possible.

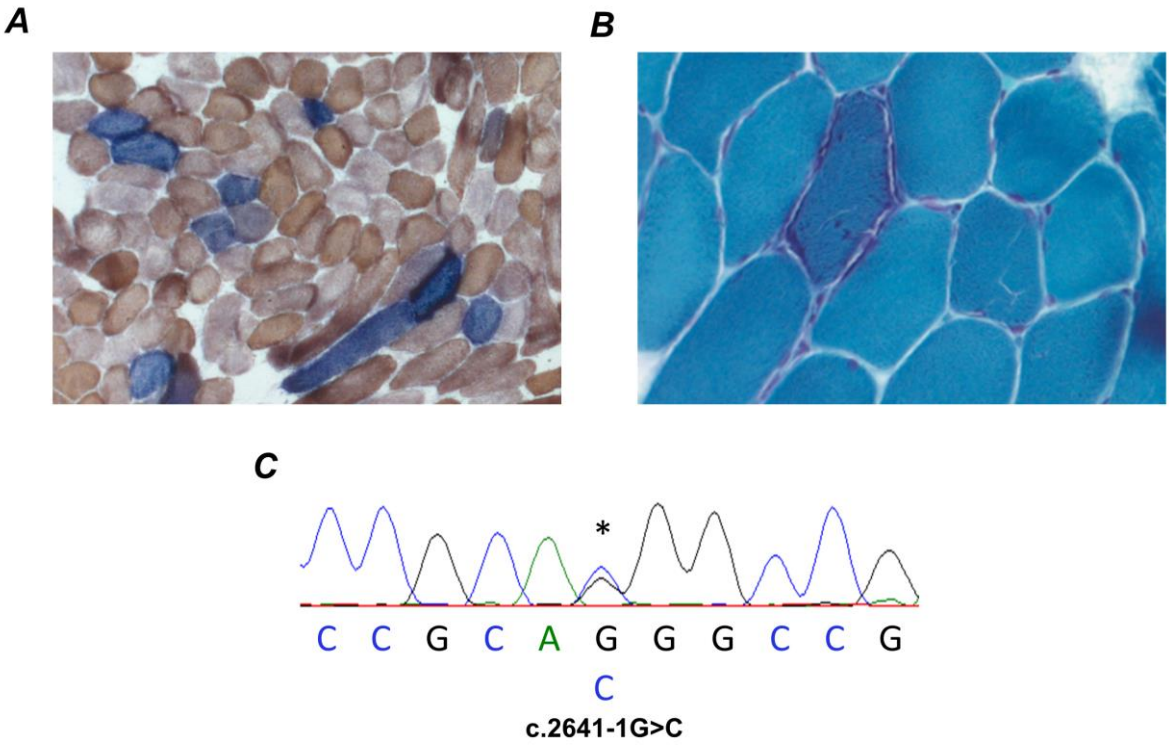


Figure 4.10 Muscle Histopathology and *POLRMT* Sanger Sequencing Confirmation for Patient 14. Patient 14 skeletal muscle was subjected to (*A*) sequential COX-SDH histochemistry and (*B*) Gomori trichrome staining. (*C*) Sanger sequencing confirmation of the c.2641-1G>C *POLRMT* splice-site mutation.

The *POLRMT* splice-site mutation was investigated using available fibroblasts from patient 14 and two controls. Western blotting with antibodies specific to POLRMT, mitochondrial ribosomal protein subunit L12 (MRPL12) and SDHA showed non-specific findings in the patient fibroblasts under proliferating and quiescent states (**Figure 4.11A**). However, there was considerable variation between the control samples. Quantitative real time PCR to assess mtDNA copy number in proliferating and quiescent fibroblasts revealed no significance between patient and control fibroblasts, based upon two repeats (**Figure 4.11B**). In contrast to patient 14 skeletal muscle, long-range PCR of quiescent fibroblasts did not reveal large-scale mtDNA rearrangements (**Figure 4.11C**).

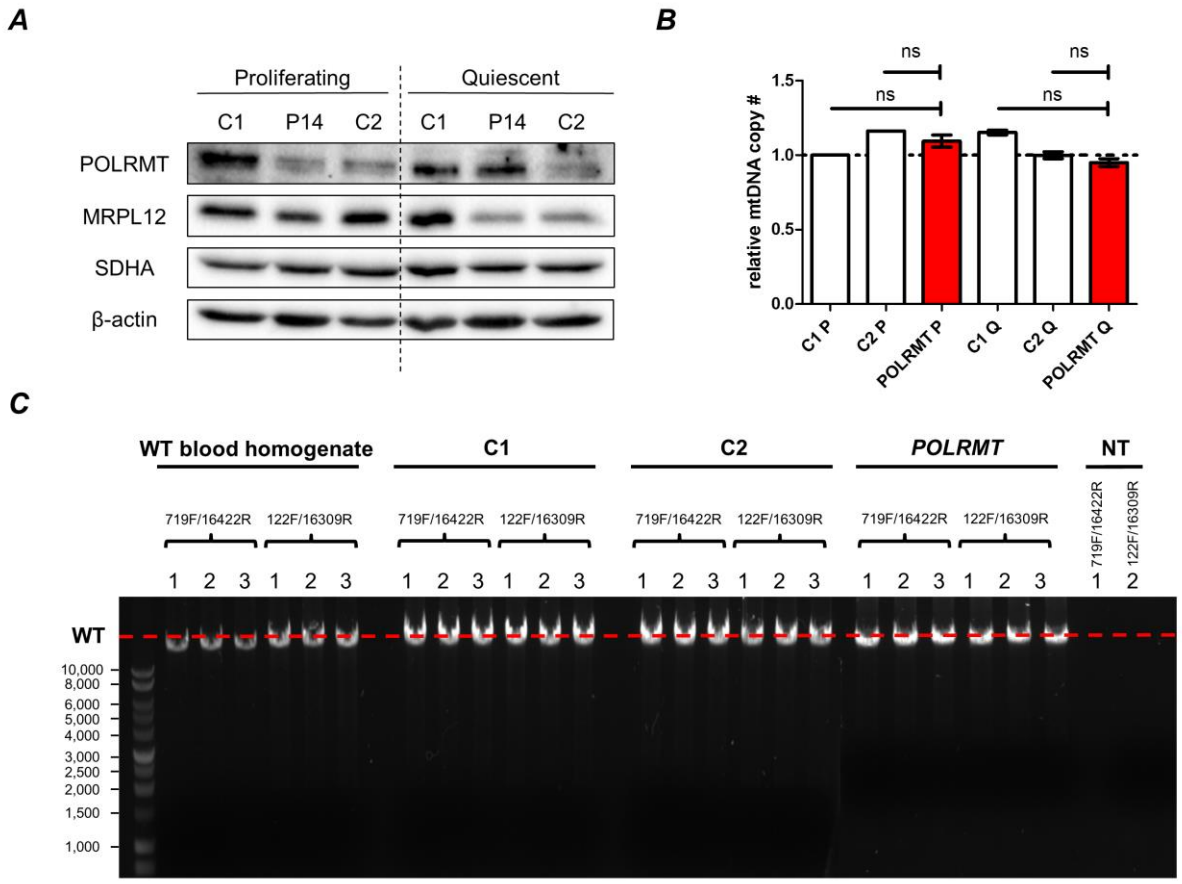


Figure 4.11 Molecular Studies of Patient 14 Fibroblasts. (*A*) Steady-state POLRMT, MRPL12 and SDHA levels in proliferating and quiescent fibroblasts of patient 14 plus two β-actin was used as a loading control. (*B*) Relative mtDNA copy number in patient 14 (red) and control fibroblasts in proliferating and quiescent state based upon two repeats only. One-way ANOVA (Kruskal-Wallis test) and Dunn's Multiple Comparisons tests were performed to compare the mean relative mtDNA copy number between proliferating and quiescent controls to patient fibroblasts. (*C*) Long-range PCR of wild-type blood homogenate, two control quiescent fibroblast (C1 and C2), patient 14 (*POLRMT*) quiescent fibroblast DNA and a no template control. Amplification of each sample was performed in triplicate with two sets of optimised forward and reverse primers. Red line indicates the wild-type mtDNA product.

4.4.15 *MGME1* (VUS) – Mitochondrial Genome Maintenance Exonuclease 1

Patient 17 was an 80 year old male who presented with PEO, ptosis and mild dysphagia. Muscle histochemistry disclosed 30% COX-deficient fibres. Diagnostic long-range PCR also detected multiple mtDNA deletions. Family history was significant; his father, sister and brother were all reported to have mild PEO.

WES analysis of heterozygous variants identified a novel c.706C>G (p.Leu236Val) missense variant of *MGME1* (20p11.23), matching the GO-Term 'mitochondr*'. The variant was absent from in-house exomes and all external databases. Sanger sequencing of all coding exons of *MGME1* was performed but did not reveal any other significant variants (**Figure**

4.12A). The Leu236 residue was conserved in all tested species except *D. rerio* (**Figure 4.12B**). Furthermore, DNA was unavailable from additional affected family members to perform segregation studies. With further studies currently not possible, the p.Leu236Val missense change is classed as a VUS.

Figure 4.12 *MGME1* Sanger Sequencing Confirmation and MGME1 MSA. (*A*) Sanger sequencing confirmation of the p.Leu236Val *MGME1* missense change. (*B*) MSA of *MGME1* Leu236 residue.

4.5 Discussion

Utilising WES with a custom filtering strategy to prioritise variants in nuclear genes encoding mitochondrial, mtDNA maintenance and DNA repair proteins, a genetic diagnosis was attained in only 2/19 (10.5%) patients. Likely causative variants were identified in a further 4/19 (21.1%) patients for mtDNA maintenance disorders (*RRM1*, *VDAC1*, *GMPR1*, *SEPT2*) and resolving the genetic aetiology of a patient with hearing loss only (*MYH14*). Potential significant VUS were also identified in 3/19 (15.8%) patients (*ABAT*, *TOP3A*, *POLRMT*).

However, for both novel causative variants and VUS, further studies are necessary to confirm the pathogenic nature. A twentieth patient was later found to not have a mtDNA maintenance disorder and instead had hearing loss only, due to a likely causative heterozygous *MYH14* mutation, hence this patient was excluded from the study.

4.5.1 *RNASEH1* Mutations are a Rare Cause of Adult-Onset mtDNA Instability

RNASEH1 encodes ribonuclease H1, a dual nuclear and mitochondrial endonuclease essential for the degradation of DNA-RNA hybrids during mtDNA replication (Cerritelli and Crouch, 1998). Homozygous *Rnaseh1* knockout mice were embryonic lethal with significant loss of mtDNA copy number, loss of COX activity and abnormal mitochondria morphology (Cerritelli *et al.*, 2003). Recessive *RNASEH1* mutations were recently implicated in 12 patients with systemic adult-onset PEO with multiple mtDNA deletions (Reyes *et al.*, 2015; Akman *et al.*, 2016). In light of this, all eight coding exons of *RNASEH1* were sequenced in 67 undiagnosed adult-onset PEO with multiple mtDNA deletions patients referred to the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory in Newcastle upon Tyne. Unfortunately, no protein coding variants were identified, including the reported pathogenic variants. Thus, it was concluded at that *RNASEH1* mutations are a rare cause of adult-onset PEO with multiple mtDNA deletions. As detailed in **3.4.17**, PEO, ptosis, myopathy with generalised muscle wasting and respiratory insufficiency are features that could direct immediate targeted *RNASEH1* sequencing in future patients.

4.5.2 Adult-Onset PEO and Multiple mtDNA Deletions Associated with TK2-Deficiency

A homozygous p.Thr108Met *TK2* missense variant was identified in patient that had been previously associated with clinically heterogeneous phenotypes, which included both severe, early-onset mtDNA depletion syndrome and late-onset myopathy with multiple mtDNA deletions (Mancuso *et al.*, 2002; Mancuso *et al.*, 2003; Wang *et al.*, 2005; Oskoui *et al.*, 2006; Chanprasert *et al.*, 2013; Martin-Hernandez *et al.*, 2016). A study of the natural history of TK2-deficiency confirmed the heterogeneous phenotypes associated with the p.Thr108Met missense change (Garone *et al*., submitted). Six patients had adult-onset myopathy with multiple mtDNA deletions, 9 had childhood-onset myopathy and six had infantile-onset myopathy with mtDNA depletion. Patient 4 was clinically similar to patients with late-onset TK2-deficiency, as outlined in **3.4.9**.

4.5.3 Somatic Mosaicism of a p.Arg374Gln *TWNK* Mutation

Somatic mosaicism is the phenomenon of a single mutation occurring in two or more populations of soma cells in one individual (Freed *et al.*, 2014). This contrasts with germline mosaic mutations, which occur only in the germ (gonadal) cells. Irrespective, mosaicism can occur at any point after first cell division of the zygote. On the other hand, a *de novo* mutation is transmitted from a father harbouring a mutation in only a single sperm cell or arises during the first cell divisions of a developing zygote and is harboured in all cells. Somatic mosaic mutations have been increasingly recognised in Mendelian human disease, including but not limited to Duchene Muscular Dystrophy (DMD) (Kesari *et al.*, 2009), Rett syndrome (Topcu *et al.*, 2002) and cancer (Fernandez *et al.*, 2016). Recent studies have also implicated low-level parental somatic mosaicism in a significant proportion of apparent *de novo* genomic disorders in children than had previously been estimated (Campbell *et al.*, 2014b; Beck *et al.*, 2016). Primary mitochondrial disease shares similarities with somatic mosaicism, in that heteroplasmic pathogenic mtDNA mutations can harboured throughout different tissues and can sometimes correlate with clinical severity (Tuppen *et al.*, 2010). Clonal expansion of somatic mtDNA deletions have also been associated with a spectrum of human disease and ageing (Schon *et al.*, 2012).

WES analysis of patient 18 identified a p.Arg374Gln *TWNK* missense change that was one of the most commonly reported pathogenic variants associated with late-onset PEO and multiple mtDNA deletions (Spelbrink *et al.*, 2001; Naimi *et al.*, 2006; Baloh *et al.*, 2007; Massa *et al.*, 2009; Martin-Negrier *et al.*, 2011; Yu-Wai-Man *et al.*, 2013; Tafakhori *et al.*, 2016) (**3.4.5**). This was unexpected since all coding exons and intronic regions of *TWNK* had been sequenced diagnostically prior to recruitment for WES. Analysis of the original electropherogram and re-sequencing of blood and muscle DNA showed that the p.Arg374Gln variant was harboured at low level somatic mosaicism but at sufficient levels to cause symptoms. Additionally it has not been possible to determine the extent of the mosaicism in other tissues, particularly in the germline population. Hence, the implications for offspring were uncertain. Probable germline mosaicism of *ANT1* and *TWNK* mutations causing adult-onset PEO with multiple mtDNA deletions have been previously described (Deschauer *et al.*, 2005; Hudson *et al.*, 2005). On the other hand, patient 18 is the first known case of somatic mosaicism associated with this disease. This finding has significant ramifications in the diagnosis of autosomal dominant Mendelian mitochondrial disease. Manual analysis and curation of the electropherogram for *TWNK* exon 1c of patient 18 blood DNA failed to note the small peak at the c.1121 site, which raises uncertainty regarding additional ‘undiagnosed’

patients. While careful curation and annotation of Sanger sequencing electropherograms could potentially resolve a handful of undiagnosed cases, this is not practical nor is Sanger sequencing sensitive to lower levels of mosaicism (Jamuar *et al.*, 2014; Qin *et al.*, 2016). One study found that the lowest range of mutant alleles detectable by Sanger sequencing was between 15-50% (Rohlin *et al.*, 2009). Rohlin *et al.* (2009) also demonstrated that massive parallel sequencing could detect somatic alleles at a frequency of 1%, although this was dependent upon high read coverage. In an era where NGS is becoming less expensive and its use more widespread, additional patients could be anticipated to harbour low level, pathogenic somatic mutations of known mtDNA maintenance disorder genes. For patient 18, WES was demonstrated as sufficient for detection of the p.Arg374Gln mutation at a level of 27.3% in blood DNA, verifiable in blood and muscle DNA by Sanger sequencing. Nonetheless, such investigations are dependent on the availability of a range of patient tissues for testing and of course, access to NGS technologies. With few diagnoses attained in this group of patients, somatic mosaicism provides a novel, alternative hypothesis for the low diagnostic yield in adult-onset PEO with multiple mtDNA deletions.

4.5.4 Novel mtDNA Maintenance Disorder Candidates Requiring Further Investigation

Likely causative variants and VUS were identified in *RRM1*, *VDAC1*, *GMPR1* (**Chapter 5**), *SEPT2*, *ABAT*, *RRM2B*, *TOP3A*, *POLRMT* and *MGME1*. All genes have been associated with either mitochondrial functions or DNA repair and maintenance pathways, but require additional functional studies to validate pathogenicity.

4.5.4.1 Dominant and Recessive *RRM1* Mutations Associated with a Novel Disorder of mtDNA Maintenance

Dominant and recessive mutations of *RRM2B* encoding the small p53R2 subunits of the ribonucleotide reductase (RNR), for the rate-limiting *de novo* synthesis of deoxyribonucleotides from ribonucleotides (Pär and Peter, 2006), have been established as causative for early-onset mtDNA depletion syndrome and adult-onset PEO with multiple mtDNA deletions (Bourdon *et al.*, 2007; Pontarin *et al.*, 2007). In this study, a heterozygous p.Asn427Lys missense change in *RRM1* encoding the large catalytic R1 subunit of the RNR was identified using the custom WES filtering strategy in patient 3. Mammalian dNTP synthesis for nuclear and mtDNA replication is detailed in **5.1.1**. To summarise, during S-phase of the cell cycle, the RNR is a tetramer comprised of homodimeric R1 subunits and smaller homodimeric R2 subunits encoded by *RRM2*. In non-dividing cells, the R2 subunit is

replaced by p53R2 (Bourdon *et al.*, 2007; Pontarin *et al.*, 2007). Given the role of *RRM2B* in dominant and recessive mtDNA maintenance disorders, the novel *RRM1* variant was considered a highly likely candidate for a novel mtDNA maintenance disorder.

The case for a *RRM1*-associated mtDNA maintenance disorder is further compelled by the identification of patients from two apparently unrelated families harbouring a homozygous c.1142G>A (p.Arg381His) *RRM1* missense change. The first patient was a 33 year old Saudi Arabian man reported at the 2016 American Academy of Neurology Meeting (Juanola-Falgarona *et al.*, 2016). He had a MNGIE-like phenotype that was comparable to one patient with recessive *RRM2B* missense variants (**3.4.8**) (Shaibani *et al.*, 2009). He presented with recurrent nausea and vomiting at 7 years old, which progressed to severe gastrointestinal dysmotility, cachexia, PEO, ptosis, axonal neuropathy and proximal limb myopathy upon clinical examination. Muscle histopathology showed COX-deficient fibres, ragged-red fibres and the presence of multiple mtDNA deletions. Unfortunately there were no further details on the second patient at the time of writing. However, the homozygous p.Arg381His missense changes were identified in both patients by WES.

The pathogenesis of an *RRM1*-related mtDNA maintenance disorder necessitates functional validation and it is of interest to determine whether this acts in a similar manner to dominant and recessive *RRM2B* mutations. Regarding the dominant heterozygous p.Asn427Lys, this could act in a dominant-negative manner, competing with the wild-type R1 subunit and leading to an inactive RNR, as proposed for dominant *RRM2B* missense and nonsense mutations (Tyynismaa *et al.*, 2009a). Pitceathly *et al.* (2011) demonstrated defective R1/p53R2 assembly by Blue-native polyacrylamide gel electrophoresis (BN-PAGE) in recessive *RRM2B* patient muscle, with no change in steady-state p53R2 levels. Given the role of the RNR for nuclear and mtDNA replication, the current prevailing mechanism could be defective RNR assembly as a consequence of the *RRM1* mutations. Since the homozygous p.Arg381His affects all R1 subunits, it is understandable that the phenotype onset appears to manifest earlier in life compared to that of patient 3 with the dominant mutation. Hence, the p.Asn427Lys and p.Arg381His mutations likely lead to reduced capacity of the RNR for dNTP synthesis, although it is essential to confirm this in future studies of patient muscle and through detailed analysis of the R1 and p53R2 interactions.

4.5.4.2 A Novel Heterozygous *VDAC1* Mutation in an adPEO Family

VDAC1 encodes one of three voltage dependent anion channels (VDAC1-3) that each form homodimers in the OMM for the movement of small molecules up to 10 kilodaltons (kDa) to

enter and exit the intermembrane space (Bayrhuber *et al.*, 2008; Shoshan-Barmatz *et al.*, 2010). Given that the VDACs are highly abundant in the OMM, it serves to highlight their importance for mitochondrial function. A novel heterozygous p.Thr80Ile missense change was identified in patient 5, who has a family history of ophthalmoparesis, prompting filtering for heterozygous variants of interest. Further support for pathogenicity of *VDAC1* as a dominant locus for human disease is given in ExAC. This shows that the gene is highly intolerant to loss-of-function (LOF) variants including nonsense or splice-site changes with a LOF probability score of 0.98, since no such *VDAC1* variants have been identified to date. However, *in silico* predictions suggested that the missense change was not highly damaging despite that the Thr80 residue was conserved almost all tested species. Since the VDACs are implicit in the transport of wide range of molecules for mitochondrial functions, it is challenging to propose a potential pathological mechanism at such an early-stage. Based on the VDAC1 structure elucidated by Geula *et al.* (2012), Thr80 is not located in either the β1- or β19-strands that are essential for oligomerisation of the two VDAC1 monomers and hence, formation of the dimer is unlikely to be impaired. Nonetheless, the Thr80 residue is located at the edge of a β-strand and hence, its substitution could still alter the secondary structure of the channel. This leads to an alternative pathological mechanism involving ROS. Under oxidative stress, mitochondria require removal of ROS to prevent damage to mtDNA, macromolecules and lipids (Ott *et al.*, 2007). Hence, the VDACs have been proposed to be gateways for the transport of ROS across the OMM to the cytosol (Han *et al.*, 2003). Tikunov *et al.* (2010) demonstrated that closing the VDACs prevented the efflux of $O_2^{-\cdot}$ anions, leading to an accumulation of this species and oxidative stress. Han *et al.* (2003) also demonstrated that VDAC inhibitors prevented the efflux of $O_2^{\cdot}$ superanions to the cytosol. Thus, the accumulation of ROS leads to mtDNA damage. As there is a reduction of ROS release to the cytosol, there may also be a reduction in ROS-induced apoptosis since cytochrome *c* cannot be released to the cytosol. Over time, this ROS accumulation and failure to degrade damaged mitochondria is thereby catastrophic. For the moment, the p.Thr80Ile variant remains an excellent candidate for patient 5, but requires confirmation that it segregates with affected family members before functional validation could commence.

4.5.4.3 *SEPT2* Mutation Links Mitochondrial Fission with PEO and Multiple mtDNA Deletions

The cytoskeleton component septin 2 has recently been characterised as an essential regulator of mitochondrial fission (Pagliuso *et al.*, 2016). Hence, of all candidates identified in this study, the novel heterozygous p.Arg330Cys *SEPT2* missense variant harboured by patient 19

was the most recent finding. Pagliuso *et al.* (2016) showed that siRNA depleted SEPT2 in HeLa cells led to elongated mitochondria with decreased rates of mitochondrial fission, but with no effect on fusion. Authors also demonstrated that SEPT2 interacted directly with Drp1 and that localisation of Drp1 to mitochondria was dependent upon SEPT2, perhaps taking the role that Fis1 plays in recruitment of Drp1 to the OMM in yeast (Lee *et al.*, 2004). Mutations of *OPA1* (**3.4.6**) and *MFN2* have associated with adult-onset, autosomal dominant mtDNA maintenance disorders (Payne *et al.*, 2004; Rouzier *et al.*, 2012). *De novo* and recessive *DNM1L* mutations have also been reported in early-onset patients with encephalopathy (Waterham *et al.*, 2007), although reduced mtDNA copy number has been noted in fibroblasts from one patient (Nasca *et al.*, 2016). Hence, this underpins the importance of proper regulation of the mitochondrial network. Impaired fusion due to *OPA1* and *MFN2* mutations may prevent the movement or passage of mtDNA replication machinery to damaged mtDNA because of inadequate content mixing, as proposed by Vielhaber *et al.* (2013). Amati-Bonneau *et al.* (2008) proposed that for OPA1 defects, the balance of dGTP pools for mtDNA maintenance could be affected by impaired GTPase activity or the mtDNA nucleoids in the matrix side of the IMM could be destabilised from defective fusion of mitochondria. However, *SEPT2* is involved in fission and hence, the pathological mechanism differs somewhat. Mitochondrial fission has shown to occur in close proximity to the mtDNA nucleoids, of which there are fewer but enlarged and clustered in the absence of Drp1 (Ban-Ishihara *et al.*, 2013). A further study has also confirmed that mtDNA nucleoid dynamics is regulated by mitochondria fission, demonstrated by severe impairment of neonatal cardiac development and nucleoid clustering in muscle-specific Drp1-knockout mice (Ishihara *et al.*, 2015). Based on this knowledge, a SEPT2 defect could impair localisation of Drp1 to mitochondria leading to an elongated network, thus impairing the segregation and distribution of the nucleoids. There could also be failure to remove damaged mitochondria via mitophagy. Hence, this necessitates future studies of patient 19 mitochondrial networks including TMRM and nucleoid staining of fibroblasts once available.

4.5.4.4 Possible Dominant Inheritance of an *MGME1* Missense Mutation

Of all VUS, only the *MGME1* p.Leu236Val missense variant harboured by patient 17 was absent from in-house and external exome databases. Even so, there are currently no dominant, pathogenic heterozygous *MGME1* variants described; all previously reported patients had homozygous variants (**3.4.12**). Furthermore, a rare c.707T>C substitution affecting the adjacent nucleotide had also been described in NHLBI ESP (5/13006 heterozygous alleles, MAF=0.0003844) and ExAC (5/121196 heterozygous alleles, MAF=0.00004126) databases,

leading to a p.Leu236Pro missense change (rs141266045). A proximal homozygous p.Tyr233Cys missense change has also been described in a single patient with a systemic PEO-plus phenotype and multiple mtDNA deletions (Kornblum *et al.*, 2013). Leu236 is moderately conserved and is the second residue of a second key motif of the PD-(D/E)XK nuclease family, (G/*h*)X*h*D, where *h* denotes a hydrophobic residue and X denotes any residue (Kornblum *et al.*, 2013). Although, the Leu236 residue could be any amino acid it is never valine in humans or orthologues. Alternative inheritance of mutations in one gene causing same phenotype has also been well characterised in adult-mtDNA maintenance disorders, including *POLG*, *RRM2B* and *SPG7* defects (**3.4.4**, **3.4.8**, **3.4.14**). Nonetheless, for patient 17 and the affected family members, segregation studies would confirm or exclude this heterozygous *MGME1* mutation as the causative variant.

4.5.4.5 Dominant Heterozygous *ABAT* Missense Mutation

Similarly, there is uncertainty regarding the heterozygous *ABAT* and *RRM2B* missense variants identified. Like *MGME1*, mutations of *ABAT* have only been associated with a recessive disorder of mtDNA maintenance to date (Besse *et al.*, 2015). Furthermore, both the *ABAT* and *RRM2B* variants have been identified in external databases, albeit at low MAFs. The p.Thr161Ile *RRM2B* missense change had also be diagnostically excluded prior to WES. Although the variants affect highly conserved residues, there is unfortunately a lack of evidence to confirm pathogenicity. Like all other identified variants in this patient cohort, it has not been possible to perform segregation studies.

4.5.4.6 *POLRMT* Splice-Acceptor Site Mutation

As described in **Chapter 1**, POLRMT is essential for initiation of mitochondrial transcription with the additional transcription initiation factors TFAM and TFB2M, plus the elongation factor TEFM. Although the identified *POLRMT* splice-site variant is an excellent and currently only candidate for patient 15, it occurs at a low MAF in ExAC. On the other hand, patient 15 presents non-progressive indolent PEO that could be perhaps be undiagnosed in the two heterozygous individuals in ExAC. Initial investigations were initiated using patient 15 fibroblasts to check steady-state levels of POLRMT and MRPL12, which is known to stabilise and coordinate mtDNA transcription with POLRMT (Wang *et al.*, 2007; Surovtseva *et al.*, 2011). Together with analysis of quiescent fibroblast mtDNA copy number and long-range PCR assay to check for large-scale rearrangements, the initial data is inconclusive in determining what effect, if any, that the *POLRMT* mutation has on mtDNA transcription in fibroblasts. This is further complicated by failure to sequence the cDNA to confirm the

splicing effect of c.2641-1C>G change, since the intronic and exonic regions flanking the mutation were GC-rich. Performing *in vitro* studies similar to Kühl *et al.* (2016) who investigated homozygous and heterozygous conditional *Polrmt*-knockout mice could confirm the pathogenic role of *POLRMT* in adult-onset PEO with multiple mtDNA deletions. Further studies that include analysis of TFAM, TFB2M and TEFM expression would provide evidence of changes to mtDNA transcription initiation. Also suggested is northern blot analysis to assess D-loop strand levels (known as 7S DNA) and mtDNA. At the time of writing, patient 15 skeletal muscle became available for research purposes and could provide a more substantial phenotype than fibroblasts.

4.5.4.7 Recessive *TOP3A* Mutations

Biallelic *TOP3A* variants were identified in patient 8, who presented a complex PEO-plus phenotype with the unusual feature of telangiectasia-like skin lesions. *TOP3A* encodes topoisomerase III-α, which is required for the transient cleavage and ligation of single-stranded nuclear and mtDNA during replication (Wu *et al.*, 2010; Chen *et al.*, 2012). This dual-localised topoisomerase is translated by a single mRNA transcript with the nuclear and mitochondrial isoforms distinguished by two initial translation codons (Wang *et al.*, 2002). The role of TOP3A in nuclear and mtDNA replication has been well-characterised, with knock-out in *D. melanogaster* causing mtDNA depletion and reduced ATP synthesis (Wu *et al.*, 2010). The p.Met100Val and p.Arg135* *TOP3A* mutations identified in patient 8 both occurred in a conserved catalytic topoisomerase-primase (Toprim) domain in the N-terminal of the encoded protein, shared with additional topoisomerases (Aravind *et al.*, 1998). However, the Met100 residue was poorly conserved in all tested species and was in fact valine in *D. melanogaster*, which led to classification of both variants as VUS. Interestingly, TOP3A is known to interact with the bloom syndrome helicase (BLM) for chromosome segregation in the nucleus (Chan *et al.*, 2007). *BLM* mutations cause the extremely rare genomic instability disorder of Bloom syndrome that is characterised by erythematous skin lesions (Bloom, 1954). Given the high similarity to the patient 8 skin lesions, the biallelic *TOP3A* variants are currently under investigation at the time of writing to validate whether these are related to the adult-onset PEO-plus phenotype or if the skin lesions as co-incidental. It is worth noting however, that one *TK2* patient had skin sarcoidosis (Tyynismaa *et al.*, 2012) (**3.4.9**).

4.5.5 Prevalence of Digenic Mendelian Disorders

4.5.5.1 Contiguous 1.4Mb 17p12 Deletion Associated with Axonal Neuropathy

Analysis of patient 1 CNVs identified a known pathogenic contiguous 1.4Mb 17p12 deletion associated with hereditary neuropathy and liability to pressure palsy (HNPP) (Li, 2012; van Paassen *et al.*, 2014). This deletion includes all coding exons of *PMP22* encoding peripheral myelin protein 22, which is essential for nerve myelination and cell proliferation (Adlkofer *et al.*, 1995). On the other hand, duplication of the same 1.4Mb 17p12 region including *PMP22* is associated with CMT type 1A (CMT1A). However, patient 1 did not manifest the HNPP phenotype and instead had an axonal neuropathy reminiscent of CMT1A. Although uncommon, patients harbouring the *PMP22* deletion presenting severe axonal neuropathy instead of HNPP have been reported (Chance *et al.*, 1993; Bergamin *et al.*, 2014). CMT disease due to *PMP22* deletion or duplication is one of the most common neurological disorders in North East England with an estimated minimum prevalence of 11.8 per 100,000 (Foley *et al.*, 2012). Given that neuropathy is a common feature of adult-onset Mendelian mtDNA maintenance disorders (**Chapter 3**), digenic disorders of mitochondrial PEO and CMT could be under recognised in this cohort of patients. However, this requires the further genetic studies of patients with PEO and neuropathy to ascertain any prevalence.

4.5.5.2 Isolated Cystinuria Type A Due to Recessive *SLC3A1* Mutations

The presence of cystinuria together with PEO and multiple mtDNA deletions in patient 19 was an unusual finding that suggested the presence of a second Mendelian disorder. Since patient 19 had received penicillamine to treat the cystinuria, it was presumed that PEO and fatigue was penicillamine-induced myasthenia gravis (Drosos *et al.*, 1993). However, this was not the case following withdrawal of penicillamine, leading to the diagnosis of mitochondrial myopathy. WES of analysis *SLC3A1* and *SLC7A9* identified the two most common pathogenic *SLC3A1* variants associated with isolated cystinuria. As of August 2016, over 150 different *SLC3A1* variants have been associated with isolated cystinuria type A (SLC3A1, 2016). To date, the p.Met467Thr missense change and exon 5-9 duplication are the most common pathogenic *SLC3A1* variants associated with isolated cystinuria type A in the United Kingdom and other Western European countries (Rhodes *et al.*, 2015; Wong *et al.*, 2015), but are not as common in South East Europe (Popovska-Jankovic *et al.*, 2013).

4.5.5.3 *MYH14* p.Gly384Cys Variant is Not Associated with Non-Syndromic Hearing Loss

In the patient 6, a heterozygous p.Gly384Cys *MYH14* missense change previously reported as pathogenic, causing autosomal dominant non-syndromic hearing loss (DFNA4, OMIM #600652). The variant had been reported as pathogenic, occurring *de novo* in a 9 year old Italian girl and was annotated as pathogenic due to the evolutionary conservation of the Gly384 residue, absence from tested controls and since the girl was the only affected family member (Donaudy *et al.*, 2004). This variant could serve to explain the history of hearing loss affecting the family of patient 6. However, thanks to NGS and the sharing of variant frequency data over the past decade it has been revealed that a number of annotated 'rare' and 'pathogenic' variants associated with various Mendelian disorders actually occur at MAFs that suggest that these are not rare within the general population. A significant re-assessment of previously reported pathogenic variants can be attributed to the ExAC database (Lek *et al.*, 2016). Re-assessment of 'pathogenic' HCM variants in 7,855 cases found that some variants were occurring at MAFs too common to cause penetrative Mendelian disease (Walsh *et al.*, 2016). Assessing the *MYH14* p.Gly384Cys missense change, it also occurs more frequently than would be expected for a disease-causing mutation. In ExAC, it occurs in 343/119994 heterozygous alleles (MAF=0.002858, 0.028%) and when further sub-categorised by ethnicity, the variant occurs in 275/66322 non-Finnish European alleles (MAF=0.004146, 0.041%). Similarly, the variant was identified in 36/12712 alleles (MAF=0.0023832, 0.024%) from NHLBI ESP and in 12/4996 alleles (MAF=0.002, 0.02%) in the 1000 Genomes Project databases. Hence, it the opinion of this study that the *MYH14* p.Gly384Cys mutation is not associated with hearing loss. Thus, this serves to highlight the importance of variant classification and the need to functionally validate candidate variants.

Although excluded from WES analysis for adult-onset PEO with multiple mtDNA deletions, a second heterozygous *MYH14* missense change (p.Arg1729Trp) was also identified in patient 13 through analysis of known autosomal dominant and recessive hearing loss genes. Therefore it was concluded that the *MYH14* change identified in patient 13 was the most likely aetiology for his SNHL since it occurred at a low MAF and affected a conserved residue.

4.5.6 Concluding Remarks

To summarise, WES of 19 patients presenting with adult-onset PEO and multiple mtDNA deletions attained a diagnostic yield of 35%, which comprised causative (10.5%). Based upon

the expected predominance of autosomal dominant mutations (**Chapter 3**), almost all proposed likely causative and VUS were heterozygous. Nonetheless, it is important to acknowledge that segregation and further functional studies are necessary to validate the pathogenicity of identified variants. The identification of a heterozygous somatic mosaic *TWNK* missense change is also suggestive of an under recognised phenomenon of mosaicism in autosomal dominant Mendelian mitochondrial disease, that is detectable by WES even at low mutational levels. Owing to this, careful curation and annotation of electropherograms generated from diagnostic targeted gene sequencing will be needed. This study has also identified the only known patient harbouring a dominant *RRM1* mutation to date, with further contributions to this emerging story anticipated. Irrespective of the custom filtering strategy that has been devised and employed, the interactome of mitochondrial function and mtDNA maintenance is not yet complete as highlighted by the identification of a novel *SEPT2* mutation. Secondary, actionable mutations causing other Mendelian disorders are also not prioritised by the filtering strategy devised here and are instead dependent upon thorough clinical phenotyping that can direct this additional variant prioritisation. Segregation studies will also greatly improve confidence in likely causative variants and VUS identified, although it is accepted that this is difficult when studying a late-onset disorder.

Chapter 5. *GMPR1* Mutation is Associated with Late-Onset PEO and Multiple mtDNA Deletions

5.1 Introduction

This chapter contains material that includes clinical, diagnostic and research support provided from colleagues and external collaborators, who are appropriately acknowledged.

5.1.1 Deoxyribonucleotide Triphosphate (dNTP) Pools for Nuclear and mtDNA Replication

The replication and maintenance of nuclear and mtDNA is dependent upon a balanced supply of all four deoxyribonucleotide triphosphates (dNTPs), the building blocks for DNA synthesis, which is intricately controlled through synthesis and degradation (Rampazzo *et al.*, 2010). An imbalance of dNTP pools, or increased or decreased precursors have been associated with human disease. Notably, genomic instability is a hallmark of cancer, which can arise due to imbalanced dNTPs leading to replication errors (Mathews, 2015). Oncogenesis has been associated with increased *de novo* synthesis of dNTPs via the ribonucleotide reductase (RNR), which is demonstrated to reduce the fidelity of DNA repair and increase mutation rates (Aye *et al.*, 2015).

The supply of dNTPs in mammalians for both nuclear and mtDNA replication is tightly coordinated by the cell cycle, involving cytosolic and mitochondrial pathways (Pica-Mattoccia and Attardi, 1972; Saada, 2009). In dividing (proliferating) cells (**Figure 5.1A**), dNTPs are predominantly generated in the cytosol by *de novo* synthesis from phosporibosyl pyrophosphate (pRpp) through ribonucleotide diphosphate (rNDP) reduction by the RNR, comprising the large R1 subunit and small R2 subunit (Pär and Peter, 2006). The active RNR is a tetramer composed of two R1 and two R2 (or p53R2) subunits, which are encoded by *RRM1*, *RRM2* and *RRM2B*. A small proportion of dNTPs are also generated by recycling through the deoxyribonucleotide (NdR) salvage pathway, which comprises three phosphorylation steps and four deoxynucleoside kinases (TK1, dCk, TK2, dGk). First, thymidine kinase 1 (TK1) phosphorylates thymidine (dT) and deoxyuridine (dU), whereas deoxycytidine kinase (dCk) phosphorylates deoxycytidine (dC), deoxyadenosine (dA) and deoxyguanosine (dG). Next, deoxyribonucleoside monophosphate kinases (NMPKs) phosphorylate deoxynucleotide monophosphates (dNMPs) to deoxynucleotide diphosphates

(dNDPs) and finally, deoxyribonucleoside diphosphate kinases (NDPKs) phosphorylate dNDPs to dNTPs. Supply of dNTPs for mitochondrial replication are imported from cytosolic pools or are to a lesser extent derived from the mitochondrial NdR salvage pathway with thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGk/DGUOK) performing the first phosphorylation step within mitochondria. TK2 phosphorylates dT, dC and dU, while dGk phosphorylates dA and dG.

In non-dividing (quiescent) cells (**Figure 5.1B**), nuclear DNA replication is halted. Hence, there is a diminished requirement for dNTPs and therefore synthesis of dNTPs and precursors in the cytosol is reduced. This is because the cytosolic enzymes TK1 and to an extent, the RNR, are S-phase specific, so are not expressed at G_0. Nonetheless, mtDNA replication continues and still relies upon a pool of dNTPs. In this quiescent state, mitochondrial dNTP synthesis becomes dependent on the rate-limiting enzymes TK2 and dGK (Rampazzo *et al.*, 2007; Eriksson and Wang, 2008), but also on limited *de novo* synthesis through the RNR that instead comprises R1 and the p53R2 small subunit encoded by *RRM2B*, instead of the R2 subunit (Tanaka *et al.*, 2000; Hakansson *et al.*, 2006; Pontarin *et al.*, 2007; Pontarin *et al.*, 2012). To this end, mitochondria are forced to rely upon its mitochondrial salvage pathway for mtDNA replication. There are also catabolic enzymes in the cytosol (NT-1) and mitochondria (NT-2) for the degradation of nucleotides, plus thymidine phosphorylase (TYMP) and purine nucleoside phosphorylase (PNP) for degrading nucleotides. Pools are also maintained by sterile alpha motif (SAM) and histidine-aspartic (HD) domain containing dNTP triphosphohydrolase 1 (SAMHD1), which has triphosphatase activity for degradation of dNTPs (Goldstone *et al.*, 2011; Beloglazova *et al.*, 2013; Franzolin *et al.*, 2013). The activity of SAMHD1 was also recently shown to contribute to the pathology of dGK-deficiency, with SAMHD1-knockout in patient fibroblasts restoring dNTP pools and mtDNA copy number (Franzolin *et al.*, 2015). Although nucleotide synthesis underpins both nuclear and mtDNA replication, the RNA content of cells is much higher than DNA and relies on the reduction of rNDPs to dNDPs by the RNR for DNA replication. Hence, the majority of nucleotides generated are rNTPs (Lane and Fan, 2015).

In vivo studies have taken advantage of the proliferating and quiescent states of patient cells to investigate the underlying pathological mechanisms of mtDNA maintenance disorders associated with defects of dNTP supply, including patients with mutations of *ABAT*, *MPV17*, *TK2* and *RRM2B* (Bianchi *et al.*, 2011; Villarroya *et al.*, 2011; Besse *et al.*, 2015; Dalla Rosa *et al.*, 2016). This has typically been achieved by lowering the serum content of culture medium from 10% FBS to approximately 0.1%. In some but not all patient cells, mtDNA

depletion occurs since non-dividing cells are reliant upon the mitochondrial salvage pathway. Owing to the underlying genetic defect of the enzymes involved in dNTP supply, this slows or stalls mtDNA replication.

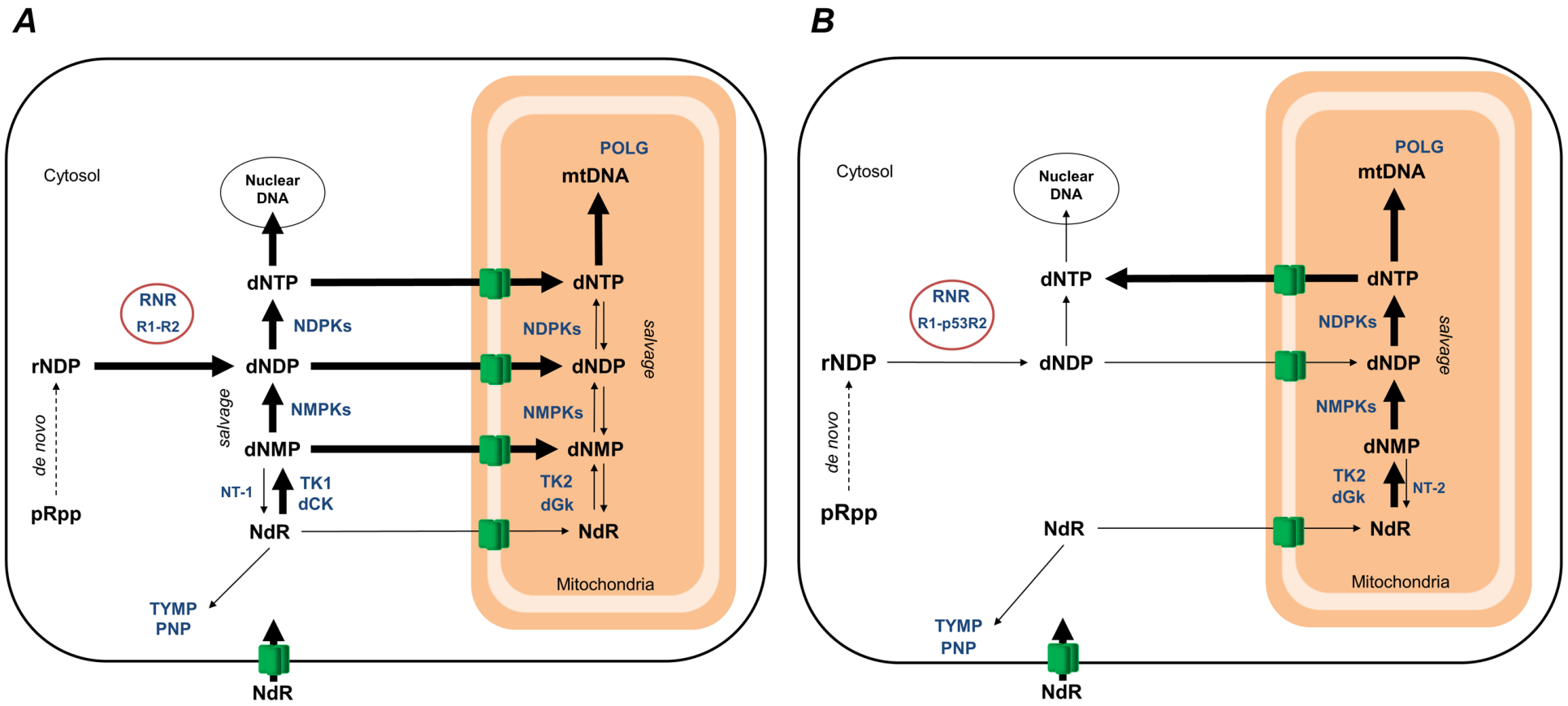


Figure 5.1 Maintenance of Cellular dNTP Supply in Dividing and Non-Dividing Cells. Cytosolic and mitochondrial dNTP synthesis in (*A*) dividing and (*B*) non-dividing cells. In dividing cells, mitochondrial dNTP pools are primarily dependent on import of dNTPs synthesised by the cytosolic salvage pathway. In non-dividing cells, cytosolic dNTP synthesis is halted and therefore mitochondrial dNTP synthesis relies on the mitochondrial salvage pathway. Figure based on Saada (2009).

5.1.2 The Interplay Between dNTP Pool Balance and mtDNA Maintenance Disorders

Both early- and late-onset mtDNA maintenance disorders have been associated with increased or decreased dNTP precursors, or a disturbance in the balance of dNTP pools for mtDNA replication (**Table 5.1**). The first gene to be implicated was *TYMP* encoding the catabolic enzyme thymidine phosphorylase, causing MNGIE with mtDNA depletion and multiple mtDNA deletions (**3.4.2**) due to increased deoxythymidine triphosphate (dTTP) levels (Nishino *et al.*, 1999b; Nishigaki *et al.*, 2003). Autosomal recessive mutations of *TK2* (**3.4.9**), *DGUOK* (**3.4.10**) and *MPV17* (**3.4.11**), dominant and recessive mutations of *RRM2B* (**3.4.8**) and potentially *RRM1* (**4.4.7**) have also been previously associated with adult-onset PEO with multiple mtDNA deletions. However, dNTP pool imbalances or alterations in precursor synthesis have been primarily associated with autosomal recessive, early-onset mtDNA depletion syndrome due to mutations of *ABAT* (Besse *et al.*, 2015), *DGUOK* (Mandel *et al.*, 2001), *MPV17* (Spinazzola *et al.*, 2006), *RRM2B* (Bourdon *et al.*, 2007), *SUCLA2* (Elpeleg *et al.*, 2005), *SUCLG1* (Ostergaard *et al.*, 2007) and *TK2* (Saada *et al.*, 2001). *SLC25A4* mutations have also been proposed to alter dNTP pool balance (Kaukonen *et al.*, 2000; Thompson *et al.*, 2016). Similar to additional forms mitochondrial disease, early-onset mtDNA depletion syndromes manifest in vast clinical and genetic heterogeneity although some patterns have emerged. *DGUOK* and *MPV17* mutations manifest in severe infantile liver failure, neurological features and liver-specific mtDNA depletion (Mandel *et al.*, 2001; Spinazzola *et al.*, 2006). Interestingly, early-onset hepatocerebal mtDNA syndromes have also been described due to recessive mutations of other genes encoding mtDNA replication machinery including *POLG* (Naviaux *et al.*, 1999), *POLG2* (Varma *et al.*, 2016) and *TFAM* (Stiles *et al.*, 2016), suggesting that faithful mtDNA replication in the liver is critical during early human development. Mutations of *SUCLA2*, *SUCLG1* and *ABAT* are unusual, since their involvement in maintaining dNTP pools is secondary to their primary functions. *SUCLA2* and *SUCLG1* encoded the α- and β-subunits of succinyl CoA synthetase that catalyses the conversion of succinate to succinyl-CoA in the TCA cycle. Yet, succinyl CoA synthetase has been linked to activity of the mitochondrial nucleoside diphosphate kinase (NDPK) known as NME4 and hence, dNTP balance for mtDNA replication (Miller *et al.*, 2011). Similarly, the GABA-transaminase encoded by *ABAT* was recently shown to interact with SUCLA2, SUCLG1, SUCLG2 and NME4 in the mitochondrial matrix, confirming a secondary role in mtDNA maintenance (Besse *et al.*, 2015).

Gene	Onset	Genotype	Phenotype	Reference(s)
ABAT	Infantile	AR	Encephalopathy, seizures, DD,	Besse *et al.* (2015)
DGUOK	Infantile	AR	Hepatocerebral Syndrome	Mandel *et al.* (2001)
	Adulthood	AR	PEO, myopathy	Ronchi *et al.* (2012a)
MPV17	Infantile	AR	Hepatocerebral Syndrome	Spinazzola *et al.* (2006)
	Adulthood	AR	PEO, myopathy, neuropathy, Parkinsonism, enlarged liver	Garone *et al.* (2012)
RRM1[a]	Adulthood	AD	PEO, OPMD-like	n.a.
	Childhood	AR	PEO, MNGIE-like, axonal neuropathy	Juanola-Falgarona *et al.* (2016)
RRM2B	Adulthood	AD and AR	PEO and PEO-plus	Tyynismaa *et al.* (2009a)
	Infantile	AR	Encephalomyopathic, proximal renal tubulopathy	Bourdon *et al.* (2007)
SUCLA2	Infantile	AR	Encephalomyopathic, DD, hearing loss	Elpeleg *et al.* (2005)
SUCLG1	Infantile	AR	Encephalomyopathic, lactic acidosis, DD	Ostergaard *et al.* (2007)
TK2	Infantile	AR	Rapidly progressive myopathy, respiratory insufficiency	Saada *et al.* (2001), Garone *et al.* (Submitted)
	Childhood	AR	Progressive myopathy, SMA-like, respiratory insufficiency	Garone *et al.* (Submitted)
	Adulthood	AR	Late-onset progressive myopathy, PEO, respiratory insufficiency	Tyynismaa *et al.* (2012), Garone *et al.* (Submitted)
TYMP	Childhood	AR	MNGIE	Nishino *et al.* (1999b)

Table 5.1 Nuclear Genes Associated with mtDNA Maintenance Disorders of dNTP Pool Balance. DD – developmental delay; MNGIE – mitochondrial neurogastrointestinal encephalopathy; OPMD – oculopharyngeal muscular dystrophy; SMA – spinal muscular atrophy. n.a. – not applicable. [a]*RRM1* mutations were putatively identified in **4.4.7**.

Several of the essential nucleotide metabolism enzymes with defects associated with mtDNA maintenance disorders (*RRM2B*, *TYMP*, *RRM1*) are localised only to the cytosol. This supports the assumption that there is a mixing of cytosolic and mitochondrial dNTP pools. Indeed, transporters traversing the IMM for the movement of nucleotides have been identified including pyrimidine nucleotide carriers 1 and 2 (PNC1, PNC2) that transport uracil, thymine,

cytosine deoxyribonucleotide mono-, di- and triphosphates (Franzolin *et al.*, 2012; Di Noia *et al.*, 2014). Equilibrative nucleotide transporter 1 (ENT1) is localised to the IMM and the plasma membrane for transporting both pyrimidine and purine dNTPs and precursor molecules to mitochondria (Lai *et al.*, 2004; Lee *et al.*, 2006a). Analogous to several nucleotide metabolism enzymes, expression of the transporters is coupled to the cell cycle. PNC1 and PNC2 are expressed in dividing cells but PNC1 is downregulated in non-dividing cells, suggesting an essential role in the supply of dNTPs for mtDNA replication from the cytosolic pool (Franzolin *et al.*, 2012). In contrast, ENT1 is upregulated in non-dividing cells.

Since there is mixing of the cytosolic and mitochondrial dNTP pools, a disturbance of either pool, or an increase or decrease in nucleotide precursors due to defects of a single nucleotide metabolism enzyme could in theory lead to a disorder of mtDNA maintenance. Given the significant number of pathways, enzymes and transcription factors needed for fine control of dNTP synthesis (Lane and Fan, 2015), it is conceivable that there are unrecognised, novel mtDNA maintenance disorders yet to be identified from within this area.

5.1.3 *De Novo* Guanosine Nucleotide Synthesis and Guanosine Monophosphate Reductase (GMPR)

The nucleotide bases essential for DNA replication and RNA biosynthesis are distinguished into two nitrogenous groups; the purines and the pyrimidines. Purines comprise the deoxyribonucleosides dA and dG, plus the ribonucleosides adenosine and guanosine. On the other hand, the pyrimidines comprise the deoxyribonucleosides dC and dT, plus the ribonucleosides cytidine and uridine. Both purines and pyrimidines can be synthesised by two independent pathways; either *de novo* or salvaged.

Purine nucleotides are synthesised in the cytosol using the pRpp as the starting nucleobase to generate the first nucleotide, inosine monophosphate (IMP), in a series of 10 intermediate enzymatic reactions requiring five ATP molecules, H_2O, CO_2, glucose, glycine, glutamine, aspartate, and a carbon unit N^{10}-formyl-TetraHydroFolate (THF) (Lane and Fan, 2015). Using IMP as a nucleotide precursor, the major purine nucleotides adenosine monophosphate (AMP) and guanosine monophosphate (GMP) can be synthesised *de novo* via two distinct pathways.

Synthesis of GMP from IMP is performed by two enzymes in the cytosol; inosine monophosphate dehydrogenase (IMPDH) and GMP synthetase (GMPS) in reversible two-step rate-limiting reactions (**Figure 5.2**). First, IMP undergoes NAD-dependent oxidation to

the intermediate nucleotide xanthosine monophosphate (XMP) by one of two catalytically indistinguishable IMPDH homologs encoded by two separate genes, *IMPDH1* and *IMPDH2*. Both genes are expressed throughout most human tissues, although *IMPDH1* appears to be less expressed and *IMPDH2* is upregulated in dividing cells (Senda and Natsumeda, 1994; Zimmermann *et al.*, 1998). The IMPDH reaction is summarised in the following equation:

$$\text{IMP} + \text{NAD}^+ + \text{H}_2\text{O} \longleftrightarrow \text{XMP} + \text{NADH} + \text{H}^+$$

Second, XMP undergoes ATP-dependent amination to GMP by GMPS (Nakamura and Lou, 1995; Tesmer *et al.*, 1996). The GMPS reaction is summarised in the following equation:

$$\text{XMP} + \text{ATP} + \text{L-Glutamine} + \text{H}_2\text{O} \longleftrightarrow \text{GMP} + \text{P}_i + \text{AMP} + \text{L-Glutamate}$$

GMP synthesised from IMP is next free to undergo conversion to guanosine diphosphate (GDP) from the addition of a phosphate group donated from ATP by guanylate kinase 1 (GUK1) (Brady *et al.*, 1996), followed by reduction to deoxyguanosine diphosphate (dGDP) via the RNR.

Although these *de novo* guanine synthesis reactions are reversible, there is an additional irreversible reaction that converts GMP back to IMP with an ammonium (NH_4^+) leaving group, thus rapidly sustaining the balance of guanine and adenine nucleotides. This NADPH-dependent amination reaction is performed by GMP reductase (GMPR) (Spector *et al.*, 1979). Currently, this is the only known reaction for the catabolism of guanine nucleotides to the IMP precursor. The GMPR reaction is summarised in the following equation:

$$\text{GMP} + \text{NADPH} + 2\text{H}^+ \longrightarrow \text{IMP} + \text{NADP}^+ + \text{NH}_4^+$$

However, Patton *et al.* (2011) also demonstrated that in the presence of sufficient ammonia, *E. coli* GMPR could directly synthesise GMP from IMP, therefore suggesting an expanded role in GMP synthesis.

Similar to IMPDH, there are two GMPR homologs encoded by separate genes humans, *GMPR1* and *GMPR2*. The two human homologs are 90% identical and exist as homotetramers that form catalytic (β/α) barrels in a square planar formation (Li *et al.*, 2006). The intron structure varies between the two genes with the *GMPR2* gene, with *GMPR2* encoding three more amino acids in the 10th exon than *GMPR1* in the 9th exon. Both GMPR1 and GMPR2 are highly expressed in the heart, skeletal muscle and kidney with low expression in the colon, thymus and blood leukocytes, whereas GMPR2 is expressed at much

higher levels in the brain, liver and placenta (Deng *et al.*, 2002). GMPR is highly conserved from bacteria to humans (Andrews and Guest, 1988) and was recently characterised in parasites (Bessho *et al.*, 2016; Smith *et al.*, 2016). The GMPR and IMPDH enzymes have opposing catalytic activities in the *de novo* guanine synthesis, suggesting that their expression is coordinated together. Indeed, during differentiation IMPDH is downregulated and GMPR is upregulated, leading to a decrease in the GMP pool (Weber *et al.*, 1992). Henceforth, IMPDH increases the guanine nucleotide pool whereas GMPR decreases the pool. Interestingly, due to their coupled expression and similar enzymatic reactions both GMPR and IMPDH share conserved active sites, a conserved GIGPGSICTT sequence motif, binding the same ligands although with different affinities (Hedstrom, 2012). The catalytic residue is conserved, corresponding to Cys186 in GMPR and Cys319 in IMPDH with this motif (Li *et al.*, 2006). A second important catalytic residue in this motif is a threonine, corresponding to Thr188 in GMPR and Thr321 in IMPDH (Hedstrom, 2012).

While the *de novo* guanine synthesis pathway is crucial to sustain the balance between guanine and adenosine nucleotides for nuclear and mtDNA replication, a direct association of GMPR or IMPDH to mitochondria has not been established. Interestingly, transcriptome profiling of skeletal muscle in patients with early-onset mtDNA depletion syndrome due to *TK2* mutations noted that *GMPR1* was significantly underexpressed, supporting its role in the balance of the guanine nucleotide pool (Kalko *et al.*, 2014).

In **Chapter 4**, a novel heterozygous p.Gly183Arg *GMPR1* missense change was identified using WES with a custom filtering strategy in patient 11, who presented with late-onset PEO with multiple mtDNA deletions. In this chapter, the novel *GMPR1* mutation is characterised to understand the potential pathological mechanism linking a disturbance of nucleotide synthesis in the cytosol to a novel mtDNA maintenance disorder.

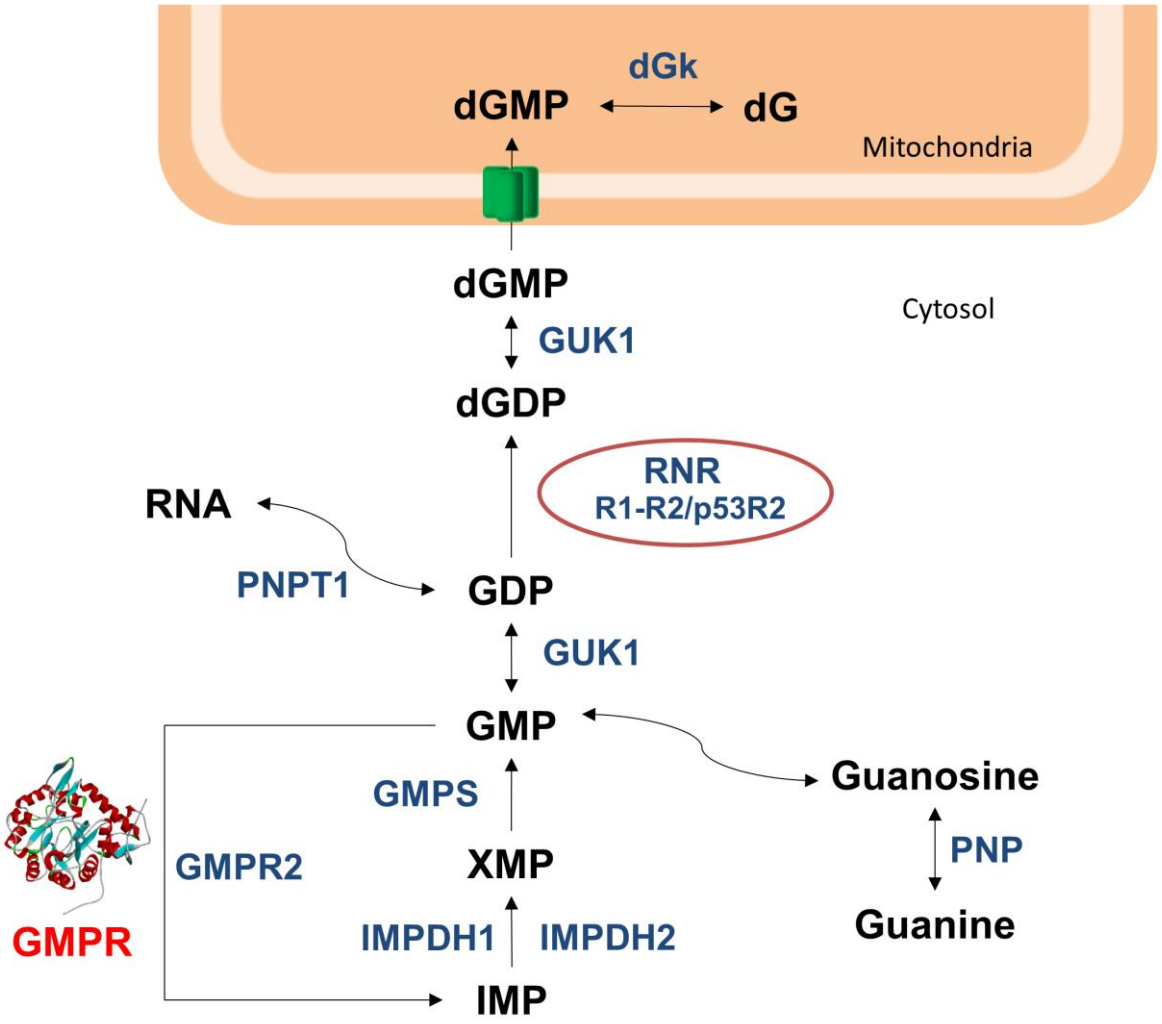


Figure 5.2 Human GMP Biosynthesis from IMP. A simplified schematic of the GMP biosynthesis pathway, beginning with the precursor IMP. GMPR is denoted in red with the resolved secondary structure (PDB ID 2BLE).

5.2 Aims

This chapter characterises the pathogenic nature of a novel heterozygous *GMPR1* missense variant in a patient presenting late-onset PEO and multiple mtDNA deletions.

5.3 Methods

5.3.1 GMPR1 Protein Structure Analysis

The full length 345 amino acid sequence of human homologs GMPR1 (NP_006868), GMPR2 (NP_001002002), paralogs IMPDH1 (NP_000874) and IMPDH2 (NP_000875), plus the secondary structure of human GMPR1 (PDB ID 2BLE) were subjected to bioinformatics analyses as outlined in **2.7**. Putative mitochondrial localisation of human GMPR1 was predicted using MitoMiner 4.0 (Smith and Robinson, 2016).

5.3.2 Cell Culture

Cultured fibroblasts were grown for patients 11 (*GMPR1*), 8 (*TOP3A*) and 15 (*POLRMT*) plus three appropriate age-matched controls. Subculturing, freezing, harvesting and generation of quiescent fibroblasts were performed as described in **2.4**.

5.3.3 Ethidium Bromide mtDNA Depletion and Recovery

Fibroblasts from patients 11, 8 and 15 plus two controls were seeded into eight T75 flasks each. Once confluent (day 0), cells were grown in MEM (10% FCS) containing 50ng ml^{-1} ethidium bromide (stock 10mg ml^{-1}) for 7 days. This was then replaced with 'quiescent' MEM (0.1% DBS) containing 50ng ml^{-1} ethidium bromide for an additional 7 days. On day 14, MEM was aspirated and replaced with fresh quiescent MEM without ethidium bromide. Quiescent MEM was immediately aspirated and replaced again. This was repeated four times for each cell line, then after 2 hours and a further 2 hours later. A single T75 flask for each cell line was harvested at days 0, 7, 14, 15, 21 and 28.

Fibroblast DNA was extracted and relative mtDNA copy number was quantified using real time PCR assay, as described in **2.3.10**.

5.3.4 Long-Range PCR of Quiescent Fibroblast DNA

DNA was extracted from quiescent fibroblasts of patient 11 and two controls. Long-range PCR of quiescent fibroblast DNA plus 1:10 control blood DNA homogenate was performed as outlined in **2.3.9**.

5.3.5 Live Cell Imaging and Confocal Microscopy

Patient 11 and two control fibroblast cell lines were prepared, imaged with fluorescent dyes TMRM for the mitochondrial network and PicoGreen for dsDNA using the Nikon A1R Invert point scanning confocal microscope and analysed as outlined in **2.6**.

5.3.6 Western Blotting

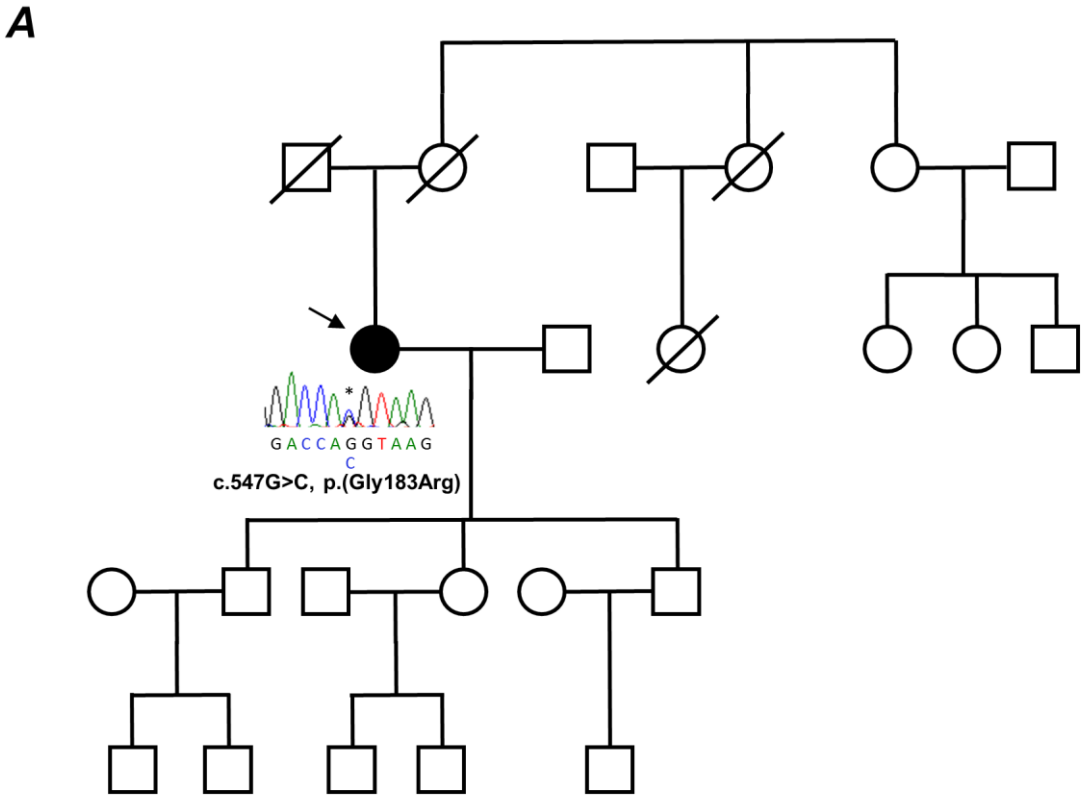
Fibroblast lysates were prepared as described in **2.5.1**. Frozen skeletal muscle from patient 11 and two age-matched controls were homogenised for western blot studies as described in **2.5.2**. Fibroblast (50µg) and muscle lysates (25-50µg) were prepared and subjected to 10% or 12% SDS-page, probed with primary and secondary antibodies (**Table 2.4**, **Table 2.5**) then detected as outlined in **2.5**. Membranes were incubated with primary antibodies specific to GMPR1, LONP1, TFAM, AK2, AK3, TK1, TK2, PNC1, PNC2, ENT1, RRM1, RRM2, p53R2/RRM2B. Primary antibodies used for OXPHOS subunits were NDUFB8, SDHA, UQCRC2, MT-COII and ATP5A. α-Tubulin, VCL and GAPDH were used as loading controls.

5.4 Results

5.4.1 Case Report

Patient 11 was a 73 year old female presenting indolent, late-onset PEO. She underwent corrective squint surgery in 2004 at 60 years old, but complained of double vision post-surgery. At 69 years old mild bilateral ptosis was noted. Upon clinical examination at 70 years old, she had marked PEO with subtle asymmetry, mild bilateral ptosis that affected predominantly the right eye, double vision, right exotropia and mild orbicularis oculi weakness. No abnormalities were detected on brain MRI scan, but there was no comment on the extraocular muscles. There was no reported family history of an eye movement disorder; she was an only child and had three asymptomatic adult children. Muscle histopathology revealed over 15% COX-deficient and 3% ragged-red fibres. Multiple mtDNA deletions were detected in skeletal muscle by long-range PCR.

Application of the WES filtering strategy prioritised a heterozygous c.547G>C (p.Gly183Arg) missense variant in *GMPR1* encoding guanosine monophosphate reductase 1 (**4.4.9**). *GMPR1* (6p22.3) matched GO-Terms 'nucleotide' and 'purine'. The variant was confirmed by Sanger sequencing with custom forward and reverse primers for *GMPR1* exon 5 (**Figure 5.3A**). Segregation studies were not possible. Gly183 was fully conserved from humans to *E. coli* (**Figure 5.3B**). The variant was also absent from in-house and external exome databases. The c.547G>C change affected the last nucleotide of exon 5, which corresponded to the first nucleotide of the Gly183 residue. The Human Splicing Finder predicted that the c.547G>C change could lead to either a broken donor or exonic splicing enhancer site, with potential implications for mRNA stability.



B

c.547G>C, p.(Gly183Arg)

*

Patient 11	LILSGADIIKVGVGP**R**SVCTTRTKTGVGYPQ
Homo sapiens	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Pan troglodytes	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Bos taurus	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Canis familiaris	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Mus musculus	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Rattus norvegicus	LILSGADIIKVGVGP**G**SVCTTRTKTGVGYPQ
Gallus gallus	LILSGADIIKVGIGP**G**SVCTTRIKTGVGYPQ
Xenopus tropicalis	LILSGADIIKVGIGP**G**SVCTTRIKTGVGYPQ
Danio rerio	LILSGADIIKVGIGP**G**SVCTTRIKTGVGYPQ
Escherichia coli	LILSGADIVKVGIGP**G**SVCTTRVKTGVGYPQ

Figure 5.3 Identification of a Novel p.Gly183Arg *GMPR1* Mutation. (*A*) Pedigree for patient 11 (solid black arrow) and Sanger sequencing confirmation of the heterozygous p.Gly183Arg *GMPR1* variant. (*B*) Multiple sequence alignment (MSA) of the GMPR1 Gly183 residue.

5.4.2 Quadruple Immunofluorescence Assay

In addition to COX-SDH histochemistry demonstrating COX-deficient fibres (**Figure 5.4A**), patient 11 skeletal muscle was subjected to a quadruple immunofluorescence assay by Mariana Rocha (Wellcome Trust Centre for Mitochondrial Research, Institute of

Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne). The protein expression profile for patient 11 was in agreement with previously investigated patients with multiple mtDNA deletions (Rocha *et al.*, 2015); there were a greater proportion of fibres with deficiency of complex I and a population of fibres with equal downregulation of both complex I and IV (**Figure 5.4B**).

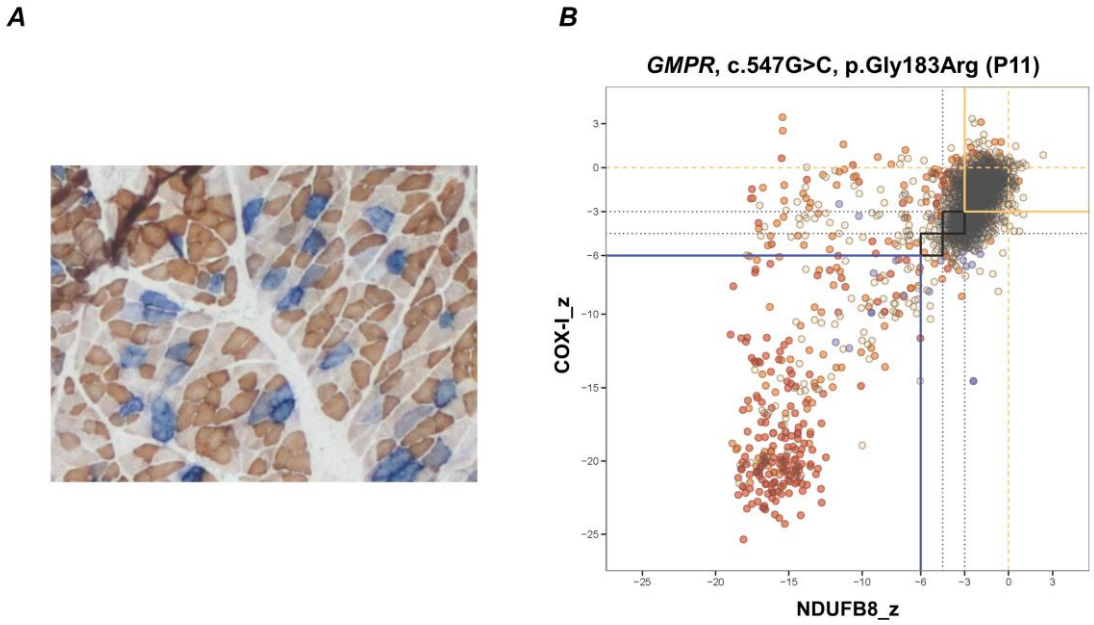


Figure 5.4 Patient 11 COX-SDH Histochemistry and Mitochondrial Respiratory Chain Protein Expression Profile. (*A*) Diagnostic sequential COX-SDH histochemistry of patient 11 skeletal muscle. (*B*) Quadruple immunofluorescence result for patient 11. Each dot represents Z-scores for complex I and complex IV protein expression for an individual muscle fibre, colour coded according to mitochondrial mass (very low: blue, low: light blue, normal: light orange, high: orange and very high: red). Thick blue lines indicate the boundaries the standard deviation limitations for classification of the fibres. Thin dashed lines indicate the mean expression levels of normal fibres.

5.4.3 *In Silico* Analysis of Human GMPR1

The secondary protein structure of human GMPR1 (hGMPR1) (PDB ID 2BLE) (**Figure 5.5A**) and the amino acid sequence (NP_006868) were obtained for *in silico* studies. InterProScan 5 identified an IMP dehydrogenase/GMP reductase signature from residues 176-188 of the hGMPR1 protein sequence. Hence, the identified p.Gly183Arg mutation was located within this region of the protein (**Figure 5.5B**), which was fully conserved between its paralog hGMPR2. MSA also showed that it was conserved between the two additional members of the IMP/GMP protein family, IMPDH1 and IMPDH2 (**Figure 5.5C**).

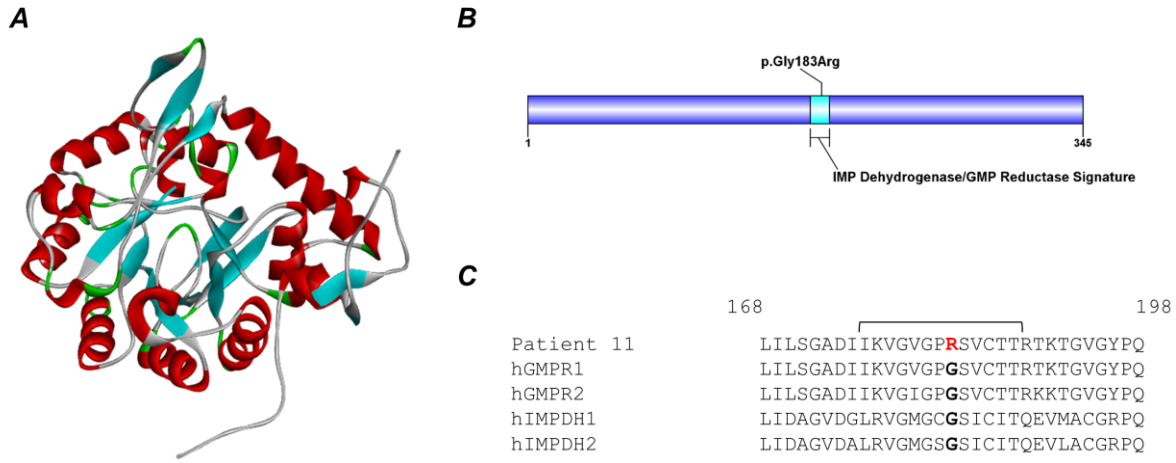


Figure 5.5 Human GMPR1 Structure. (*A*) The secondary structure of human GMPR1 monomer (PDB ID 2BLE). (*B*) The IMP dehydrogenase/GMP reductase domain within GMPR1 and the location of the p.Gly183Arg missense change. (*C*) MSA of the patient 11 GMPR1, wild-type GMPR1, GMPR2, IMPDH1 and IMPDH2 demonstrating conservation of the Gly183 residue. Parentheses indicate the conserved IMP dehydrogenase/GMP reductase domain.

Analysis of the secondary GMPR1 monomer structure showed that the Gly183 residue was an α-helix coil within IMP dehydrogenase/GMP reductase signature, which formed a loop structure within the core. Consistent with Li *et al.* (2006), an oxygen atom of the Gly183 residue was hydrogen bonded to nitrogen atom of Cys186, but at a distance of 3.10Å (**Figure 5.6A**). Nonetheless, the 'loop' structure interacted with the bound GMP and is therefore the likely active site. . Gly183 formed one of two carbon hydrogen bonds, with the fourth oxygen atom of bound GMP, at a distance of 3.66Å (**Figure 5.6B**).

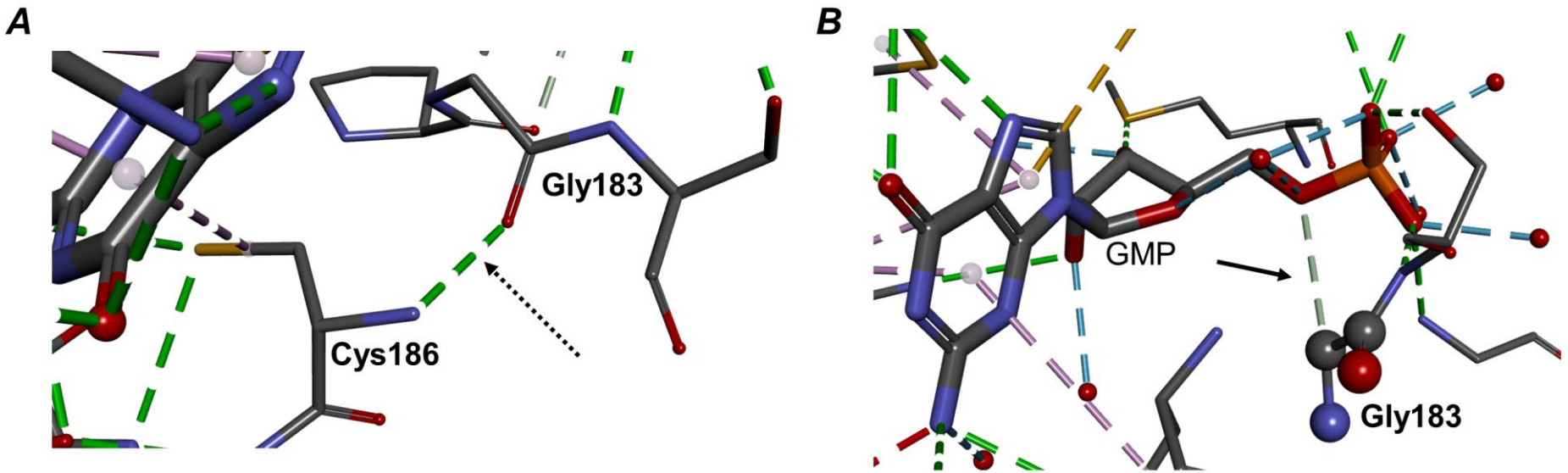


Figure 5.6 Intramolecular Interactions of hGMPR1 Gly183. hGMPR1 forms two intramolecular interactions. (*A*) A hydrogen bond (dashed green line) between an oxygen atom of Gly183 and a nitrogen atom of Cys186. (*B*) A carbon hydrogen bond between a carbon atom of Gly183 and oxygen atom of bound GMP in the hGMPR1 active site loop. The solid arrow denotes the carbon hydrogen bond.

GMP (**Figure 5.7A**) also formed a number of additional significant intramolecular interactions when bound to GMPR1 (**Figure 5.7B**). This included pi-alkyl interactions, hydrogen bonds, carbon hydrogen bonds and pi-sulphur interactions. The second carbon hydrogen bond of the fourth oxygen atom of GMP involved Asn220 at a distance of 3.16Å, also with the fourth oxygen atom of GMP.

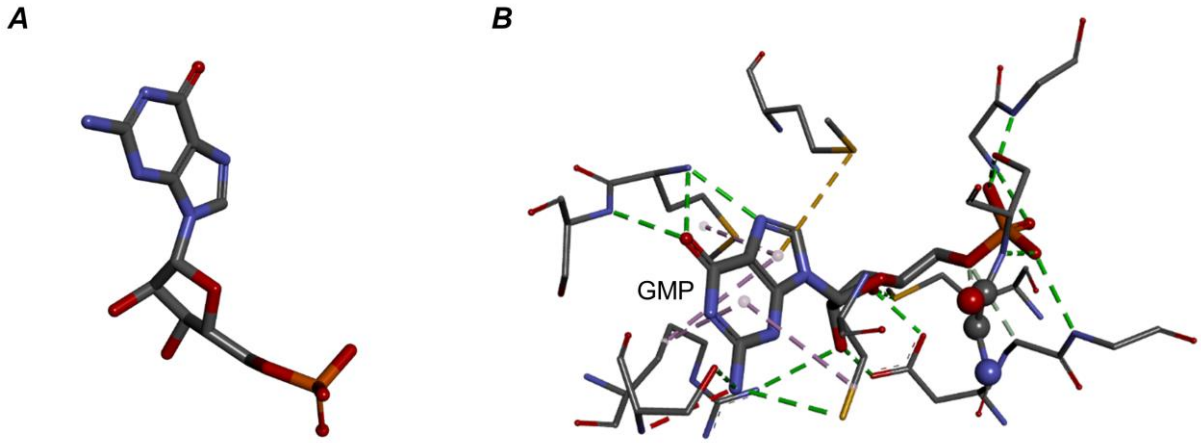


Figure 5.7 GMP Ligand Interactions. (*A*) GMP is the substrate of hGMPR1 and hGMPR2. (*B*) Bound GMP forms several intramolecular bonds with GMPR1 residues, including with Gly183.

Substitution of glycine with arginine at position 183 abolished the carbon hydrogen bond with bound GMP, although the hydrogen bond with Cys183 remained intact. Nonetheless, a number of unfavourable interactions between Arg183 and GMP atoms were introduced as a consequence the substitution, suggesting either that binding of GMP to the active site loop is significantly impaired during to interference from the bulky arginine or that GMP is inefficiently bound to GMPR1 (**Figure 5.8**).

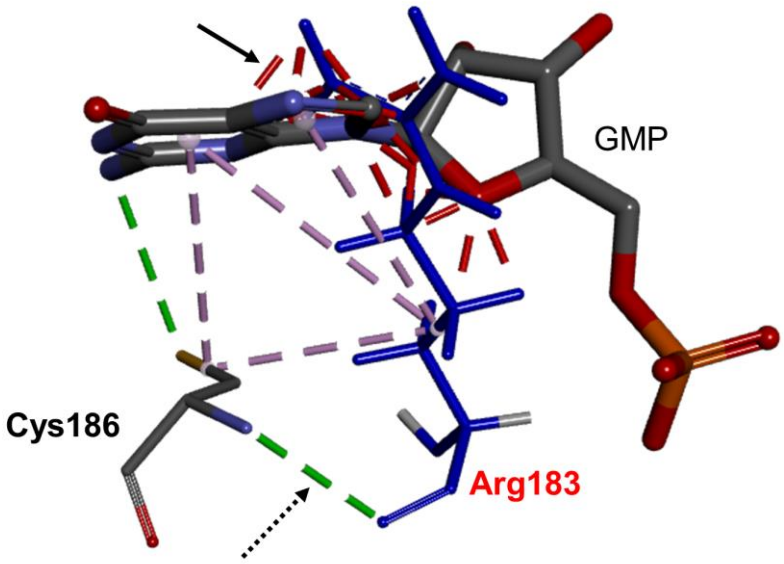


Figure 5.8 Substitution of Gly183 with Arginine. Binding of GMP to hGMPR1 is likely impaired due to the introduction of unfavourable interactions (red lines denoted by the solid black arrow) and abolishment of a carbon hydrogen bond that existed between Gly183 and GMP. However, the hydrogen bond between Cys186 and mutated Arg183 is retained (dashed arrow). For clarity, the mutated Arg183 residue is shown in dark blue.

5.4.4 Genetic Variation within the GMPR1 Active Site Loop

Amino acid variations within the active site loop, residues 176-188, of hGMPR1 and hGMPR2 were studied within ExAC (**Table 5.2**). In hGMPR1, one synonymous and one non-synonymous change was identified. None of the conserved residues were mutated. On the other hand, there were two synonymous, 11 non-synonymous changes and one frameshift mutation affecting the hGMPR2 active site loop were listed that included all conserved residues. Nonetheless, all changes were rare.

Residue	Human GMPR1 Variation Variant	MAF	Human GMPR2 Variation Variant	MAF
Ile176	p.Gly179Gly	0.00001648	p.Ile176Met	0.000008284
Lys177	p.Val180Ala	0.000008243		
Val178			p.Val178Met	0.000008284
			p.Val178Val	0.00003313
Gly179			p.Gly179Arg	0.00003313
			p.Gly179Glu	0.000008283
Val180				
Gly181			p.Gly181Glu	0.000008283
Pro182				
Gly183	p.Gly183Arg	Ø	p.Gly183Ser	0.00001657
Ser184			p.Ser184Cys	0.00002486
Val185			p.Val185_Cys186insGlu	0.000008287
			p.Val185Val	0.00001658
Cys186			p.Cys186Gly	0.000008288
			p.Cys186Arg	0.000008288
			p.Cys186Phe	0.000008288
Thr187				
Thr188			p.Thr188Ile	0.00001658

Table 5.2 Human GMPR1 and GMPR2 Amino Acid Variation. List of hGMPR1 and hGMPR2 variants listed in ExAC and the minor allele frequencies (MAF). Synonymous changes are shown in green. The novel *GMPR1* missense change identified in patient 11 is shown in red. Conserved residues between hGMPR1, hGMPR2, hIMPHD1 and hIMPDH2 are italicised. Ø denotes absence of the p.Gly183Arg missense change in ExAC.

5.4.5 Sublocalisation of GMPR1

In MitoMiner 4.0, GMPR1 was predicted to localise to mitochondria by iPSORT (1.0), but was predicted unlikely by TargetP (0.36), MitoProt (0.357) and MitoFates (0.003). To investigate localisation of GMPR1, subfractionation and immunoblotting of HEK293 and HeLa cells was performed by Francesco Bruni (Wellcome Trust Centre for Mitochondrial Research, Newcastle upon Tyne). In both HeLa and HEK293 cells, GMPR1 was detectable in total cell lysate and the post-mitotic supernatant but not in isolated mitochondrial subfractions. Hence, GMPR1 localised only to the cytosol (**Figure 5.9**).

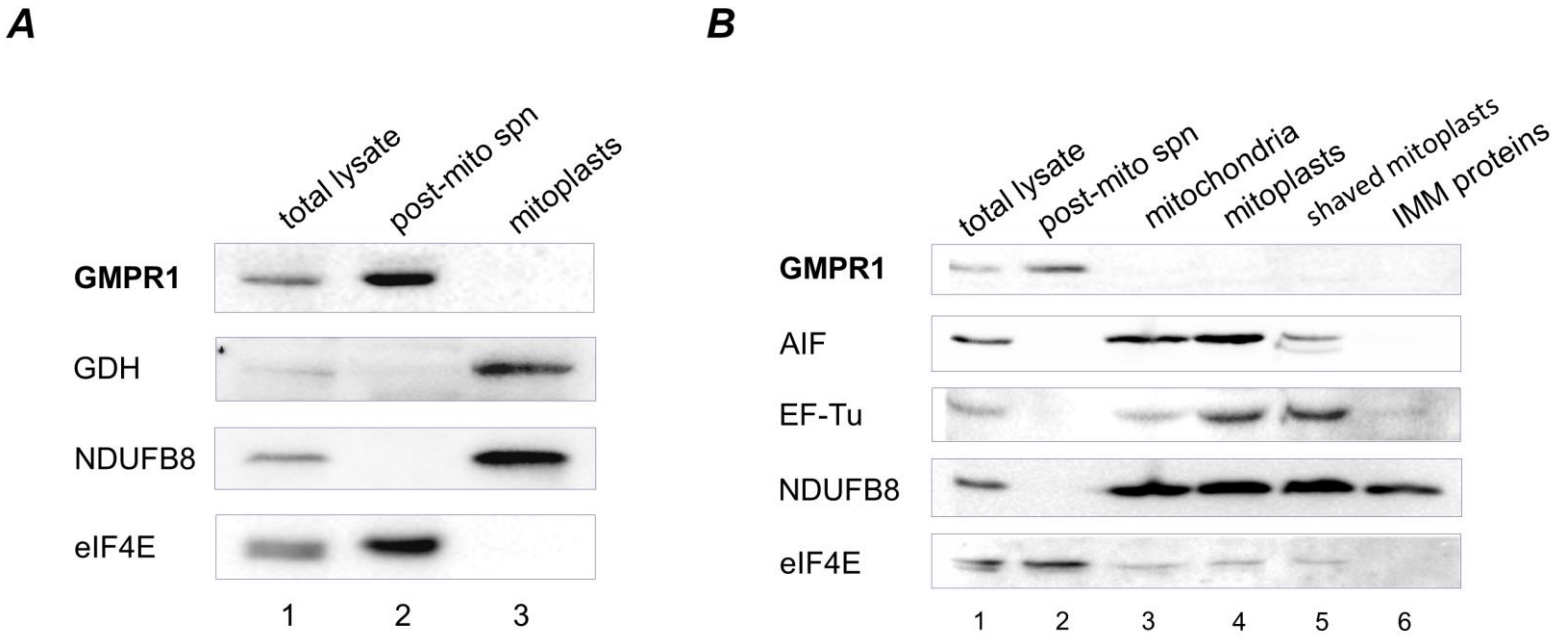


Figure 5.9 Sublocalisation of GMPR1. Subfractionation of (*A*) HeLa and (*B*) HEK293 cells subjected to immunoblotting with a GMPR1 antibody and markers for each subfraction: GDH – mitochondrial matrix; AIF – mitochondrial inner membrane space; NDUFB8 – mitochondrial inner membrane; EF-Tu – mitochondrial inner membrane; eIF4E – cytosol. All subfractions were prepared from the same HeLa or HEK293 lysate.

5.4.6 Relative mtDNA Copy Number in Proliferating and Quiescent *GMPR1* Fibroblasts

From the same passage, relative mtDNA copy number of patient 11 proliferating and quiescent fibroblasts were compared by Ilaria Dalla Rosa (MRC Mill Hill Laboratory, London, United Kingdom). Based on one replicate, control and patient 11 fibroblast mtDNA copy number increased from proliferating to quiescent state (**Figure 5.10**).

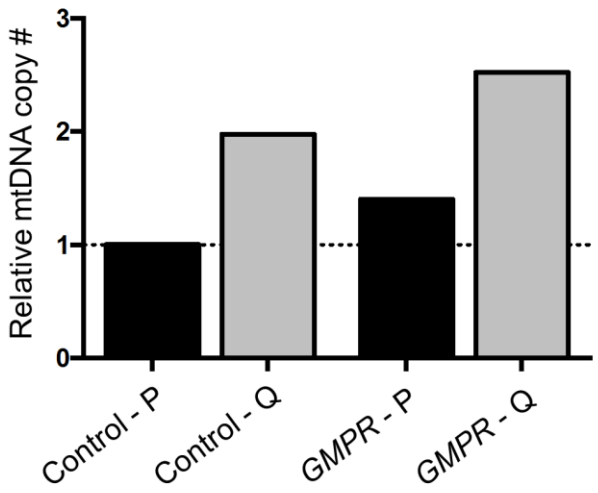


Figure 5.10 Relative mtDNA Copy Number of Patient 11 Proliferating and Quiescent Fibroblasts. The relative mtDNA copy number of patient 11 and control fibroblasts from the same passage in proliferating and quiescent states, with only one replicate.

5.4.7 Ethidium Bromide Depletion and Recovery of mtDNA

Patient 11 (*GMPR1*), patient 8 (*TOP3A*) and patient 15 (*POLRMT*) fibroblasts were depleted of mtDNA with ethidium bromide for 14 days. After removal of ethidium bromide and the change of proliferating to quiescent MEM, there was no significant difference in the recovery of mtDNA compared to control fibroblasts (**Figure 5.11**). Both control and patient fibroblasts did not recover mtDNA to the relative levels at the start (day 0) of depletion.

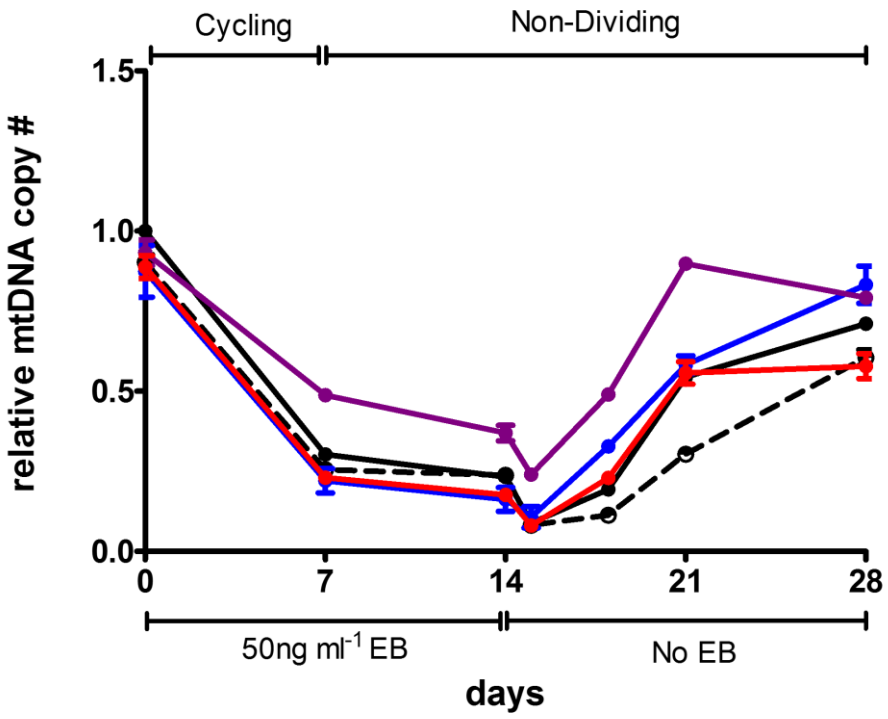


Figure 5.11 Ethidium Bromide Induced mtDNA Depletion of Multiple mtDNA Deletion Patient Fibroblasts. Fibroblasts from three adult-onset PEO with multiple mtDNA deletions patients and two controls were depleted of mtDNA with ethidium bromide, followed by removal and recovery of copy number. Control 1- solid black line. Control 2 – dashed black line. Patient 11 (*GMPR*) – solid blue line. Patient 8 (*TOP3A*) – solid purple line. Patient 15 (*POLRMT*) – solid red line.

5.4.8 Long-Range PCR of Quiescent Fibroblast DNA

In contrast to the skeletal muscle, no mtDNA rearrangements were detected in extracted quiescent fibroblast DNA from patient 11 and two controls, using two sets of forward and reverse primers for mtDNA amplification (**Figure 5.12**).

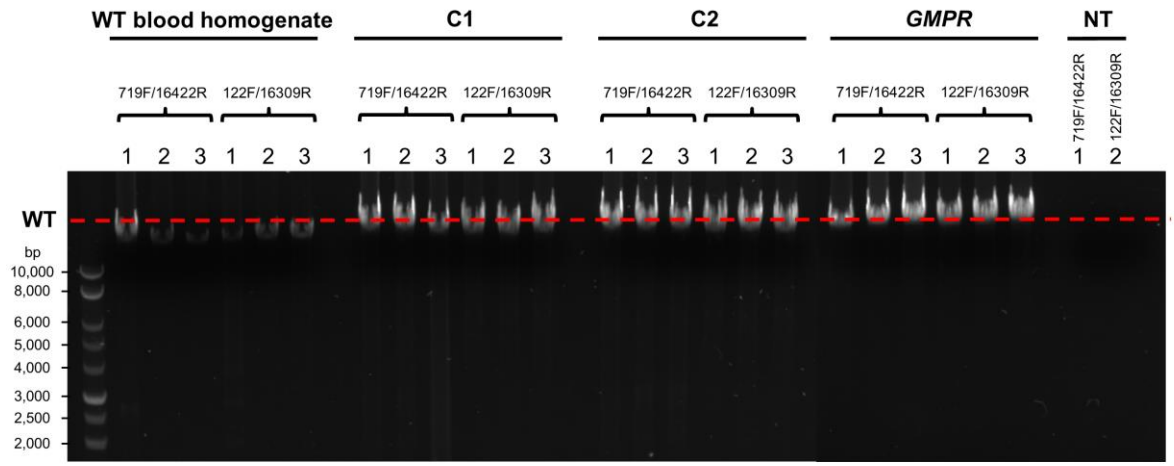


Figure 5.12 Long-Range PCR of Patient 11 Quiescent Fibroblast DNA. Long-range PCR of wild-type blood homogenate, two control quiescent fibroblast (C1 and C2), patient 11 (*GMPR*) quiescent fibroblast DNA and a no template control. Amplification of each sample was performed in triplicate with two sets of optimised forward and reverse primers. Red line indicates the wild-type mtDNA product.

5.4.9 Live Cell Imaging and Confocal Microscopy

Live cell imaging of proliferating fibroblasts was performed together with Maria-Eleni Anagnostou (Wellcome Trust Centre for Mitochondrial Research, Newcastle upon Tyne) (**Figure 5.13**). TMRM staining showed no fragmentation or elongation of the mitochondrial dynamic network in patient 11 fibroblasts. PicoGreen staining of dsDNA showed also no abnormalities in nucleoid morphology.

Additional confocal microscopy of fixed quiescent fibroblasts from patient 11 was performed by Ilaria Dalla Rosa, using anti-DNA to stain dsDNA and anti-TOM20 to stain the mitochondrial network (**Figure 5.14**). This also showed no fragmentation or elongation of the mitochondrial network and no abnormal nucleoid morphology.

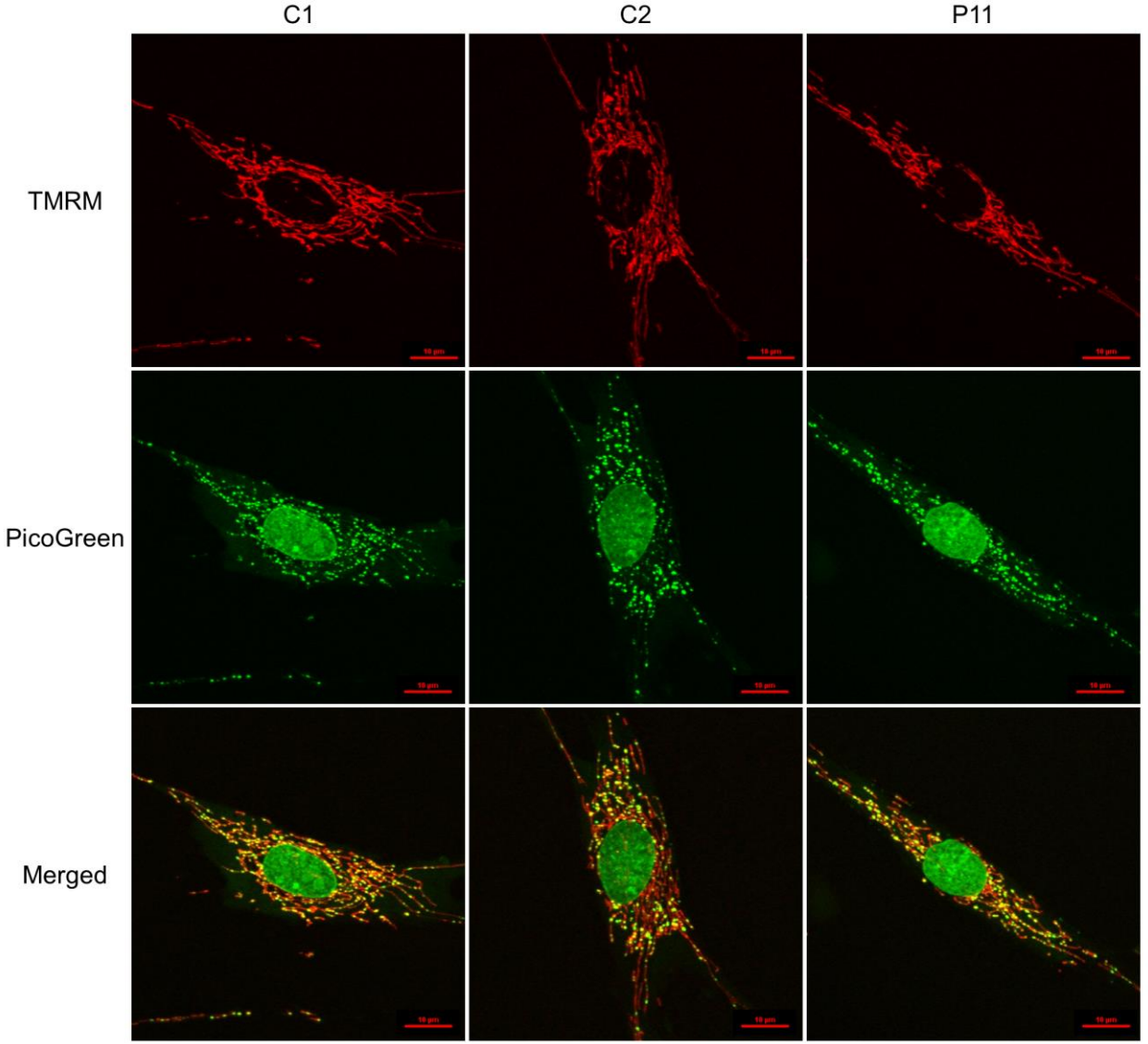


Figure 5.13 Analysis of the Mitochondrial Network and Nucleoids in Patient 11 Proliferating Fibroblasts. TMRM and PicoGreen staining of patient 11 fibroblasts and two controls was performed using the Nikon A1R Confocal microscope.

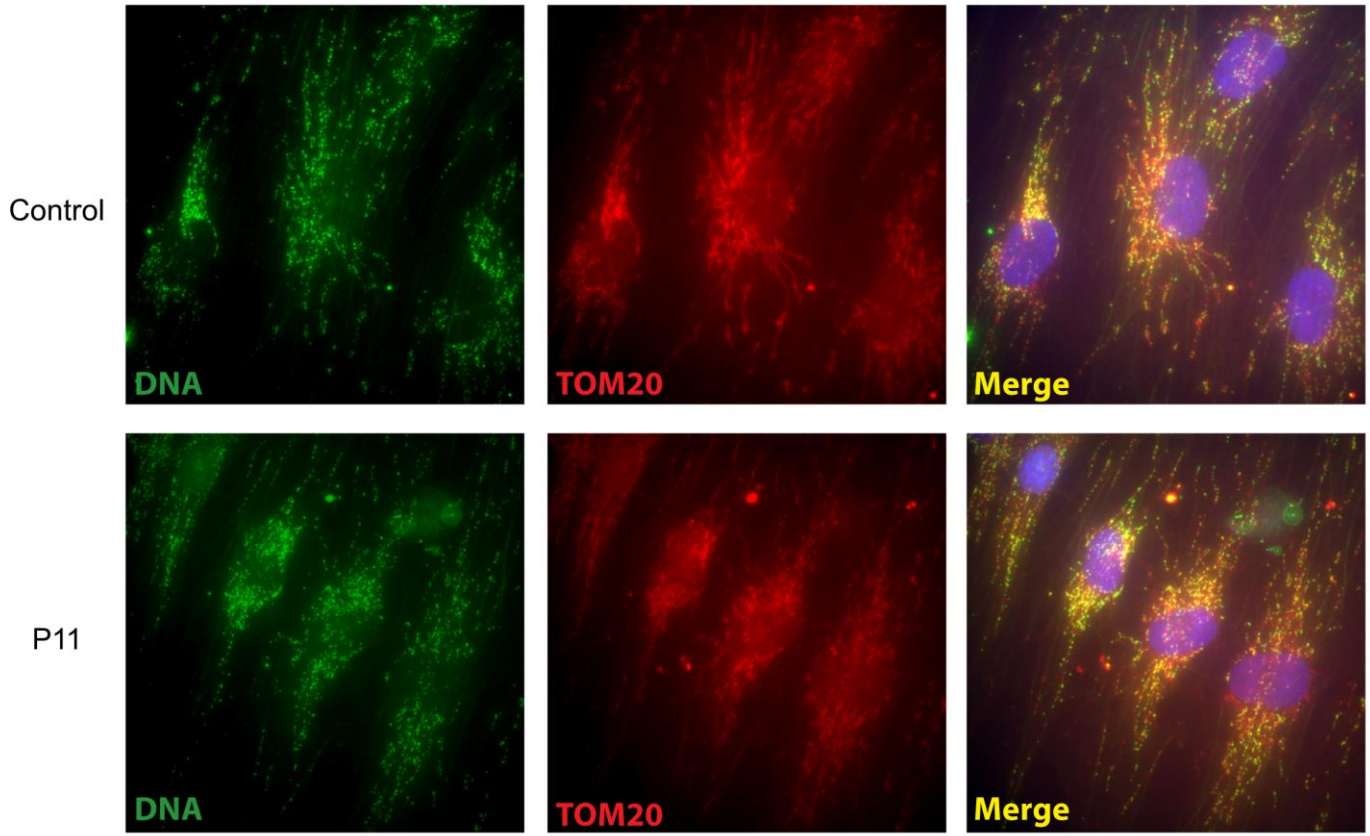


Figure 5.14 Analysis of the Mitochondrial Network and Nucleoid Morphology in Patient 11 Quiescent Fibroblasts. Quiescent patient 11 and control fibroblasts were incubated with primary antibodies for anti-DNA and TOM20 overnight, probed with corresponding HRP-conjugated secondary antibodies for 2 hours and fixed to glass slides for confocal microscopy.

5.4.10 Nucleotide Homeostasis

Steady-state of OXPHOS subunits, mtDNA replication machinery, dNTPs synthesis enzymes and nucleotide transporters were investigated in patient 11 and control fibroblasts.

There was a minor decrease of steady-state GMPR1 levels in patient 11 fibroblasts under proliferating and quiescent states (**Figure 5.15A**). Nonetheless, the relative expression of GMPR1 was decreased in patient and control fibroblasts in quiescence compared to proliferating cells. Mild decreases in NDUFB8 (complex I) and MT-COI (complex IV) levels were also noted in patient 11 fibroblasts.

Expression of LONP1 was slightly decreased in patient 11 proliferating and quiescent fibroblasts, but there was no change in TFAM levels (**Figure 5.15B**).

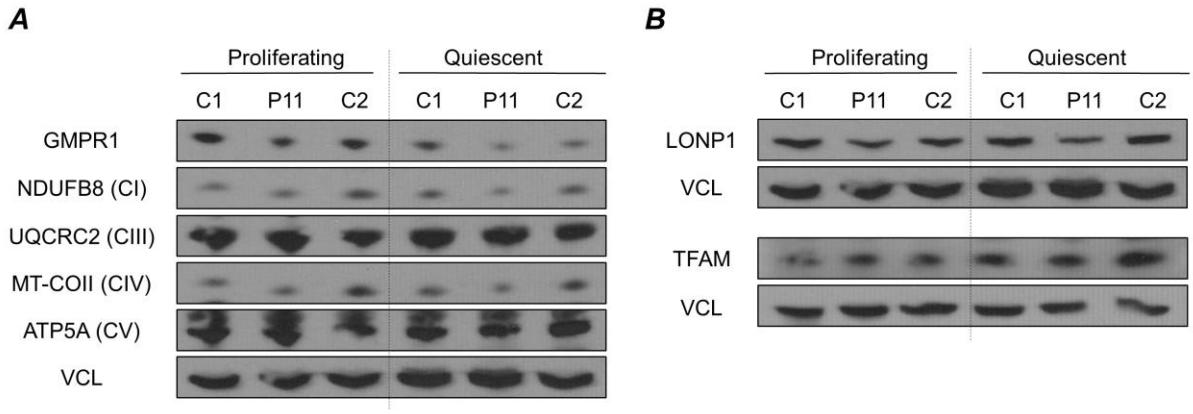


Figure 5.15 Western Blot Analysis of GMPR1, OXPHOS subunits and mtDNA Replication Factors LONP1 and TFAM in Fibroblasts. Steady-state levels of (*A*) GMPR1 and OXPHOS subunits, plus (*B*) LONP1 and TFAM.

Next, additional nucleotide metabolism enzymes were analysed (**Figure 5.16A**). Adenylate kinase 2 (AK2) was expressed in both proliferating and quiescent fibroblasts but did not appear to be significantly altered patient 11 fibroblasts. On the other hand, adenylate kinase 3 (AK3) was expressed only in quiescent fibroblasts and appeared to be decreased in patient 11 fibroblasts. As anticipated, TK1 was expressed in proliferating fibroblasts only, while TK2 was upregulated in quiescence. However, its expression was decreased compared to quiescent controls.

Expression of steady-state RNR subunits (R1, R2, p53R2) were also analysed (**Figure 5.16B**). R1 was expressed in both proliferating and quiescent fibroblasts. R2 was upregulated in proliferating cells and decreased slightly in quiescence. p53R2 expression was low in

proliferating fibroblasts and increased in quiescence. However, expression of the RNR subunits were not significantly altered in patient 11 fibroblasts. Nonetheless, there was variation between controls, including apparently decreased R1 levels in C1 and multiple bands observed for R2 in quiescence.

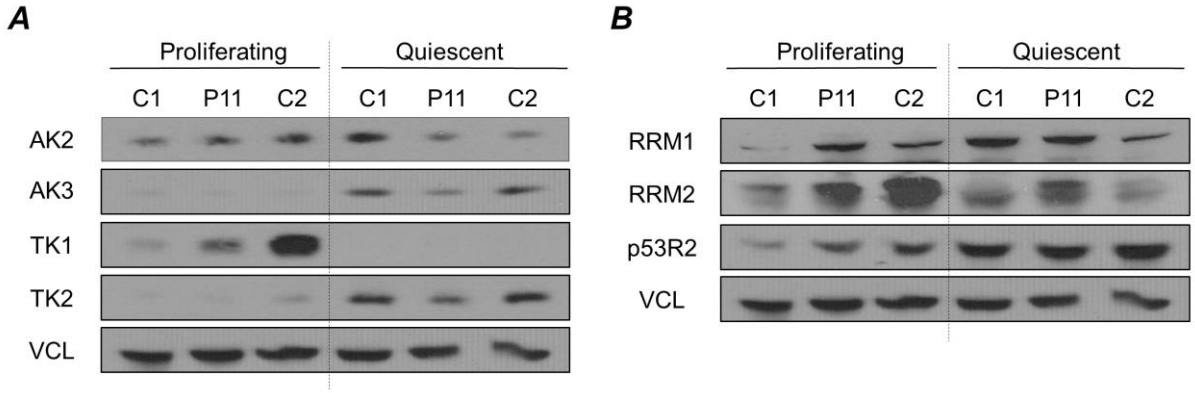


Figure 5.16 Western Blot Analysis of Nucleotide Metabolism Enzymes and the RNR Subunits. Steady-state levels of (*A*) nucleotide metabolism enzymes and (*B*) RNR subunits.

Nucleotide transporters PNC1, PNC2 and ENT1 were also investigated (**Figure 5.17**). As expected, PNC2 was downregulated in quiescent fibroblasts, while PNC1 and ENT1 were expressed during both states. Although PNC2 was downregulated in quiescence, there was an increase in patient 11 fibroblasts compared to controls. Again, there was variation in the controls, with C2 apparently showing decreased steady-state levels of PNC1.

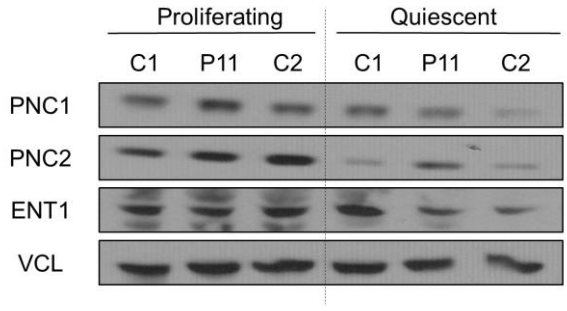


Figure 5.17 Western Blot Analysis of Nucleotide Transporters. Steady-state levels of IMM nucleotide transporters PNC1, PNC2 and ENT1.

Initial investigation of patient 11 and control skeletal muscle also commenced. Steady-state GMPR1 levels were greatly decreased in patient 11 relative to controls (**Figure 5.18A**). TFAM was also downregulated in the muscle, although LONP1 levels were not significantly altered (**Figure 5.18B**). NDUFB8 (complex I) was also markedly decreased in patient 11 (**Figure 5.18C**).

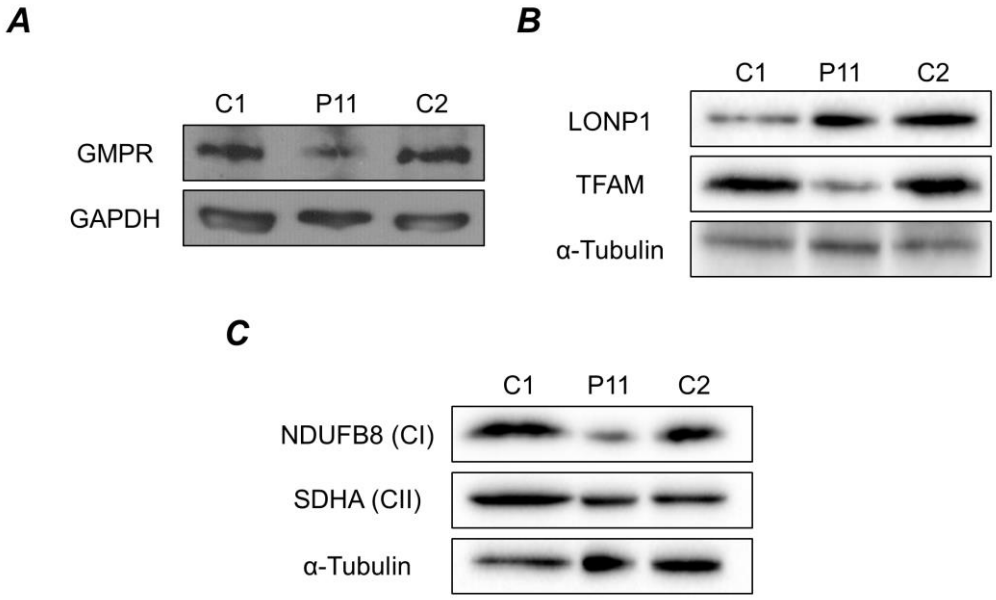


Figure 5.18 Western Blot Analysis of GMPR1, OXPHOS subunits and mtDNA Replication Factors LONP1 and TFAM in Skeletal Muscle. Steady-state levels of (*A*) GMPR1, (*B*) LONP1 and TFAM, plus (*C*) OXPHOS subunits NDUFB8 (complex I) and SDHA (complex II).

5.4.11 Mitochondrial Protein Synthesis in Fibroblasts

Mitochondrial translation products in proliferating and quiescent fibroblasts were subjected to ^{35}S-methionine labelling by Ilaria Dalla Rosa using a well-established protocol (Chomyn, 1996). This demonstrated an exceptionally subtle decrease of nascent mitochondrial proteins in patient 11 proliferating and quiescent fibroblasts compared to controls (**Figure 5.19**).

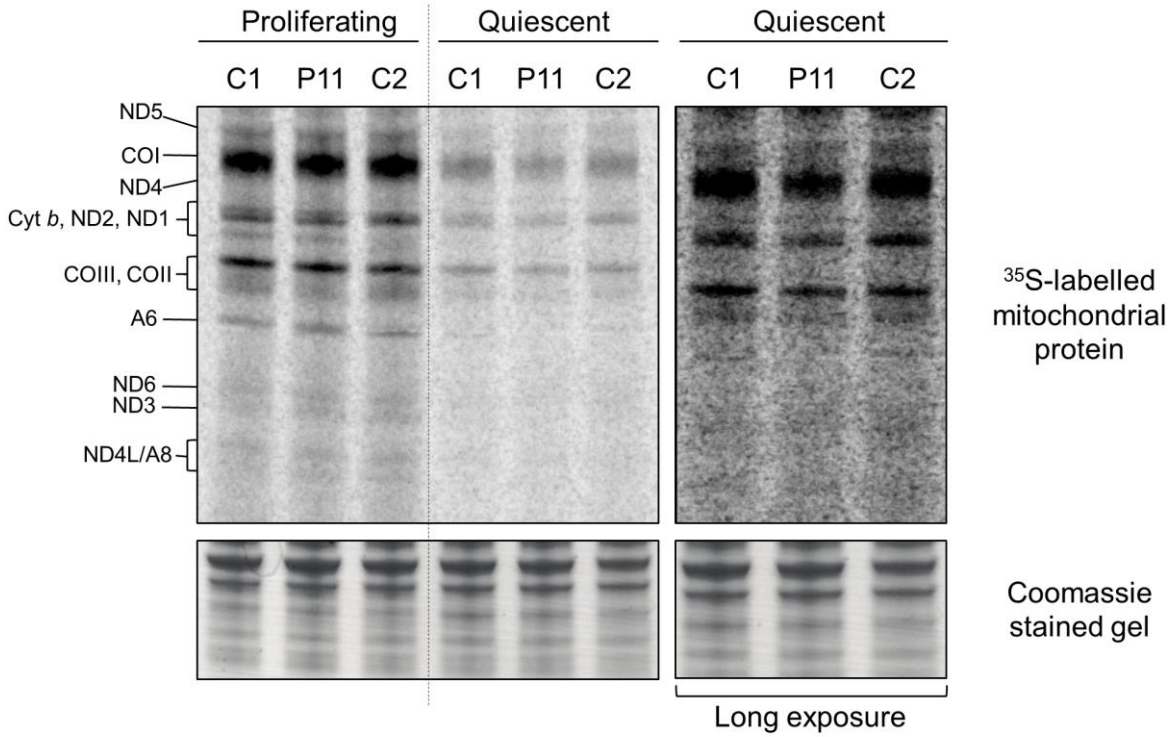


Figure 5.19 ^{35}Methionine-Labelling of Mitochondrial Proteins in Proliferating and Quiescent Fibroblasts. Mitochondrial proteins were separated by 12% PAGE. Radiolabelled proteins were detected by PhosphorImaging. Fibroblasts were placed in quiescence for 10 days. Performed by Ilaria Dalla Rosa.

5.5 Discussion

Using the custom WES filtering strategy for adult-onset PEO with multiple mtDNA deletions outlined in **Chapter 4**, a novel heterozygous p.Gly183Arg missense change was identified in *GMPR1* encoding GMP reductase 1 for the catabolism of the ribonucleotide GMP to the guanine and adenine precursor nucleotide IMP and hence, maintaining the balance of the purine nucleotides. In this chapter, evidence has been provided to demonstrate *GMPR1* as a novel candidate adPEO gene, through the combination of *in silico* analysis, study of patient fibroblasts and muscle. Together with *TYMP*, *SLC25A4*, *RRM2B*, *DGUOK*, *MPV17*, *TK2* and *RRM1*, *GMPR1* potentially expands the list of genes involved in dNTP nucleotide homeostasis that are associated with adult-onset PEO and multiple mtDNA deletions.

5.5.1 Impaired GMP Binding Within the GMPR1 Active Site

With the available human GMPR1 secondary structure (PDB ID 2BLE), it was possible to model the effect of the p.Gly183Arg mutation on protein structure and function. As first noted by Li *et al.* (2006), in GMPR1 and GMPR2 Gly183 forms a hydrogen bond with the critical

catalytic Cys186 residue. Both residues are within a conserved IMP/GMP dehydrogenase domain shared between all IMP/GMP protein family members that comprises an active site binding loop conformation for GMP binding. The conversion of GMP to IMP by GMPR has been shown to consist of two chemical transformations; a deamination step and hydride transfer step (Patton *et al.*, 2011). In the first deamination step, the crucial Cys186 residue 'attacks' the second carbon atom of GMP, which creates a covalent thiomidate intermediate; *E*-XMP*. In the second step there is a hydride transfer of NADP, which forms IMP. A mutation of this conserved cysteine residue has been shown to inactivate GMPR activity in *E. coli* (Li *et al.*, 2006; Min *et al.*, 2008). However, *in silico* modelling shows that the human p.Gly183Arg mutation does not abolish the hydrogen bond with this crucial residue. On the other hand, this mutation abolishes a critical intramolecular bond for GMP binding and introduces unstable interactions with GMP, perhaps as a consequence of the bulky size of arginine. Therefore it is probable that the p.Gly183Arg mutation causes an impairment of GMP binding either from blocking the active site or due to unstable binding.

The evolutionary conservation of the GMPR1 active site region was next investigated further. Overall, the GMPR1 amino acid sequence is 90% identical to its homolog GMPR2 (Deng *et al.*, 2002), suggesting that the latter arose from duplication of the ancestral GMP reductase gene (Becerra and Lazcano, 1998). This includes the IMP dehydrogenase/GMP reductase signature shared between the two other IMP/GMP enzymes IMPDH1 and IMPDH2. Given the conservation of this region and the Gly183 residue in GMPR1, all identified variants in this region were examined from ExAC. Remarkably, the p.Gly183Arg missense change is only the second missense variant identified in this region, the first being a p.Val180Ala variant at a low MAF. However, Val180 is not conserved between GMPR1 and GMPR2, nor with IMPDH1 or IMPDH2. Regarding GMPR2, there is an abundance of missense changes, albeit at low MAFs, that affect conserved residues between GMPR1, IMPDH1 and IMPDH2. This suggests high evolutionary significance for GMPR1 and supports the notion that GMPR1 is the ancestral gene that underwent duplication to conceive GMPR2, but also probably IMPDH1 and IMPDH2. The evolutionary significance is highlighted by Hedstrom (2012), who notes that since life likely emerged from an ammonia-rich environment (Zahnle *et al.*, 2010), the ancestral gene is likely GMPR with some GMP synthesis activity. Such activity is supported by Patton *et al.* (2011), who demonstrated that *E. coli* GMPR could synthesis GMP from IMP in sufficient ammonia levels. It is also worth noting that there is currently only one known protein coding hGMPR1 transcript, whereas there are seven known protein coding hGMPR2 transcripts. Thus there is high evolutionary constraint against variance in the

IMP/GMP dehydrogenase region in human *GMPR1*, which supports the view that this is dominant human disease gene rather than recessive when compared with *GMPR2*. However, it is uncertain whether reduced activity or binding of GMP with GMPR1 is counteracted with increased GMPR2 expression. To investigate this further, a collaboration with Dr Liz Hedstrom and Dr Masha Rosenberg (Brandeis University, Massachusetts, USA) to express and purify human GMPR2 harbouring the p.Gly183Arg mutation from *E. coli* to measure the catalytic conversion of GMP to IMP is currently underway.

5.5.2 Nucleotide Homeostasis is Altered in *GMPR1* Patient Fibroblasts

Decreased GMPR1 expression was clearly evident in both patient fibroblasts and significantly more so in the skeletal muscle. Additionally, GMPR1 expression was mildly decreased in non-dividing control and patient fibroblasts. This study has shown for the first time that GMPR1 remains active during quiescence and continues to play an important role in the synthesis of guanine ribonucleotides, although this is not surprising since the RNR continues to reduce rNDPs in non-dividing cells (Tanaka *et al.*, 2000; Hakansson *et al.*, 2006; Pontarin *et al.*, 2007; Pontarin *et al.*, 2012). Nonetheless, it should be noted that there was clear variation between the control samples used in both proliferating and quiescent states.

Since GMPR1 reduces GMP to IMP, the precursor of purine nucleotide synthesis, expression of the *de novo* adenine synthesis enzymes, adenylate kinase 2 and 3 (AK2, AK3), were studied. Both kinases catalyse the reversible conversion of AMP to ADP, although AK2 is localised to the mitochondrial intermembrane space, whereas AK3 is localised to the matrix (Noma, 2005). AK2 was expressed in both patient and control proliferating and quiescent cells, while AK3 is upregulated in quiescence suggesting a prominent role in the mitochondrial salvage pathway. In quiescent patient fibroblasts though, there was a decrease in AK3 suggesting that the mitochondrial purine salvage pathway is downregulated. Surprisingly, TK2 was also decreased in quiescence, which indicates that mitochondrial pyrimidine salvage is also repressed. There was also no apparent change in the expression of the RNR subunits in proliferating and quiescent state, implying that the reduction of rNDPs from *de novo* nucleotide synthesis is not altered. Regarding the nucleotide transporters, PNC2 was downregulated in quiescent control and patient fibroblasts but was mildly elevated in patient cells. No observable changes in PNC1 and ENT1 expression were noted. Taken together, this suggests that mitochondria are attempting to re-balance the dNTP pools through a combination of mitochondrial purine and pyrimidine salvage repression, while also attempting to redress the balance by up-taking *de novo* synthesised and free purine

nucleotides from the cytosolic pools. Interestingly the pattern of purine and pyrimidine repression together with the upregulated PNC2 transporter is comparable to that of MPV17-deficient fibroblasts, which demonstrate an interesting link to guanosine synthesis (Dalla Rosa *et al.*, 2016). Given that the precise role of MPV17 in nucleotide homeostasis and mtDNA maintenance disorders has yet to be elucidated, further examination of the effect of the *GMPR1* mutation on MPV17 expression in fibroblasts and skeletal muscle could provide an invaluable contribution to the debate.

In light of the mild changes to nucleotide homeostasis in fibroblasts, studies are currently underway to measure the total cellular and mitochondrial dNTP pools in both proliferating and quiescent states, as has been performed in MPV17-deficient fibroblasts (Dalla Rosa *et al.*, 2016). Based on the reduced GMPR1 expression and repressed mitochondrial purine and pyrimidine salvage pathways, it is expected that the total cellular and mitochondrial dGTP pools will be depleted. Moreover, if GMP is not readily catabolised back to IMP, this to some degree could also inhibit *de novo* adenosine synthesis. Therefore, the putative pathogenic mechanism of the *GMPR1* mutation could be depleted dGTP pools, impairing mtDNA replication. Despite this, uptake of dNTPs from the cytosol through PNC2 would be minimal since cytosolic *de novo* synthesis is low in quiescent fibroblasts.

Owing to the association of genomic instability with oncogenesis due to nucleotide pool imbalances (Mathews, 2015), it is important to take a cautious approach regarding defects of these essential enzymes. Concerning GMPR1, Wawrzyniak *et al.* (2013) demonstrated that its activity supressed melanoma cell invasion and the formation of tumorigenic cells, by depleting the intracellular GTP pools. Interestingly, there is no known history of melanoma or cancer for patient 11 or the immediate family. At the age of 73, no apparent clinical manifestations associated with nuclear genomic stability such as cancer have arisen. Nonetheless, this has yet to be evaluated at a cellular level.

5.5.3 mtDNA Replication is Not Stalled in Adult-Onset mtDNA Maintenance Patient Fibroblasts

To determine whether mtDNA replication may be slowed or stalled, the intercalating agent ethidium bromide was used to deplete the mtDNA in fibroblasts from three adult patients with multiple mtDNA deletions for 14 days. This was then removed to allow copy number to recover. Ethidium bromide induced mtDNA depletion has been used previously for the study of mtDNA maintenance disorders in fibroblasts (Stewart *et al.*, 2011; Dalla Rosa *et al.*, 2016),

since it is known to inhibit mtDNA replication without affecting the nuclear DNA (Leibowitz, 1971; Seidel-Rogol and Shadel, 2002). This was studied with fibroblasts from three patients (patients 8, 11, 15) from the adult-onset PEO with multiple mtDNA deletions WES cohort, together with two controls. While there was almost complete restoration of mtDNA after 14 days of treatment, there was no slowing or stalling of mtDNA replication in all fibroblast lines. This was somewhat unsurprising since a previous study that used ethidium bromide to deplete mtDNA in fibroblasts from a patient harbouring a homozygous *TK2* mutation restored mtDNA at a similar rate to controls (Stewart *et al.*, 2011). On the other hand, MPV17-deficient fibroblasts demonstrated a severe stalling of mtDNA replication following treatment (Dalla Rosa *et al.*, 2016). This perhaps suggests that not all defects of dNTP pools affect the rate of mtDNA replication, although there are presently limited replicates to confirm this. In quiescence, there was no effect on the relative mtDNA copy number compared to control cells. Additionally, there was no abnormal morphology, aggregation or depletion of the nucleoids. Thus, this confirms that the *GMPR1* mutation is a qualitative mtDNA maintenance disorder.

Nonetheless, there was a slight unexpected decrease of LONP1 in proliferating and quiescent patient fibroblasts accompanied by no apparent change in the steady-state levels of TFAM. LONP1 is known to bind to transcription promoters of the mtDNA (Fu and Markovitz, 1998), while also acting synergistically with TFAM to regulate mtDNA maintenance, with *Lon*-knockdown in *Drosophila melanogaster* causing increased TFAM and mtDNA copy number (Matsushima *et al.*, 2010). Hence, the decreased steady-state LONP1 levels in the *GMPR1* patient fibroblasts would be suggestive of increased mtDNA proliferation perhaps as a compensatory mechanism. Although the apparently unchanged levels of TFAM and mtDNA copy number suggest otherwise, the increased expression of PNC2 for uptake of dNTPs for mtDNA replication advocates this hypothesis. On the other hand, a marked decrease of TFAM was observed in the patient skeletal muscle but without altered LONP1 expression. In this instance, the significant loss of TFAM expression is indicative of decreased mtDNA replication but the unaltered LONP1 levels perhaps implies limited selective degradation of TFAM.

5.5.4 Late-Onset mtDNA Maintenance Disorders Express Null or Subtle Cellular Phenotypes

Although a reduction of GMPR1 was observed in the patient fibroblasts and muscle, the expressed cellular phenotype was otherwise mild or subtle despite taking advantage of the

proliferating and quiescent states. Moreover, restoration of mtDNA copy number in patient fibroblasts indicated that mtDNA replication was neither slow nor stalled; this was also observed in fibroblasts from patients 8 and 15 with VUS in *TOP3A* and *POLRMT*. A subtle reduction in mitochondrial protein synthesis was also noted in both proliferating and quiescent fibroblasts, most likely due to the accumulation of mtDNA deletion species. Even in muscle, though decreased TFAM expression is a clear indicator of a mtDNA maintenance defect this is not specific to a single nuclear gene defect. Quadruple immunofluorescence assay to quantify complex I and IV protein expression in skeletal muscle demonstrated an expression pattern that matched that of previously studied multiple mtDNA deletion patients (Rocha *et al.*, 2015). While this novel technique is highly valuable in the diagnostic process, the expression profile is not indicative for a specific nuclear gene defect. Consequently, validating the pathogenicity the novel *GMPR1* mutation within these tissues remains a challenge. Hence, the use of *in silico* modelling has been crucial to confirm the pathogenic nature of the p.Gly183Arg mutation. This reflects the nature of autosomal dominant, late-onset mtDNA maintenance disorders. Though candidates including *GMPR1* have been proposed in this study (**Chapter 4**), the validation process is immediately compounded by the inability to perform segregation studies with unaffected and/or affected relatives. Since multiple mtDNA deletions associated with disturbed mtDNA maintenance are skeletal muscle restricted, additional study of the *GMPR* patient 11 muscle including analysis of nucleotide homeostasis could reveal a more insightful view of the pathogenic nature of the mutation. Nonetheless, it has been demonstrated here that techniques and assays should be specific to the gene of interest.

5.5.5 Concluding Remarks

To summarise, this study has identified a novel heterozygous *GMPR1* p.Gly183Arg missense change in a patient presenting indolent, late-onset PEO with multiple mtDNA deletions. The phenotype was reminiscent of adPEO since the disease onset was late in life, although it is acknowledged that there is no known family history suggestive of dominant inheritance. In the absence of additional candidate gene variants, this study proposes that *GMPR1* is a novel candidate locus for adult-onset PEO and multiple mtDNA deletions. GMPR1 has been confirmed as a cytosolic enzyme for the catabolism of GMP to IMP, thus maintaining the balance of guanine and adenine nucleotides. The p.Gly183Arg mutant likely acts in a dominant-negative manner by competing with wild-type (and potentially GMPR2) for GMP binding and activity. Nonetheless, *GMPR1*-mutant fibroblasts and skeletal muscle are a paradigm of late-onset mtDNA maintenance disorders due to the subtle cellular phenotypes

that provide challenges in elucidating its pathogenic nature. Measurements of the total cellular and mitochondrial dNTP pools in proliferating and quiescent fibroblasts are also currently underway that seek to confirm the effect of due to repressed mitochondrial salvage pathways. Together with further studies of nucleotide metabolism and mtDNA replication in the skeletal muscle, these will provide further insights of the pathological nature of the mutation. Finally, identification of a second patient with disturbed mtDNA maintenance and one or more *GMPR1* mutation would provide further validation of pathogenicity.

Chapter 6. WES of Mitochondrial Respiratory Chain Complex Deficiency

6.1 Introduction

This chapter contains material published in *Journal of Inherited Metabolic Disorders Reports* (Oliveira and Sommerville, 2016) and *American Journal of Human Genetics* (Kopajtich *et al.*, 2014). Additional clinical, diagnostic and research support are provided from colleagues and external collaborators who are appropriately acknowledged.

6.1.1 Mitochondrial Respiratory Chain (RC) Deficiency

Mitochondrial respiratory chain (RC) disorders are among the most common early-onset metabolic disorders with an estimated minimum prevalence of 1 in 5000 live births (Skladal *et al.*, 2003). These defects affect one or more of the five multi-subunit enzymes that comprise the OXPHOS system, manifesting in vast clinical and genetic heterogeneity. Pathogenic mtDNA mutations account for an estimated 15-30% of patients with RC deficiency (DiMauro and Davidzon, 2005; Kirby and Thorburn, 2008), hence the remaining patients have a suspected nuclear aetiology.

Attaining a genetic diagnosis is challenging, since approximately 1,200 proteins encoded by the nuclear genome (Lopez *et al.*, 2000; Calvo *et al.*, 2006) are required for the coordination of mitochondrial processes. Mendelian RC defects can be divided into (i) isolated and (ii) combined deficiencies. Isolated RC deficiency is predominantly associated with mutations of RC complex subunits or assembly factors (Loeffen *et al.*, 2000; Diaz, 2010; Hoekstra and Bayley, 2013; Hejzlarova *et al.*, 2014; Fernández-Vizarra and Zeviani, 2015). On the other hand, combined RC deficiencies are typically associated with defects of mtDNA maintenance, protein synthesis, OXPHOS cofactors, dynamics and metabolism. This genetic and clinical heterogeneity is reflected in approximately 245 nuclear genes currently associated with RC deficiency (Mayr *et al.*, 2015).

6.1.2 NGS Approaches in Mitochondrial RC Disease Diagnosis

While clinical features, patient muscle histology and biochemical studies may be indicative of mitochondrial disease, findings are often non-specific to one genetic aetiology. Unless a clear phenotype-genotype correlation has been delineated, targeted screening of a subset of nuclear genes have mostly proved ineffective in diagnosing suspected autosomal recessive

mitochondrial RC disease, providing diagnostic yields up to 11% (Kemp *et al.*, 2011; Neveling *et al.*, 2013).

Over the past decade, various NGS approaches in the diagnosis of paediatric mitochondrial RC deficiency have been described throughout the literature (Vasta *et al.*, 2009; Calvo *et al.*, 2012; Vasta *et al.*, 2012; DaRe *et al.*, 2013; Lieber *et al.*, 2013; Neveling *et al.*, 2013; Taylor *et al.*, 2014; Wortmann *et al.*, 2015; Kohda *et al.*, 2016; Legati *et al.*, 2016; Pronicka *et al.*, 2016). In contrast to late-onset mtDNA maintenance disorders, the vast majority of early-onset RC disease patients are expected to harbour autosomal recessive (compound heterozygous and homozygous) or X-linked (particularly in males) variants. Hence, the focus of the majority of NGS studies of Mendelian mitochondrial disease have been on this cohort of patients.

Nonetheless, NGS approaches have varied between published studies. A so-called 'MitoExome' approach whereby a panel of nuclear genes encoding mitochondrial proteins are targeted, ranging from ~400 to 1000 genes, was utilised by DaRe *et al.* (2013), (Vasta *et al.*, 2012) and Calvo *et al.* (2012). Furthermore, Calvo *et al.* (2012) combined the exome panel of ~1000 genes with whole mitochondrial genome sequencing. As perhaps expected, the highest yield was attained by Calvo *et al.* (2012) with 55% of patients identified with causative or likely causative variants. More recently, large cohort studies have used WES or WGS for prioritisation of candidate variants in all nuclear genes, including the ~1,200 genes encoding mitochondrial proteins. This has given rise to somewhat higher diagnostic yields compared to 'MitoExome' gene panels, varying between 34.5% and 60%. On the other hand, Legati *et al.* (2016) studied 152 patients by first using a small 132 gene 'MitoExome' panel, attaining a diagnostic yield of 15.2% before next turning to WES for only 10 patients and identifying causative variants in six patients (60%). However, it should be noted that the size of patient cohorts varied dramatically between studies, making it difficult to ascertain the true diagnostic yield in early-onset mitochondrial disease. Crucially, filtering of called variants could also have varied between studies, with more efficient and concise prioritisation leading to an improved diagnostic yield. Further to this, there are variations of the criteria and proportion of patients with isolated RC versus multiple RC defects who were recruited in previous studies.

Although autosomal recessive or X-linked variants are expected, challenges still remain due to the possible implication of *de novo* dominant (heterozygous) variants (Harel *et al.*, 2016; Thompson *et al.*, 2016) and in the identification and prioritisation of candidate variants in genes with no known function. Similar to mtDNA maintenance factors as discussed in

Chapter 4, there are likely additional nuclear genes encoding proteins involved in RC function that have yet to be functionally validated. This is highlighted by the identification of previously unknown RC regulators using mitochondrial protein interaction mapping and gene editing combined with proteomics analysis to identify complex I assembly factors (Floyd *et al.*, 2016; Stroud *et al.*, 2016). Though ~245 nuclear genes have been associated with mitochondrial RC disease, there are likely to be additional genes found that are not currently associated with human disease. Secondary RC defects have also been described in muscle biopsies from patients with other neuromuscular or neurological disorders with phenotypic overlap with mitochondrial disease, due to mutations in genes encoding non-mitochondrial proteins including but not limited to *SOD1*, *TARDBP*, *STXBP1*, *MECP2*, *ANO10*, *CAPN3* and *COLQ* (Crugnola *et al.*, 2010; Keogh *et al.*, 2015; Pyle *et al.*, 2015; Kohda *et al.*, 2016). Thus, this can be a misleading finding that complicates variant filtering and prioritisation.

In light of the diagnostic yields attained, WES is an attractive tool in the diagnosis of early-onset mitochondrial RC disorders. The previously published WES filtering strategies for this group of patients, focusing on nuclear genes encoding mitochondrial proteins with autosomal recessive or X-linked inheritance means that establishing similar approaches with appropriate custom modifications is possible. In this chapter, a custom filtering strategy for the prioritisation of candidate gene variants is designed with previous strategies in mind and applied to 20 patients with undiagnosed paediatric mitochondrial RC deficiency and a suspected nuclear aetiology.

6.2 Aims

This chapter aims to attain genetic diagnoses using WES for 20 patients with clinically heterogeneous phenotypes and mitochondrial RC complex deficiency.

6.3 Methods

6.3.1 Recruitment of Patients

Patients were clinically examined at the NHS Highly Specialised Service for Rare Mitochondrial Disorders or at separate clinics in the UK or internationally, with diagnostic testing performed by the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory in Newcastle upon Tyne. There were 20 unrelated patients (14 male, 6 female) with genetically undetermined early-onset RC disease recruited for WES. Diagnostic investigations were performed as described in **2.2.3**.

6.3.2 WES Filtering and Analysis

The WES filtering strategy for mitochondrial RC deficiency patients was similar to the filtering strategy outlined for adult-onset PEO with multiple mtDNA deletions patients in **4.3.5**.

Called variants that passed quality score filtering were restricted to exonic (coding) or splice-site variants, with a minor allele frequency (MAF) equal to or less than 0.01 (1%) of in-house exomes or external exome databases.

Next, variants were filtered using GO-Terms associated with mitochondrial-localisation and mitochondrial protein synthesis. GO-Terms employed were the wildcard term 'mitochondr*', 'tRNA' and 'translation'. This allowed inclusion of nuclear genes encoding proteins involved in mitochondrial translation, but also all mitochondrial-targeted proteins. Hence, this provided a means of identifying candidate variants in known nuclear genes associated with mitochondrial RC deficiency and the prioritisation of novel candidate genes. Copy number variations (CNVs) were also analysed using the same employed GO-Terms.

Lists of genes and called variants were categorised according to gene role and function, expected inheritance and association with human disease (if previously known). For patients born to consanguineous parents, homozygous variants were prioritised. For patients born to non-consanguineous parents, compound heterozygous variants were prioritised.

To assess the potential pathogenicity of called variants, a simplified scoring system was used (**Table 6.1**). Since trios comprising the proband and parents were not sequenced, segregation of variants was not included in variant prioritisation. The effect of missense on protein function were predicted using PolyPhen2, Align-GVGD and SIFT. However, *in silico* predictions were purely advisory and were not used to directly excluded candidates using the scoring system. Splice-site or nonsense loss-of-function (LOF) variants were also considered but not scored.

Mutation(s)	Genotype	GO-Terms			Mitochondrial Disease Gene?			Score
		Mitochondr*	tRNA	Translation	Y/N	Allele-1	Allele-2	
c.?, p.?								

Table 6.1 Basic WES Variant Scoring System for Early-Onset RC Deficiency Patients. Genotype – 1 point for a heterozygous variant, 2 points for a recessive (homozygous, compound heterozygous) or hemizygous variant(s). Since mitochondrial disease was confirmed in biochemical and histopathological studies, 2 points were given for mitochondr* hits, while 1 point was given each for 'tRNA' and 'translation'. 1 point was given if they gene was previously associated with mitochondrial disease. 1 point was given to each known pathogenic variant (compound heterozygous) or 2 points for a known pathogenic homozygous or hemizygous variant. CNVs were also included in analysis.

Owing to the scores attained, variants could be classified into categories. Category-1 (≥ 7 points) comprised the highest level candidates. These variants met the following criteria:

- Autosomal recessive variant(s) – homozygous, compound heterozygous or hemizygous (2 points).
- GO-Terms matched 'mitochondr*' (2 points) **and** 'translation' **or** 'tRNA' (1 point for each term).
- Known causative gene associated with mitochondrial RC deficiency (1 point).
- One or more previously reported causative mutation(s) (1-2 points).

Category-2 (5-6 points) was comprised of medium prioritised candidates. These variants met the following criteria:

- Autosomal recessive variant(s) – homozygous, compound heterozygous or hemizygous (2 points).
- GO-Terms matched 'mitochondr*' (2 points) **or** 'translation' **or** 'tRNA' (1 point for each term).
- Known causative **and** previously unreported genes (0-1 point).
- One or more previously reported causative mutation(s) (1-2 points).

Variants in category-3 (3-4 points) were considered low priority and met the following criteria:

- Autosomal recessive variant(s) – homozygous, compound heterozygous or hemizygous (2 points).
- GO-Terms matched ‘mitochondr*’ (2 points) **or** ‘translation’ **or** ‘tRNA’ (1 point for each term).
- No known association with mitochondrial RC disease (0 points).

The final category were variants of unknown significance (VUS) (≤ 3 points). In this instance, no appropriate candidate variants in nuclear genes encoding mitochondrial localised proteins were identified. Upon this failure, *all* recessive and dominant VUS were evaluated individually. All listed VUS in unsolved cases were reviewed at least every 2-3 months to ensure up-to-date findings from the literature were reflected.

- Autosomal dominant (1 point) **or** autosomal recessive (homozygous, compound heterozygous) or hemizygous (2 point).
- No GO-Terms matched (0 points).
- No known association with mitochondrial RC disease or other known human pathology (0 points).

A schematic of WES filtering for mitochondrial RC disease is given in **Figure 6.1**.

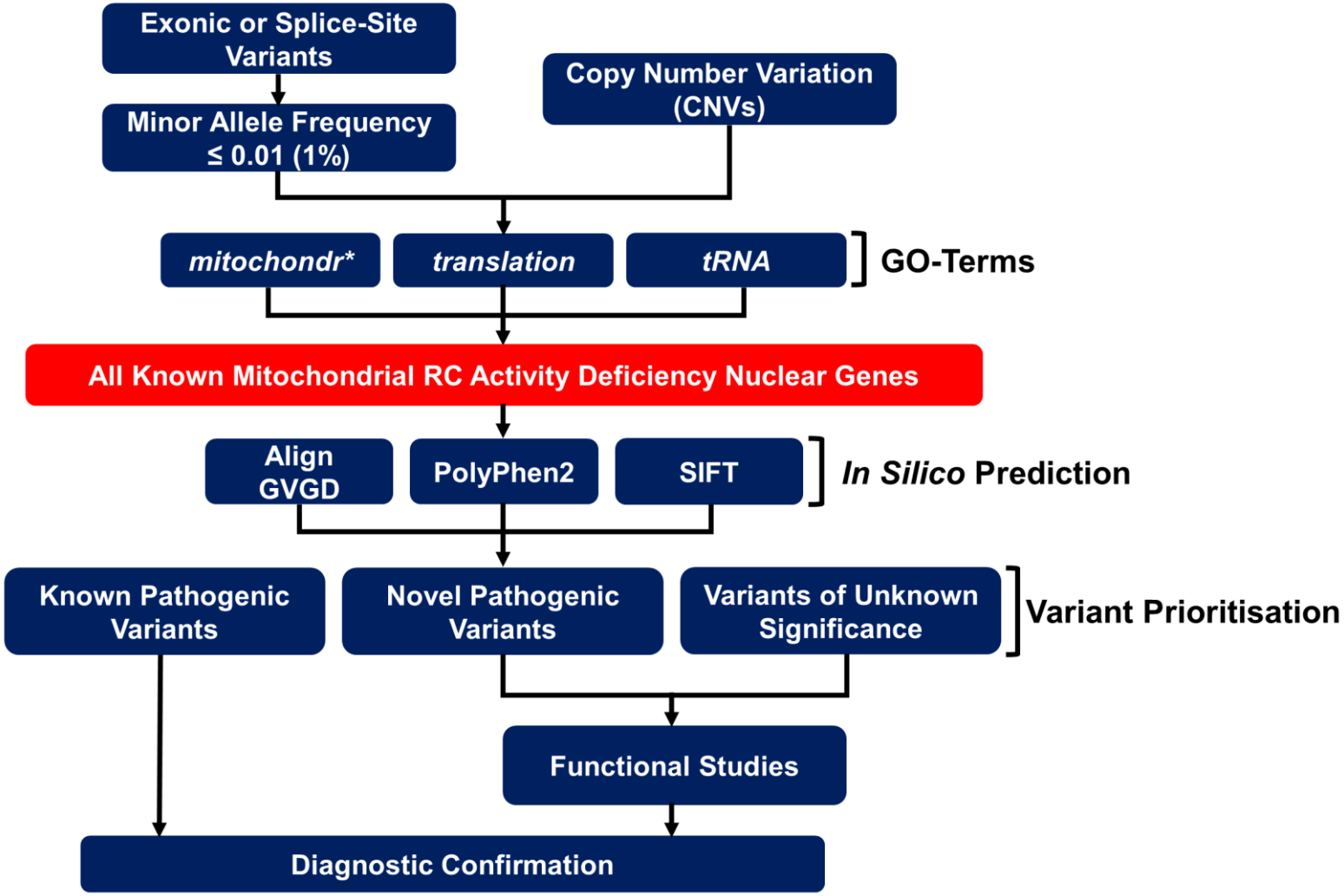


Figure 6.1 Mitochondrial RC Deficiency WES Filtering Strategy.

6.3.3 Sanger Sequencing Confirmation

Custom primer design for candidate variants (**Appendix A**), PCR amplification, Sanger sequencing confirmation and analysis were performed as described in **2.3**. If already available, primers designed within the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory were used.

6.3.4 Cell Culture

Cultured fibroblasts were grown for patient 21 and three appropriate age-matched controls. Subculturing, freezing and harvesting of cells were performed as described in **2.4**.

6.3.5 Western Blotting

Patient 21 and control fibroblast lysates were prepared and subjected to 12% SDS-page, probed with primary and secondary antibodies (**Table 2.4**, **Table 2.5**) then detected as outlined in **2.5**. Membranes were incubated with primary antibodies specific to PTPIP51/RMDN3, plus OXPHOS subunits NDUFB8, SDHA, UQCRC2, MT-COI, MT-COII and ATP5B. β-actin was used as a loading control.

6.3.6 TMRM Staining and Live-Cell Imaging

Patient 21 and two control fibroblast cell lines were prepared, imaged with fluorescent dyes TMRM for the mitochondrial network using the Nikon A1R Invert point scanning confocal microscope then analysed as outlined in **2.6**.

6.4 Results

6.4.1 Clinical and Molecular Features

The clinical, histochemical and biochemical features of the 20 patients recruited for WES are summarised in **Table 6.2**. Metabolic acidosis including lactic acidosis and elevated blood serum lactate (15/20, 60%), HCM (9/20, 45%) and neurological impairment including abnormal brain MRI and seizures (9/20, 45%) were the most common features in the cohort. Additional features were muscle weakness or exercise intolerance (6/20, 30%), liver dysfunction (5/20, 25%), failure to thrive (FTT) (5/20, 25%), developmental delay or regression (5/20, 25%) and renal impairment (2/20, 10%). Ataxia, limb spasticity, ptosis, 3-methylglutatonic and methylglutaric acidosis were confined to single cases. Consanguinity was reported for 6/20 (30%). Onset was predominantly from birth (7/20, 35%) and almost always during the first year of life (16/20, 80%). Age of onset was not determined for two patients.

Muscle biochemical studies of mitochondrial RC activities were performed for all patients, comprising 9/20 (45%) patients with complex I and IV deficiency, 4/20 (20%) with complex I, III and IV deficiency, 2/20 (10%) patients with isolated complex I deficiency, 3/20 (15%) patients with isolated complex IV deficiency and 1/20 (5%) patient with complex I and II deficiency. One patient had normal RC activities. Muscle histopathological studies were performed in 16/20 (80%) patients and showed variable findings that included global COX-deficiency (5/20, 25%), COX-mosaic or some COX-deficient fibres (4/20, 20%), lipid accumulation (3/20, 15%) and increased fat content (1/20, 5%).

Patient	Sex	AO	Consanguinity	Clinical Features	Muscle Studies Histochemistry	RC Deficiency
21	M	Birth	+	Progressively worsening muscle weakness, gastrointestinal reflux, Trisomy 21 (Down's Syndrome), mild cerebellar atrophy and thinning of corpus callosum on brain MRI	COX-deficient fibres, lipid accumulation	I, IV
22†	F	8 Months	-	Developmental regression, cerebellar atrophy on brain MRI, limb spasticity, sunken eyes, thick spindle fingers, elbow and shoulder contractures, increased lower limb tone, absent reflexes, scoliosis	Fibre atrophy, secondary myopathic changes, denervation atrophy	I, IV
23	M	Day 2	-	Hypertonia, profound head lag, bulbar weakness, recurrent apnoeic episodes, desaturation, bradycardia	n.d.	IV
24†	M	Birth	-	Metabolic acidosis, renal impairment, developmental delay, hearing impairment, Wolf Parkinson White Syndrome, seizures, denate nucleus abnormalities on brain MRI	Mosaic COX-deficient fibres	I, IV
25	F	Childhood	-	Motor and speech delay, spastic jerky ataxia, pulmonary hypertension	Occasional transitional or intermediate COX-activity	I, IV
26†	M	Birth	-	Congenital lactic acidosis	Global COX-deficiency, lipid accumulation, neonatal myosin positive fibres	I, III, IV
27†	M	4 Weeks	+	HCM, lactic acidosis, FTT, Leigh-like changes on brain MRI	Global COX-deficiency	I, IV
28†	M	Birth	-	IUGR, severe lactic acidosis, renal impairment, hypotonia, central apnoea, anaemia, liver disease (coagulopathy), dysmorphic (smooth philtrum, hypospadias), VSD, PDA, lissencephaly	Normal	I, IV

Patient	Sex	AO	Consanguinity	Clinical Features	Muscle Studies Histochemistry	RC Deficiency
29†	F	Day 7	-	Leigh-like syndrome, hypotonia, lactic acidosis, apnoeic episodes, encephalopathy, liver dysfunction, hypogenesis of the corpus callosum, bulbus, nucleus, cerebellum and subthalmic lesions on brain MRI	COX-deficient fibres Ragged red fibres	I, IV
30†	M	Birth	-	HCM, severe myopathy, lactic acidosis, respiratory failure	COX-deficient fibres, lipid accumulation	I, III, IV
31	F	Birth	+	Liver failure (hepatomegaly), hypotonia, growth retardation, FTT, abnormal ECG (details unknown), possible high signal in globus pallidus	Normal	I, II
32	F	6 Months	-	HCM, lactic acidosis, myopathy (exercise intolerance), developmental delay, seizures, short stature	Global COX-deficiency	I, IV
33†	M	Birth	-	Persistent acidosis, developmental delay and regression, 3-methylglutatonic and methylglutaric acidosis, constipation, gastroenteritis, vomiting, FTT	Increased fat content	I, III, IV
34†	M	2 Weeks	+	Congenital lactic acidosis	n.d.	IV
35	M	Neonatal	-	Congenital lactic acidosis, muscle weakness, Leigh-like changes on brain MRI	Increased SDH activity	I, IV
36†	M	Infantile	+	Congenital lactic acidosis, HCM, encephalopathy	Global COX-deficiency	IV
37	M	1 Week	+	Encephalopathy, congenital lactic acidosis, liver disease, FTT, basal ganglia injury on brain MRI	Global COX-deficiency	I, III, IV
38	F	n.d.	-	Myopathy, lactic acidosis	n.d.	I
39†	M	2 Years	-	HCM	n.d.	I
40	M	n.d.	-	Seizures, developmental delay, hypotonia, choreoathetoid movement disorder, ptosis, FTT, hepatomegaly, periventricular leukomalacia, white	Normal	Normal

Patient	Sex	AO	Consanguinity	Clinical Features	Muscle Studies Histochemistry	RC Deficiency
				matter loss, thinning of corpus callosum on brain MRI		

Table 6.2 Clinical and Molecular Features of the Mitochondrial RC Deficiency Cohort for WES. AO – Age of onset; ECG – echocardiogram; FTT – failure to thrive; HCM – hypertrophic cardiomyopathy; IUGR - intrauterine growth restriction; PDA - patent ductus arteriosus; VSD - ventricular septic defect. † - deceased; * - '+' – yes; '-' – no. 'n.a.' – not applicable; 'n.d.' – not determined.

WES Read Coverage and Depth Statistics

WES read coverage and depth statistics of the mitochondrial RC deficiency cohort were calculated for 32,947,520 exome consensus coding sequence (CCDS) bases (bp) (**Appendix O**). The mean depth per exome consensus coding sequence (CCDS) bases was 69-fold. The mean percentage of CCDS bases at 20-fold coverage was 78.69%.

6.4.2 WES Analysis

Using the simplified scoring system devised to advise the prioritisation of candidates, causative or likely causative variants and VUS are listed in **Table 6.3** with MAF data and *in silico* predictions. Causative (7/20, 35%) and likely causative variants (2/20, 10%) were identified in 9 patients, providing a diagnostic yield of 45%. VUS were also identified in two patients.

Causative mutations were identified in six nuclear genes previously associated with mitochondrial RC deficiency for six patients (24, 27-31 and 34) in *MRPS22* (NM_020191), *PDHA1* (NM_001173454), *EARS2* (NM_001083614), *AARS2* (NM_020745), *TRMU* (NM_018006) and *SCO1* (NM_004589.3). *GTPBP3* (NM_001195422) was confirmed as the seventh nuclear gene associated with RC deficiency following the identification of additional patients. Of these, 10 variants were identified of which eight were novel or had not been previously reported as causative. Three patients had homozygous variants, one patient had a hemizygous (X-linked) variant and the remaining three patients had compound heterozygous variants. All variants were confirmed by Sanger sequencing using custom or diagnostic forward and reverse primers. Where possible, segregation studies confirmed parental or familial carrier status. After evaluation and confirmation, variants were diagnostically reported.

Likely causative variants were identified in two patients (21 and 22) in *PTPIP51* (NM_018145.1) and *CTBP1* (NM_001012614.1). However, these variants require additional validation including segregation and molecular studies to confirm pathogenicity.

VUS were identified in two patients (32 and 35) in *MTO1* (NM_012123.3) and *LONP1* (NM_004793). The candidate genes were previously associated with Mendelian mitochondrial disease but pathogenicity was uncertain due to the genotype and unusual phenotype compared to previously reported patients.

Analysis of 9 patients (23, 26, 32, 33 and 36-40) did not identify causative or likely causative variants. VUS were listed for these patients (**Appendix P**) but the functional or potential pathological role in human disease is currently uncertain.

To attain a genetic diagnosis, patient 33 genomic DNA was prepared and sent for WGS to the Human Genome Sequencing Center at the Baylor College of Medicine in Houston, Texas. At the time of writing, the dataset from WGS was not available for filtering and analysis.

Patient	Clinical Features	Gene	Mutations		Minor Allele Frequency			*In Silico* Predictions		
			cDNA Change	Amino Acid Change	ExAC	NHLBI ESP	1000G	PolyPhen2	Align-GVGD	SIFT
Causative Variants										
24†	Metabolic acidosis, renal impairment, developmental delay, hearing impairment, Wolf Parkinson White Syndrome, seizures, denate nucleus abnormalities on brain MRI	*MRPS22*	Homozygous c.509G>A	Homozygous p.Arg170His (rs119478059)	0.00005795	0.0003488	Ø	1.000	Class 25	0.00
27†,[a]	HCM, lactic acidosis, FTT, Leigh-like changes on brain MRI	*GTPBP3*	Homozygous c.424G>A	Homozygous p.Glu142Lys	Ø	Ø	Ø	1.000	Class 15	0.02
28†	IUGR, severe lactic acidosis, renal impairment, hypotonia, central apnoea, anaemia, liver disease (coagulopathy), dysmorphic (smooth philtrum, hypospadias), VSD, PDA, lissencephaly	*PDHA1*	Hemizygous c.1033_1035dup	Hemizygous p.Glu345dup	Ø	Ø	Ø	n.a.	n.a.	n.a.
29†,[b]	Leigh-like syndrome, hypotonia, lactic acidosis, apnoeic episodes, encephalopathy, liver dysfunction, hypogenesis of the corpus callosum, bulbus, nucleus, cerebellum and subthalmic lesions on brain MRI	*EARS2*	c.1A>G c.184A>T	p.Met1? p.Ile62Phe	Ø Ø	Ø Ø	Ø Ø	n.a. 0.006	n.a. Class 0	n.a. 0.04

30†	HCM, severe myopathy, lactic acidosis, respiratory failure	*AARS2*	c.1088dupT c.1738C>T	p.Asp337* p.Arg580Trp	0.00000851 0.00002474	Ø Ø	Ø Ø	n.a. 0.996	n.a. Class 0	n.a. 0.02
31	Liver failure (hepatomegaly), hypotonia, growth retardation, FTT, abnormal ECG (details unknown), possible high signal in globus pallidus	*TRMU*	c.747_752del c.827C>T	p.Asp249_Lys251delinsGlu p.Pro276Leu	Ø 0.00000825	Ø Ø	Ø Ø	n.a. 0.879	n.a. Class 35	n.a. 0.02
34†	Congenital lactic acidosis	*SCO1*	Homozygous c.881T>C	Homozygous p.Met294Thr	Ø	Ø	Ø	0.989	Class 25	0.04
Likely Causative Variants										
21	Progressively worsening muscle weakness, gastrointestinal reflux, Trisomy 21 (Down's Syndrome), mild cerebellar atrophy and thinning of corpus callosum on brain MRI	*PTPIP51*	Homozygous c.1352C>T	Homozygous p.Thr451Met	0.00004942	Ø	0.000197	1.000	Class 0	0.02
22†	Developmental regression, cerebellar atrophy on brain MRI, limb spasticity, sunken eyes, thick spindle fingers, elbow and shoulder contractures, increased lower limb tone, absent reflexes, scoliosis	*CTBP1*	c.991C>T/=	p.Arg331Trp, Heterozygous	Ø	Ø	Ø	0.996	Class 15	0.03
Variants of Unknown Significance (VUS)										

32	HCM, lactic acidosis, myopathy (exercise intolerance), developmental delay, seizures, short stature	*MTO1*	c.292G>A/=	p.Gly98Ser, Heterozygous	Ø	Ø	Ø	1.000	Class 55	0.00
35	Congenital lactic acidosis, muscle weakness, Leigh-like changes on brain MRI	*LONP1*	c.1693T>C c.2197G>A	p.Tyr565His (rs144125085) p.Glu733Lys	0.00000844 0.00001671	Ø Ø	Ø Ø	0.997 0.006	Class 0 Class 0	0.00 0.36

Table 6.3 Causative, Likely Causative Variants and VUS Identified in the Mitochondrial RC Cohort. Prioritised variants following application of the WES filtering strategy. FTT – failure to thrive; HCM – hypertrophic cardiomyopathy; IUGR - intrauterine growth restriction; PDA - patent ductus arteriosus; VSD - ventricular septic defect. 'Ø' denotes that a variant was absent from external databases. n.a. – not applicable. [a]Published in (Kopajtich *et al.*, 2014). [b]Published in (Oliveira and Sommerville, 2016).

6.4.3 *MRPS22* – Mitochondrial Ribosomal Protein S22

Patient 24 was a boy born to non-consanguineous parents who required peritoneal dialysis to treat severe metabolic acidosis shortly after birth. He manifested with Wolf-Parkinson-White syndrome, seizures, developmental delay, severe hearing loss and renal involvement. Erythropoietin treatment was given for renal anaemia but he developed end-stage renal failure. Muscle biochemical studies revealed mild complex I and IV deficiency (**Figure 6.2A**). Muscle histochemistry revealed mosaic COX-deficient fibres. RC activity was normal in fibroblasts. Family history was significant; an affected female sibling died 3 days after birth from severe metabolic acidosis and the mother had three earlier miscarriages.

WES filtering identified a homozygous c.509G>A, p.Arg170His (rs119478059) missense variant in *MRPS22* encoding Mitochondrial Ribosomal Protein S22. *MRPS22* (3q23) matched the GO-Term 'mitochondr*'. Sanger sequencing using custom forward and reverse primers for *MRPS22* exon 4 confirmed the variant in both patient 24 and his affected sibling. The p.Arg170His missense change was absent from in-house exomes, but was present in 7/120796 non-Finnish European alleles (MAF=0.00005795) in ExAC and 3/13006 alleles (MAF=0.00023007) in the NHLBI ESP, all in heterozygous state. Arg170 was fully conserved in all tested species and occurred in a highly conserved region of the protein (**Figure 6.2B**).

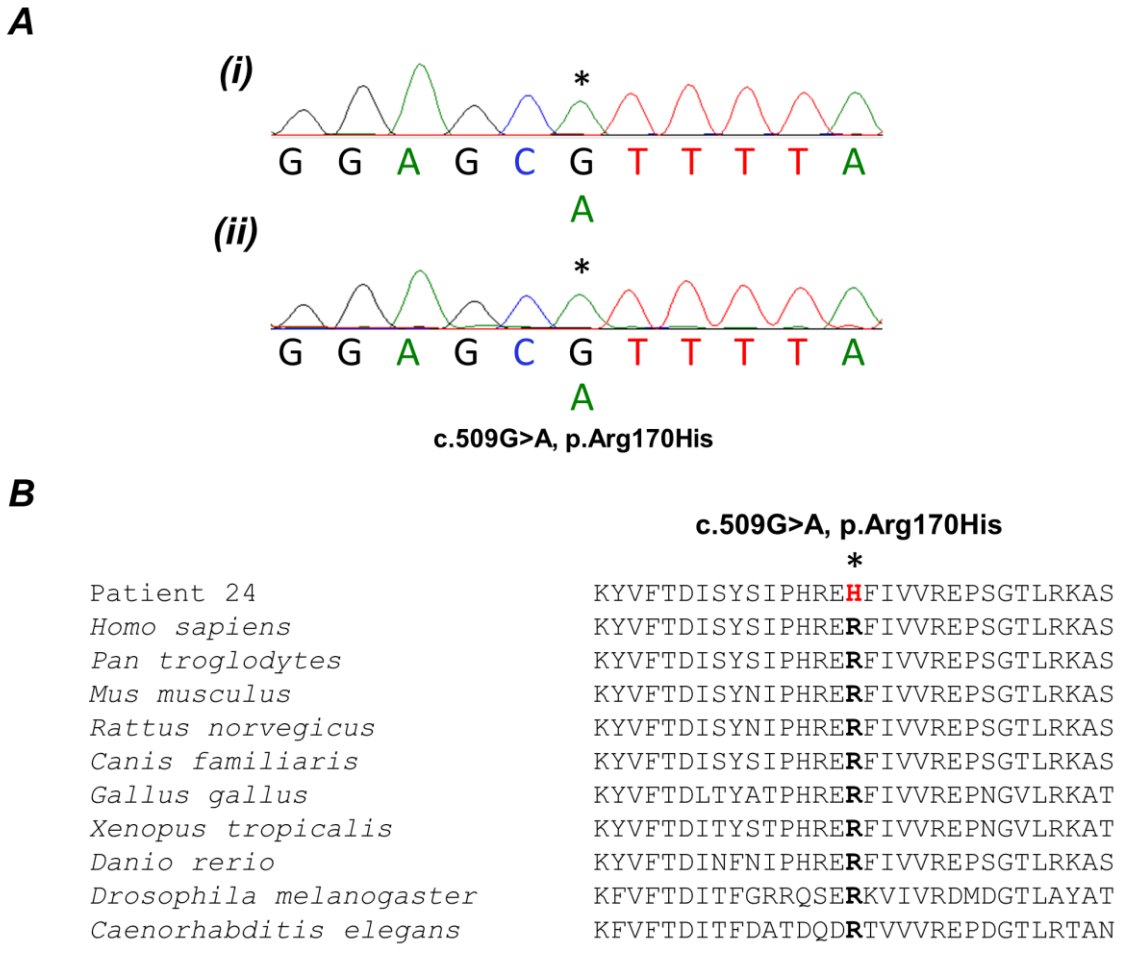
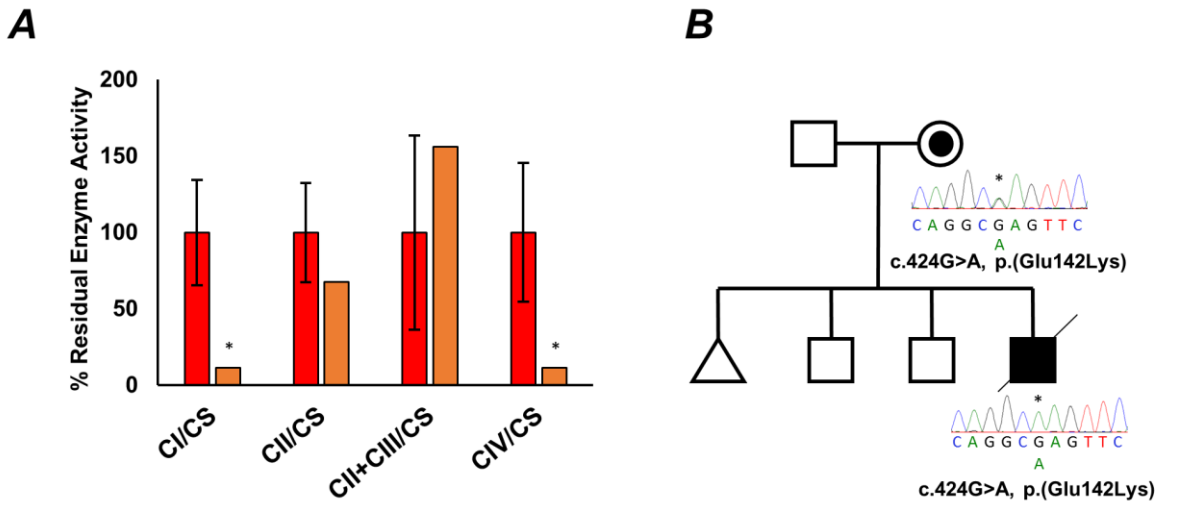


Figure 6.2 Sanger Sequencing Confirmation and MSA of the Homozygous *MRPS22* Mutation in Patient 24. (*A*) Sanger sequencing confirmation of the homozygous p.Arg170His *MRPS22* mutation in (*i*) patient 24 and (*ii*) the affected female sibling. (*B*) MSA of the MRPS22 Arg170 residue.

6.4.4 *GTPBP3* - GTP Binding Protein 3

Patient 27 was a boy born prematurely at 34 weeks gestation to apparently non-consanguineous Romanian parents. At day 25 of life, he was admitted to hospital with weight-loss, hypothermia, jaundice and metabolic acidosis. Due to presumed sepsis a course of intravenous antibiotics (details not known) was prescribed. Blood serum lactate was also elevated (11.0mmol/L; normal <2.5mmol/L). Echocardiography revealed concentric left ventricular hypertrophy. Cerebrospinal fluid (CSF) lactate was also markedly elevated (12.4mmol/L; normal 0.9-2.4mmol/L). On these findings, bicarbonate treatment was given. Brain MRI showed an abnormal T2 signal in the midbrain and basal ganglia bilaterally, plus diffusion abnormalities of the subthalamic nuclei extending down to the brain stem. There were no dysmorphic features but the patient was thin, jaundiced and had swollen feet. Patient 27 was fed through a nasogastric feeding tube but was not responsive despite a high calorie

intake. At 5 weeks old he presented apnoeic episodes and subsequently died. Muscle biochemical studies revealed complex I (12%) and IV (12%) deficiency compared to controls (**Figure 6.3A**). Patient 27 was the fourth pregnancy and the third child born. The first pregnancy ended in miscarriage, while two older male siblings were unaffected.



C

	c.424G>A, p.Glu142Lys *
Patient 27	P------GLRPAEAG**K**FTRRAFANGKLNLTE
Homo sapiens	P------GLRPAEAG**E**FTRRAFANGKLNLTE
Macaca mulatta	P------GLRPAEAG**E**FTRRAFANGKLNLTE
Rattus norvegicus	P------GLRPAKAG**E**FTRRAFAHGKLSLTE
Mus musculus	P------GLRPAEAG**E**FTRRAFAHGKLSLTE
Canis familiaris	P------GLRPAEAG**E**FTRRAFAHGKLSLTE
Xenopus tropicalis	Q------MLRPAEPG**E**FTKRAFQNGKLDLTE
Drosophila melanogaster	D------GLRPAEPG**E**FTKRAFFGGKLDLTE
Caenorhabditis elegans	Q------NVREAKRG**E**FTRRAFHNGKLSISE
Saccharomyces cerevisiae	HDRSSGKDIRFALPGDFSRRAFQNGKFDLTQ

Figure 6.3 Biochemical and Genetic Features of Patient 27. Measurements of mitochondrial RC activities normalised to citrate synthase (CS) of complex I (CI/CS), complex II (CII/CS), CII and CIII (CII+CIII/CS) and CIV (CIV/CS) from patient 27 (orange) compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Family pedigree and Sanger sequencing confirmation of the homozygous p.Glu142Lys *GTPBP3* mutation. (*C*) MSA of the GTPBP3 Glu142 residue.

WES filtering revealed a homozygous c.424G>A (p.Glu142Lys) missense variant of *GTPBP3* encoding GTP Binding Protein 3. *GTPBP3* (19p13.11) matched the GO-Terms 'mitochondr*' and 'tRNA'. The variant was absent from in-house exomes and external databases. Sanger sequencing with custom forward and reverse primers for *GTPBP3* exons 4+5 confirmed that patient 27 was homozygous for the novel p.Glu142Lys missense change, while the mother

was a heterozygous carrier (**Figure 6.3B**). Paternal DNA was not available but due to the maternal carrier status, it was expected that the father was also a heterozygous carrier. Glu142 was conserved in all species except *Saccharomyces cerevisiae* (**Figure 6.3C**).

At the time of identification, a further 10 patients from eight pedigrees with autosomal recessive *GTPBP3* mutations from diagnostic centres in Germany, Belgium, Italy, France, Israel and Japan were identified independently of this study. Mutations were identified using WES for 9 patients, while targeted sequencing found mutations in the tenth patient. All patients presented within the first decade of life, although usually within the first year of life, with a core phenotype of HCM, encephalopathy and lactic acidosis. However, two patients apparently did not have HCM. Six patients died before the age of one, while almost all patients who survived beyond the first year of life had delayed development or intellectual disability. Including patient 27, brain MRI in four patients showed signal abnormalities in the brainstem and basal ganglia comparable with Leigh syndrome. Combined complex I and IV deficiency were found in skeletal muscle of eight patients; one patient had normal RC activity. Muscle histochemistry also revealed COX-deficient fibres in all tested patients. A total of 13 different *GTPBP3* variants were identified, including missense, in-frame and frameshift variants. Only patient 27 harboured the p.Glu142Lys variant.

Following the identification of *GTPBP3* mutations as a novel disorder of mitochondrial tRNA modification, a second patient presenting severe lactic acidosis and HCM with multiple OXPHOS defects was referred to the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory in Newcastle upon Tyne. All 9 *GTPBP3* coding exons were sequenced with custom forward and reverse primers in this patient, but no causative or likely causative variants were identified.

6.4.5 *PDHA1* - Pyruvate Dehydrogenase E1 Subunit

Patient 23 was a boy born at 32 weeks gestation to non-consanguineous parents. Antenatal ultrasound showed intrauterine growth restriction (IUGR), echogenic foetal bowel and decreased movements. The patient presented from birth with severe lactic acidosis, renal impairment, hypotonia, central apnoea, anemia, liver dysfunction (coagulopathy) and died at 2 days old. He was dysmorphic with a smooth philtrum and hypospadias. He had a ventricular septic defect (VSD) and patent ductus arteriosus (PDA) resulting in congestive heart failure. He also had lissencephaly and grey matter abnormalities of the brain. Serum lactate was also significantly elevated (22.9mmol/L, normal <2.5mmol/L). He was an only child. However,

there was apparently a family history of cardiomyopathy; his maternal grandmother was affected. However, it was not known if this was HCM or dilated cardiomyopathy. Muscle biochemistry revealed mild complex I and IV deficiency (**Figure 6.4A**). Quantitative real-time PCR assay also detected severe mtDNA depletion. Targeted screening of *NDUFS1*, *NDUFS*2, *NDUFS*3, *NDUFS*4, *NDUFS*7, *NDUFS*8, *NDUFAB1*, *NDUFV1*, *NDUFV2*, *TWNK*, *POLG*, *RRM2B*, *TK2*, *DGUOK* and *MPV17* were negative for causative mutations.

WES filtering revealed a hemizygous in-frame c.1033_1035dup (p.Glu345dup) duplication of *PDHA1* encoding the Pyruvate Dehydrogenase E1-α Subunit. *PDHA1* (Xp22.12) matched the GO-Term 'mitochondr*'. The variant was absent from in-house exomes and external databases. Sanger sequencing with custom forward and reverse primers for *PDHA1* exon 12 confirmed the in-frame duplication. Segregation studies showed that the variant was absent from both parents (**Figure 6.4B**), although possible maternal germline mosaicism could not be excluded. Additionally, comparative genomic hybridisation (CGH) array had not been performed, in light of the dysmorphic features.

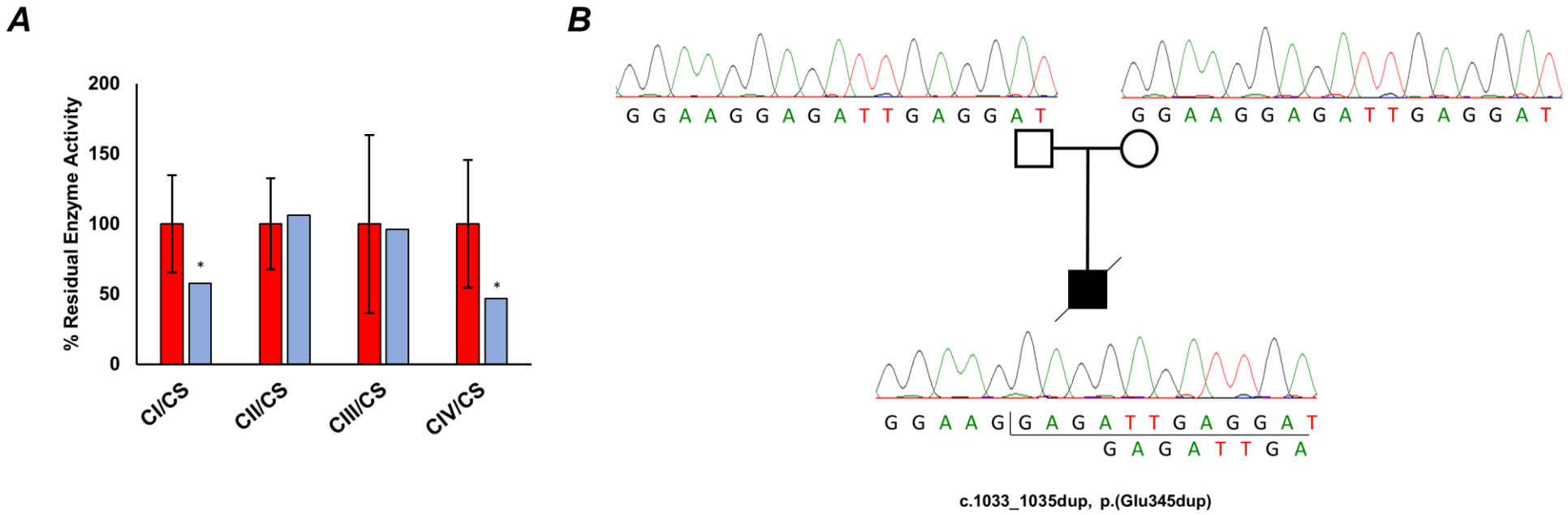


Figure 6.4 Biochemical and Genetic Features of Patient 23. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase of complex I (CI/CS), complex II (CII/CS), complex III (CIII/CS) and complex IV (CIV/CS) from patient 23 (blue) skeletal muscle compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Family pedigree and Sanger sequencing confirmation of the *de novo* p.Glu345dup *PDHA1* duplication.

6.4.6 *EARS2* – Mitochondrial Glutamyl tRNA Synthetase

Application of the WES strategy for patient 29 revealed biallelic variants of *EARS2* encoding mitochondrial glutamyl-tRNA synthetase (mt-GluRS). The clinical, genetic and molecular features of patient 29 are outlined in **7.4.2** together with additional patients harbouring mutations of the mt-aaRS.

6.4.7 *AARS2* – Mitochondrial Alanyl tRNA Synthetase

Application of the WES strategy for patient 30 revealed biallelic variants of *AARS2* encoding mitochondrial alanyl-tRNA synthetase (mt-AlaRS). The clinical, genetic and molecular features of patient 30 are outlined in **7.4.1**, together with additional patients harbouring mutations of the mt-aaRS.

6.4.8 *TRMU* - tRNA 5-Methylaminomethyl-2-Thiouridylate Methyltransferase

Patient 31 was a girl born to consanguineous parents who presented from birth with significant liver failure, FTT, hypotonia and had growth retardation. Echocardiogram (ECG) was abnormal but no details were given. Brain MRI revealed high signal in the globus pallidus. Serum lactate was elevated (4.4mmol/L; normal <2.5mmol/L), as was CSF lactate (4.4mmol/L; normal 0.9-2.4mmol/L). At 18 months old, the overseeing clinician noted a remarkable recovery in weight and satisfactory developmental progress. Muscle histochemistry was normal, but biochemical studies revealed a severe complex II (4%) and mild complex I (40%) defect compared to controls (**Figure 6.5A**). Targeted screening of *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF1* and *SDHAF2* were negative for causative mutations.

WES filtering identified compound heterozygous variants in *TRMU* encoding tRNA 5-Methylaminomethyl-2-Thiouridylate Methyltransferase (MTU1); a frameshift c.747_752del (p.Asp249_Lys251delinsGlu) indel and a c.827C>T (p.Pro276Leu) missense variant. *TRMU* (22q13.31) matched the GO-Terms ‘mitochondr*’ and ‘tRNA’. Sanger sequencing with diagnostic forward and reverse primers for *TRMU* exons 6+7 and 8 confirmed both variants (**Figure 6.5B**). Segregation studies were not possible. The p.Asp249_Lys251delinsGlu variant was absent from in-house exomes and external databases. The p.Pro276Leu missense change was also absent from in-house exomes, but had been reported in 1/121172 heterozygous South Asian allele (MAF= 0.000008253) in ExAC. Pro276 was moderately conserved and occurred in a moderately conserved region of the protein (**Figure 6.5C**).

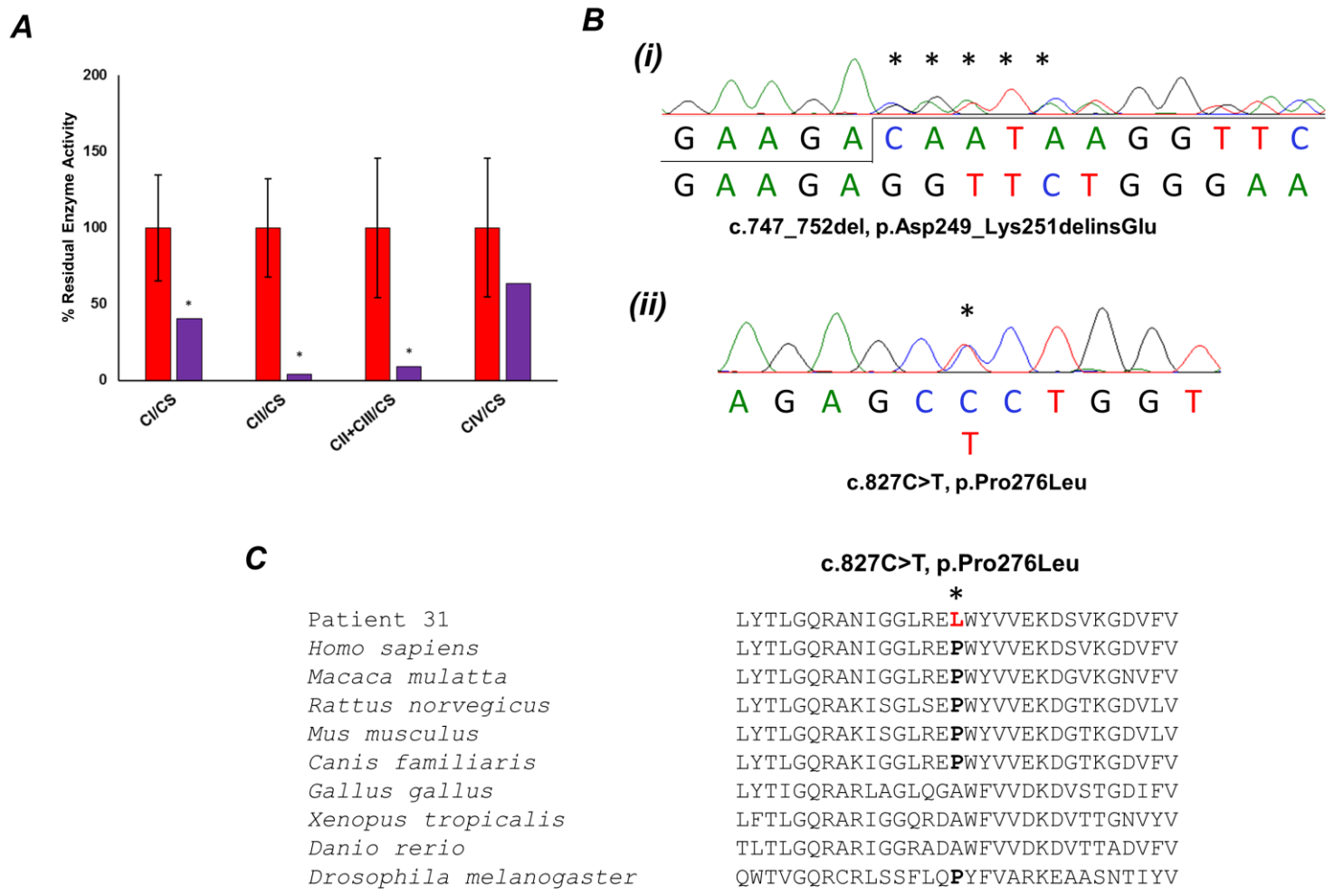


Figure 6.5 Biochemical and Genetic Features of Patient 31. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase (CS) of complex I (CI/CS), complex II (CII/CS), complex II and complex III (CII+CIII/CS) and complex IV (CIV/CS) from patient 31 (purple) skeletal muscle compared to controls (red). Complex I, II and II+III defects are denoted with an asterisk ('*'). (*B*) Sanger sequencing confirmation of *TRMU* variants (*i*) p.Asp249_Lys251delinsGlu and (*ii*) p.Pro276Leu. (*C*) MSA of the MTU1 Pro276 residue.

6.4.9 *SCO1* - SCO1 Cytochrome *C* Oxidase Assembly Protein

Patient 34 was a boy born to consanguineous British-Pakistani parents who had severe early-onset lactic acidemia and died at 2 weeks old. Muscle biochemical studies showed isolated COX deficiency. There was a significant history of mitochondrial disease in additional branches of this highly consanguineous family. Previous WES and diagnostic targeted screening identified two affected members of one branch who harboured a homozygous c.1349G>C (p.*450Serext*32) (rs115079861) extension variant of *RMND1* (NM_017909) encoding Required for Meiotic Nuclear Division Protein 1, which may be essential for assembly or stability of the mitoribosome (Janer *et al.*, 2012; Janer *et al.*, 2015). In another branch, one affected member was homozygous for a c.431A>G (p.Tyr144Cys) (rs397514610) missense change in *FARS2* (NM_006567) encoding mitochondrial phenyl-tRNA synthetase (mt-PheRS), which aminoacylates phenylalanine with its cognate mitochondrial $tRNA^{Phe}$ for mitochondrial protein synthesis. Patient 34 was neither homozygous nor heterozygous for either causative *RMND1* or *FARS2* variant.

WES filtering for patient 34 revealed a homozygous c.881T>C (p.Met294Thr) missense variant in *SCO1* encoding SCO1 Cytochrome *c* Oxidase Assembly Protein. *SCO1* (17p13.1) matched the GO-Term 'mitochondr*'. Sanger sequencing using custom forward and reverse primers for *SCO1* exon 6 confirmed the missense change (**Figure 6.6A**). The parents of patient 34 were also diagnostically confirmed as heterozygous carriers. The p.Met294Thr missense change was absent from in-house exomes including affected family members who underwent WES or diagnostic targeted screening and were homozygous for either the *RMND1* or *FARS2* variant. The missense variant was also absent from external databases. Met294 was highly conserved except in *Caenorhabditis elegans* and *S. cerevisiae*, occurring in a moderately conserved region of the protein (**Figure 6.6B**).

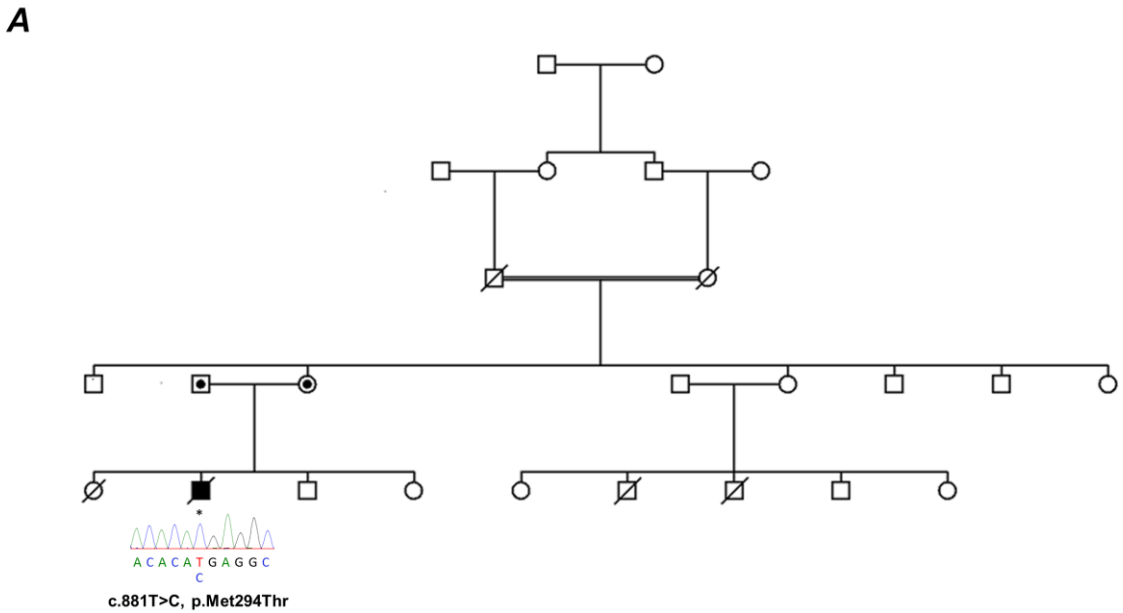
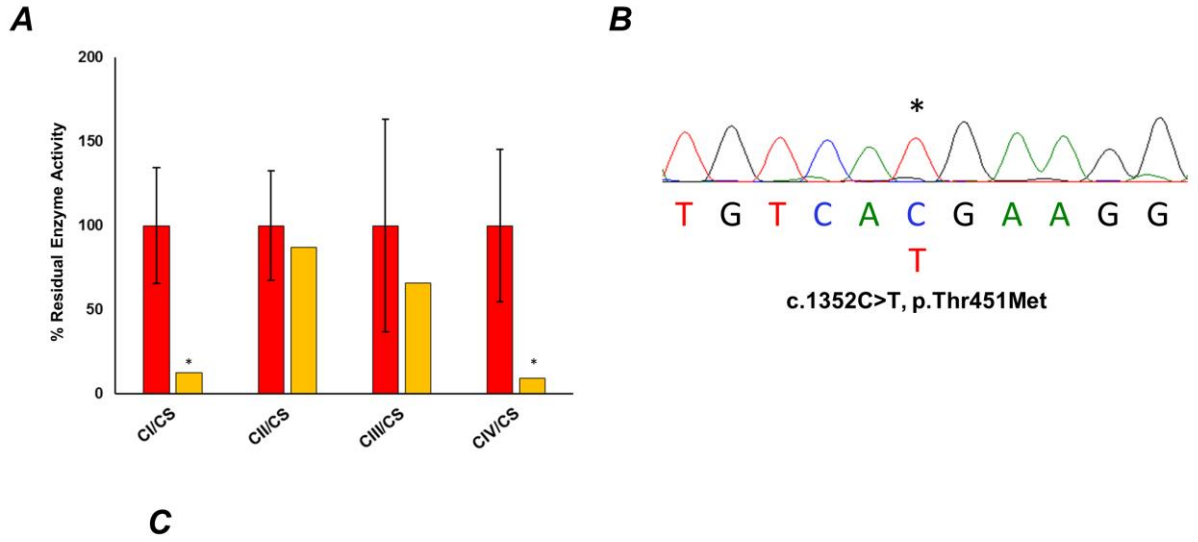


Figure 6.6 Family Pedigree for Patient 34, Sanger Sequencing Confirmation and MSA of the Homozygous *SCO1* Mutation. (*A*) Family pedigree and Sanger sequencing confirmation of the homozygous p.Met294Thr *SCO1* mutation in patient 34. (*B*) MSA of the SCO1 Met294 residue.

6.4.10 *PTPIP51* - Protein Tyrosine Phosphatase-Interacting Protein 51

Patient 21 was a boy born to consanguineous parents who had Trisomy 21 (Down's syndrome), was floppy from birth and had progressively worsening muscle weakness. Following respiratory infection and subsequent sepsis at 3 weeks old, he became continuous positive airway pressure (CPAP) dependent. He also suffered from severe gastrointestinal reflux, had poor diaphragmatic movement and some distal antigravity movement of the distal limbs. Brain MRI revealed mild cerebellar atrophy and thinning of the corpus callosum consistent with Down's syndrome. Muscle histochemistry revealed COX-deficient fibres and lipid accumulation. Muscle biochemical studies showed severe complex I and IV defect (**Figure 6.7A**). Targeted screening of *TRMU* mutations was negative for causative mutations.



C

c.1352C>T, p.Thr451Met

```
                                  *
Patient 21                        EARWWMKLALELPDVMKEDLAIQKDLEELEV
Homo sapiens                      EARWWMKLALELPDVTKEDLAIQKDLEELEV
Pan troglodytes                   EARWWMKLALELPDVTKEDLAIQKDLEELEV
Canis familiaris                  EARQWLELALELPDITNEDSAFQKDLEELKV
Mus musculus                      EARKWMKLAQELPDVTNEDSAFQKDLEELEV
Gallus gallus                     AALLWMNLASELPVNTKEDSETERELEEMRS
Bos taurus                        EAKEWMKLALELPNVTKEDSAFQKDLEELEV
Physeter macrocephalus            EAKQWMKLALELPNVTKEDSAFQKDLEELEV
Danio rerio                       EARNWVQLALNTPAGANEDSDVAALQTELKA
Pygoscelis adeliae                AAVLWMSLASELPVNTKEDAESERELEEMRS
Xenopus tropicalis                TAAHWLKLAEELPNVTQEDRECAATIEEMLP
```

Figure 6.7 Biochemical and Genetic Features of Patient 21. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase of complex I (CI/CS), complex II (CII/CS), complex III (CIII/CS) and complex IV (CIV/CS) from patient 21 (orange) skeletal muscle compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Sanger sequencing confirmation of the homozygous p.Thr451Met *PTPIP51* missense variant. (*C*) MSA of the PTPIP51 Thr451 residue.

WES filtering revealed a homozygous c.1352C>T (p.Thr451Met) missense variant in *PTPIP51* encoding Protein Tyrosine Phosphatase-Interacting Protein 51, also referred to as Regulator Of Microtubule Dynamics 3 (RMND3). *PTPIP51* (15q15.1) matched the GO-Term 'mitochondr*'. CNV analysis showed a partial duplication of chromosome 21 (chr21:10,475,517-48,084,864), confirming the diagnosis of Trisomy 21. Sanger sequencing with custom forward and reverse primers for *PTPIP51* exon 12 confirmed the p.Thr451Met variant (**Figure 6.7B**). Segregation studies were not possible. The p.Thr451Met missense change was present at a MAF of 0.002392344 from in-house exomes and in 6/121412 alleles (MAF=0.00004942) in ExAC, comprising five South Asians and one non-Finnish European, all in heterozygous state. Thr451 was conserved in all tested species except *Danio rerio* (**Figure 6.7C**).

Western blot analysis of patient 21 fibroblasts did not reveal changes in steady-state PTPIP51 levels compared to controls (**Figure 6.8A**). However, porin/VDAC1 was significantly increased compared to controls. Furthermore, there were no changes in steady-state OXPHOS subunits levels (**Figure 6.8B**). Skeletal muscle was not available for analysis.

TMRM staining of the mitochondrial network in patient 21 fibroblasts was also performed but this did not show elongation or fragmentation compared to controls (**Figure 6.9**).

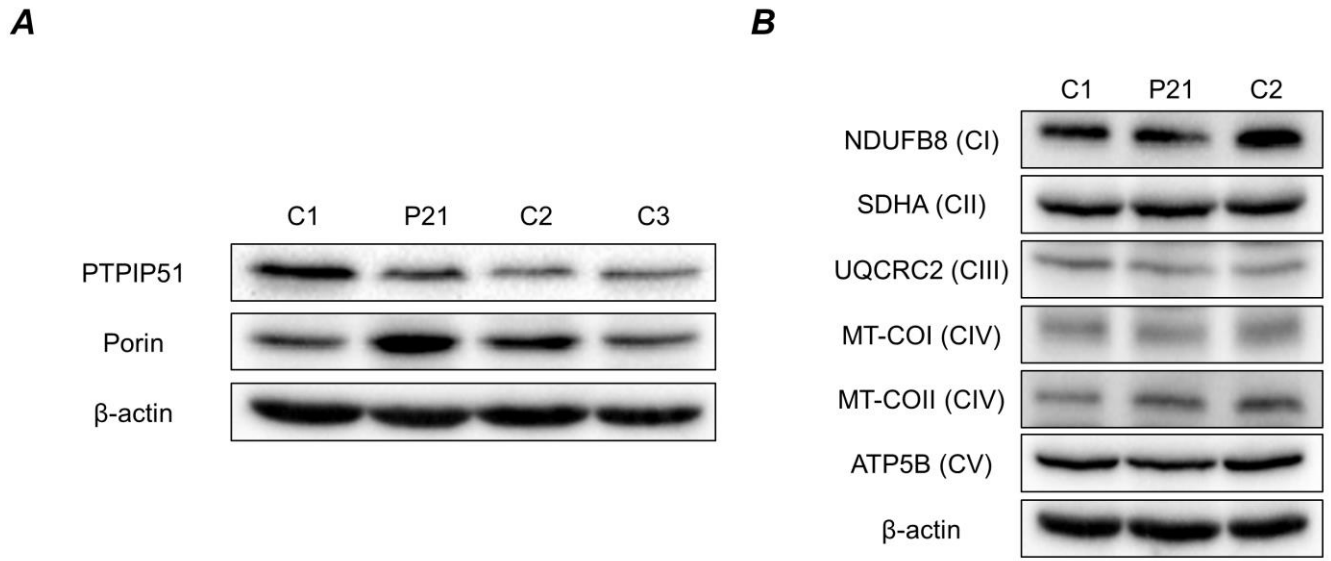


Figure 6.8 Western Blot Analysis of Patient 21 Fibroblasts. Western blot analysis of (*A*) steady-state PTPIP51 and Porin/VDAC1, plus (*B*) OXPHOS subunit protein levels in patient 21 fibroblasts.

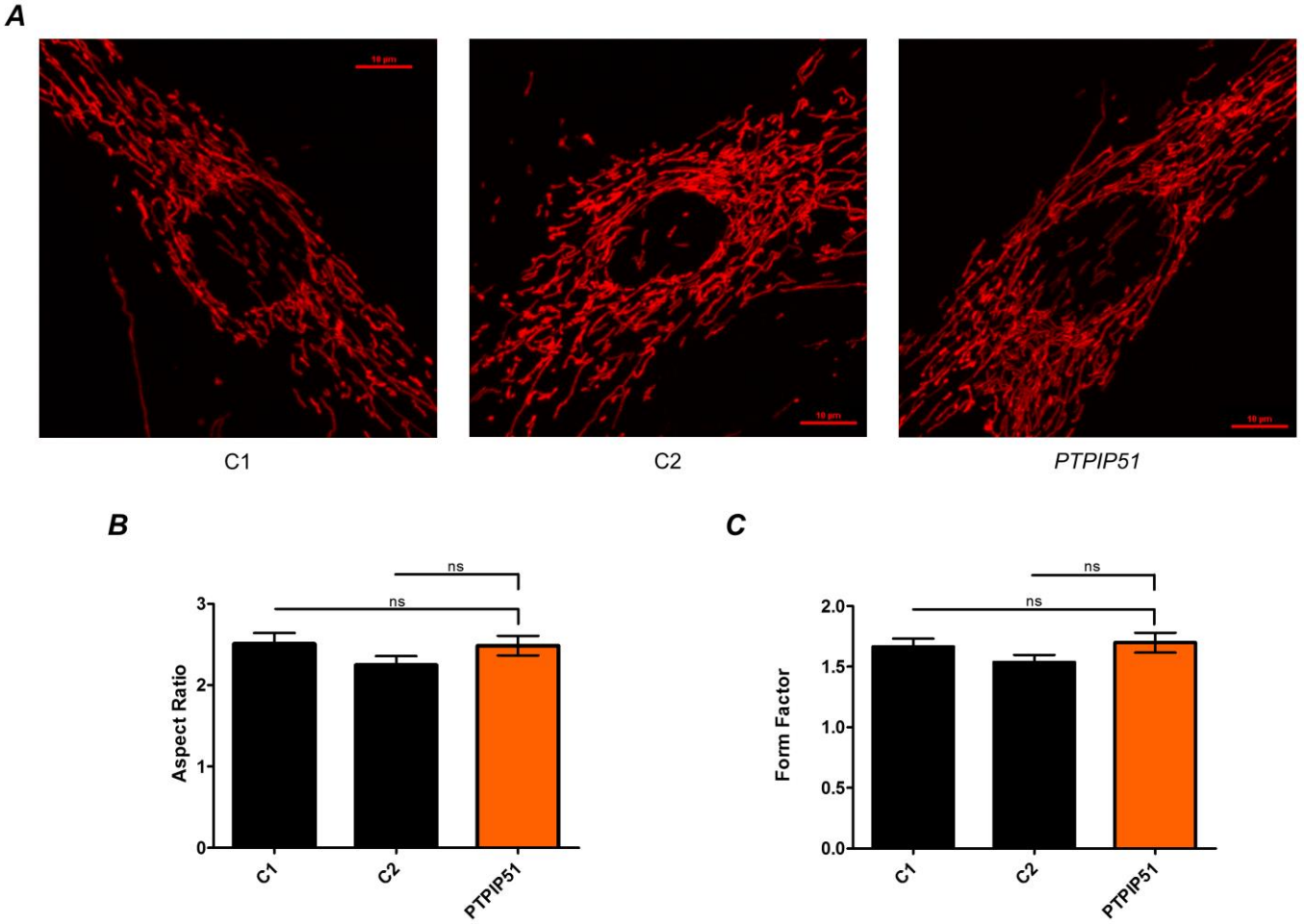


Figure 6.9 Analysis of the Mitochondrial Network in Patient 21 Fibroblasts. (*A*) TMRM staining of patient 21 fibroblasts and two controls was performed using the Nikon A1R Confocal microscope. Mitochondrial morphological features were quantified by (*B*) aspect ratio (AR) and (C) form factor (FF). Student's t test and Dunn's Multiple Comparison tests were performed to compare the median AR and FF between patient 21 fibroblasts and controls.

6.4.11 *CTBP1* – C-Terminal Binding Protein 1

Patient 22 was a girl born at term to non-consanguineous parents who presented a complex phenotype and died at 16 years old due to acute respiratory failure. Initial development was normal for the first 8 months of life but she then manifested with a delay in walking. Rapid progressive scoliosis developed by 10 years old that necessitated corrective surgery. She suffered a respiratory tract infection at 14 years old that required resuscitation, following which she was weaker, became dependent on a percutaneous endoscopic gastrostomy (PEG) tube for feeding and was noted to subsequently develop contractures of the wrists and elbows. Clinical examination showed bradykinesia, unusually cold lower limbs with raised papular skin texture, marked spinal scoliosis and increased tone at the elbows, wrists, knees and ankles. Muscle mass was generally decreased. The patient also had slightly sunken eyes and thin spindle fingers. Brain MRI at 13 years old revealed mild cerebellar and brain stem atrophy. Muscle biochemical studies at 6 years old revealed complex I and IV deficiency (**Figure 6.10A**). Muscle histopathology disclosed fibre atrophy, secondary myopathic changes and evidence of denervation atrophy. Diagnostic targeted gene screening of *PLA2G6* and *APTX* were negative for causative mutations.

WES filtering failed to identify likely autosomal recessive candidate variants and hence, single heterozygous variants were then prioritised. A rare heterozygous c.452A>G (p.Lys151Arg) *EARS2* missense change was identified. All coding and intronic regions of *EARS2* were sequenced but no second likely causative variant was identified. There were no additional heterozygous variants in nuclear genes encoding mitochondrial proteins of interest identified. Hence, all rare recessive and dominant variants were listed for future evaluation.

Beck *et al.* (2016) reported four patients with severe developmental delay, intellectual disability, hypotonia, ataxia, problems with weight gain and tooth enamel defects who all shared a *de novo* heterozygous c.991C>T (p.Arg331Trp) missense variant in *CTBP1*, encoding C-terminal binding protein 1 (CtBP1). Re-analysis of candidate variants for patient 22 identified the p.Arg331Trp *CTBP1* variant. Sanger sequencing with custom forward and reverse primers for *CTBP1* exon 9 confirmed the variant (**Figure 6.10B**). *CTBP1* (4p16.3) did not match the prioritised GO-Terms. The missense change was absent from in-house and external databases. The p.Arg331Trp variant occurred in the PLDLS C-terminal binding domain (**Figure 6.10C**), with Arg331 fully conserved in all tested species except *S. cerevisiae* (**Figure 6.10D**). Segregation studies were not possible.

Following identification of the p.Arg331Trp missense change in patient 22, all in-house WES datasets were analysed for additional *CTBP1* variants. A total of 19 rare (MAF≤0.01) were identified from all in-house exomes, including the novel p.Arg331Trp variant. It was confirmed that the p.Arg331Trp was harboured only by patient 22. Additional missense variants were evaluated but were not deemed causative for other cases.

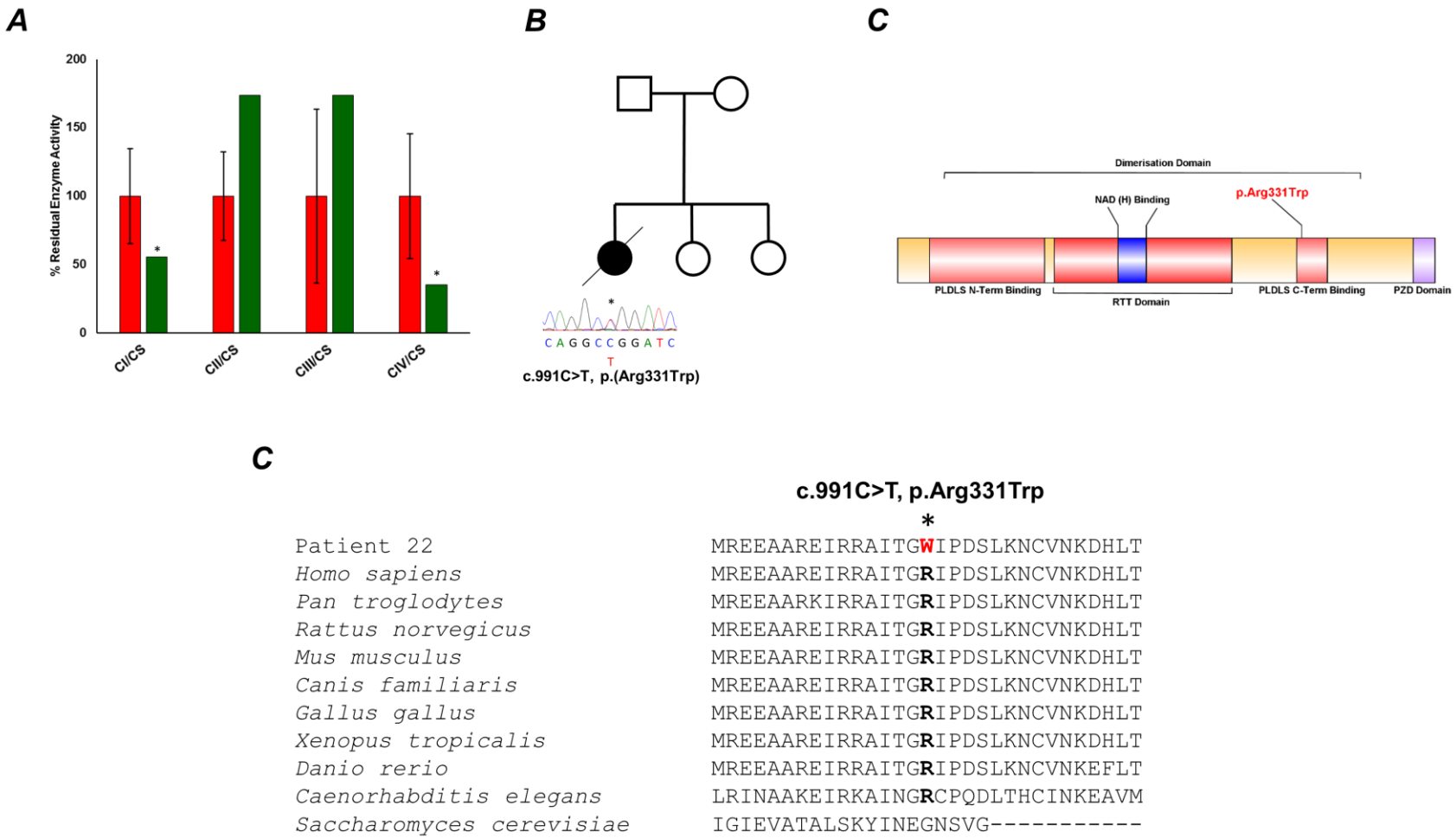


Figure 6.10 Biochemical and Genetic Features of Patient 22. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase of complex I (CI/CS), complex II (CII /CS), complex III (CIII/CS) and complex IV (CIV/CS) from patient 22 (green) skeletal muscle compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Family pedigree with sequencing confirmation of a heterozygous p.Arg331Trp missense change in patient 22. (*C*) CtBP1 domain architecture with the p.Arg331Trp variant denoted (red) in the PLDLS C-terminal binding domain. (*C*) MSA showing conservation of the CtBP1 Arg331 residue.

6.4.12 *MTO1* (VUS) – Mitochondrial tRNA Translation Optimisation 1

Patient 32 was a 35 year old female and a long-standing, unresolved case first referred to NHS Highly Specialised Service for Rare Mitochondrial Disorders in the late 1980s. From 6 months old she apparently had difficulty raising her head. At 8 years old, HCM was diagnosed. She also had exercise intolerance, persistent lactic acidosis, developmental delay, suffered from headaches and had seizures. At 10 years old, serum lactate was also elevated (3.3mmol/L; normal <2.5mmol/L). Muscle histochemistry showed striking global COX-deficiency. Muscle biochemical studies revealed severe complex I and IV defects, but also a significant increase of complex II, III and V (**Figure 6.11A**). Quantitative real time PCR of skeletal muscle DNA also showed a significant increase in mtDNA copy number. Intriguingly, her mother also reported some muscle fatigue since childhood and in her 50s reported difficulty opening her eyes during the night and occasionally in the morning. Clinical examination revealed slight jerky eye movements, but no PEO or ptosis. She had neck muscle weakness (Medical Research Council (MRC) muscle strength grade 4/5) and mild shoulder and hip weakness; she was unable to stand from a crouched position. She also had HCM. Interestingly, the proband's maternal aunt (her mother's sister) had marked PEO and ptosis but did not have muscle weakness. Histochemical studies of muscle from the proband's mother were performed externally and apparently showed COX-deficient fibres, but no specific details were available. Patient 32 also had a sister who had problems with depression but was otherwise asymptomatic.

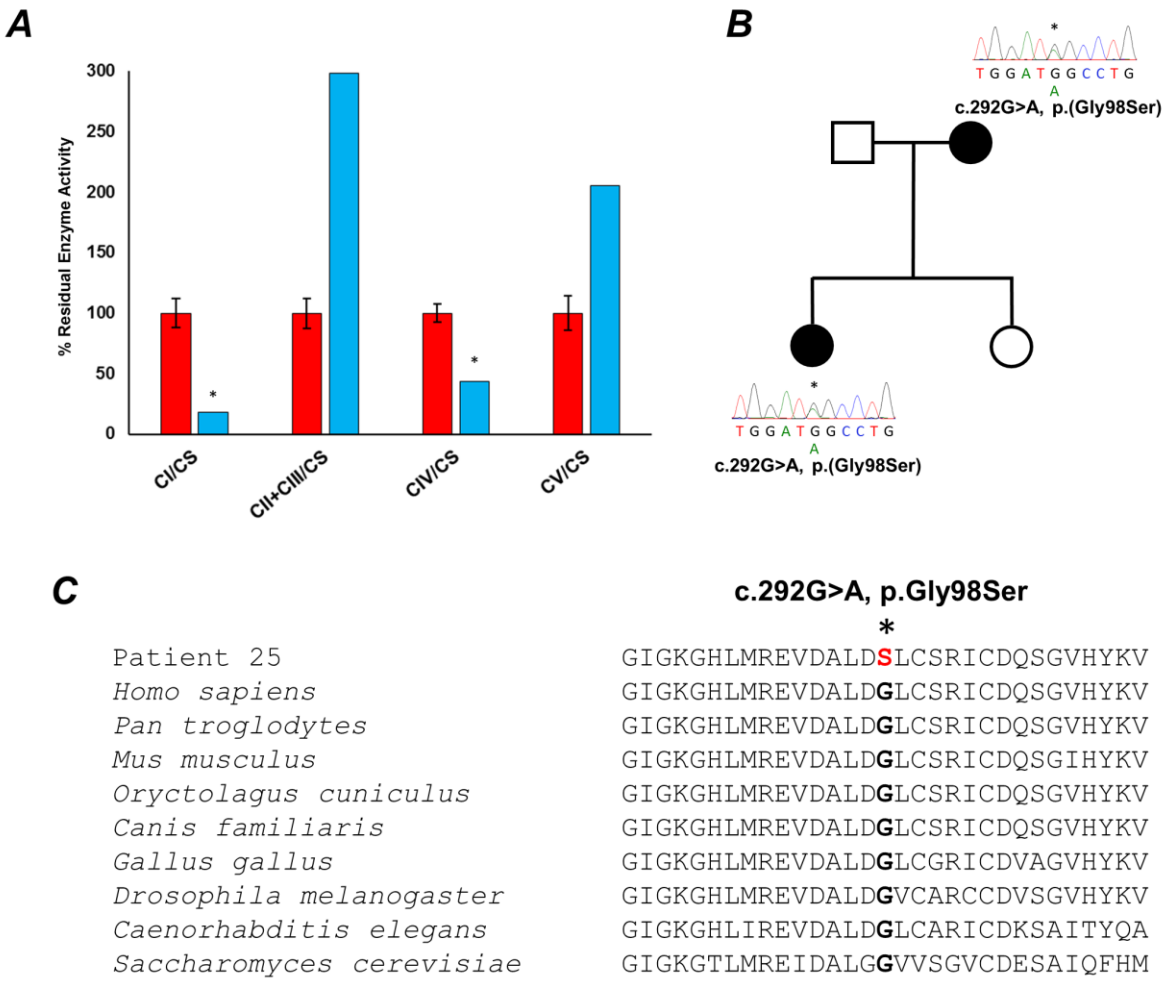


Figure 6.11 Biochemical and Genetic Features of Patient 32. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase of complex I (CI/CS), complex II+III (CII+III/CS), complex IV (CIV/CS) and complex V (CV/CS) from patient 27 (blue) skeletal muscle compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Sanger sequencing confirmation of the novel heterozygous p.Gly98Ser *MTO1* missense variant in patient 32 and her mother. (*C*) MSA of the MTO1 Gly98 residue.

Utilising the WES filtering strategy to prioritise both recessive and dominant variants, a novel heterozygous c.292G>A, p.Gly98Ser missense variant was identified in *MTO1* encoding mitochondrial tRNA translation optimisation 1. *MTO1* (6q13) matched the GO-Terms 'mitochondr*' and 'tRNA'. Using diagnostic forward and reverse primers for *MTO1* exons 2+3, the variant was confirmed in both patient 32 and her mother (**Figure 6.11B**). Genomic DNA was not available from the asymptomatic sister or father. The missense change was absent from in-house exomes and external databases. It affected a fully conserved residue in a moderately conserved region of the protein (**Figure 6.11C**). In the absence of a second causative variant the read coverage of *MTO1* was examined, revealing three coding regions with no bases at 20-fold coverage. Targeted sequencing of all 14 *MTO1* exons using

diagnostic forward and reverse primers did not reveal a second likely causative variant in either patient 32 or her mother.

6.4.13 *LONP1* (VUS) – Lon Peptidase 1

Patient 35 was a male born to non-consanguineous parents who presented from birth with congenital lactic acidosis and muscle weakness. Brain MRI showed a Leigh-like Syndrome pattern. Muscle histochemistry showed increased SDH activity but no COX-deficient fibres. Muscle biochemical studies revealed complex I and IV defects (**Figure 6.12A**). Severe mtDNA depletion was also detected by quantitative real time PCR. Targeted screening of *SLC25A4*, *MPV17*, *SUCLG1*, *SUCLA2*, *TK2* and *RRM2B* were negative for causative mutations.

WES filtering identified compound heterozygous missense variants in *LONP1* encoding Lon peptidase 1; c.1693T>C (p.Tyr565His) (rs144125085) and c.2197G>A (p.Glu733Lys). Both variants were sequenced and confirmed diagnostically. The p.Tyr565His variant was present in 1/118492 non-Finnish European allele (MAF=0.000008439) in ExAC, while the p.Glu733Lys variant was present in 2/119658 alleles (MAF=0.00001671), a South Asian and non-Finnish European in ExAC, all in heterozygous state. Both variants were absent from in-house exomes and external databases. The p.Tyr565His missense change occurred within the AAA+ ATPase domain, whereas the p.Glu733Lys variant occurred within the peptidase C-terminal domain near the boundary of the ATPase domain (**Figure 6.12B**). Tyr565 was also fully conserved in all tested species. On the other hand, Glu733 was poorly conserved, occurring in a moderately conserved region of the protein (**Figure 6.12C**). Autosomal recessive *LONP1* mutations had been previously associated with a multisystem syndrome of cerebral, ocular, dental, auricular and skeletal abnormalities, known as CODAS syndrome (Dikoglu *et al.*, 2015; Strauss *et al.*, 2015). As this did not fit the phenotype, the missense changes were classed as VUS.

At the time of writing, patient 35 and the *LONP1* missense variants were under investigation by Christie Waddington (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne).

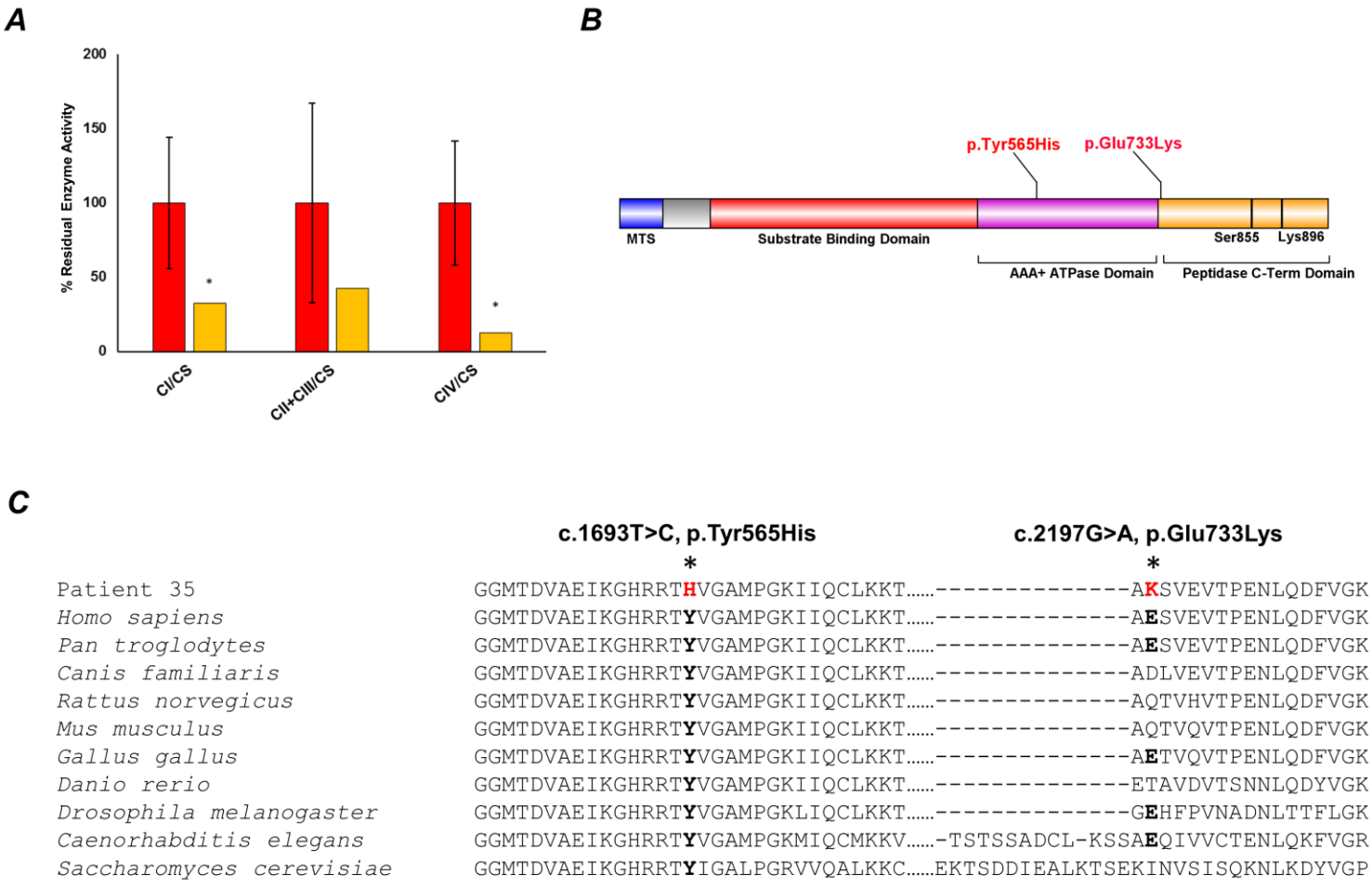


Figure 6.12 Biochemical Features of Patient 35, LONP1 Domain Architecture and MSA. (*A*) Measurements of mitochondrial RC activities normalised to citrate synthase of complex I (CI/CS), complex II+III (CII+III/CS) and complex IV (CIV/CS) from patient 35 (orange) skeletal muscle compared to controls (red). Complex I and IV defects are denoted with an asterisk ('*'). (*B*) Domain architecture of LONP1 with the p.Tyr565His and p.Glu733Lys missense changes noted (red). (*C*) MSA of LONP1 Tyr565 and Glu733Lys residues. '……' denotes the space between the regions where Tyr565 and Glu733 are located.

6.5 Discussion

Utilising WES and a custom filtering strategy to prioritise variants in nuclear gene encoded mitochondrial proteins, genetic diagnoses were attained in 7/20 (35%) patients. Additionally, 2/20 (10%) patients had likely, novel causative nuclear genes (*CTBP1*, *PTPIP51*) associated with RC deficiency. Potential significant VUS were identified in 2/20 (10%) patients (*MTO1*, *LONP1*). This is comparable to the diagnostic yield attained by Wortmann *et al.* (2015), but not as high as 60% attained by Calvo *et al.* (2012), Taylor *et al.* (2014), Pronicka *et al.* (2016) and Legati *et al.* (2016).

6.5.1 Disorders of Mitochondrial Translation

Of the seven patients confirmed genetic diagnoses attained, five had mutations in nuclear genes encoding factors in mitochondrial protein synthesis involved in mitoribosome structure (*MRPS22*), mt-tRNA aminoacylation (*EARS2*, *AARS2*) and tRNA modification (*TRMU*, *GTPBP3*). In **Chapter 7**, the two patients with *EARS2* and *AARS2* mutations plus the pathological mechanisms are described.

6.5.1.1 A Homozygous *MRPS22* Mutation Affecting Mitoribosome Assembly and Stability

MRPS22 mutations had previously been reported in five patients, including three siblings who were also homozygous for the p.Arg170His missense change identified in patient 24 (Saada *et al.*, 2007; Smits *et al.*, 2011b; Baertling *et al.*, 2015). HCM was shared in all patients but the phenotypic spectrum was broad. Three siblings reported by Saada *et al.* (2007) harbouring the homozygous p.Arg170His mutation presented with neonatal skin oedema, hypotonia and had a tubulopathy. The patient reported by Baertling *et al.* (2015) with a homozygous c.1032_1035dup (p.Leu346Asnfs*21) also presented a severe neonatal phenotype with multiple organ system failure leading to death at 3 days old. Contrastingly, the patient reported by Smits *et al.* (2011b) with a homozygous c.644T>C (p.Leu216Pro) missense variant presented with mild, Cornelia de Lange-like dysmorphic features and brain abnormalities manifesting as corpus callosum hypoplasia. Hypogenesis of the corpus callosum was also shared with the patient reported by Baertling *et al.* (2015).

MRPS22 encodes a small 28S subunit of the mitoribosome but its exact location within the structure had been unknown and it had no bacterial, eukaryotic cytoplasmic or yeast mitochondrial homolog. Recently, Amunts *et al.* (2015) and Greber *et al.* (2015) resolved the

mitoribosome structure using cryo-electron microscopy, which demonstrated S22 of the 28S subunit was located in the lower body domain on the solvent side. Both publications also modelled the p.Arg170His mutation, suggesting that it impaired assembly of the 28S subunit. Functional analysis of p.Arg170His patient fibroblasts showed a substantial decrease in 12S rRNA levels and decreased S11 and S16 levels in mitochondria, suggesting the 28S subunit cannot assemble in the absence of S22 (Emdadul Haque *et al.*, 2008). Despite the broad phenotypes, when taken together with the resolved structure of the mitoribosome, the S22 subunit is confirmed as a critical component of 28S subunit stability, assembly or both and hence, mitochondrial translation. Additional mitoribosome subunit mutations have also been reported in *MRPL3 (*Galmiche *et al.*, 2011*), MRPL12* (Serre *et al.*, 2013), *MRPL44* (Carroll *et al.*, 2013), *MRPS16* (Miller *et al.*, 2004) and *MRPS23* (Kohda *et al.*, 2016), highlighting the importance of mitoribosome assembly and stability for mitochondrial protein synthesis.

6.5.1.2 Mutations of Mitochondrial tRNA Modification Factors

Mutations were also identified in three mt-tRNA modification factors; autosomal recessive mutations in *TRMU* (patient 31) and *GTPBP3* (patient 27) (**6.5.1.3**), plus a potential causative heterozygous *MTO1* (patient 32) mutation. Interestingly, all three mt-tRNA modification factors are involved in modifying the uridine 34 (U34) wobble-base of mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Trp}$, $tRNA^{Lys}$, $tRNA^{Gln}$ or $tRNA^{Glu}$. Modification of the U34 wobble-base in mitochondria is evolutionarily conserved, with both yeast and bacteria possessing homologs of MTU1 (MnmA in bacteria), MTO1 (GidA in bacteria) and GTPBP3 (Mss1 in yeast, MnmE in bacteria) (Elseviers *et al.*, 1984; Colby *et al.*, 1998; Cabedo *et al.*, 1999; Scrima *et al.*, 2005; Villarroya *et al.*, 2008). The first modification is 5-taurinomethyluridine (τm^5U) at position 5 of U34 of $tRNA^{Leu(UUR)}$, $tRNA^{Trp}$, $tRNA^{Lys}$, $tRNA^{Gln}$ or $tRNA^{Glu}$ that is proposed to be synthesised by a GTPBP3-MTO1 complex (Colby *et al.*, 1998). However in yeast and bacteria, MTO1-Mss1 (yeast) and GidA-MnmE (bacteria) synthesise a 5-carboxymethylaminomethyluridine ($cmnm^5U$) modification of U34 instead of τm^5U (Elseviers *et al.*, 1984). A second modification of τm^5U, 2-thiouridine (τm^5s^2U) is also synthesised at the second position of the U34 wobble-base of $tRNA^{Lys}$, $tRNA^{Gln}$ and $tRNA^{Glu}$ by MTU1 (Umeda *et al.*, 2005). Regarding mt-tRNA mutations and the U34 wobble-base, cybrid cells from patients harbouring the mt-$tRNA^{Leu(UUR)}$ m.3243A>G and m.3271T>C MELAS mutations lacked τm^5U modification (Yasukawa *et al.*, 2000). In contrast, tissue from patients harbouring different mt-$tRNA^{Leu(UUR)}$ mutations had normal taurinomethylation of U34 (Kirino *et al.*, 2005), which correlates the MELAS phenotype with the specific lack of τm^5U modification.

TRMU mutations have been previously reported in patients presenting acute infantile-onset liver failure (AILF) and RC deficiency, with onset between birth and 6 months of life (Zeharia *et al.*, 2009; Kemp *et al.*, 2011; Schara *et al.*, 2011; Uusimaa *et al.*, 2011; Gaignard *et al.*, 2013; Taylor *et al.*, 2014; Grover *et al.*, 2015). Consistent with previous cases, patient 31 had severe acute liver failure and hepatomegaly from birth, with cardiac involvement and possible signal changes in the globus pallidus, which were rare manifestations of *TRMU* patients. Normal muscle histology was also true in previous cases. The p.Asp249_Lys251delinsGlu and p.Pro276Leu variants harboured by patient 31 had not been previously described in affected patients. However, the p.Pro276Leu missense change was proximal to previously reported c.815G>A (p.Gly272Asp) and c.835G>A (p.Val279Met) variants. Although further details were not available, patient 31 appeared to have some recovery at around 18 months of age, as seen in other patients who survived beyond the first year of life.

Since its activity is thiouridylase and not methyltransferase, MTU1 is the accepted term for the encoded enzyme by *TRMU*. The pathological nature of *TRMU* mutations may be due to loss of MTU1 thiouridylation activity causing failure to modify the U34 wobble-base in mitochondrial $tRNA^{Lys}$, $tRNA^{Gln}$ and $tRNA^{Glu,}$ and hence impaired mitochondrial translation. Accumulation of non-thiourylated $tRNA^{Lys}$, $tRNA^{Gln}$ and $tRNA^{Glu}$ in patient fibroblasts has been found (Zeharia *et al.*, 2009), but defective mitochondrial translation associated with MTU1-deficiency was not demonstrated in patient fibroblasts, MTU1-knockout HEK293 cells or myoblasts and there were non-specific decreases in mt-tRNAs (Sasarman *et al.*, 2011). Sasarman *et al.* (2011) proposed that although MTU1 is essential for 2-thiouridylation of the U34 wobble-base for mitochondrial $tRNA^{Lys}$, $tRNA^{Gln}$ and $tRNA^{Glu}$, it is dispensable since mitochondrial protein synthesis was normal. Furthermore, knockout of *TRMU* in MELAS and MERRF patient fibroblasts, for which the translation defects are ascribed to failed modification of the mt-$tRNA^{Leu(UUR)}$ (MELAS) and mt-$tRNA^{Lys}$ (MERRF) U34 wobble-base, did not cause further impairment of mitochondrial protein synthesis. To explain this, it was hypothesised that only loss of steady-state mt-tRNA levels for thiouridylation together with deficient MTU1 could impair translation. When steady-state levels of mt-tRNAs are normal, MTU1 is not required for mitochondrial translation. This has yet to be studied and confirmed in *TRMU* patient liver, which could provide further evidence to support this hypothesis and the tissue-specific nature of this disease.

Unexpectedly, patient 31 had a severe complex II defect (4% activity compared to controls) in skeletal muscle, despite all complex II subunits being encoded by the nuclear genome. A

complex II defect was also noted in one patient with compound heterozygous *TRMU* mutations; p.Val279Met and a splice-site c.248+1G>A variant (Gaignard *et al.*, 2013). In agreement with Gaignard *et al.* (2013), this unusual finding indicates a secondary role for MTU1 that is independent of mitochondrial protein synthesis. In bacteria and yeast homologs, 2-thiouridylation of the U34 wobble-base is dependent on a sulphur relay system to provide sulphur, encoded by five genes unconserved between the species (Kambampati and Lauhon, 2003; Noma *et al.*, 2009). In humans, bacteria and yeast the essential sulphur donor for 2-thiouridylation is the amino acid L-cysteine, which is critical for early human development. During the neonatal period, the availability of L-cysteine is reduced because its synthesis from methionine is restricted by the rate-limiting enzyme cystathionase, which is expressed at low levels from birth and increases slowly during the first few months of life (Zlotkin and Anderson, 1982; Levonen *et al.*, 2000). The low availability of L-cysteine as a sulphur donor together with *TRMU* mutations has been proposed as a pathological mechanism for severe liver failure and recovery or stabilisation in several patients over time (Zeharia *et al.*, 2009). Concerning the complex II defect, Sasarman *et al.* (2011) noted that MTU1-deficient HEK293 cells and *TRMU* patient fibroblasts had a subcomplex of complex II appearing as a 'doublet' when using an antibody for the 70kDa subunit SDHA, which was consistent in all knockdowns and patient fibroblasts. In MTU1-deficient HEK293 cells the 30kDa subunit SDHB that contained the iron-sulphur (Fe-S) clusters was absent. Authors proposed that addition of the Fe-S clusters to this complex II subunit was compromised due to MTU1 deficiency, resulting in an unassembled subcomplex containing only the 70kDa subunit. Hence, MTU1 may have a secondary role in obtaining the sulphur it requires for 2-thiouridylation and for the addition of Fe-S clusters to the complex II 30kDa subunit. Why only two *TRMU* patients have been noted to have a complex II defect however remains intriguing, particularly as the clinical course of the patient reported by Gaignard *et al.* (2013) was fatal, unlike that of patient 31, warranting further investigation of the secondary function of MTU1.

6.5.1.3 A Novel Disorder of Defective mt-tRNA Modification Due to *GTPBP3* Mutations

The third nuclear gene associated with mt-tRNA modification and with mutations in patients with multiple RC deficiency was *GTPBP3*. The homozygous *GTPBP3* mutation harboured by patient 27 was identified independently by this study on patients with early-onset RC deficiency. Although the parents reported no consanguinity, the possibility of a founder effect within Romania could not be excluded but no further details on the family were available. Following the sharing of WES results with diagnostic centres worldwide, a cohort of 11

patients from 9 pedigrees with autosomal recessive *GTPBP3* mutations was collated, including patient 27, suggesting that *GTPBP3* mutations are an important cause of mitochondrial RC disease. All patients including patient 27 (corresponding to patient #81471) have been published in Kopajtich *et al.* (2014), all presenting a core phenotype of HCM, encephalopathy and lactic acidosis. When performed, brain MRI revealed abnormalities of the brainstem and basal ganglia suggestive of Leigh syndrome that included patient 27. Based on these core features, targeted *GTPBP3* sequencing was performed in an additional undiagnosed patient referred to the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory in Newcastle upon Tyne but did not reveal any likely causative variants.

Although it has been proposed that the τm^5U modification at position 5 of U34 of mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Trp}$, $tRNA^{Lys}$, $tRNA^{Gln}$ or $tRNA^{Glu}$ is synthesised by cooperation of GTPBP3 and MTO1 (Colby *et al.*, 1998), there is currently no functional confirmation of this. While *GTPBP3* patient fibroblasts demonstrate a mitochondrial translation defect and multiple RC deficiency that could be rescued by lentiviral transfection, there were no changes in the steady-state mt-tRNA levels, including mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Trp}$, $tRNA^{Lys}$, $tRNA^{Gln}$ or $tRNA^{Glu}$ to validate the role of GTPBP3 in τm^5U modification. On the other hand, analysis of mt-tRNAs in *MTO1* patient fibroblasts showed altered mt-$tRNA^{Glu}$ modification but not mt-$tRNA^{Lys}$ or mt-$tRNA^{Gln}$ (Tischner *et al.*, 2015), providing evidence for a pathological mechanism due to defective τm^5U modification. This could also be a shared mechanism in *GTPBP3* patients but this has yet to be examined. Activity of the proposed MTO1-GTPBP3 complex is also coupled to the 2-thiouridylation activity of MTU1 (Umeda *et al.*, 2005). Patients with *TRMU* and *MTO1* mutations manifest liver failure or HCM. On the other hand, patients with *GTPBP3* and *MTO1* mutations present striking similarities of HCM, lactic acidosis and multiple RC defects (Ghezzi *et al.*, 2012; Kopajtich *et al.*, 2014), which provides some support for the shared mechanism of τm^5U modification.

6.5.2 A Novel *SCO1* Mutation Causing Isolated COX Deficiency in a Consanguineous Family with 'Triple Threat' Mitochondrial Disease

Isolated COX deficiency is a prevalent form of mitochondrial disease that can occur due to mutations of any of the three mtDNA encoded structural subunits and some nuclear encoded structural subunits, or COX assembly factors (DiMauro *et al.*, 2012). In a highly consanguineous British Pakistani family with mitochondrial disease affecting several branches of the pedigree, a novel homozygous *SCO1* mutation was identified in a single

affected patient with fatal congenital lactic acidosis and isolated COX deficiency. Causative homozygous *RMND1* and *FARS2* mutations were identified in the other branches of this family. The *RMND1* p.*450Serext*32 extension variant had been previously reported homozygous in several affected patients of Pakistani origin (Taylor *et al.*, 2014; Ng *et al.*, 2016). The p.Tyr144Cys *FARS2* missense change was previously reported in homozygous in a Saudi girl presenting with mitochondrial encephalopathy (Shamseldin *et al.*, 2012). Consanguineous families are at a greater risk of Mendelian disease due to common founder mutation within the family or local population (Hamamy, 2012). Nonetheless, the presence of three segregating forms of Mendelian mitochondrial disease in one consanguineous family is exceptional.

SCO1 and its paralog *SCO2* encode copper chaperones, with autosomal recessive mutations in either gene causing isolated COX deficiency (Papadopoulou *et al.*, 1999; Jaksch *et al.*, 2000; Valnot *et al.*, 2000; Salviati *et al.*, 2002; Tarnopolsky *et al.*, 2004; Verdijk *et al.*, 2008; Stiburek *et al.*, 2009; Leary *et al.*, 2013a), though *SCO1* mutations have been previously reported in only three pedigrees. Both SCO proteins have high sequence identity, particularly in the C-terminal and share a CxxxC motif and histidine residue that are indispensable for copper binding, which is also shared with the yeast sco1 homolog (Balatri *et al.*, 2003; Horng *et al.*, 2005). Although yeast has conserved sco1 and sco2 homologs, only yeast sco1 is essential for COX assembly (Glerum *et al.*, 1996). In humans, together in cooperation with SCO2 and additional copper chaperones that include recently characterised COX20 and COA6 (Bourens *et al.*, 2014; Stroud *et al.*, 2015), SCO1 is required for the delivery of two copper ions to the catalytic core of COX and metalation of the Cu_A copper site of MT-COII during the early stage of COX assembly, forming a complex that facilitates MT-COII insertion, maturation and assembly into the COX holoenzyme (Leary *et al.*, 2004). Therefore failure to maturate and incorporate MT-COII leads to its degradation and ultimately, COX deficiency. Significant tissue- and cell-specific copper deficiency due to an inability to retain cellular copper content and improper mitochondrial redox signalling have also been demonstrated in affected *SCO1* and *SCO2* patient tissue, highlighting a critical role for the SCO proteins in cellular copper homeostasis (Leary *et al.*, 2007; Leary *et al.*, 2013b). Furthermore, a fraction of SCO1 is physically associated with fully assembled COX in human muscle mitochondria, suggesting a link between COX and cellular copper homeostasis (Stiburek *et al.*, 2009).

SCO1 and *SCO2* mutations appear to show tissue- and cell-specificity despite ubiquitous expression. *SCO2* mutations have been associated with fatal cardioencephalomyopathy with

heart and muscle tissues demonstrating severe COX-deficiency in comparison to other tissues (Papadopoulou *et al.*, 1999). The *SCO1* patient reported by Leary *et al.* (2013a) who had fatal lactic acidosis and harboured compound heterozygous *SCO1* (p.Met294Val and c.425+6T>C) is comparable to patient 34 who also had fatal infantile lactic acidosis and harboured a novel missense variant affecting the same residue, p.Met294Thr. In contrast, previously reported *SCO1* patients had neonatal liver failure or HCM (Valnot *et al.*, 2000; Stiburek *et al.*, 2009). The pathological mechanisms to explain the distinct tissue specific phenotypes were investigated by Leary *et al.* (2013a), who compared fibroblasts from the patient with the p.Met294Val variant to a patient with a c.520C>T (p.Pro174Leu) variant. Fibroblasts expressing the p.Met294Val variant had a milder COX-defect, more stable MT-COII and an absent intermediate S2 subcomplex previously observed in *SCO1* patient fibroblasts as a consequence of stalled COX assembly. Despite lower expression of SCO1, this evidence suggests that *SCO1* expressing the p.Met294Val variant retained residual ability for COX assembly. Authors proved this by overexpressing the p.Met294Val mutation, which resulted in fully recoverable COX activity. Nonetheless, the copper deficiency of patient fibroblasts harbouring the p.Met294Val mutation was similar to second *SCO1* patient. Overall, Leary *et al.* (2013a) proposed that the defect caused by p.Met294Val is due to the cellular copper deficiency, instead of impaired COX assembly as demonstrated in *SCO1* patients with different mutations. Hence, tissue specificity of *SCO1* mutations is dependent upon the particular function that is impaired, either COX assembly or cellular copper homeostasis. Although the amino acid substitution of patient 34 is different, from methionine to threonine, the proposed mechanism likely stands true as both patients presented fatal lactic acidosis and isolated COX deficiency.

6.5.3 Pyruvate Dehydrogenase Deficiency Due to a *De Novo PDHA1* Mutation

A novel *de novo* p.Glu345dup *PDHA1* mutation was identified in patient 23, who presented severe neonatal metabolic acidosis, renal impairment, hypotonia, central apnoea, anemia, liver disease and a coagulopathy. Mutations of *PDHA1*, which encodes the E1-α subunit of the pyruvate dehydrogenase complex (PDHc), are a common cause of mitochondrial disease and that are typically characterised by neonatal- or infantile-onset lactic acidosis and pyruvate dehydrogenase deficiency with a poor outcome, although it is known to have a broad phenotypic spectrum (DeBrosse *et al.*, 2012). Diagnosis of PDHc deficiency is typically made using an assay for measuring activity, normally with patient fibroblasts but can also include skeletal muscle, liver or lymphocytes (Kerr *et al.*, 1987; Kerr *et al.*, 2012).

The pyruvate dehydrogenase complex (PDHc) is an approximately 9.5 megadalton (MDa), nuclear encoded mitochondrial matrix multi-enzyme complex required for the irreversible conversion of pyruvate to acetyl co-enzyme A (CoA) (Patel and Korotchkina, 2006). Hence, the PDHc provides this vital link with glycolysis (Harris *et al.*, 2002; Patel and Korotchkina, 2006). It is comprised of five essential enzymes, one of which is pyruvate dehydrogenase (E1) that is composed of 20-30 E1 units. Each unit is a heterotetrameric complex comprising two E1-α subunits encoded by *PDHA1* and two E1-β subunits encoded by *PDHB (Patel and Korotchkina, 2006)*. *PDHB* mutations have also been implicated in pyruvate dehydrogenase deficiency but are uncommon compared to *PDHA1* (Okajima *et al.*, 2008; DeBrosse *et al.*, 2012). The active site of the E1 subunit is within a deep cleft at the interface of the α- and β-subunits (Gray *et al.*, 2014).

Missense, in-frame and frameshift *PDHA1* mutations have been identified in patients with pyruvate dehydrogenase deficiency, which have been proposed to affect either (i) targeting to mitochondria, (ii) binding to thiamine pyrophosphate co-factor or (iii) assembly of the pyruvate dehydrogenase complex (Gray *et al.*, 2014). As the p.Glu345dup in patient 23 did not introduce a frameshift, it is unclear how this impacts the PDHc activity or function. Furthermore, patient 23 PDHc activity was not measured, perhaps due to early demise. Nonetheless since the p.Glu345dup variant apparently occurred *de novo* or was germline mosaic and that in-frame *PDHA1* mutations have been previously reported, it is highly likely to be the definitive diagnosis. Given the critical role of the PDHc in generating acetyl CoA for the TCA cycle in mitochondria and hence, the dependency for OXPHOS this would explain the low complex I and IV activities in patient muscle. Severe mtDNA depletion in patient 23 muscle was also an intriguing finding. This could be linked to the activity of succinyl-CoA in the TCA cycle, given its association with early-onset mtDNA depletion syndrome (Carrozzo *et al.*, 2007; Ostergaard *et al.*, 2007). In patient 23, mtDNA depletion may be secondary to a disturbance of the PDHc leading to deficiency of acetyl CoA feeding into TCA cycle, therefore affecting pathways downstream, disrupting the interaction between succinyl CoA synthetase and NDPK. Currently, patient 23 is currently the only known *PDHA1* patient known to have mtDNA depletion and therefore it is not clear if this is a rare phenomenon or if it is an under recognised feature of PDHc deficiency.

6.5.4 *CTBP1* Mutation and the Emergence of *De Novo* Dominant Mitochondrial RC Disease

Surprisingly, two novel heterozygous missense variants were identified in two early-onset patients; p.Arg331Trp *CTBP1* in patient 22 and p.Gly98Ser *MTO1* in patient 32. Of the two variants, the *CTBP1* missense change is the most likely causative variant due to the identification of four patients who were also *de novo* for the same missense change with similar phenotypes (Beck *et al.*, 2016). All patients including patient 22 had hypotonia, developmental delay and varying muscle weakness. Cerebellar atrophy was noted in patient 22 and two reported patients.

Contrastingly, patient 22 had limb spasticity, whereas the reported patients had difficulty walking due to ataxia. Muscle biopsies also showed non-specific findings in the four reported patients. Additionally, RC defects were not reported but it is unclear whether skeletal muscle was analysed given the neurological phenotypes presented by the four patients were not dissimilar to some early-onset patients with mitochondrial disease. One patient was apparently investigated for mitochondrial mutations, but no further details were given. The four reported patients had enamel hypoplasia or discolouration. According to the clinical notes, patient 22 had normal teeth but there was no description on the enamel. It is not clear though when these defects manifested, or if it was the deciduous or permanent teeth that were affected. Finally, it was not possible to determine whether the p.Arg331Trp missense change occurred *de novo* in patient 22. At the time of writing, segregation studies had not been performed. Interestingly, the mother of one of the four reported patients was low-level somatic mosaic for the p.Arg331Trp variant, but did not present any neurological impairment.

To date, CtBP1 is not a known mitochondrial protein nor predicted to possess a MTS. It was first identified as an E1A oncogene binding protein (Schaeper *et al.*, 1995), but has since been characterised as a transcriptional co-repressor that associates with DNA-specific repressors during human development and tumorigenesis (Bergman and Blaydes, 2006; Chinnadurai, 2007). Hence it regulates gene expression and DNA repair. Mammalian CtBP1 has overlapping gene expression roles with its paralog CtBP2, whereby knockout of CtBP1 resulted in smaller offspring and 25% lethality by day 20 of life in CtBP1-mutator mice and knockout of CtBP2 is embryonic lethal (Hildebrand and Soriano, 2002). Despite no mitochondrial targeting, CtBP1 has been identified as a key regulator of mitochondrial morphology and function. Knockout of CtBP1 in mouse embryonic fibroblasts (MEFs) results in elongated mitochondria, swollen cristae, decreased cellular ATP, decreased oxygen consumption and decreased mitochondrial membrane potential (Kim and Youn, 2009).

Altered mitochondrial morphology and function were attributed to the regulation of the *Bax* (Bcl-2-associated X protein) gene by CtBP1. BAX promotes mitochondrial apoptosis and has been proposed to associate with mitochondrial fission and fusion sites (Wu *et al.*, 2011). Kim and Youn (2009) demonstrated that mitochondrial-dependent apoptosis in response to glucose deprivation was regulated by the *Bax* gene, for which expression was modulated by the association of CtBP1 to the *Bax* promotor. This association was proven when both *Bax* and *Ctbp1* were knocked out together in MEFs, which led to an increase in ATP production, oxygen consumption rate and membrane potential. RC deficiency demonstrated in patient 22 skeletal muscle suggests that mitochondrial morphology has been disrupted due to impaired regulation of *Bax*. Without the proper fusion and fission of mitochondria there is a failure to target and clear damaged mitochondria via mitophagy, including factors involved in mitochondrial protein synthesis or assembly of the RC complexes. Further investigation of RC activities in the muscle of the four reported patients could provide further insights. Given the neurological phenotypes and the function of CtBP1, it is likely that the p.Arg331Trp mutation acts in a dominant-negative manner on *Bax* expression.

The role of CtBP1 in the regulation of gene expression is reflected in the spectrum of clinical features. The tooth enamel defects in the four reported patients are the most unusual yet specific feature of this disorder. Development of teeth and enamel is dependent on tight gene regulation (Paine *et al.*, 2001; Balic and Thesleff, 2015). Non-syndromic and syndromic forms of Mendelian orodental disorders affecting tooth development and the oral cavity have described with a significant proportion of nuclear encoding transcriptional regulators with some phenotypic overlap between the *CTBP1* patients (Bergendal *et al.*, 2011; Prasad *et al.*, 2016; Shahid *et al.*, 2016). This suggests a key role of CtBP1 in regulating enamel structure. Since the p.Arg331Trp missense change likely acts in a dominant-negative manner, the expression of genes critical for mineralisation or formation of the enamel could be significantly downregulated.

De novo heterozygous mutations are widely associated with early onset autism and epileptic encephalopathies (Epi and Epilepsy Phenome/Genome, 2013; Iossifov *et al.*, 2014). In contrast, *de novo* mutations of nuclear encoded mitochondrial proteins causing mitochondrial disease are limited. Recently, five patients with global developmental delay, hypotonia, optic atrophy, axonal neuropathy and HCM sharing a *de novo* c.1582C>T (p.Arg528Trp) *ATAD3A* mutation were reported (Harel *et al.*, 2016). *De novo* c.239G>A (p.Arg80His) and c.703C>G (p.Arg235Gly) *SLC25A4* mutations were also reported in seven patients with neonatal-onset respiratory insufficiency and severe mtDNA depletion in skeletal muscle (Thompson *et al.*,

2016). Dominant *SLC25A4* mutations are further complicated though by patients with late-onset PEO and multiple mtDNA deletions, as described in **3.4.3**. These recent publications suggest that *de novo* nuclear gene mutations in early-onset mitochondrial disease patients could be considerably underrepresented. Hence, full re-evaluation of candidate variants in unsolved cases may increase diagnostic yield.

6.5.5 Novel Mitochondrial RC Disease Candidates Requiring Further Investigation

Likely causative variants and VUS were identified in *PTPIP51*, *MTO1* and *LONP1*. Although all three genes encode mitochondrial proteins that have been characterised to varying extents, the potential mechanisms associated with RC deficiency are currently unclear.

6.5.5.1 A Homozygous *PTPIP51* Missense Mutation

A novel homozygous p.Thr451Met missense variant in *PTPIP51* was identified in a boy with progressive muscle weakness, complex I and IV defects who was born to consanguineous parents. Given consanguinity, the homozygous *PTPIP51* variant was a strong candidate as the causative variant in this case. PTPIP51 is a scaffold protein that localises in different cellular locations has been associated with several cellular processes including regulation of the microtubule cytoskeleton, photoreceptor differentiation, cell morphology and mitosis (Yu *et al.*, 2008; Brobeil *et al.*, 2012). Regarding mitochondrial function, Lv *et al.* (2006) first demonstrated localisation of PTPIP51 to mitochondria by identifying a putative N-terminal MTS and showing that overexpression in HEK293 and HeLa cells induced apoptosis, the release of cytochrome *c* and a decrease in mitochondrial membrane potential. De Vos *et al.* (2012) further demonstrated that PTPIP51 localises to the OMM and interacts with the endoplasmic reticulum (ER) membrane protein VAPB to regulate calcium exchange between the two organelles. Knockout of either PTPIP51 or VAPB caused increased cytosolic and decreased mitochondrial calcium levels that was comparable to knockout of mitofusin-2 (MFN2), which is also proposed to tether mitochondria to the endoplasmic reticulum (de Brito and Scorrano, 2008), although it was recently proposed that MFN2 acts antagonistically in mitochondria-ER contacts (Filadi *et al.*, 2015). OMM proteins including MFN1, MFN2, OPA1 and Drp1 are also critical for mitochondrial fusion or fission (Pitts *et al.*, 1999; Chen *et al.*, 2003; Cipolat *et al.*, 2004). To determine if PTPIP51 had any role in mitochondrial morphology, TMRM staining of patient fibroblasts was performed but this did not reveal any elongation or fragmentation of the network. Steady-state PTPIP51 levels were also unchanged, demonstrating the difficulty of functional investigations in fibroblasts. Curiously

there was a significant upregulation in VDAC1/porin, which is also an OMM channel protein that allows the movement of metabolites, cations, adenine nucleotides and calcium into the intermembrane space (Bayrhuber *et al.*, 2008; Shoshan-Barmatz *et al.*, 2010). This upregulation suggests a possible compensatory mechanism, although whether this is related to disturbed mitochondrial calcium homeostasis is not clear. Unfortunately, skeletal muscle is unavailable and no second patient has yet been identified with *PTPIP51* mutations to allow for further investigations.

6.5.5.2 An Atypical Phenotype with Severe mtDNA Depletion Due to Compound Heterozygous *LONP1* Mutations

Compound heterozygous *LONP1* mutations were identified in patient 35 who had congenital lactic acidosis, muscle weakness, a Leigh-like imaging pattern on brain MRI and severe mtDNA depletion. The Lon protease is known to be involved in the protein quality control by degrading proteins damaged by oxidative stress (Venkatesh *et al.*, 2012). Lon also binds to mtDNA to regulate expression but it also binds and degrades TFAM to control the ratio of TFAM-mtDNA, hence regulating mtDNA transcription (Venkatesh *et al.*, 2012; Lu *et al.*, 2013). Given the severe mtDNA depletion in patient 35 muscle, an autosomal recessive mtDNA depletion disorder was suspected and hence, *LONP1* strongly fitted the criteria due to its role in mtDNA maintenance. Despite this, recessive *LONP1* mutations have been previously associated with a multisystem syndrome of cerebral, ocular, dental, auricular and skeletal abnormalities, known as CODAS syndrome (Dikoglu *et al.*, 2015; Strauss *et al.*, 2015). CODAS syndrome did not fit the phenotype for patient 35. Furthermore, there was no evidence of mtDNA depletion in muscle of affected CODAS syndrome patients. On the other hand, the clinical and genetic heterogeneity of mitochondrial disease suggest that multiple phenotypes associated with causative mutations of the same nuclear gene are to be expected, due to independent pathological mechanisms. The p.Tyr565His and p.Glu733Lys missense changes were also proximal to previous reported causative *LONP1* variants, but require further investigation.

6.5.5.3 An Autosomal Dominant *MTO1* Mutation with Reduced Penetrance?

Finally, a novel heterozygous *MTO1* p.Gly98Ser mutation was identified in a family with possible autosomal dominant inheritance of a mitochondrial disorder. Both patient 32 and her affected mother harboured the p.Gly98Ser missense change with no second likely causative *MTO1* variant identified in either patient. Clinically, patient 32 fits the previously described *MTO1* phenotype well. On the other hand, her mother is not affected to the same degree,

although muscle histochemistry performed externally apparently confirmed a mitochondrial myopathy. To date, all patients harbouring *MTO1* mutations have homozygous or compound heterozygous variants, hence not only would this be the first causative dominant inherited *MTO1* variant, but also the first dominant mitochondrial translation disorder. Quite how this mutation acts requires investigation into the τm^5U modification of mt-tRNAs. The p.Gly98Ser mutation could be acting in a dominant-negative manner with reduced penetrance in the affected mother. Phenotypic variability between affected family members with dominant heterozygous PEO with multiple mtDNA deletions mutations are not uncommon (**Chapter 3**). In the absence of other possible candidates, the *MTO1* variant remains the best candidate. Further segregation studies, particularly of the apparently unaffected sibling could support the likelihood of this novel *MTO1* mutation.

6.5.6 Concluding Remarks

To summarise, WES of 20 early-onset patients with mitochondrial RC deficiency provided a higher diagnostic yield for causative and likely causative variants (45%) than the adult-onset PEO with multiple mtDNA deletions cohort (35%). This is due to the undeniable prominence of causative autosomal recessive variants, versus the prevalence of dominant variants in adult-onset mtDNA maintenance disorders. However, diagnostic targeted gene screening from the early-onset RC cohort was limited to one or two genes before recruitment to WES increasingly the likelihood of a diagnosis and identifying variants in known disease genes, whereas all common nuclear genes associated with PEO and multiple mtDNA deletions were excluded in the late-onset cohort. Furthermore, the identification of a heterozygous *CTBP1* mutation associated with combined RC defects suggests a growing significance for (presumed) *de novo* or germline mosaic variants in early-onset Mendelian mitochondrial disease. The clinical and genetic heterogeneity of mitochondrial RC disease is highlighted in that only single cases for each gene were identified. This study advocates (i) reporting of all recessive *and* heterozygous candidate variants in unsolved cases and (ii) periodic re-evaluation, which may ultimately lead to an increase in diagnostic yield. The identification of variants in genes predominantly involved in mitochondrial translation indicate that prioritisation of these genes is appropriate but nonetheless still allow for additional factors in complex assembly, dynamics and metabolism to be listed. Finally, attaining diagnoses in paediatric cases is paramount for the parents and families, that will help inform future reproductive options to include pre-natal or pre-implantation diagnosis.

Chapter 7. Expanding the Clinical, Genetic and Molecular Features of Mitochondrial Amino-Acyl tRNA Synthetase Mutations

7.1 Introduction

This chapter contains material published in *Journal of Inherited Metabolic Disorders Reports* (Oliveira and Sommerville, 2016) and *JAMA Neurology* (Sommerville *et al.*, 2017). The expansion of the clinical, molecular and genetic features of mitochondrial amino-acyl tRNA synthetase (mt-aaRS) mutations is a large on-going collaborative study. Additional clinical, diagnostic and research support are provided from colleagues and external collaborators who are appropriately acknowledged.

7.1.1 The mt-aaRS and Mitochondrial Translation

Integrity of mitochondria is dependent upon accurate and efficient translation of the genetic code in the form of mRNA to produce the correct, corresponding amino acid sequence. Following post-transcriptional modification, it is vital that amino acids are aminoacylated (attached) with their specific cognate tRNA, as dictated by the codon sequence of the mRNA. This attachment is catalysed by the mt-aaRS, an essential set of enzymes encoded by the nuclear genome and imported to mitochondria. These are distinguished from the cytosolic 'aaRS' by the prefix 'mt-' or the suffix '2'. For example, AlaRS refers to the cytosolic alanyl-tRNA synthetase, whereas mt-AlaRS or AlaRS2 refers to mitochondrial alanyl-tRNA synthetase. On the other hand, the nuclear genes are referred to as '*aaRS*' for aaRS or '*aaRS2*' for mt-aaRS.

There are 19 mt-aaRS each encoded by a single nuclear gene distinct from the aaRS with the exceptions of *GARS* and *KARS*, which encode dual-localised cytosolic and mitochondrial synthetases (Suzuki *et al.*, 2011). The cytosolic and mitochondrial GlyRS isoforms are encoded by a single mRNA transcript with alternate translation initiation sites (Shiba *et al.*, 1994; Mudge *et al.*, 1998). The LysRS isoforms are generated by alternative splicing of exons 1, 2 and 3 (Tolkunova *et al.*, 2000). Cytosolic LysRS is generated by splicing of exons 1 and 3, while the mitochondrial LysRS is generated from inclusion of all three exons. Additionally, there is no mammalian mitochondrial homolog of GlnRS. Instead, Nagao *et al.* (2009) discovered that mt-GluRS efficiently misaminoacylates $tRNA^{Gln}$ to form Glu-$tRNA^{Gln}$ in mitochondria.

Each mt-aaRS catalyses the attachment of an amino acid to its specific cognate tRNA by a two-step reaction (Ibba and Söll, 2000) (**Figure 7.1**). First, the amino acid is activated with ATP at the mt-aaRS active site to form an intermediate aminoacyl-adenlyate species, with an inorganic pyrophosphate leaving group. Second, the amino-acyl group is transferred to the 3' acceptor stem of the cognate tRNA by 3'-esterification, generating an AMP leaving group. To discriminate between the mt-aaRS, mt-tRNAs contain structural elements that are often unique, ensuring that aminoacylation occurs with its correct mt-aaRS (Ibba and Söll, 2000). Low levels of misaminoacylated tRNAs have been shown to be lethal in bacteria and led to cerebellar Purkinje cell loss, ataxia and tremors in mice (Beebe *et al.*, 2003; Lee *et al.*, 2006b). However, the mischarging of tRNAs with incorrect amino acids can occasionally occur due to discrete structural differences in the active site. For example, PheRS can mischarge tyrosine to generate an incorrect Tyr-tRNA^{Phe} species (Ling *et al.*, 2007) and IleRS can mischarge valine to generate Val-tRNA^{Ile} (Fukai *et al.*, 2000). Maintenance of mitochondrial translation fidelity from the mischarging of amino acids is provided by an editing domain acquired by several mt-aaRS or by the mitochondrial elongation factor mtEF-Tu, which forms a complex with the aminoacylated tRNA and GTP following aminoacylation. Mischarged mammalian Ser-tRNA^{Gln} was demonstrated to have a lower affinity for mt-EFTu than Glu-tRNA^{Gln} and Ser-tRNA^{Ser} (Nagao *et al.*, 2007), confirming a mechanism for ensuring accuracy of mitochondrial protein synthesis.

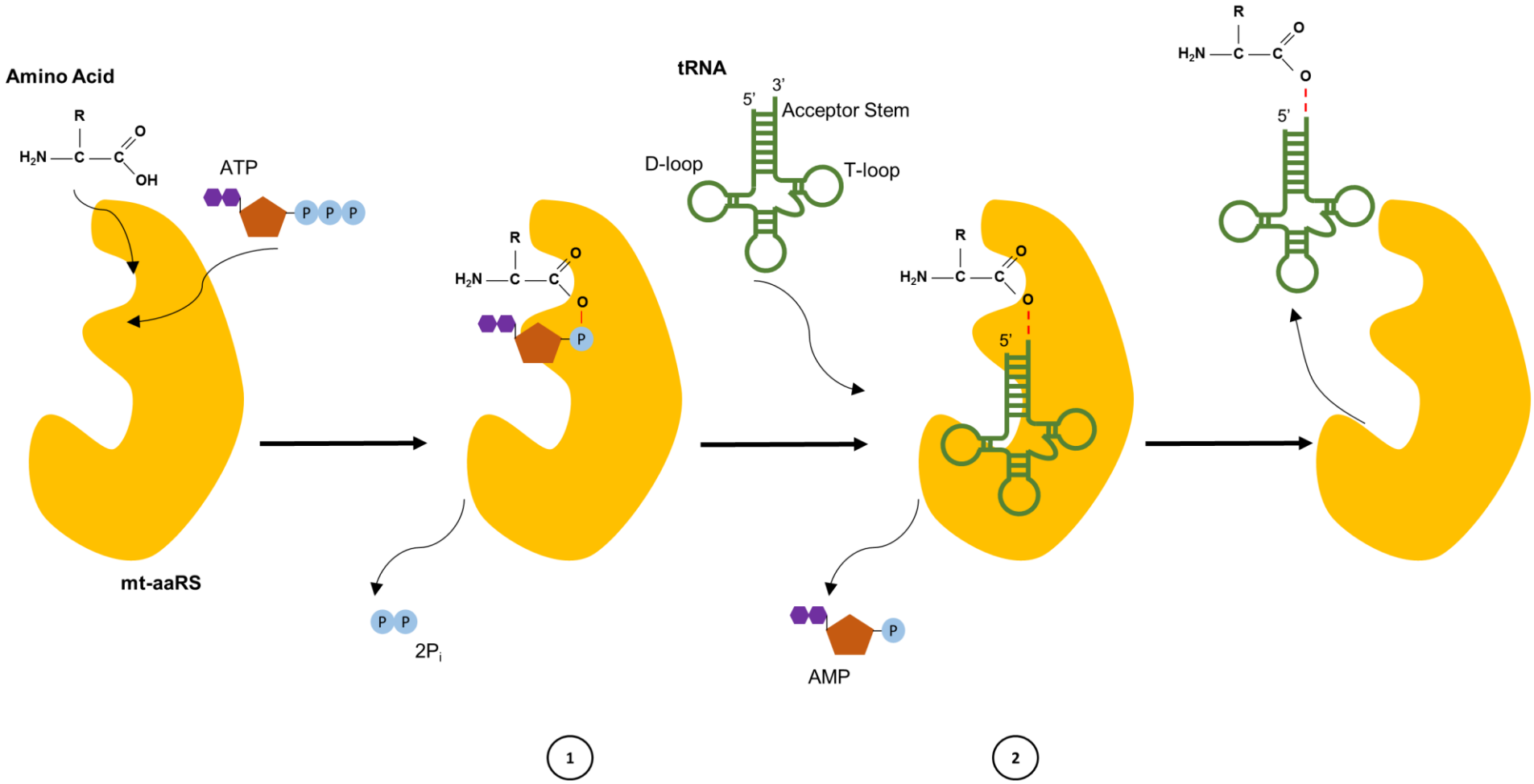


Figure 7.1 Aminoacylation of Cognate mt-tRNAs with their Specific Amino Acid. Aminoacylation of mt-tRNAs is a two-step reaction whereby (1) the amino acid is activated to form an intermediate amino-adenylate species and (2) the charged amino acid is attached to the 3' acceptor stem of its corresponding tRNA by 3'-esterification.

The mt-aaRS are structurally diverse but typically possess a catalytic domain for the two-step aminoacylation reaction and an anti-codon binding domain for binding of the cognate tRNA. Several aaRS and mt-aaRS have also acquired a second active site referred to as the editing domain for proofreading of mischarged amino acids. This includes mt-AlaRS since glycine and serine are also able to bind to the synthetase, hence requiring correcting (Beebe *et al.*, 2008; Guo *et al.*, 2009). Much like their cytosolic counterparts, mt-aaRS are also divided into two distinct, unrelated classes; Class I synthetases (mt-ArgRS, mt-CysRS, mt-GluRS, mt-IleRS, mt-LeuRS, mt-MetRS, mt-TrpARS, mtValRS) function as either monomeric or dimeric structures and Class II synthetases (mt-AlaRS, mt-AsnRS, mt-AspRS, GlyRS, mt-HisRS, LysRS, mt-PheRS, mt-ProRS, mt-SerRS, mt-ThrRS, mt-TyrRS) function as either dimeric or tetrameric structures. Class I and II synthetases are distinguished by structural differences of the active site and by the presence of distinct sequence motifs. Class I synthetases have a Rossmann fold for the binding of co-factors such as nucleotides, whereas class II synthetases have an anti-parallel β-fold and three characteristic motif sequences (Ibba and Söll, 2000). Due to the structural differences in the active sites, ATP binds in an extended form in class I synthetases and in a bent formation in class II synthetases (Arnez and Moras, 1997). Nonetheless, the charging of cognate amino acids is performed by virtually the same two-step reaction in all mt-aaRS.

7.1.2 The mt-aaRS and Human Disease

All genes encoding the mt-aaRSs except *WARS2* have been associated with an autosomal recessive disorder of mitochondrial translation with multiple respiratory chain complex activity deficiency (**Table 7.1**). Of the dual-localised synthetases, recessive *GARS* mutations have been reported in two patients with systemic mitochondrial disease (McMillan *et al.*, 2014; Taylor *et al.*, 2014). Recessive *KARS* mutations have been associated with hearing impairment or peripheral neuropathy (McLaughlin *et al.*, 2010; Santos-Cortez *et al.*, 2013). However, the potential pathological mechanisms of the dual-localised synthetases with mitochondrial defects has not be fully explored. Mt-aaRS mutations typically manifest in early-onset phenotypes with clinical, genetic and molecular heterogeneity. However, notable tissue and cell-type specific phenotypes have been described for some mt-aaRS associated disorders (**Table 7.1**), with the nervous system (NS) most frequently affected. Although mtDNA disease can present some degree of tissue specificity due to varying heteroplasmy in cells, mt-aaRS defects are intriguing since mutations are expressed in all cells. Furthermore, mutations of the mt-aaRS cause phenotypes that do not correspond with mutations of their corresponding cognate mtDNA-encoded tRNA.

	OMIM #	Genotype	Affected Tissue(s)	Onset	Phenotype	Reference
Mitochondrial Amino-Acyl tRNA Synthetases						
AARS2	614096	AR	Heart	Infantile	HCM	Götz *et al.* (2011)
	615889	AR	NS, Reproductive	Childhood	Leukoencephalopathy, POF (females)	Dallabona *et al.* (2014)
CARS2	616672	AR	NS	Infantile	EIEE	Hallmann *et al.* (2014)
DARS2	611105	AR	NS	Variable	LBSL	Scheper *et al.* (2007)
EARS2	614924	AR	NS	Antenatal-Infantile	LTBL	Steenweg *et al.* (2012)
FARS2	614946	AR	NS	Infantile	EIEE	Almalki *et al.* (2014)
	n.a.	AR	NS, Liver	Infantile	Alpers-like	Elo *et al.* (2012)
	617046	AR	NS	Childhood	SPG77	Yang *et al.* (2016)
	n.a.	AR	NS	Infantile	DD	Vernon *et al.* (2015)
HARS2	614926	AR	Inner Ear, Reproductive	Childhood	Perrault Syndrome-2	Pierce *et al.* (2011)
IARS2	616007	AR	NS, Endocrine	Infantile	CAGSSS	Schwartzentruber *et al.* (2014)
	n.a.	AR	NS, Muscle	Neonatal	Leigh Syndrome	Schwartzentruber *et al.* (2014)
LARS2	615300	AR	Inner Ear, Reproductive	Childhood	Perrault Syndrome-4	Pierce *et al.* (2013)
	617021	AR	Blood, Liver	Neonatal	HLASA	Riley *et al.* (2015)
MARS2	611390	AR	NS	Variable	SPAX3	Bayat *et al.* (2012)
	616430	AR	NS, Endocrine	Neonatal	DD, Growth Retardation, Hearing Loss	Webb *et al.* (2015)
NARS2	616239	AR	NS	Infantile	Alpers-like	Sofou *et al.* (2015)
	616239	AR	NS	Childhood	ID, Seizures	Vanlander *et al.* (2015)
	616239	AR	Muscle	Childhood	Myopathy, Fatigue, CPEO	Vanlander *et al.* (2015)

	OMIM #	Genotype	Affected Tissue(s)	Onset	Phenotype	Reference
	n.a.	AR	Inner Ear	Infantile	DFNB94	Simon *et al.* (2015)
	n.a.	AR	NS	Infantile	Leigh Syndrome	Simon *et al.* (2015)
PARS2	n.a.	AR	NS, Liver	Infantile	Alpers-like	Sofou *et al.* (2015)
RARS2	611523	AR	NS	Infantile	PCH6	Edvardson *et al.* (2007)
SARS2	613845	AR	Renal, Pulmonary	Infantile	HUPRA	Belostotsky *et al.* (2011)
	n.a.	AR	NS	Infantile	Spastic Paresis	Linnankivi *et al.* (2016)
TARS2	615918	AR	NS	Neonatal	Encephalopathy	Diodato *et al.* (2014)
VARS2	615917	AR	NS	Infantile	Encephalopathy, Myopathy, CPEO	Diodato *et al.* (2014)
YARS2	613561	AR	Muscle, Blood	Variable	MLASA	Riley *et al.* (2010)
Dual-Localised Amino-Acyl tRNA Synthetases						
GARS	n.a.	AR	NS	Neonatal-Childhood	Cardiomyopathy, Leukoencephalopathy, Myalgia	McMillan *et al.* (2014)
	601472	AD	NS	Variable	CMT2D	Antonellis *et al.* (2003)
	600794	AD	NS	Variable	HMN5A	Antonellis *et al.* (2003)
KARS	613641	AR	NS	n.a.	CMTRIB	McLaughlin *et al.* (2010)
	613916	AR	Inner Ear	Infantile	DFNB89	Santos-Cortez *et al.* (2013)
Cytosolic Mitochondrial Amino-Acyl tRNA Synthetases						
AARS	613287	AD	NS, Inner Ear	Childhood-	CMT2N	Latour *et al.* (2010)

	OMIM #	Genotype	Affected Tissue(s)	Onset	Phenotype	Reference
				Adulthood		
	616339	AR	NS	Infantile	EIEE	Simons *et al.* (2015)
DARS	615281	AR	NS	Infantile	HBSL	Taft *et al.* (2013)
HARS	616625	AD	NS	Variable	CMT2W	Vester *et al.* (2013)
	614504	AR	Inner Ear, Ocular	Childhood	USH3B	Puffenberger *et al.* (2012)
IARS	617093	AR	NS, Endocrine, Liver	Childhood	GRIDHH	Kopajtich *et al.* (2016)
LARS	615438	AR	Liver	Infantile-Childhood	IFLS1	Casey *et al.* (2012)
MARS	616280	AD	NS	Adulthood	CMT2U	Gonzalez *et al.* (2013)
	615486	AR	Liver, Pulmonary	Infantile-Childhood	ILLD	van Meel *et al.* (2013)
QARS	615760	AR	NS	Neonatal-Infantile	MSCCA	Zhang *et al.* (2014)
RARS	616140	AR	NS	Infantile	HLD9	Wolf *et al.* (2014)
VARS	n.a.	AR	NS	Infantile	Microcephaly, Cortical Atrophy	Karaca *et al.* (2015)
YARS	608323	AD	NS	Childhood-Adulthood	CMTDIC	Jordanova *et al.* (2006)
	n.a.	AR	NS, Liver	Infantile	Leukoencephalopathy	Nowaczyk *et al.* (2016)

Table 7.1 The Amino-Acyl tRNA Synthetases and Human Disease. Summary of the mt-aaRS and aaRS associated with human disease. CAGSSS - Cataracts, Growth Hormone Deficiency, Sensory Neuropathy, Sensorineural Hearing Loss and Skeletal Dysplasia; CMT – Charcot-Marie-Tooth; CMTDIC – CMT Dominant Intermediate Type C; CMTRIB - Charcot-Marie-Tooth Recessive Intermediate, B; DD – Developmental Delay; DFNB89 - Deafness, Autosomal Recessive 89; EIEE – Early Infantile-Onset Epileptic Encephalopathy; GRIDHH - Growth Retardation, Intellectual Developmental Disorder, Hypotonia and Hepatopathy; HBSL - Hypomyelination with Brainstem and Spinal Cord Involvement and Lactate Elevation; HCM – hypertrophic cardiomyopathy; HLASA – Hydrops, Lactic Acidosis and Sideroblastic Anaemia; HLD9 - Hypomyelinating Leukodystrophy-9; HMN5A - Neuropathy, Distal Hereditary Motor, Type VA; HUPRA - Hyperuricemia, pulmonary hypertension, renal failure, and alkalosis; IFLS1 -

Infantile Liver Failure Syndrome 1; ILLD - Interstitial Lung and Liver Disease; LBSL - Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation; LTBL - leukoencephalopathy with thalamus and brainstem involvement and high lactate; MLASA – myopathy, lactic acidosis and sideroblastic anaemia; MSCCA - Microcephaly, Progressive Seizures and Cerebral and Cerebellar Atrophy; PCH – Pontocerebellar Hypoplasia; POF – Premature Ovarian Failure; SPAX3 – Spastic Ataxia 3; SPG77 - Spastic Paraplegia 77, Autosomal Recessive; USH3B – Usher Syndrome Type 3B. AD – Autosomal Dominant; AR – Autosomal Recessive. 'n.a.' – not available.

Regarding cytosolic aaRS defects, there are some similarities and differences between the mt-aaRS. In contrast to the mt-aaRS, some aaRS have been associated with autosomal dominant Charcot-Marie Tooth (CMT) disease (Antonellis *et al.*, 2003; Jordanova *et al.*, 2006). Autosomal recessive mutations have also been described with overlapping phenotypes or features due to mt-aaRS mutations without biochemical evidence of mitochondrial dysfunction, which includes overlap between some specific aaRS and mt-aaRS. Despite the specific brain imaging pattern, leukoencephalopathy is a shared feature between recessive *DARS2* and *DARS* mutations (Taft *et al.*, 2013), suggesting a possible shared pathological mechanism. However, this is not strictly established between all mt-aaRS and their corresponding aaRS.

Several mt-aaRS mutations have been identified in only a few or isolated patients, therefore delineating the genotype-phenotype correlations is challenging. The use of WES and NGS have further broadened the phenotypic spectrum to include patients with differential tissue specificity. A homozygous *SARS2* c.1347G>A splicing variant was identified in a patient with progressive spastic paresis other than HUPRA syndrome (Linnankivi *et al.*, 2016). Authors demonstrated that mt-tRNA$^{Ser(AGY)}$ was significantly more destabilised than in HUPRA patients and mt-tRNA$^{Ser(UCN)}$ largely unaffected in both types of patients, suggesting a possible influence for the differential tissue specificity. Additionally, *FARS2* variants have now been associated with many overlapping phenotypes; Alpers-like (Elo *et al.*, 2012; Shamseldin *et al.*, 2012), epileptic encephalopathy (Almalki *et al.*, 2014; Raviglione *et al.*, 2016; Walker *et al.*, 2016), developmental delay (Vernon *et al.*, 2015) and HSP (Yang *et al.*, 2016). Several hypotheses have been proposed in attempting to explain the tissue- and cell-specific manifestations. Complete loss of a single mt-aaRS is likely to be incompatible with life. However, a decrease in the steady-state levels of a single mt-aaRS, severe or partial loss of aminoacylation activity may not be immediately lethal and could modulate the phenotype. Loss of aminoacylation activity for a single mt-aaRS in some tissue or cell types may be insufficient to cause defective mitochondrial translation. Mt-aaRS could also have secondary, non-translational functions similar to the aaRS that could contribute to the apparent cell specificity (Yao and Fox, 2013). For example, the yeast homolog of the dual-localised human LysRS has been shown to import a small proportion of cytosolic tRNA^{Lys} into mitochondria (Martin *et al.*, 1979). Nonetheless, the identification of additional patients is needed to further investigate these proposed these hypotheses.

As described in **6.4.7** and **6.4.8**, two patients with biallelic *AARS2* (patient 30) and *EARS2* (patient 29) variants were identified by WES, utilising the variant filtering strategy for

multiple respiratory chain deficiency. Together with six adult patients with childhood-onset mitochondrial myopathy referred to the NHS Highly Specialised Mitochondrial Diagnostic Service for Rare Mitochondrial Disorders in Newcastle upon Tyne harbouring *YARS2* variants identified previously by WES or targeted *YARS2* gene screening, this chapter depicts and expands the clinical, genetic and molecular features of mt-aaRS mutations and defective mitochondrial translation.

7.1.3 Mitochondrial Alanyl-tRNA Synthetase (*AARS2*)

Mutations of *AARS2* encoding the class II mitochondrial alanyl-tRNA synthetase (mt-AlaRS) are a notable example of two different phenotypes and differential tissue effects associated with one *aaRS2* gene. Unlike other class II synthetases, mt-AlaRS is monomeric. The first patients described presented HCM leading to death within the first year of life (Götz *et al.*, 2011). A second distinct phenotype of childhood- to adult-onset leukoencephalopathy with premature ovarian failure (POF) in females was also described in several patients with conspicuous absence of cardiac involvement (Dallabona *et al.*, 2014; Hamatani *et al.*, 2016; Lynch *et al.*, 2016; Szpisjak *et al.*, 2016). Interestingly, all patients presenting with fatal infantile HCM harboured a recurrent c.1774C>T (p.Arg592Trp) missense variant, either homozygous or in compound with a second variant, that was absent in patients with leukoencephalopathy. Leukoencephalopathy was also described in patients harbouring recessive variants of the cytosolic homolog *AARS* manifesting as deficient myelination on MRI, although accompanied by severe infantile-onset epileptic encephalopathy (Simons *et al.*, 2015). This possibly suggests a similar pathological mechanism, especially since cardiac involvement was also absent from these patients. Euro *et al.* (2015) proposed that the two distinct phenotypes and tissue specificity could be explained by the location of the mutations within the mt-AlaRS structure with differential effects on the alanyl-aminoacylation activity due to the possible combinations of mutations. *AARS2* missense variants were classified as moderate, severe or loss of function. Missense changes occurring in the mt-AlaRS editing domain including the recurrent p.Arg592Trp variant were restricted to patients with fatal infantile HCM due to loss of aminoacylation activity, impaired tRNA binding or misaminoacylation with serine or glycine. In contrast, missense changes associated with childhood- to adult-onset leukoencephalopathies led to only partial reduction in aminoacylation activity.

7.1.4 Mitochondrial Glutamyl-tRNA Synthetase (*EARS2*)

Mutations of *EARS2* encoding the class I mitochondrial glutamyl-tRNA synthetase (mt-GluRS) cause a unique neonatal- or childhood-onset neurological phenotype known as Leukoencephalopathy with Thalamus and Brainstem involvement and high Lactate (LTBL) (Steenweg *et al.*, 2012). Previous clinical and genetic diagnoses of *EARS2* patients have relied upon a distinct brain MRI pattern of leukoencephalopathy, abnormal signal changes in the thalamus and brainstem with magnetic resonance (MR) spectroscopy disclosing a lactate peak. Although brain imaging of *EARS2* patients shows remarkable consistency, Steenweg *et al.* (2012) noted two bi-phasic courses; (i) a mild or intermediate phenotype with developmental delay preceding severe regression within the first year of life, followed by some stabilisation that is evident by improved brain magnetic resonance imaging (MRI); (ii) a severe phenotype with neurological impairment from birth (or shortly after) and clinical stabilisation over time, dependent on the initial neurological damage. Since the initial report, patients harbouring *EARS2* mutations have consistently grouped into one of these two bi-phasic courses.

7.1.5 Mitochondrial Tyrosyl-tRNA Synthetase (*YARS2*)

Mutations of *YARS2* encoding the class II mitochondrial tyrosyl-tRNA synthetase (mt-TyrRS) have been associated with childhood-onset myopathy, lactic acidosis and sideroblastic anaemia, known as MLASA syndrome (Riley *et al.*, 2010; Sasarman *et al.*, 2012; Riley *et al.*, 2013; Shahni *et al.*, 2013; Nakajima *et al.*, 2014; Ardissone *et al.*, 2015b). Sideroblastic anaemia is a conspicuously rare feature of mitochondrial disease more commonly associated with Pearson's syndrome due to single large-scale mtDNA deletions (Pearson *et al.*, 1979; McShane *et al.*, 1991). Prior to *YARS2*, mutations of *PUS1* encoding pseudouridylate synthase 1 were associated with a phenotypically similar mitochondrial translation disorder (Bykhovskaya *et al.*, 2004; Fernandez-Vizarra *et al.*, 2009; Cao *et al.*, 2015). Therefore, MLASA syndrome has been useful in guiding diagnostic investigations. On the other hand, this has been complicated by the recent identification of similar presentations in patients with different genetic aetiologies (**Table 7.2**).

Gene	Mutation(s)	Onset	Phenotype	Reference(s)
MtDNA Mutations				
Single Large-Scale Deletion		Infantile	Pearson's Syndrome	Pearson *et al.* (1979), McShane *et al.* (1991)
MT-ATP6	m.8969G>A	Neonatal	MLASA Syndrome, DD, Hearing Loss, Epilepsy	Burrage *et al.* (2014)
Nuclear-Encoded Genes				
NDUFB11	c.276_278del, p.Phe93del	Neonatal-infantile	X-Linked Congenital Sideroblastic Anaemia	Lichtenstein *et al.* (2016), Torraco *et al.* (2016)
PUS1	Variable	Variable	MLASA Syndrome	Bykhovskaya *et al.* (2004), Patton *et al.* (2005), Zeharia *et al.* (2005), Fernandez-Vizarra *et al.* (2009), Cao *et al.* (2015), Metodiev *et al.* (2015)
TRNT1	Variable	Variable	Sideroblastic anaemia, Immunodeficiency, DD	Chakraborty *et al.* (2014), Sasarman *et al.* (2015), Wedatilake *et al.* (2016)
YARS2	Variable	Variable	MLASA Syndrome	Riley *et al.* (2010), Sasarman *et al.* (2012), Riley *et al.* (2013), Shahni *et al.* (2013), Nakajima *et al.* (2014), Ardissone *et al.* (2015b), Sommerville *et al.* (2017)

Table 7.2 Sideroblastic Anaemia in Mitochondrial Disease. A list of mt-DNA and nuclear genes currently associated with sideroblastic anaemia.

To date, *YARS2* mutations have predominantly been identified in patients from Lebanon with either a homozygous c.137G>A (p.Gly46Asp) or c.156C>G (p.Phe52Leu) missense change. Despite harbouring the same missense change, patients presented variable phenotypes including childhood- to adult-onset and the absence of some clinical features, including sideroblastic anaemia. Taken together, this suggests that *YARS2*-associated mitochondrial disease could be underdiagnosed outside of the Middle East.

7.2 Aims

This chapter expands the clinical, genetic and molecular features of patients harbouring *AARS2, EARS2* or *YARS2* mutations.

7.3 Methods

7.3.1 Patients

This study comprised 10 patients (29, 30 and 41-46.2) referred to the NHS Highly Specialised Service for Rare Mitochondrial Disorders or to external clinics.

7.3.2 Diagnostic Histochemical, Biochemical and Molecular Studies

Histochemical, biochemical and molecular studies of patient skeletal muscle were performed by the NHS Highly Specialised Mitochondrial Diagnostic Service in Newcastle upon Tyne or externally, as described in **2.2.3**.

7.3.3 *YARS2* mtDNA Haplogroups

Haplogroups of *YARS2* patients (43.1 and 44-46.1) were defined from all mtDNA polymorphisms using HaploGrep2 (Kloss-Brandstätter *et al.*, 2011).

7.3.4 Genetic Studies

Patients 29 (*EARS2*) and 30 (*AARS2*) were diagnosed using WES by this research (**6.4.7** and **6.4.8**) utilising the WES filtering strategy for the mitochondrial respiratory chain complex deficiency cohort. Identified *EARS2* (NM_001083614) and *AARS2* (NM_020745.2) variants were confirmed by Sanger sequencing using forward and reverse primers for the respective exons (**Appendix A**). Patients 41 and 42 (*AARS2*) were diagnosed previously using WES, corresponding to patients #11 and #7 from Taylor *et al.* (2014).

YARS2 (NM_001040436.2) variants were identified previously in patients 43.1, 44-46.1 using WES or targeted screening of all exons and intronic regions of *YARS2*. Patient 46.1 was diagnosed first by WES and corresponded to patient 22 from Taylor *et al.* (2014). Patients 44 and 45 were also diagnosed previously using WES, analysed using a different WES bioinformatics pipeline (Haack *et al.*, 2012). Patient 43.1 was diagnosed by targeted *YARS2*

screening and had been initially described in 1974 together with his sibling, patient 43.2 (Rawles and Weller, 1974).

7.3.5 Cell Culture

The subculturing, freezing and harvesting of cells was performed as described in **2.4**. Cultured fibroblasts were grown for patients 30, 41, 42 (*AARS2*), patient 29 (*EARS2*) and patient 45 (*YARS2*), together with at least two appropriate age-matched controls. Cultured myoblasts for patient 45 *(YARS2*) and two age-matched controls were also grown.

7.3.6 Western Blotting

Fibroblast and myoblast lysates were prepared as described in **2.5.1**. Frozen skeletal muscle from patient 29 and two age-matched controls were homogenised as described in **2.5.2**. All fibroblast (50µg), myoblast (50µg) and muscle lysates (25µg) subjected to 12% SDS-page, probed with primary and secondary antibodies (**Table 2.4**, **Table 2.5**) then detected as outlined in **2.5**. Membranes were incubated with primary antibodies specific to mt-AlaRS, mt-GluRS or mt-TyrRS, plus OXPHOS subunits NDUFB8, SDHA, UQCRC2, MT-COI, MT-COII and ATP5B. α-Tubulin, β-Actin and VDAC1/Porin were used as loading controls.

7.4 Results

7.4.1 A Novel *AARS2* Editing Domain Mutation Causing Fatal Infantile HCM and Respiratory Failure

Clinical data for patient 30 were obtained and diagnostic investigations were performed by Andrew A. M. Morris (Willink Biochemical Genetics Unit, Manchester Centre for Genomic Medicine, Central Manchester University Hospitals NHS Foundation Trust, Manchester), Robert McFarland, and Robert W. Taylor (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne). *In silico* and functional studies were performed in collaboration with Liliya Euro and Svetlana Konovalova, Henna Tyynismaa (Research Programs Unit, Molecular Neurology, Biomedicum Helsinki and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland).

7.4.1.1 Case Report

Patient 30 was a boy born to non-consanguineous British parents who presented with severe myopathy, metabolic acidosis, respiratory insufficiency that required non-invasive ventilation (NIV) from birth and died at 7 weeks of age. Initially absent, cardiomyopathy developed by 5 weeks of age, manifesting as mild biventricular hypertrophy and a significant conduction defect. Whole mitochondrial genome sequencing did not identify any pathogenic or likely pathogenic mutations. Muscle biochemical studies revealed decreased complex I, III and IV activities compared to controls (**Figure 7.2A**), while muscle histopathology disclosed over 50% COX-deficient fibres and increased lipid. Patient 30 was the fifth pregnancy of the parents and the third child born (**Figure 7.2B**). The first-born child (female) died 24 hours after birth from severe lactic acidemia and coagulopathy, with post mortem showing pulmonary hypoplasia. The second born child (male) was healthy but the third and fourth pregnancies resulted in miscarriages.

The clinical, molecular and genetic features of the *AARS2* patients (30, 41 and 42) included in this study are provided in **Table 7.3**. To summarise, all three patients presented with fatal HCM within the first year of life. Patients 30 and 42 also had myopathy and lactic acidosis. Respiratory involvement was confined to patient 30 and central nervous system (CNS) involvement to patient 42 only. All patients had COX-deficient fibres and multiple respiratory chain complex deficiency.

Patient	30	41	42
Sex	M	F	M
Age of Onset	Birth	6 months	Birth
Death	7 weeks	11 months	6 weeks
Ethnicity	White	White	White
Consanguinity	No	No	No
Family History	Yes (one sibling)	No	No
Clinical Signs			
HCM	+	+	+
Myopathy	+ (severe)	-	+
Lactic acidosis	+	-	+
Other Features	Respiratory insufficiency	-	CNS involvement
Muscle Studies			
Histochemistry	>50% COX-deficient fibres, increased lipid	COX-mosaic	COX-mosaic
RC Deficiency	I, III, IV	I, IV	I, III, IV
AARS2 **Variants**			
cDNA Change	c.1088dupT/c.1738C>T	Homozygous c.1774C>T	c.1774C>T/c.2882C>T
Amino Acid Change	p.Asp337*/p.Arg580Trp	Homozygous p.Arg592Trp	p.Arg592Trp/p.Ala961Val

Table 7.3 Clinical, Molecular and Genetic Features of Studied *AARS2* Patients. '+' – present; '-' – absent; RC – mitochondrial respiratory chain.

7.4.1.2 WES Analysis

Implementation of the WES filtering strategy (**6.4.8**) in patient 30 revealed biallelic variants in *AARS2*; c.1008dupT, p.Asp337* and c.1738C>T, p.Arg580Trp (**Figure 7.2B**) as the only recessive variants in genes encoding mitochondrial-targeted proteins. *AARS2* (6p21.1) matched the GO-Terms 'mitochondr*', 'tRNA' and 'translation'. Sanger sequencing with diagnostic primers for *AARS2* exons 6+7 and 11+12 confirmed both variants. However, segregation studies were not possible. Both variants were absent from in-house exomes. The p.Asp337* nonsense variant was present in 1/117540 heterozygous non-Finnish European allele (MAF=0.000008508) in ExAC but was absent in other external exome databases. The

p.Arg580Trp missense variant was present in 3/121260 heterozygous alleles (MAF=0.00002474), two South Asians and one non-Finnish European, in ExAC but was absent in other external databases. The Arg580 residue was highly conserved in vertebrate species, but not in invertebrate species *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (**Figure 7.2C**). It was predicted to be damaging by both PolyPhen 2 (0.996) and SIFT (0.02) but not by Align-GVGD (Class 0).

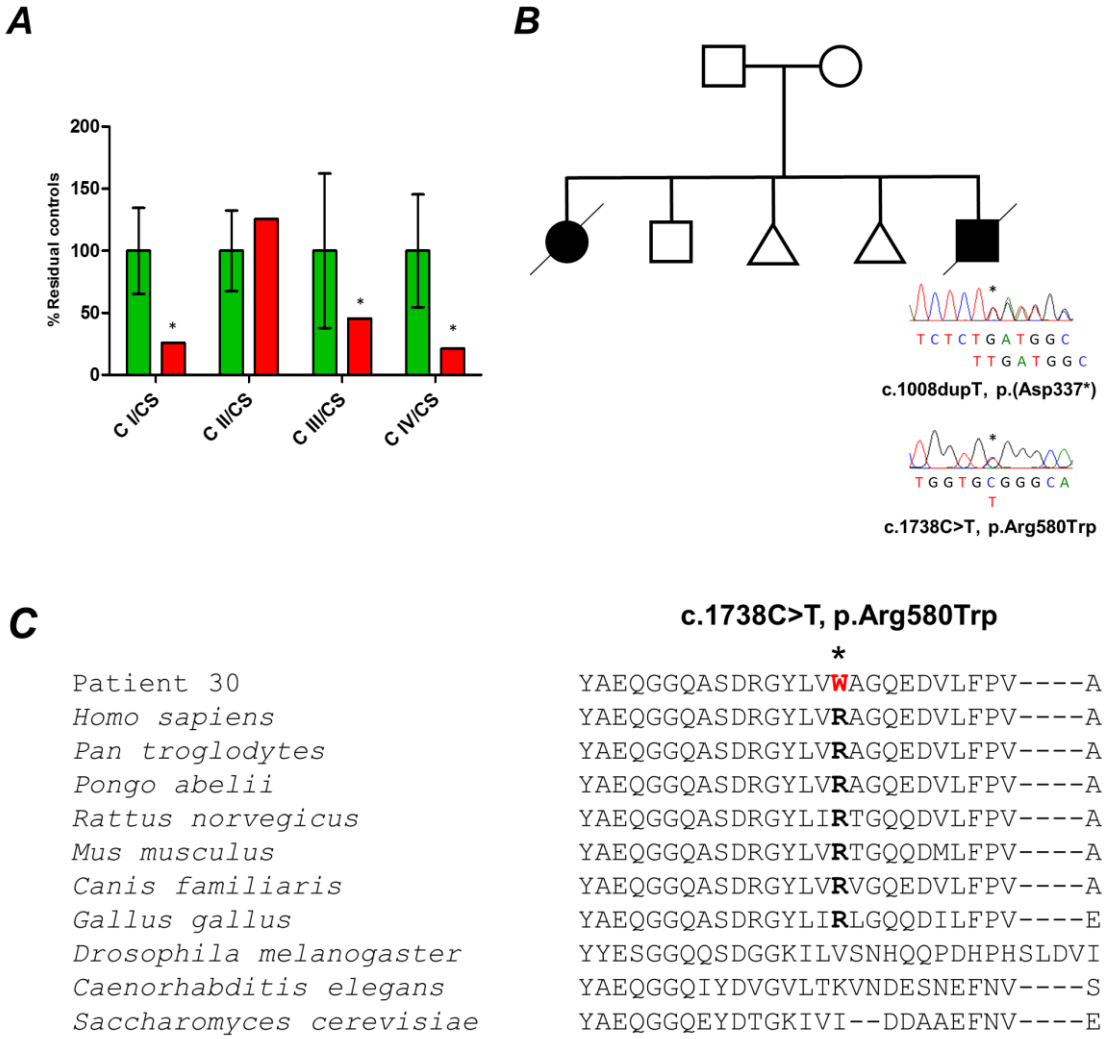


Figure 7.2 Biochemical and Genetic Features of Patient 30. (*A*) Measurements of mitochondrial respiratory chain activities normalised to citrate synthase of complex I (CI/CS), II (CII/CS), III (CIII/CS) and IV (CIV/CS) from patient 30 (red) skeletal muscle compared to controls (green). (*B*) Family pedigree and Sanger sequencing confirmation of the p.Asp337* and p.Arg580Trp *AARS2* variants. (*C*) Multiple sequence alignment (MSA) of the mt-AlaRS Arg580 residue.

From Taylor *et al.* (2014), patient 41 was homozygous for the recurrent p.Arg592Trp missense change and patient 42 had compound heterozygous p.Arg592Trp and c.2882C>T, p.Ala916Val *AARS2* missense variants.

7.4.1.3 *In Silico* Structural Modelling of p.Arg580Trp

In silico modelling of the p.Arg580Trp missense change on human mt-AlaRS was performed by Liliya Euro (Research Programs Unit, Molecular Neurology, Biomedicum Helsinki and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland) to predict possible effects on structure and aminoacylation activity. Modelling was performed using the resolved human mt-AlaRS structure as described previously (Euro *et al.*, 2015).

In human mt-AlaRS, Arg580 is a surface exposed residue at the top of the β-barrel in the editing domain. Other exposed residues of the editing domain mutated in patients presenting fatal infantile cardiomyopathy were Tyr539 and Arg592. However, Arg580 was localised nearer the top of the β-barrel structure (**Figure 7.3A**). Given the position of Arg580 and the side chains of possible residues, it was suggested that Arg580 would have a role in the enzyme structure. Substitution with tryptophan was predicted to affect either enzyme stability, the surface of the β-barrel or both (**Figure 7.3B**). Previous modelling of human mt-AlaRS demonstrated the presence of a linker between the aminoacylation and editing domains referred to as the 'safety belt', with the editing domain acting as a 'buckle' when $tRNA^{Ala}$ is bound to the aminoacylation domain (Euro *et al.*, 2015). Side chains of tryptophan may propagate along the entire β-barrel surface, affecting areas in direct contact with the 'safety belt'. The Arg580 residue was predicted to be associated in electrostatic, hydrophobic and hydrogen bonds (**Figure 7.3C**), though it was not predicted to interact directly with the 'safety belt'. Substitution with tryptophan was predicted to abolish approximately half of the interactions (**Figure 7.3D**). Several of these interactions were predicted to be involved the stability of the β-barrel domain, which may affect both folding of the β-barrel domain and stability of the whole protein structure. Furthermore, absence of several side chain interactions, including one required for when tRNA is bound for aminoacylation was predicted to be abolished, possibly affecting the rate of tRNA aminoacylation.

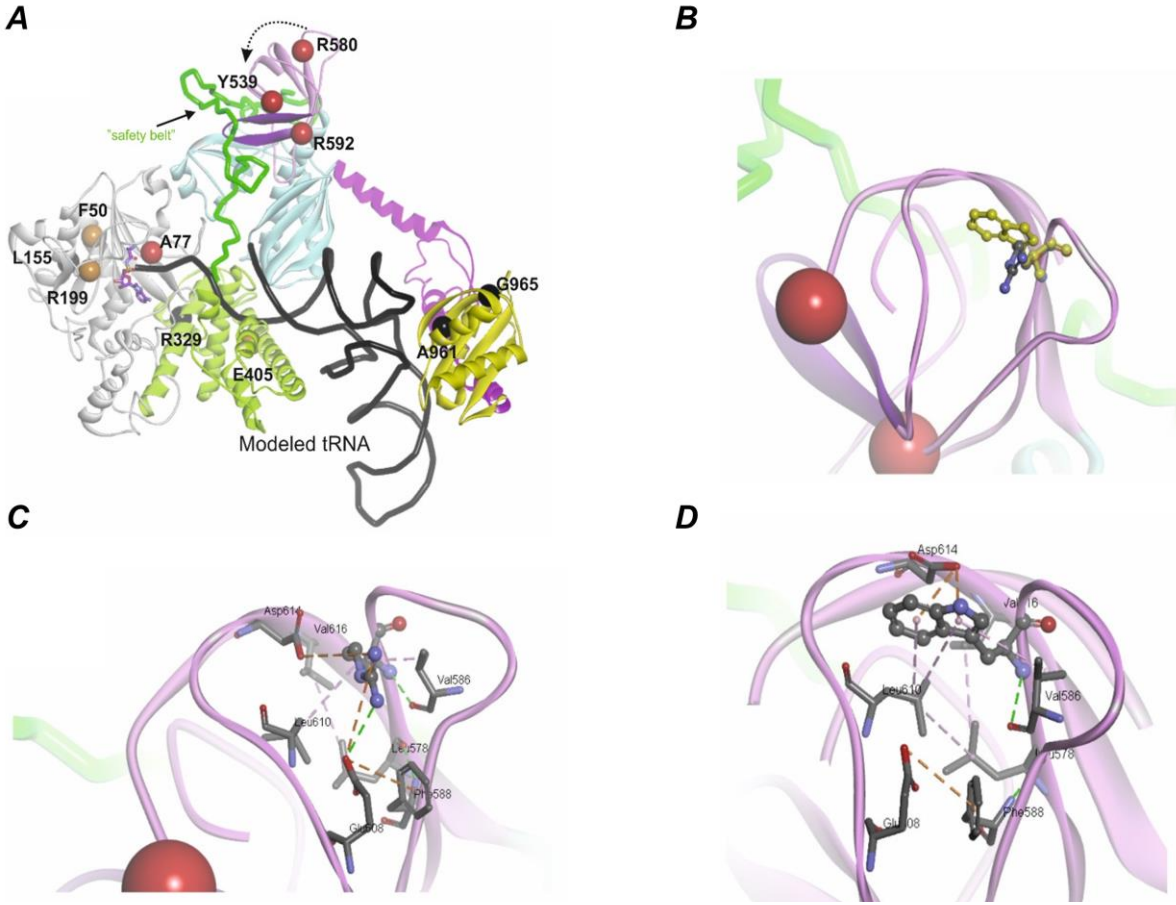


Figure 7.3 *In silico* Modelling of the Human mt-AlaRS p.Arg580Trp Missense Change. *(A*) Location of the Arg580 residue within the β-barrel of the human mt-AlaRS editing domain, together with additional residues mutated in *AARS2* patients. The 'safety belt' is shown in green, denoted by the solid arrow. The dashed arrow denotes the direction of propagation due to p.Arg580Trp variant. (*B*) Comparison of the possible side chains with Arg580 or Trp580. (*C*) Intramolecular interactions of the Arg580 residue. (*D*) Changes in intramolecular interactions due to the p.Arg580Trp missense variant. Hydrogen bonds – green dashed lines; electrostatic bonds – orange dashed lines; hydrophobic bonds – magenta dashed lines.

7.4.1.4 Western Blot Analysis

Fibroblasts from all three studied *AARS2* patients showed a marked decrease in steady-state mt-AlaRS levels, as predicted by the *in silico* modelling of the p.Arg580Trp variant and previous modelling of the p.Arg592Trp and p.Ala961Val missense variants. However, levels of OXPHOS subunits were not decreased in all patients compared to controls (**Figure 7.4**).

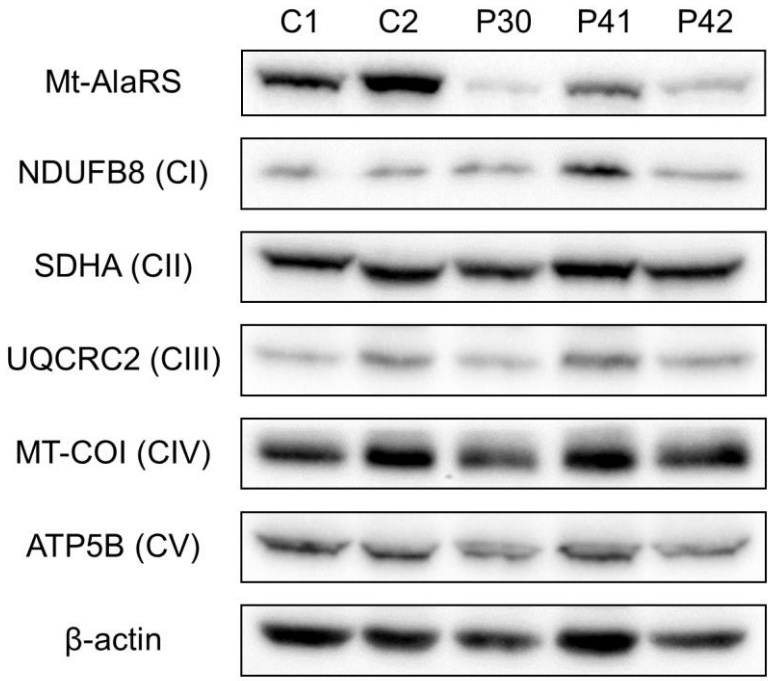


Figure 7.4 Western Blot Analysis of *AARS2* Patient Fibroblasts. Steady-state mt-AlaRS and OXPHOS subunit protein levels in fibroblasts of patients 30, 41 and 42.

7.4.1.5 Northern Blot and Aminoacylated tRNA Analysis

Northern blot analysis of tRNAs were performed by Svetlana Konovalova (Research Programs Unit, Molecular Neurology, Biomedicum Helsinki and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland), with total RNA extracted from fibroblasts of patient 30 and a second patient homozygous for the recurrent p.Arg592Trp variant. RNA was hybridised with probes for $tRNA^{Ala}$ and $tRNA^{Arg}$ (loading control) species. However, this did not show changes in the abundance of either species (**Figure 7.5A and B**). Analysis of aminoacylated $tRNA^{Ala}$ showed the presence of charged Ala-$tRNA^{Ala}$ and Arg-$tRNA^{Arg}$ with no uncharged tRNAs (**Figure 7.5C**).

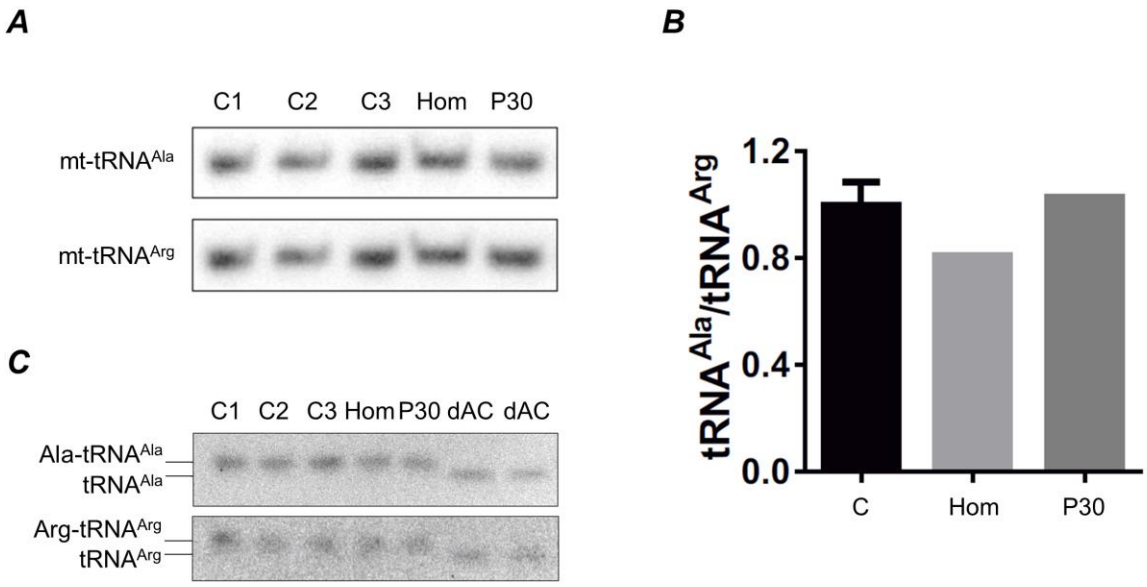


Figure 7.5 Northern Blot Analysis of *AARS2* Patient Fibroblasts. (*A*) Northern blot analysis of mt-tRNA^{Ala} levels in fibroblasts from patient 30 and a patient homozygous for the recurrent p.Arg592Trp *AARS2* missense change, using mt-tRNA^{Arg} as a loading control. (*B*) Quantification of tRNA^{Ala} normalised to tRNA^{Arg}. (*C*) Northern blot analysis of charged (aminoacylated) Ala-tRNA^{Ala} and Arg-tRNA^{Arg} with uncharged (deacylated) tRNAs. The lower bands in the dAC lanes denote uncharged tRNA species.

7.4.2 Lethal Neonatal Leukoencephalopathy with Thalamus and Brainstem Involvement with High Lactate (LTBL) Caused by *EARS2* mutations

Clinical data for patient 29 were obtained and diagnostic investigations were performed by Renata Oliveira (Human Genetics Department, Centro Hospitalar de São João, Porto, Portugal), Joana Nunes (Imaging Department, Centro Hospitalar Vila Nova de Gaia/Espinho, Vila Nova de Gaia, Portugal), Manuela Grazina (CNC - Center for Neuroscience and Cell Biology, Laboratory of Biochemical Genetics, University of Coimbra, Coimbra, Portugal; Faculty of Medicine, University of Coimbra, Coimbra, Portugal), Luísa Diogo, Paula Garcia (Metabolics Unit - Child Development Center, Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal), Kyle Thompson and Robert W. Taylor (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne).

7.4.2.1 Case Report

Patient 29 was a female born at term to non-consanguineous Portuguese parents. Mild ventriculomegaly was noted on ultrasound at 21 weeks of gestation. This was confirmed by brain MR at 22 weeks, but a further brain MR at 24 weeks was normal. Birth weight, length

and occipital frontal circumference were normal. Her first admission was 7 days after birth because of FTT, severe feeding problems and hypotonia. On further admissions she had signs of undernutrition and subsequently developed encephalopathy. She had persistent lactic acidosis and liver dysfunction, but no respiratory or cardiac involvement. Newborn screening was also normal. Brain MRI was performed at 2 months of age, which showed delayed myelination and symmetrical lesions of the brainstem, cerebellum and thalami (**Figure 7.6A-G**). Elevated lactate peak was observed on brain MRI. Blood plasma ammonia levels were normal and there was no ketonuria. Muscle histochemistry demonstrated COX-deficient (**Figure 7.7A**) and ragged-red fibres. Mitochondrial respiratory chain complex activities were measured in muscle and liver, showing decreased complex I (19%) and complex IV (11%) activity in the muscle compared to controls (**Figure 7.7B**). Respiratory chain activity in the liver was within normal ranges. Histopathology of the liver demonstrated sinusoid distension, mixed steatosis of the peri-centrilobulbar and hemosiderosis. Whole mitochondrial genome sequencing of patient blood, muscle and liver DNA excluded mtDNA mutations. Additionally, mtDNA depletion and rearrangements were excluded by quantitative real time PCR and long range PCR assays. Sanger sequencing and Multi Ligation Probe Amplification (MLPA) also excluded *SURF1* and *POLG* variants.

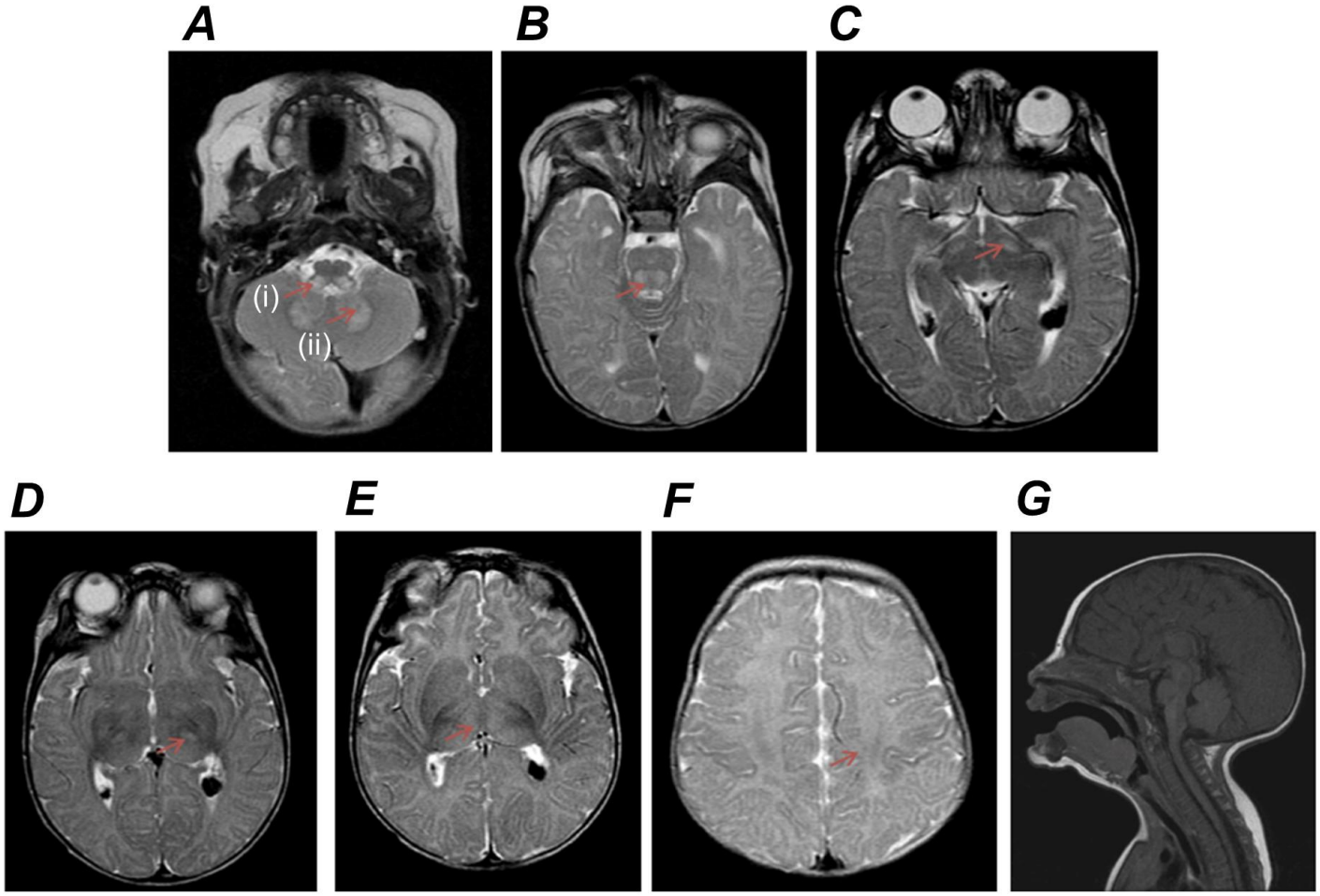


Figure 7.6 Brain MRI of Patient 29. Brain MRI was performed at 2 months old. T2-weighted images showed abnormally high bilateral and symmetrical signals in (*A*) (i) posterior part of the medulla oblongata and (ii) cerebellar white matter and dentate nucleus, (*B*) posterior part of the pons, (*C*) cerebral penducles and (*D*, *E*) subthalamus and thalamus. (*F*) Delayed myelination in pericentral regions. (*G*) Hypogenesis of the corpus callosum. Adopted and amended from (Oliveira and Sommerville, 2016).

7.4.2.2 WES Analysis

Implementation of the WES filtering strategy (**6.4.7**) identified biallelic *EARS2* variants; a c.1A>G (p.Met1?) start-loss variant and a c.184A>T (p.Ile62Phe) missense variant. *EARS2* (16p12.2) matched the GO-Terms 'mitochondr*', 'tRNA' and 'translation'. Both variants were confirmed by Sanger sequencing using primers for *EARS2* exons 1 and 2. Segregation studies confirmed that p.Met1? was inherited from the mother and p.Ile62Phe from the father (**Figure 7.7C**). Both p.Met1? and p.Ile62Phe were absent from in-house exomes and external databases. However, p.Met1? was previously identified in a patient of Portuguese ancestry harbouring biallelic *EARS2* variants (Steenweg *et al.*, 2012), suggesting a possible founder effect in this population. Since the first nucleotide of the *EARS2* start codon was substituted, this was predicted to eliminate translation of the protein on one allele. The novel p.Ile62Phe missense variant affected a moderately conserved residue in a highly conserved region of mt-GluRS (**Figure 7.7D**). It was predicted benign by PolyPhen2 (0.006) and Align-GVGD (Class 0), but was predicted to affect protein function by SIFT.

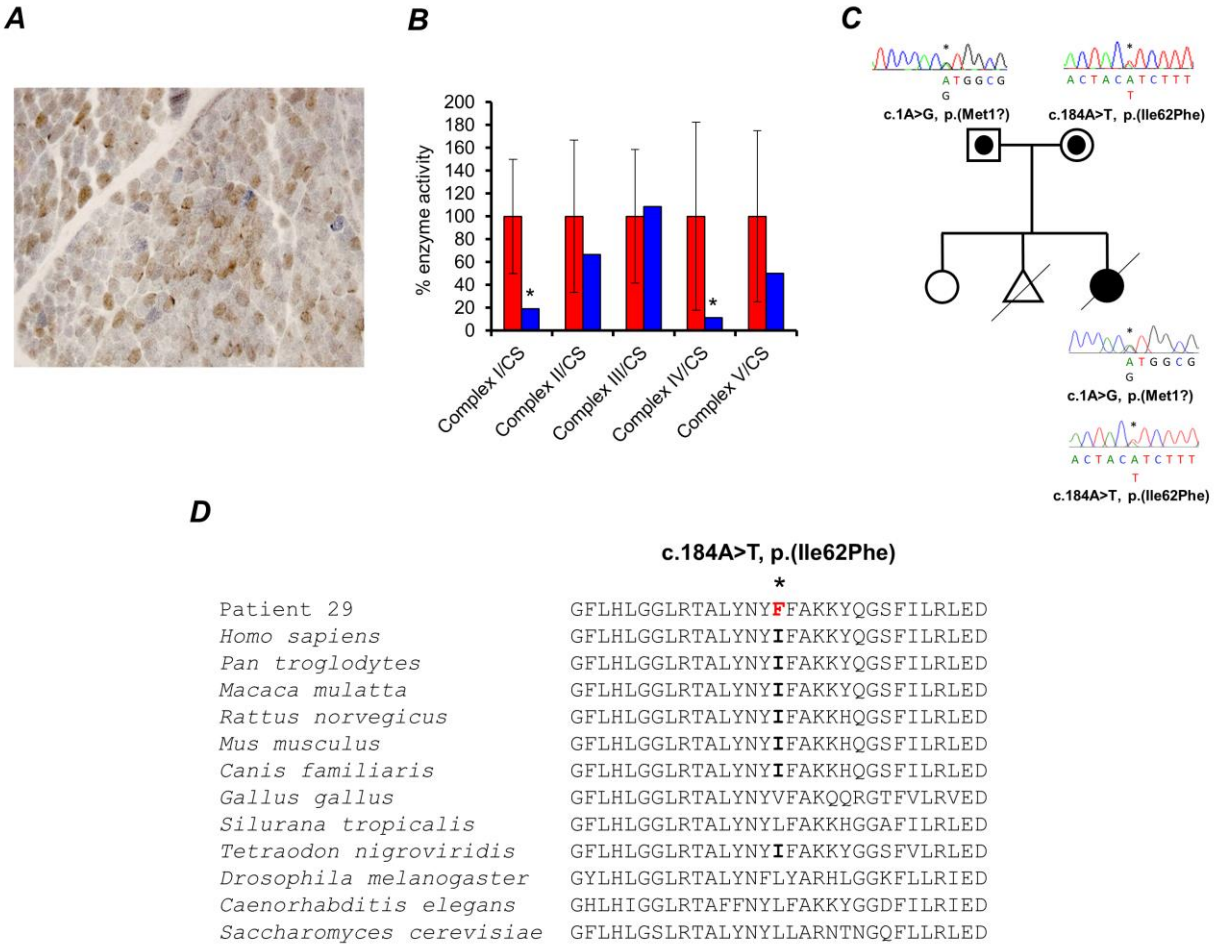


Figure 7.7 Histochemical, Biochemical and Genetic Features of Patient 29. (*A*) Sequential COX-SDH histochemistry of patient 29 skeletal muscle. (*B*) Biochemical analysis of mitochondrial respiratory chain activities in skeletal muscle of patient 29 (blue) compared to controls (red), normalised to citrate synthase of complex I (CI/CS), II (CII/CS), III (CIII/CS), IV (CIV/CS) and V (CV/CS). (*C*) Family pedigree and Sanger sequencing confirmation of parental carrier status of the p.Met1? and p.Ile62Phe *EARS2* variants. (*D*) MSA of the Ile62 mt-GluRS residue. Adopted from (Oliveira and Sommerville, 2016).

7.4.2.3 Western Blot Analysis

Fibroblasts and muscle homogenate were studied to inspect mt-GluRS protein levels and OXPHOS subunits. Kyle Thompson (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne) performed western blotting of control and patient muscle homogenates. Both patient fibroblasts and muscle homogenate showed marked decreases in steady-state mt-GluRS levels (**Figure 7.8**), confirming elimination of the translation initiation site due the p.Met1? start-loss variant and suggesting that p.Ile62Phe leads to unstable mt-GluRS protein structure. A reduction in the abundance of steady-state mt-GluRS levels had been previously demonstrated in fibroblasts of a patients harbouring compound heterozygous *EARS2* variants (Danhauser *et al.*, 2016). Decreased levels of MT-COI, MT-COII (complex IV) and NDUFB8 (complex I) were observed only in patient muscle homogenate, which correlated with the complex I and IV activity deficiencies (**Figure 7.8B**).

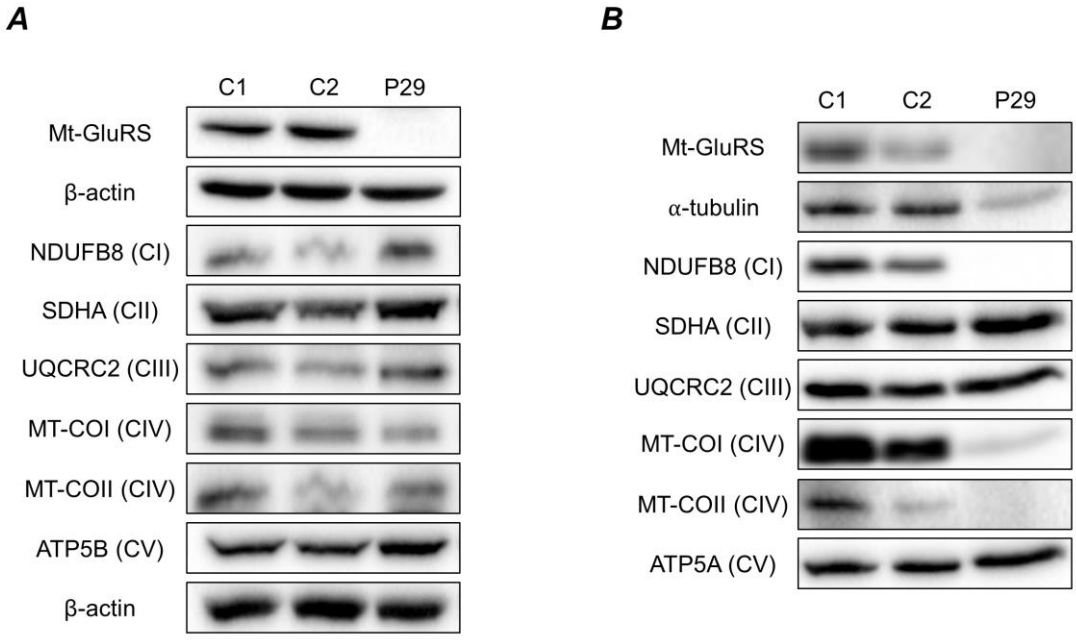


Figure 7.8 Western Blot Analysis of Patient 29 Fibroblasts and Muscle Homogenate. Steady-state mt-GluRS and OXPHOS subunit protein levels in patient 29 (*A*) fibroblasts and (*B*) muscle homogenate. Adopted and amended from (Oliveira and Sommerville, 2016).

7.4.3 Adults with *YARS2*-Associated Mitochondrial Myopathy

Clinical data for six patients with *YARS2* mutations were obtained, diagnostic investigations and functional studies were performed by Yi Ng, Charlotte L. Alston, Langping He, Charlotte Knowles, Sophie L. Chin, Andrew M. Schaefer, Gavin Falkous, Robert McFarland, Douglass

Turnbull, Robert W. Taylor, Gráinne S. Gorman (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne), Cristina Dallabona, Micol Gilberti, Claudia Donnini (Department of Life Sciences, University of Palma, Italy), David Murdoch (Department of Cardiology, Queen Elizabeth University Hospital, Glasgow), Cheryl Longman (West of Scotland Regional Genetics Service, Queen Elizabeth University Hospital, Glasgow), Marianne de Visser (Department of Neurology, Academic Medical Centre, Amsterdam, Netherlands), Laurence A. Bindoff (Department of Clinical Medicine (K1), University of Bergen, Norway; Department of Neurology, Haukeland University Hospital, Bergen, Norway), John M. Rawles (Formerly Department of Medicine, University of Aberdeen, Aberdeen), John C. S. Dean (Department of Medical Genetics, Medical School Building, University of Aberdeen, Aberdeen), Richard K. Petty, Maria E. Farrugia (Institute of Neurological Sciences, Queen Elizabeth University Hospital, Glasgow), Tobias B. Haack and Holger Prokisch (Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany; Institute of Human Genetics, Technische Universität München, Munich, Germany).

7.4.3.1 Case Reports

The clinical, genetic and molecular features of six patients diagnosed with *YARS2*-associated mitochondrial myopathy are summarised in **Table 7.4**. This was comprised of four pedigrees with two sets of affected siblings (43.1 and 43.2, 46.1 and 46.2). Patients 43.1 and 43.2 were first reported in Rawles and Weller (1974). Patient 46.1 was reported in Taylor *et al.* (2014), corresponding to patient #22.

All six studied patients presented during childhood and had varying degrees of myopathy, but only four patients had lactic acidosis. Three patients had sideroblastic anaemia, but only patient 46.1 was transfusion dependent. All patients survived into adulthood. However, three patients were deceased (43.1, 43.2 and 46.1); they had respiratory insufficiency that required NIV and/or HCM that preceded death. Patient 44 was the oldest surviving patient, who at 73 years old is currently alive having presented progressive myopathy without lactic acidosis and sideroblastic anaemia.

All patients had striking global-COX deficiency (**Figure 7.9**), while patient 45 also had ragged-red fibres. Biochemical analysis of muscle respiratory chain activities in patients 43.1, 44-46.1 revealed multiple respiratory chain deficiency; patients 44 and 46.1 had decreased complex I and IV activities, while patients 43.1 and 45 had decreased complex I, III and IV

activities. Additionally, mtDNA copy number was substantially increased in patient 45, while a modest increase was also seen in patient 46.1.

Patient	**43.1**	**43.2**	**44**	**45**	**46.1**	**46.2**
Sex	M	M	F	M	M	M
Age of Onset	12	2	Childhood	4	<10	<10
Course	52†	45†	Alive, 73	Alive, 23	33†	Alive, 35
Ethnicity	White	White	White	White	Arab	Arab
Consanguinity	No	No	Yes	No	No	No
Family History	Yes (43.2)	Yes (43.1)	No	No	Yes (46.2)	Yes (46.1)
Clinical Signs						
Myopathy (MRC)	+ (4/5)	+	+ (4-/5, wheel chair at age 59)	+ (4/5)	+ (4/5)	+ (3/5)
Lactic Acidosis	+	+	-	+	+	+
SA	+	+	-	-	+	n.d.
Transfusion Dependent	-	-	-	-	+	n.d.
Respiratory Insufficiency	n.a.	n.a.	+ (NIV at age 55)	+ (severe restrictive pattern)	+	+ (NIV at age 33)
HCM	+	+	-	+	+	-
Other Features	-	-	Facial weakness	-	-	Scapular winging
Muscle Studies						
Histochemistry	Global COX	n.a.	Global COX	Global COX	Global COX	Ragged Red Fibres

	deficiency		deficiency	deficiency, Ragged Red Fibres	deficiency	
RC Deficiency	I, III, IV	n.a.	I, IV	I, III, IV	I, IV	n.d.
***YARS2* Variant**						
cDNA Change	Homozygous c.1175T>C	n.d.	Homozygous c.1175T>C	c.1106G>A/ c.1147_1164dup	Homozygous c.137G>A	n.d.
Amino Acid Change	Homozygous p.Leu392Ser	n.d.	Homozygous p.Leu392Ser	p.Cys369Tyr/ p.Val383_Glu388dup	Homozygous p.Gly46Asp	n.d.
mtDNA Haplogroup	H1m1	n.d.	G2a3a	T2e1a	HV13	n.d.

Table 7.4 Clinical, Molecular and Genetic Features of Studied *YARS2* Patients. † – deceased; '+' – present; '-' – absent; HCM – hypertrophic cardiomyopathy; MRC - Medical Research Council scale for muscle strength; SA – sideroblastic anaemia. 'n.a.' – not available; 'n.d.' – not determined. Adopted and amended from (Sommerville *et al.*, 2017).

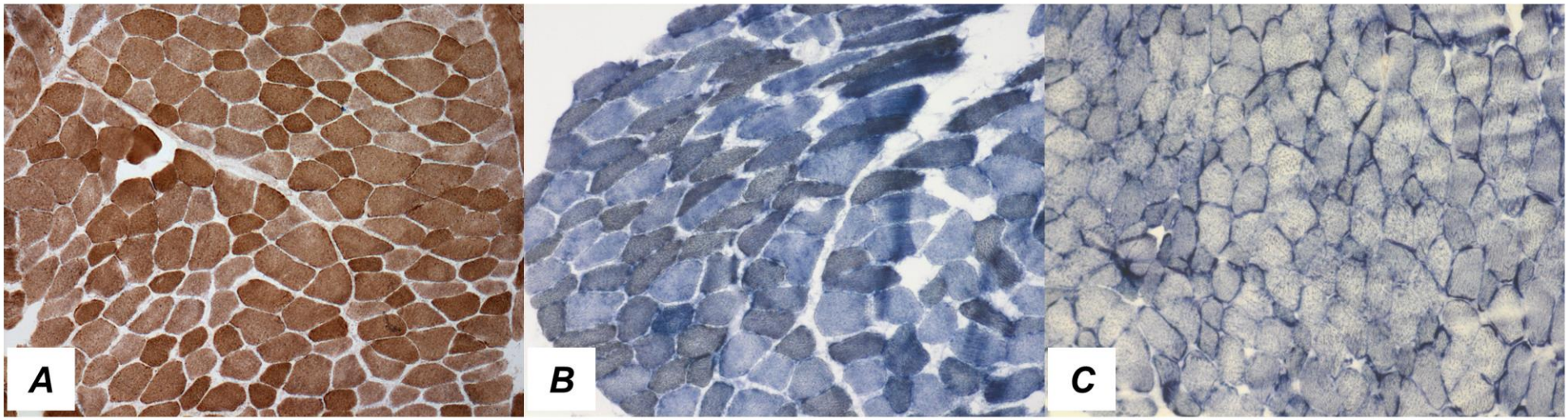


Figure 7.9 Sequential COX-SDH Muscle Histochemistry of *YARS2* Patients. Sequential COX-SDH histochemistry of skeletal muscle from (*A*) a healthy control adult, (*B*) patient 44 and (*C*) patient 46.1. Adopted from (Sommerville *et al.*, 2017).

7.4.3.2 Identification of *YARS2* Mutations

Patients 43.1 and 44 both harboured the same homozygous p.Leu392Ser missense change and were apparently from two unrelated families of Scottish ancestry. It was not possible to confirm the missense change the affected sibling, patient 43.2. However, his unaffected sibling did not harbour the missense change. Patient 44 was born to first cousin parents. The p.Leu392Ser missense variant was reported in 1/121354 non-Finnish European allele (MAF=0.000008240) in ExAC. The Leu392 residue was a highly conserved residue in all species except *D. melanogaster* and occurred in a moderately conserved region of mt-TyrRS (**Figure 7.10A**). Patient 45, who was also of Scottish ancestry, harboured compound heterozygous p.Cys369Tyr and p.Val383_Glu388dup variants. The p.Cys369Tyr variant was absent from all external exome databases. The Cys369 residue was also highly conserved except in *D. melanogaster* and *S. cerevisiae*, occurring in the same moderately conserved region of mt-TyrRS as the Leu392 residue (**Figure 7.10A**). The p.Val383_Glu388dup in-frame duplication was reported in 2/121360 non-Finnish European alleles (MAF=0.00001648) in ExAC. Patient 46.1 was Jordanian who was homozygous for the previously reported p.Gly46Asp missense change (Sasarman *et al.*, 2012). He had an affected sibling (patient 46.2), but segregation studies were not possible.

7.4.3.3 Microsatellite Genotyping of the p.Leu392Ser Missense Change

To determine a possible founder effect between patient 43.1 and 44 who shared the homozygous p.Leu392Ser missense change, microsatellite genotyping of dinucleotide repeat regions flanking the *YARS2* gene (**Figure 7.10B**) was performed by Charlotte Alston (Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne). This confirmed a founder effect, with both patients 43.1 and 44 sharing a T-1-2-3 haplotype that consisted of the p.Leu392Ser variant and three markers (**Figure 7.10C**). Analysis of an unaffected sibling of patient 43.1 confirmed inheritance of wild-type alleles i.e. not harbouring the p.Leu392Ser missense change. Family pedigrees for the *YARS2* patients are also given (**Figure 7.10C**).

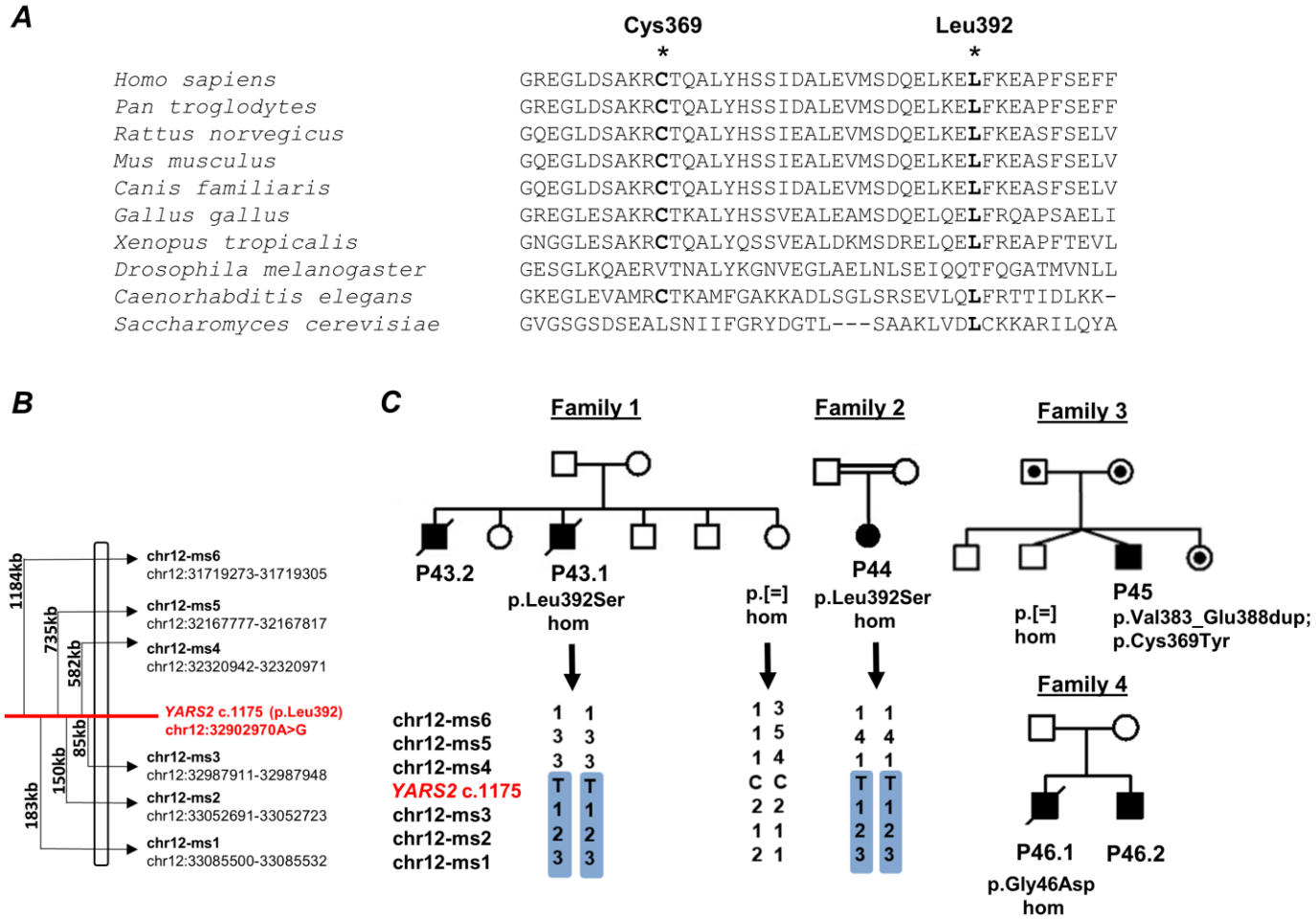


Figure 7.10 *YARS2* MSA, Microsatellite Genotyping and Pedigrees. (*A*) MSA of mt-TyrRS demonstrating conservation of the human Cys369 and Leu392 amino acids. (*B*) Locations of dinucleotide regions flanking *YARS2* used for microsatellite genotyping. (*C*) Pedigrees of the six *YARS2* with microsatellite genotyping results for families 1 and 2 with the shared haplotype (blue) and the novel Scottish p.Leu392Ser founder mutation (red). Adopted and amended from (Sommerville *et al.*, 2017).

7.4.3.4 mtDNA Haplogroups

MtDNA haplogroups had been previously proposed to influence the clinical heterogeneity of patients with *YARS2*-related mitochondrial disease (Riley *et al.*, 2013). Using the full list of mtDNA polymorphisms from whole mitochondrial genome sequencing of *YARS2* patients 43.1, 44-46.1 (**Appendix Q**), mtDNA haplogroups were investigated. All patients were found to belong to different mtDNA haplogroups. Patient 43.1 was H1m1; patient 44 was G2a3a; patient 45 was T2e1a and patient 46.1 was HV13 (**Table 7.4**).

7.4.3.5 Western Blot Analysis

Fibroblasts and myoblasts from patient 45 were investigated for steady-state mt-TyrRS levels and OXPHOS subunits (**Figure 7.11**). Both fibroblasts and myoblasts showed no decrease in steady-state mt-TyrRS protein levels, despite the in-frame duplication and a possible splicing defect due to the p.Cys369Tyr missense variant. There was a mild reduction of MT-COI (complex IV) in fibroblasts and myoblasts. However, NDUFB8 (complex I) was markedly decreased in the myoblasts only.

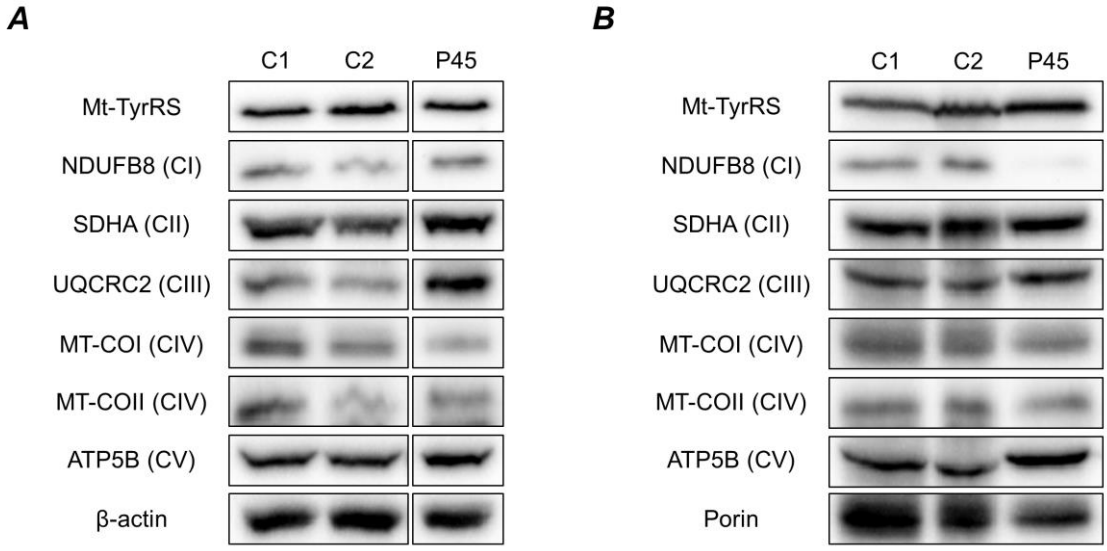


Figure 7.11 Western Blot Analysis of Patient 45 Fibroblasts and Myoblasts. Steady-state mt-TyrRS and OXPHOS subunit protein levels in patient 45 (*A*) fibroblasts and (*B*) myoblasts. Adopted and amended from (Sommerville *et al.*, 2017).

7.4.3.6 *In Silico* Analysis of mt-TyrRS Protein Structure

The human mt-TyrRS protein structure (PDB ID 2PID) was analysed to observe the p.Cys369Tyr missense variant, as resolved by (Bonnefond *et al.*, 2007). However, this was not the full length mt-TyrRS as it did not include the S4-like domain where the Leu392

residue is located, due to previous difficulties of modelling this domain in other species. Therefore, the p.Leu392Ser missense change was not analysed.

The structure of human mt-TyrRS has been examined in detail by (Bonnefond *et al.*, 2007). Mt-TyrRS is the only class II mt-aaRS that exists as a dimer. The Cys369 residue is located in the α15 helix of the anti-codon binding region (**Figure 7.12**), comprising five α-helices at the distal regions of the synthetase. Substitution with tyrosine does not affect the secondary helix structure. Given the residue conservation and position, binding of mt-TyrRS to the anticodon loop of $tRNA^{Tyr}$, recognition or both may be affected by the p.Cys369Tyr substitution.

Figure 7.12 Human mt-TyrRS Protein Structure and Spatial Location of p.Cys369Tyr Missense Variant. The resolved dimeric structure of human mt-TyrRS (Bonnefond *et al.*, 2007). The anticodon binding domain comprising the five α-helices (α11-15) are indicated in orange at the distal end of one mt-TyrRS monomer. The Cys369 residue located with the anticodon binding domain is indicated. The resolved structure does not contain the S4-like domain where the Leu392 residue is located. Adopted and amended from PDB ID 2PID.

7.4.3.7 Yeast Modelling of *YARS2* Missense Variants

Yeast modelling of the unreported *YARS2* missense variants (p.Cys369Tyr and p.Leu392Ser) with *S. cerevisiae* was performed by Cristina Dallabona, Micol Gilberti and Claudia Donnini (Department of Life Sciences, University of Palma, Italy) to demonstrate pathogenicity. Yeast strains were generated to express mutant alleles that corresponded to Cys369Tyr and Leu392Ser, using previously described methods (Ho *et al.*, 1989; Bonneaud *et al.*, 1991; Wach *et al.*, 1994; Gietz and Schiestl, 2007; Baruffini *et al.*, 2010). The Leu392 amino acid was conserved, corresponding to Leu411 in yeast (msy^{L411S}) (**Figure 7.13A**). However the human Cys369 residue was not conserved in yeast (**Figure 7.13A**). Therefore, a humanised mutant was generated (msy^{hL391C}) with the corresponding yeast leucine amino acid substituted with cysteine. The mutant human allele, a substitution with tyrosine, was then also generated (msy^{L391Y}). Oxidative growth and consumption of yeast strains expressing mutant alleles were compared to the wild-type strain at 28^{O}C and 36^{O}C, as previously described (Ardissone *et al.*, 2015b). Strains expressing the msy^{L411S} and humanised msy^{hL391C} alleles had oxidative growth that was similar to the wild-type strain. On the other hand, the mutant msy^{L391Y} strain had reduced growth that was more evident at 36^{O}C (**Figure 7.13B**). All mutant strains also showed a reduction in oxygen consumption compared to the wild-type (**Figure 7.13C**). The humanised msy^{hL391C} strain was similar to the wild-type, whereas the mutant msy^{L391Y} strain showed a 27% reduction in respiration. The mutant msy^{L411S} showed a slightly milder 21% reduction in respiration.

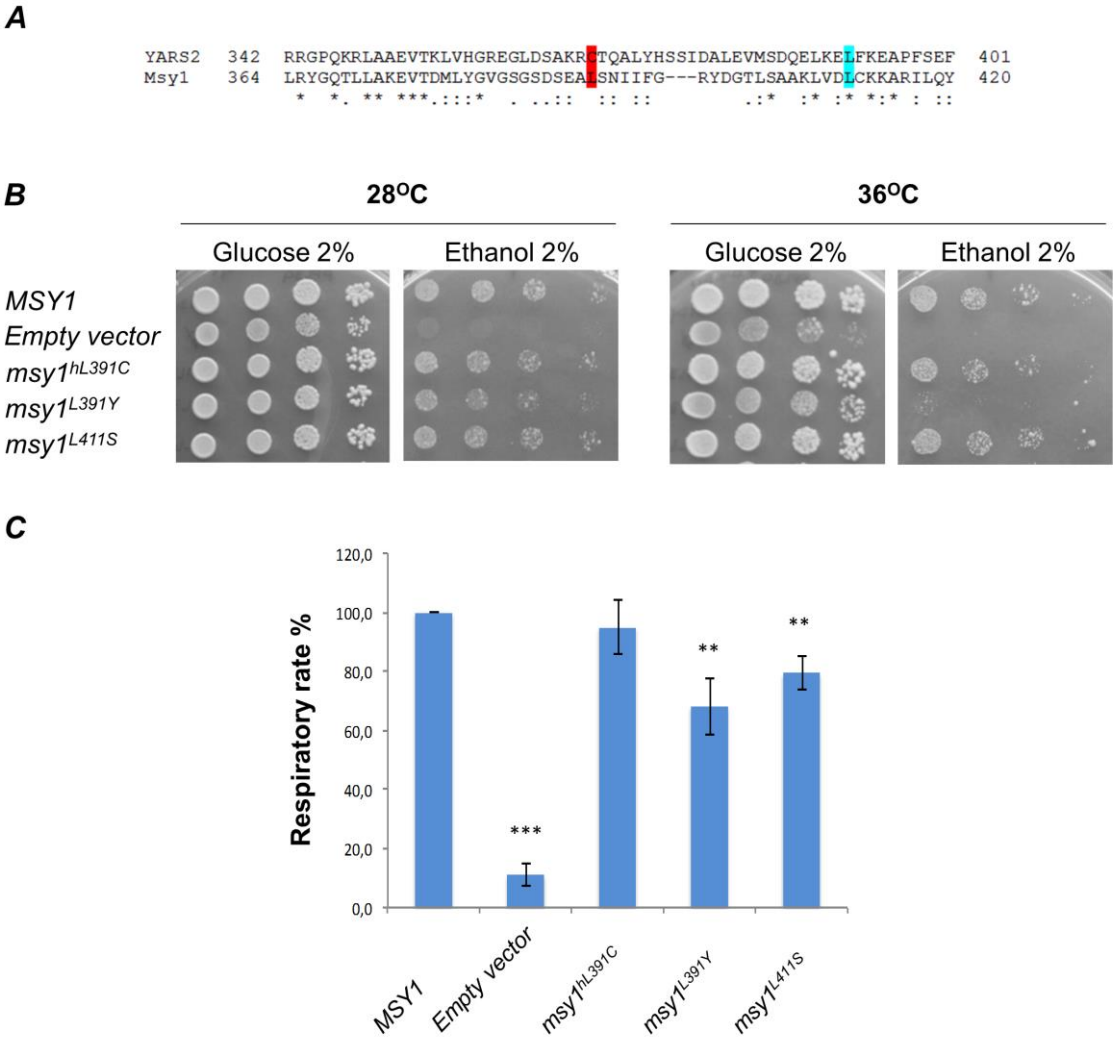


Figure 7.13 Yeast Modelling of *YARS2* Missense Variants. The unreported *YARS2* p.Cys369Tyr and p.Leu392Ser missense variants were modelled in *S. cerevisiae*. *MSY1* corresponds to the wild-type strain. (*A*) MSA showing conservation of Leu392 residue between human and yeast (blue), but not the Cys369 residue (red). (*B*) Oxidative growth of the wild-type, empty vector, msy^{hL391C}, msy^{L391Y} and msy^{L411S} strains at 28^{O}C and 36^{O}C. (*C*) Oxygen consumption of strains relative to the wild-type *MSY1* strain. Two-paired student's t test was used to compare strains using 0.05 or less as the value of statistical significance. Adopted and amended from (Sommerville *et al.*, 2017).

7.5 Discussion

Here, the clinical, genetic and molecular features of *AARS2*, *EARS2* and *YARS2* mutations are expanded by delineating two patients presenting fatal, neonatal mitochondrial disease and six adult patients with childhood-onset *YARS2*-associated mitochondrial myopathy. Although the clinical phenotypes of the *AARS2* and *EARS2* patients were consistent with previously reported cases (Götz *et al.*, 2011; Steenweg *et al.*, 2012), *YARS2* patients presented striking phenotypic variability that included three patients who did not have sideroblastic anaemia.

The use of *in silico* models, yeast modelling and functional studies of fibroblasts and muscle were invaluable in demonstrating the pathological nature of novel mutations. While it is clear that the mechanisms surrounding the tissue and cell specificity of the mt-aaRS remain unresolved, this data provides a further contribution to the hypotheses surrounding this intriguing conundrum.

7.5.1 A Novel *AARS2* Editing Domain Mutation Leads to Misaminoacylation of mt-tRNA^{Ala}

Autosomal recessive *AARS2* mutations were first associated with fatal infantile HCM (Götz *et al.*, 2011). However, patients with childhood- to adult-onset leukoencephalopathy are now equally prominent (Dallabona *et al.*, 2014; Hamatani *et al.*, 2016; Lynch *et al.*, 2016; Szpisjak *et al.*, 2016). Currently, patient 30 is the 11th patient reported with a fatal infantile HCM phenotype. However, he is the first known patient not to harbour the recurrent p.Arg592Trp editing domain founder mutation and instead had a different editing domain missense change, p.Arg580Trp. *In silico* modelling of p.Arg580Trp predicted that aminoacylation would be severely compromised, as would potentially the entire protein structure. Western blot analysis of steady-state mt-AlaRS levels in fibroblasts, together with two patients harbouring the p.Arg592Trp variant on at least one allele, was in agreement with previous *in silico* modelling (Euro *et al.*, 2015) and of p.Arg580Trp. Patients 30 and 42 demonstrated slightly more decreased mt-AlaRS compared to patient 41, likely due to the combination of the nonsense p.Asp337* (patient 30) and a p.Ala961Val (patient 42) missense change predicted to result in an unstable protein (Euro *et al.*, 2015). OXPHOS was apparently unaffected. Similarly, northern blot analysis of patient fibroblasts did not demonstrate loss of aminoacylation activity, suggesting a lower requirement for OXPHOS and hence, lower aminoacylation activity of mt-AlaRS is typical regardless of the *AARS2* mutations. Taken together, the unreported p.Arg580Trp editing domain variant fits into the 'severe' *AARS2* missense change category with other editing domain variants p.Arg592Trp and p.Tyr539Cys proposed by Euro *et al.* (2015), due to (i) the position in the β-barrel of the editing domain, (ii) the predicted significant loss of aminoacylation activity and (iii) proofreading errors due to increased misaminoacylation of tRNA^{Ala} with serine or glycine. Further to the p.Arg592Trp and p.Tyr539Cys variants, p.Arg580Trp also may lead to misfolding of the β-barrel subdomain and hence, stability of the entire protein structure. This continues to support the proposal that the two distinct *AARS2*-associated phenotypes are due to the differential effects on

aminocylation activity and that cardiac cells during early development are especially susceptible to the dramatic loss of mt-AlaRS activity.

7.5.2 Lethal Neonatal LTBL Due to *EARS2* Mutations

Similar to *AARS2*, biallelic *EARS2* mutations harboured by patient 29 caused a lethal mitochondrial translation disorder that was phenotypically consistent with previously reported cases. The presence of a specific brain imaging pattern is comparable to that *DARS2* (Scheper *et al.*, 2007), although even with CNS involvement specific neuronal cells appear to be more receptive to loss of mt-GluRS or mt-AspRS aminoacylation activities. Unlike previously published *EARS2* patients however, brain imaging of patient 29 at 2 months of age revealed an atypical pattern. There were 26 published patients (including patient 29) harbouring *EARS2* variants (Steenweg *et al.*, 2012; Talim *et al.*, 2013; Biancheri *et al.*, 2015; Danhauser *et al.*, 2016; Gungor *et al.*, 2016; Kevelam *et al.*, 2016; Kohda *et al.*, 2016; Oliveira and Sommerville, 2016; Pronicka *et al.*, 2016; Sahin *et al.*, 2016; Taskin *et al.*, 2016). Of these, brain MR imaging was available from 11 patients for comparison with phenotypes varying from the severe to mild and intermediate forms of LTBL. Patient 29 presented the severe form that was similar to patients described by Danhauser *et al.* (2016) and Talim *et al.* (2013). However, there was no brain imaging available from these patients to compare. Although bilateral and symmetrical lesions that predominantly affected the grey structures and brainstem were indicative of Leigh syndrome, it was insufficient to indicate LTBL. An important determinant for this was the patient's age, which at 2 months old the brain would not be fully developed. The rapid, fatal disease course and delayed myelination also prevented further delineation of the brain MR changes. Therefore, the cerebral white matter lesions were not as apparent compared to published patients and leukoencephalopathy or additional white matter changes may not have been evident, but in spite of this there was sparing of the periventricular rim as reported previously (Steenweg *et al.*, 2012; Biancheri *et al.*, 2015; Gungor *et al.*, 2016; Kevelam *et al.*, 2016; Sahin *et al.*, 2016; Taskin *et al.*, 2016). Despite her age, the brainstem, thalami and cerebral lesions were features typical of LTBL. Hypogenesis of the corpus callosum was also evident as described previously (Steenweg *et al.*, 2012; Biancheri *et al.*, 2015; Gungor *et al.*, 2016; Kevelam *et al.*, 2016), including the severely affected patients reported by Danhauser *et al.* (2016) and Talim *et al.* (2013). WES of patient 29 was performed shortly after the first report of patients with biallelic *EARS2* mutations (Steenweg *et al.*, 2012). Given the clinical, histochemical and biochemical features together with the brain imaging, irrespective of the inconspicuous white matter lesions, this would prompt targeted *EARS2* gene screening. Regarding *EARS2* variants, there was no correlation

with location or whether variants were homozygous or compound heterozygous (Oliveira and Sommerville, 2016), which contrasted with *AARS2* mutations. The complete loss of mt-GluRS activity due to the near or complete loss of steady-state mt-GluRS levels demonstrated in by western blotting in fibroblasts and muscle show that this incompatible for life. Differential effects due to other *EARS2* mutations may also lead to complete or partial loss of mt-GluRS, loss of aminoacylation activity or both. Currently though, no correlation between the effects of mutations and the clinical phenotypes have been ascertained, although Sahin (2016) proposed that the combination of a mild and a severe *EARS2* mutation could contribute to the milder phenotypes.

7.5.3 Phenotypic Spectrum of *YARS2* Mutations Due to Differential Effects on mt-TyrRS Activity

YARS2-related mitochondrial disease has been associated with MLASA that includes the distinct presence of sideroblastic anaemia; a rare manifestation of mitochondrial disease more commonly associated in Pearson's Syndrome due to single, large scale mtDNA deletions (Pearson *et al.*, 1979; McShane *et al.*, 1991). All six *YARS2* patients identified through WES or targeted *YARS2* gene screening presented during childhood with myopathy, commonly manifesting as exercise intolerance with survival into adulthood. However, disease progression varied from complete MLASA syndrome to slowly progressive mitochondrial myopathy. Additionally, four patients of Scottish ancestry were identified including two pedigrees sharing a novel *YARS2* Scottish p.Leu392Ser founder mutation. To date, *YARS2*-related mitochondrial disease have predominantly been described in patients from the Middle East presenting severe early-onset phenotypes, harbouring either a homozygous p.Phe52Leu or p.Gly46Asp missense change (Riley *et al.*, 2010; Sasarman *et al.*, 2012; Riley *et al.*, 2013; Shahni *et al.*, 2013). Given the broad phenotypes observed between the six adult patients, this indicates that *YARS2*-related mitochondrial myopathy may be underdiagnosed outside of the Middle East, especially in Western populations who do not harbour the known, frequently reported variants.

Despite varied clinical severity between the six *YARS2* patients, impressive global COX-deficiency in skeletal muscle was demonstrated in all tested patients that may be diagnostic for adults with *YARS2*-related myopathy. This was examined further by immunoblotting of fibroblasts and myoblasts from patient 45, which revealed minimal decreases in MT-COI and MT-COII (complex IV) in both tissues, but a significant decrease of NDUFB8 (complex I) in the myoblasts. This was an intriguing finding due to the profound COX-deficiency

demonstrated histochemically and biochemically, which may reflect the tissue specificity of mt-TyrRS since mitochondrial protein synthesis differs between tissues and may also explain the stable expression of OXPHOS subunits in the *AARS2* patient fibroblasts. Furthermore, no changes in steady-state mt-TyrRS levels were detected. Prior immunoblotting of fibroblasts, myoblasts or MyoD-forced myotubes from two patients also did not disclose any loss of mt-TyrRS (Riley *et al.*, 2010; Riley *et al.*, 2013). In contrast, mt-TyrRS was undetectable in myoblasts and myotubes from a third patient, but increased with differentiation of healthy cultured muscle cells from myoblasts to myotubes (Sasarman *et al.*, 2012). Mitochondrial proliferation during myogenesis resulting in increased respiratory chain complex subunits was demonstrated between healthy control myotubes and myofibres, but not in *YARS2* patient muscle cells (Riley *et al.*, 2013). Furthermore, upregulation of mtDNA proliferation was also observed between control myoblasts and myotubes. In contrast, a substantial increase in mtDNA copy number was observed in patient 45 skeletal muscle and a modest increase in patient 46.1. Taken together, this suggests an increased requirement for *YARS2* during muscle proliferation and that increased mitochondrial proliferation is a response to overcome the respiratory chain defects in muscle cells, providing a link between mt-TyrRS requirement in muscle cells and the myopathy and exercise tolerance presented in affected patients. Despite mt-TyRS requirement in the muscle the phenotypic variability between patients is stark, suggesting additional contributing mechanisms. Riley *et al.* (2013) hypothesised that mtDNA haplogroups of *YARS2* patients could be one explanation. Haplogroup T appeared to align with a severe fatal infantile form of the disease due to HCM and haplogroup H aligning with a mild clinical phenotype. Haplogroup T has been previously associated with increased susceptibility of HCM in the Spanish population (Castro *et al.*, 2006), but has not been verifiable in the Danish population (Hagen *et al.*, 2013). Using HaploGrep2 to define haplogroups (Kloss-Brandstätter *et al.*, 2011), patient 45 was found to belong to haplogroup T and also developed HCM, but presented with a milder phenotype. Additionally, patient 43.1 belonged to haplotype H and similarly presented with a mild disease course. Cybrids with haplogroup H were shown to have increased mitochondrial protein synthesis compared to cybrids of haplogroups J and U (Gómez-Durán *et al.*, 2010; Gómez-Durán *et al.*, 2012). Interestingly, cybrids of haplotype T have been shown to have higher mtDNA copy number and be less susceptible to oxidative stress than haplotype H cybrids (Mueller *et al.*, 2012). However, this did not correlate with patient phenotypes; patient 44 presented with the mildest phenotype of all six *YARS2* patients and belonged to haplogroup G. Together with the previous assessment of *YARS2* patient mtDNA haplogroups (Riley *et al.*, 2013), the

heterogeneous phenotypes including the presence of absence of sideroblastic anaemia are likely due to additional genetic factors that are yet to be disclosed.

Yeast modelling of the unreported *YARS2* missense changes (p.Cys369Tyr and p.Leu392Ser) was performed to demonstrate pathogenicity. A previously reported p.Asp311Glu *YARS2* variant was modelled in yeast (msy^{D333E}) and shown to have a severe OXPHOS defect (Ardissone *et al.*, 2015b). Oxidative growth and respiration of mutant strains expressing the p.Cys369Tyr (msy^{L391Y}) and p.Leu392Ser (msy^{L411S}) both had milder OXPHOS defects, though msy^{L391Y} was slightly more severe. Comparing OXPHOS defects of all mutant yeast strains including msy^{D333E} showed a direct correlation with the severity of *YARS2* human patient phenotypes. Finally, to better understand the relationship between the secondary structure and missense variants, the available human mt-TyrRS protein structure was used to analyse the p.Cys369Tyr variant (Bonnefond *et al.*, 2007). The p.Cys369Tyr change occurred in the α-15 helix of the distal anti-codon binding domain, but this substitution did not affect the overall secondary structure. Although it was not possible to analyse the p.Leu392Ser change using the resolved secondary mt-TyrRS structure, it was proximal to p.Cys369Tyr and could also potentially be associated with $tRNA^{Tyr}$ anti-codon binding. Furthermore, missense changes within this region of mt-TyrRS were not previously reported. One hypothesis could be that patients harbouring either p.Cys369Tyr or p.Leu392Ser have a stable mt-TyrRS with partial reduction in aminoacylation activity due to impaired binding, recognition or both of the $tRNA^{Tyr}$ anti-codon loop. The unchanged steady-state levels of mt-TyrRS in patient 45 fibroblasts and myoblasts lends support this hypothesis. However, how sideroblastic anaemia in two of four patients harbouring either p.Cys369Tyr or p.Leu392Ser remains unclear. Also, it is not possible to be absolutely certain regarding the differences between the S4-like domain variants p.Leu392Ser with survival into adulthood and a homozygous *YARS2* p.Ser435Gly missense change that led to a severe MLASA phenotype with death within the 6 months of life (Nakajima *et al.*, 2014).

7.5.4 Tissue and Cell Specificity of mt-aaRS Defects

Mt-aaRS mutations associated with defective translation are a paradigm of mitochondrial disease, reflected in the vast clinical heterogeneity manifested in affected patients and intriguing tissue and cell specificity. Absence of one or more features, such as sideroblastic anaemia in *YARS2*-related mitochondrial myopathy, abnormal brain imaging in LTBL due to *EARS2* mutations or two different phenotypes due to *AARS2* mutations provides challenges in

attaining a genetic diagnosis and understanding the pathological mechanisms underpinning tissue specific phenotypes.

Varying thresholds in each tissue- or cell-type whereby levels of misaminoacylated tRNAs or uncharged mt-tRNAs are intolerable or toxic, thus having a greater degree of impact on mitochondrial protein synthesis could account for the high level of apparent specificity. Due to the significant number of mt-aaRS defects associated with NS and brain involvement, it appears that specific neuronal cells types are significantly affected. Together with other developing tissues in early life such as cardiac cells, these could be highly susceptible to either a severe reduction of mt-aaRS activity and are sensitive to misaminoacylated mt-tRNAs or uncharged mt-tRNAs. Tissue- or cell-type thresholds are not are unknown in mitochondrial disease; mtDNA mutations manifest in tissue-specific phenotypes when the mutational load reaches a particular level, known as the 'phenotypic threshold effect' (Rossignol *et al.*, 2003). Variation in the threshold of misaminoacylated or uncharged mt-tRNAs between individuals could account for some of this phenotypic and tissue specific variability. Furthermore, the variability of mt-tRNA expression in specific tissues or cells will perhaps impact the aminoacylation activity of mt-aaRS. Interestingly, the brain has been found to express higher levels of mt-tRNAs than any other tested tissue (Dittmar *et al.*, 2006), emphasising a greater need for mitochondrial translation fidelity. Levels of misaminoacylated or uncharged tRNAs in tissues and cells appear to be intrinsically linked to the differential effects of the synthetase mutations. As demonstrated for mt-AlaRS, editing domain mutations including the novel p.Arg580Trp missense variant likely cause $tRNA^{Ala}$ to be misaminoacylated with serine or glycine, together with a severe reduction of aminoacylation activity. On the face of it however, *EARS2* mutations do not appear to show a genotype and phenotype correlation. Indeed, both homozygous and compound heterozygous mutations contribute to both the severe and mild or intermediate phenotypes. As noted though by Sahin *et al.* (2016), the combination of a mild and a severe *EARS2* mutation led to a milder clinical course for their patient. On the other hand, complete loss of mt-GluRS expression on one allele together with a severe mutation contributed to a severe, lethal course. This was true for patient 29, who harboured a start-loss p.Met1? variant together with p.Ile62Phe missense change, which is likely detrimental to mt-GluRS stability as evidenced by loss of mt-GluRS expression in patient 29 fibroblasts and muscle on western blotting. Initial investigations of *YARS2* missense variants including the p.Cys369Tyr variant appear to show some structural correlation with the vast phenotypic variability observed in patients, like that of *AARS2* mutations. The p.Phe52Leu and p.Gly46Asp mutations are located near the binding site for

the mt-tRNA^{Tyr} acceptor stem, with both variants associating with a more severe phenotype that was strongly associated sideroblastic anaemia requiring transfusion, suggesting a decreased binding affinity with mt-tRNA^{Tyr}. On the other hand a p.Cys369Tyr variant associated with a milder phenotype and investigated in this study is located within the anti-codon binding domain, suggesting impairment on mt-tRNA^{Tyr} due to impaired recognition of the anti-codon loop instead. However, there are no additional *in silico* or functional studies of *EARS2* or *YARS2* missense changes to the extent that *AARS2* missense changes have been investigated (Euro *et al.*, 2015). Thresholds in tissues could also change over time and with or without differential effects of mt-aaRS mutations, may lead to broadened phenotypes between individuals, as seen with *AARS2* mutations. Nonetheless, findings from this study and previous suggest that a reduction of mt-GluRS activity is a key mediator of the LTBL phenotype in neuronal cells of the brainstem and thalamus.

It is also conceivable that mt-aaRS have secondary functions in addition to their aminoacylation activities like the aaRS (Yao and Fox, 2013). Several nuclear encoded regulators of mitochondrial function have been associated with possible secondary roles, including MTU1 in sulphur trafficking (Sasarman *et al.*, 2011) and SCO1 in cellular copper homeostasis (Leary *et al.*, 2007). Given the presence of sideroblastic anemia in a significant proportion of *YARS2* patients, there may be an as yet undisclosed role for mt-TyrRS in haematopoiesis; the production of all blood cell types in each individual throughout their entire lifetime. Inhibition of mitochondrial translation as a therapeutic strategy for treatment of acute myeloid leukaemia highlights the importance of mitochondria for blood cell production in haematopoietic stem cells in the bone marrow (Škrtić *et al.*, 2011). Currently, potential secondary functions of the mt-aaRS are not well characterised compared to their cytosolic counterparts, with the exception of dual-localised LysRS (Martin *et al.*, 1979).

7.5.5 Concluding Remarks

To summarise, there are likely to be varying and multiple pathological mechanisms between each mt-aaRS defect that explain tissue and cell specificity. Although *AARS2* mutations have a clear link between differential mutations and aminoacylation activity, the same proposed mechanism does not fit for *EARS2* and *YARS2*, or indeed other mt-aaRS defects. Further studies of mt-aaRS mutations on aminoacylation activity, secondary roles of mt-aaRS and of their cytoplasmic aaRS counterparts could provide insights. In spite of this, *in silico* and yeast modelling of missense changes were demonstrated as powerful tools to confirm pathogenicity, with an impressive correlation demonstrated between *YARS2* variants and

clinical severity. When considering the clinical, histochemical and biochemical features together, it may be possible to direct targeted gene screening. Identification of additional patients using this approach and NGS will also likely further expand the phenotypic spectrum of mt-aaRS defects.

Chapter 8. Final Discussion

The past decade has ushered in an exciting era of targeted NGS technologies that includes WES and WGS, which have sought to enhance the diagnosis and understanding of complex genetic disorders. Mitochondrial disease poses a particular challenge for both clinicians and researchers in the laboratory, due to vast clinical and genetic heterogeneity. This is compounded by a potential defect in either mtDNA or one of approximately 1,200 nuclear encoded proteins that are essential for mitochondrial functions (Lopez *et al.*, 2000; Calvo *et al.*, 2006). Establishing a genetic diagnosis is essential for expanding our understanding of the pathological nature of mitochondrial disease, which is seminal for the development of therapeutic strategies to mitigate the devastating symptoms that impact on health-related quality of life. For parents and families of paediatric patients, a genetic diagnosis informs reproductive options for future pregnancies, including pre-natal or pre-implantation diagnosis.

The foundation of all studies in my thesis centred on the use of WES as a cost-effective tool for the diagnosis of suspected Mendelian mitochondrial disease, while building upon previously described approaches to enhance the diagnostic yield.

8.1 Whole Exome Sequencing (WES) in Mitochondrial Disease

The fundamental challenge of WES is to discriminate pathogenic or causative variants from thousands of called variants, which include common, benign or neutral polymorphisms. To this end, it is essential to devise and employ filtering strategies to sift through and prioritise the most likely candidate causal variants. In seeking to advance WES filtering strategies, 40 undiagnosed deep-phenotyped mitochondrial disease patients presenting extreme genotypic and phenotypic heterogeneity were recruited. These were sub-grouped into (i) adult-onset PEO with multiple mtDNA deletions (n=19) and (ii) early-onset mitochondrial RC deficiency (n=20).

Adult-onset Mendelian PEO with secondary, clonally expanded skeletal muscle restricted multiple mtDNA deletions (Zeviani *et al.*, 1989) is a well-recognised disorder of mtDNA maintenance. Mutations of 16 nuclear genes have now been associated with Mendelian PEO with mtDNA instability (Sommerville *et al.*, 2014). In general, more than 50% of these patients could receive a genetic diagnosis if all known genes are investigated. Nuclear genes associated with this disorder predominantly encode proteins involved in mtDNA maintenance.

Anticipating the potential difficulties of WES in this particular patient cohort, I comprehensively reviewed all published patients (**Chapter 3**), confirming the considerable heterogeneity of disorder. The review also noted that autosomal dominant mutations were the most common inheritance pattern in adult-onset mtDNA maintenance disorders and hence, this knowledge was applied to the WES strategy. Utilising a custom filtering strategy (**Chapter 4**), a confirmed diagnostic yield of just 10.5% was achieved. Nonetheless, primarily heterozygous candidate variants and VUS (as anticipated) were also identified in nuclear genes encoding mitochondrial proteins involved in a range of functions that included mtDNA replication (*POLRMT*, *TOP3A*), mitochondrial fission (*SEPT2*), nucleotide homeostasis (*ABAT*, *GMPR1*, *RRM1*, *RRM2B*) and metabolite transport (*VDAC1*).

On the other hand, early-onset mitochondrial RC disorders arise due to defects affecting one or more of the five multi-subunit enzymes comprising the OXPHOS system and are among the most common early-onset metabolic disorders with an estimated minimum prevalence of 1 in 5,000 live births (Skladal *et al.*, 2003), compared to adult-onset mtDNA maintenance disorders, early-onset RC deficiency has been extensively studied using NGS approaches and is associated with defects in approximately 245 nuclear genes encoding proteins involved in mtDNA maintenance, but also protein synthesis, OXPHOS cofactors, dynamics and metabolism (Mayr *et al.*, 2015). With a second custom filtering strategy specific to this cohort (**Chapter 6**), I identified causative mutations in known and novel mitochondrial disease genes plus one putative non-mitochondrial nuclear gene, giving a causative and likely causative diagnostic yield of 45%. Significantly, I identified mostly autosomal recessive mutations in nuclear genes encoding mitochondrial protein synthesis machinery (*AARS2*, *EARS2*, *GTPBP3*, *MRPS22*, *MTO1*, *TRMU*), indicating that biased filtering for this group of genes in early-onset RC deficiency is appropriate, while still prioritising other genes encoding mitochondrial proteins.

Prioritisation of candidate variants was also dependent on deep phenotyping of patients with suspected mitochondrial disease. Biochemical or histochemical evidence of mitochondrial dysfunction ensured that variant filtering and prioritisation of candidate variants was focused on nuclear genes encoding mitochondrial proteins. This had a profound impact within my WES cohorts, particularly emphasised in patients with early-onset RC deficiency. Of the five patients with isolated deficiency, a definitive diagnosis made was in only one case. On the other hand, of the 14 patients with multiple RC deficiency, causative or likely causative variants were identified in eight; a diagnostic yield of 57%; higher than 45% yield attained for the full early-onset RC cohort.

However, I accept that my filtering strategies are limited to nuclear genes encoding known mitochondrial proteins. Despite advances in NGS technological capabilities, functional examination of the human proteome is on-going. I was unable to prioritise the novel heterozygous *SEPT2* mutation without knowledge of its recently published involvement in mitochondrial fission (Pagliuso *et al.*, 2016). Therefore, there remain uncharacterised proteins that could be essential for mitochondrial function and associated with mitochondrial disease, yet cannot be prioritised without functional knowledge. This advocates the need for continued re-evaluation of apparently unsolved and future exomes as the human proteome continues to be characterised.

8.1.1 *De Novo* and Dominant Mutations

Prior studies of undiagnosed early-onset mitochondrial disease cohorts using NGS have warned that the inheritance pattern could sometimes wrongly be assumed to be recessive, as Kohda *et al.* (2016) most recently acknowledged. This was accentuated by the recent identification of *de novo ATAD3A* and *SLC25A4* mutations in early-onset mitochondrial disease patients (Harel *et al.*, 2016; Thompson *et al.*, 2016). Hence, substantial emphasis was placed on the potential prominence of causative dominant or *de novo* heterozygous mutations, despite expectations that the majority of inheritance would be recessive and that no trios underwent WES. I was exonerated by the identification of a possible *de novo CTBP1* mutation (Beck *et al.*, 2016) and the first prospective dominant inherited *MTO1* mutation within this cohort. While RC deficiency due to the *CTBP1* mutation is probably secondary, it by no means precludes the identification of other patients with RC deficiency due to a mutation(s) of a non-mitochondrial disease gene. My observations further serve to reinforce the need for continued re-evaluation of heterozygous variants in unsolved and future exomes, since these are likely to be under recognised in mitochondrial disease.

8.1.2 Is Somatic Mosaicism an Under-Recognised Phenomenon?

To my knowledge, I identified the first case of somatic mosaicism associated with adult-onset PEO and multiple mtDNA deletions in a single patient harbouring a known heterozygous pathogenic *TWNK* p.Arg374Gln mutation (Spelbrink *et al.*, 2001; Naimi *et al.*, 2006; Baloh *et al.*, 2007; Massa *et al.*, 2009; Martin-Negrier *et al.*, 2011; Yu-Wai-Man *et al.*, 2013; Tafakhori *et al.*, 2016). This demonstrated the efficiency of WES to detect low level mosaicism. Significantly, somatic mosaicism may provide an explanation for the failure to attain a diagnosis in a small proportion of patients with mtDNA maintenance disorders.

However, difficulties could arise from the availability of tissues for testing or failure to amplify DNA in one or more tissues. Sanger sequencing is also insufficient to detect mutation loads of less than 15% (Rohlin *et al.*, 2009). Until NGS is established as routine in the diagnosis of prospective mitochondrial disorders, the impact of somatic mosaicism remains an open question. In the interim, this requires careful curation and annotation of electropherograms generated from diagnostic targeted screening of mtDNA maintenance disorder-associated genes.

8.1.3 Guanosine Monophosphate Reductase 1 (*GMPR1*)

I pursued investigations of a novel heterozygous *GMPR1* p.Gly183Arg missense change harboured by patient 11 to determine if it was the causal mutation leading to PEO and multiple mtDNA deletions (**Chapter 5**). Despite the known role of GMPR1 in *de novo* guanosine synthesis, it had not been previously associated with human disease nor was there evidence demonstrating a direct link to mtDNA maintenance. Collaborating with Dr Antonella Spinazzola and Dr Ilaria Dalla Rosa (MRC Mill Hill Laboratory, London), altered nucleotide homeostasis in proliferating and quiescent patient fibroblasts was noted, emphasised by decreased steady-state GMPR1 protein expression and further underpinned by a decrease in patient skeletal muscle. However, the subtle cellular phenotype was not specific to directly implicate the *GMPR1* mutation, underscoring the difficulties in understanding the pathological mechanisms of dominant, late-onset mtDNA maintenance disorders.

Nonetheless, continued investigation of the *GMPR1* mutation is on-going. Expression and purification of mutant GMPR harbouring the p.Gly183Arg mutation from *Escherichia coli* for measuring enzymatic activity is to be performed by Dr Liz Hedstrom and Dr Masha Rosenberg (Brandeis University, Massachusetts, USA). Simultaneously, Dr Spinazzola and Dr Dalla Rosa are extracting and measuring the dNTP pools from patient fibroblasts.

8.1.4 Tissue Specificity in Mitochondrial Disease

Tissue- and cell-specificity was a significant theme throughout my thesis. It was especially prominent in mutations of the mt-aaRS (**Chapter 7**), although tissue-specificity was also noted in the *SCO1* patient with fatal lactic acidosis (Valnot *et al.*, 2000; Stiburek *et al.*, 2009), plus the *TRMU* patient with acute infantile-onset liver failure (AILF) (Zeharia *et al.*, 2009; Kemp *et al.*, 2011; Schara *et al.*, 2011; Uusimaa *et al.*, 2011; Gaignard *et al.*, 2013; Taylor *et al.*, 2014; Grover *et al.*, 2015). It is also reasonable to consign mtDNA maintenance disorders in this classification, since mtDNA instability is restricted to the skeletal muscle.

This thesis expanded the clinical, molecular and genetic spectrum of mt-aaRS defects (**Chapter 7**). Once thought to be key to directing targeted gene screening, the absence of one or more clinical features in mt-aaRS defects provides new challenges in diagnosing early-onset RC deficiency. Between the three mt-aaRS defects studied in this thesis and likely for all other mt-aaRS, the rules governing tissue- and cell-specificity differ. Secondary roles of the mt-aaRS also offer explanations, as proposed for MTU1 and SCO1 (Leary *et al.*, 2007; Sasarman *et al.*, 2011). Nevertheless, further examination and the identification of additional patients is necessary to provide more detailed insights.

8.2 Genetic Diagnoses to Therapeutic Strategies

Attaining genetic diagnoses and understanding disease mechanisms can allow for the development of treatments and therapeutic strategies for mitochondrial disease, although these are currently in the early-stages (Nightingale *et al.*, 2016). Indeed, I identified mutations in genes that are potential targets for treatments. Notably, dTMP and dCMP supplementation has been trialled in *Tk2* p.His126Asn knock-in mouse models as a means to overcome pool imbalances causing mtDNA depletion (Garone *et al.*, 2014). Given the emergence of nucleotide metabolism defects associated with mtDNA maintenance disorders in this cohort, dNTP supplementation therapy is emerging as a significant avenue of further study. Supplementation could be beneficial in *GMPR1* patient fibroblasts. However, it is too early to evaluate the potential of this strategy until dNTP measurements are completed. Given the subtle cellular phenotype observed so far, the effect of supplementation may not be valuable.

Another significant finding was the identification of defects in the modification or processing of mt-tRNA^{Glu}; *EARS2*, *TRMU*, *MTO1* and *GTPBP3*. Reversible infantile RC deficiency (RIRCD) is associated with a homoplasmic m.14674T>C or m.14674T>G mutation in the mt-tRNA^{Glu} gene, manifesting as severe encephalomyopathy in the first months of life followed by spontaneous recovery (Horvath *et al.*, 2009; Mimaki *et al.*, 2010; Uusimaa *et al.*, 2011). Strikingly, spontaneous recovery of some patients harbouring *EARS2* and *TRMU* mutations have been noted (Steenweg *et al.*, 2012), while partial recovery of patients with *MTO1* mutations has also been described (Ghezzi *et al.*, 2012). This was not true for patient 29 (*EARS2*), but the clinical course for patients 31 (*TRMU*) and 32 (*MTO1*) indeed appeared to stabilise. Recovery has been linked to the availability of L-cysteine, expressed at low levels from birth and slowly increase throughout the first few months of life (Zlotkin and Anderson, 1982; Levonen *et al.*, 2000). Thus, *in vitro* supplementation of L-cysteine or N-acetyl-

cysteine in fibroblasts from patients with *TRMU* and *MTO1* mutations show incredible restoration of RC activities (Boczonadi *et al.*, 2013; Bartsakoulia *et al.*, 2016).

8.3 WES or WGS?

Regrettably, I was unable to identify causative or likely causative variants in 17/40 (42.5%) of patients in this thesis. Though this was significant proportion, failure to identify causative mutations is a common outcome in WES studies throughout the literature. This poses the question of how to proceed these cases. It could be that the causative mutation(s) were missed by WES or lie in non-coding regions of the genome, such as the deep-intronic or untranslated regions. Thus, WGS signifies a potential solution for both problems and has some significant advantages over WES in the diagnostic setting (Belkadi *et al.*, 2015; Meienberg *et al.*, 2016).

WES throughout this study relied upon amplicon-based library preparation for adequate coverage of the human genome. Poor or uneven read coverage from WES likely stems from the PCR-amplification steps (Kebschull and Zador, 2015), which could be attributed to GC-rich regions and the use of short read lengths (Kozarewa *et al.*, 2009; Veal *et al.*, 2012). Therefore, inadequate and uneven read coverage of the genome could have led to causative variants being missed, as has been proposed previously (Calvo *et al.*, 2012). Detection of CNVs is also challenging in WES, again due to uneven coverage and the use of short reads that are insufficient to cover large rearrangements (Tattini *et al.*, 2015). This includes expanded and triplet repeats, which are associated with several neurological conditions including Huntington's disease, Friedreich's ataxia and spinocerebellar ataxia (Orr *et al.*, 1993; Campuzano *et al.*, 1996; Walker, 2007). On the other hand, PCR-free WGS has been demonstrated to provide more uniform coverage of the human genome since it is less sensitive to GC-rich regions (Meienberg *et al.*, 2016). Owing to greater read lengths, this also allows more accurate identification of large genomic structural rearrangements (Belkadi *et al.*, 2015).

Non-coding mutations are increasingly reported throughout the literature in neuromuscular and neurological diseases (Ruggieri *et al.*, 2015; Elsaid *et al.*, 2016; Gonorazky *et al.*, 2016; Kremer *et al.*, 2016), which would not be called by WES. However, if the genetic aetiology of a mitochondrial disease patient is potentially non-coding, how does one interpret pathogenicity from a list of thousands of non-coding changes called by WGS? Recently, RNA-based transcript analysis (RNAseq) has been used in tandem with PCR-free WGS to evaluate RNA expression or processing for patients who have not received a genetic diagnosis

via WES (Ku *et al.*, 2012). RNAseq has only been applied to mitochondrial disease once so far; Kremer *et al.* (2016) identified three families with an intronic exon-creating variant in *TIMMDC1* associated with complex I deficiency. On these early studies, RNAseq appears to be more beneficial only when combined with PCR-free WGS.

Irrespective of the additional, essential bioinformatics processing that would be required for PCR-free WGS and RNAseq analysis, these technologies will remain out of reach for many public-funded diagnostic centres owing to the costs involved. While sequencing of the human genome continues to become rapidly less expensive, it has yet to reach a level that can be widely afforded. While I whole-heartedly agree that PCR-free WGS with RNAseq should and will ultimately be the first-tier choice of genetic investigation in the diagnosis of Mendelian disease, the current costs remain out of reach. Meanwhile, WES currently remains the most viable and cost-efficient tool for diagnostic services, including the NHS Highly Specialised Mitochondrial Diagnostic Service Laboratory. Thus, PCR-free WGS and RNAseq are currently options of last-resort.

8.4 Concluding Remarks

To summarise, my thesis has demonstrated the power of WES for the identification of causative and likely causative variants in two cohorts of suspected Mendelian mitochondrial disease manifesting broad clinical and genetic heterogeneity. The clinical, molecular and genetic spectrum of known mitochondrial disease genes were expanded, while novel candidates were proposed. Alas, I concede that further functional validation of the novel genes is essential and on-going. As NGS technologies including WES, PCR-free WGS and RNAseq become less expensive and more accessible for diagnostic purposes, we are entering a new age of '*Now*-Generation Sequencing' in mitochondrial disease diagnosis.

Appendix

Appendix A – Sanger Sequencing Primers

Primer	Sequence	Fragment size (bp)	Position of primer	Optimal conditions
AARS2_Ex6+7	F-ttctggatgtggttggcttt R-atttagggtggggacctctg	694	Chr6:44274548-44275241	
AARS2_Ex11+12	F-cgaggctgaagtcccact R-agcctttccctccccttg	705	Chr6:44272301-44273005	
ABAT_Ex13	F-aattcagcgagattgggtgt R-gaatgggatggagggatagg	506	Chr16:8870041-8870546	
APEX1_Ex5	F-gcctgaactcttcaaaaccaa R-tcccaggctcaaagtgattt	655	Chr14:20925043- 20925697	
BCL2L13_Ex7a	F-tttgctttccagattgatgttt R-agggctggatttctcaactg	591	Chr22:18209368- 18209958	
BCL2L13_Ex7b	F-agctcccttgcttccacata R-gctgctggaggttcaaattc	508	Chr22:18209868- 18210375	
C10orf2_Ex1c	F-acaatctgtttggattaccactg R-aacccacttgcttttgtcacc	578	Chr10:102748701-102749278	
CTBP1_Ex9	F-agtgcccagagagacaggac R-cttccaagaggccctggt	456	Chr4:1206524-1206979	62^{O}C
EXD2_Ex7	F-tggttaggccccagttagaa R-tgagggttaatgtccctggt	504	Chr14:69704192- 69704695	
FDXR_Ex4	F-ggtttggagactttggtcaag R-tcagcaccagcactgatagg	477	Chr17:72862402- 72862878	
EARS2_Ex1	F-tgtgactttaaggggcaagg R-aacacaagaaactcctggcaat	447	Chr16:23568326-23568772	DMSO
EARS2_Ex2	F-gtgagccaatgtgtcctgct R-ccagtcttggagctgtgtttc	483	Chr16:23563271-23563753	
EARS2_Ex3	F-ggacattgtactaatgccaacc R-ttgcttgtggctttgagaga	497	Chr16:23555661-23556157	
EARS2_Ex4	F-ggtcacacagctatgacagagg R-aggactgtctgagccaaagc	631	Chr16:23546134-23546764	
EARS2_Ex5	F-gaagacaggctggaggattg R-tgtgccagggctatacaaga	536	Chr16:23543728-23544263	
EARS2_Ex6+7	F-tttccctgggggagagac R-ccagccctgaaggaaagag	551	Chr16:23540741- 23541291	
EARS2_Ex8	F-gtcccttggtggtttttcct R-cctaacacaatgcttgcaca	516	Chr16:23536380-23536895	
EARS2_Ex9	F-ggctcttaggacccatcctt R-cccagccaattgacaaaaac	442	Chr16:23535503-23535944	
GMPR1_Ex5	F-ttctcatgccaacagctcac R-acttttcatcaggggcttcc	451	Chr6:16274451-16274901	DMSO
GNL3L_Ex11	F-tcacccctcctccaactcta R-ttcttcttcttccccaaacg	499	ChrX:54577852 - 54578350	
GTPBP3_Ex1	F-cagccaatggaagcgagtat R-gactgtgaggccagaaggag	409	Chr19:17448246-17448654	
GTPBP3_Ex2	F-gtctccttctggcctcacag R-gaaaggggaaactgagtcat	489	Chr19:17448633-17449121	
GTPBP3_Ex3	F-tctggttcccaggtgagg R-gtcaggttcagcttcccatt	374	Chr19:17449053- 17449426	
GTPBP3_Ex4+5	F-catgactcagtttccccttc	501	Chr19:1744910	64^{O}C

Primer	Sequence	Fragment size (bp)	Position of primer	Optimal conditions
	R-gttgcacagatggggtctct		1-17449601	
GTPBP3_Ex6+7	F-cacctcatatcagccctcaaa R-cagggagaaaaggcagagaa	605	Chr19:1744986 6-17450470	
GTPBP3_Ex8	F-taactgtccctccacccagt R-atggtctctcccgacctttt	488	Chr19:1745178 1-17452268	
GTPBP3_Ex9	F-tccctcaacttcaatttctgaac R-cctgcagggatctcactacc	489	Chr19:1745218 3-17452671	
GUF1_Ex12	F-tgattacagctggagggatttt R-tcagtatgctcagcagaaagaaa	344	Chr4:44692636- 44692979	
LETM2_Ex5a	F-aaaatgaaacaagacagggttttt R-gagaaattgggatggggttg	359	Chr8:38251518- 38251876	
LETM2_Ex6	F-ggcttctttttacttgctctgg R-aagacaggcttttctgcaaac	497	Chr8:38257617- 38258113	
MGME1_Ex2	F-ataaaggccttcgaccgttg R-cattcttttgacaccgttgc	633	Chr20:1795045 4-17951086	62^{O}C, DMSO
MGME1_Ex3	F-ttgtattgtttcagggcgttt R-tggctcttctccttgtacctg	479	Chr20:1795623 4-17956712	
MGME1_Ex4	F-tggtgaataagagcaatggtca R-ggcccggagttatagttttaag	322	Chr20:1796868 5-17969006	
MGME1_Ex5	F-tcttgtgtcctggtgcagtc R-gggcttttcagtcttttgg	504	Chr20:1797041 9-17970922	
MYH14_Ex11	F-ccacacgtgacctctgtcc R-ggaagggggatgctctctac	226	Chr19:5074746 8- 50747693	
MYH14_Ex35	F-agaggtgagcacaggtcagg R-gtgccttgcctcacactaca	475	Chr19:5079629 5- 50796769	
MIEF2_Ex2	F-ggtgtcctcccctgtggat R-cagggcaaagcagaccag	492	Chr17:1816585 0- 18166341	64^{O}C
MRPL9_Ex1	F-attctaggcgtccgtcacc R-gcgcctccagaaacaatact	395	Chr1:15173574 5- 151736139	62^{O}C
MRPL9_Ex3	F-agcagtctcttaaatcttgttcct R-ggcttctgcattcctgttct	412	Chr1:15173472 3- 151735134	
MRPS12_Ex2	F-cggagggactttctgttagc R-acacgcagtagccctccat	406	Chr19:3942176 1- 39422166	
MRPS12_Ex3	F-tgcctaaaacaccaaaattga R-agcatcggaggaccctgt	527	Chr19:3942289 4- 39423420	
MRPS22_Ex4	F-gcacagcagtgattagtgaagg R-atcaaggcacaaagccaact	415	Chr3:13906882 8-139069242	
MTO1_Ex1	F-gccctgcagattgtctcttg R-cctgatccttgcgagacact	459	Chr6:74171494- 74171952	HS PCR
MTO1_Ex2+3	F-attgggtctttgggattttctt R-tccaggtagagaaaatgcaaca	682	Chr6:74175774- 74176455	HS PCR
MTO1_Ex4	F-ctggcgatagagtgagacttca R-cagtacttcctgctgtgaatagc	466	Chr6:74182975- 74183440	HS PCR
MTO1_Ex5+6	F-cgtatcatgtggaatttatttgga R-gctaaggcctcctcactgac	547	Chr6:74189374- 74189374	HS PCR
MTO1_Ex8	F-gccactgctcctgacctaaata R-atctccaaacaccctgaaacat	396	Chr6:74190277- 74190672	HS PCR
MTO1_Ex9	F-tttctccttttaatgtctgttgtg R-atcctattattggctgggcaa	409	Chr6:74190579- 74190987	HS PCR
MTO1_Ex10	F-ggcaatacttgctttcttcctg R-gggaagggagaaagagaatataatg	519	Chr6:74191650- 74192168	HS PCR
MTO1_Ex11	F-taatccaagcaatttgagagacca R-ccaaaaacaagctacattcatttg	449	Chr6:74192020- 74192468	HS PCR
MTO1_Ex12	F-tacagttgatgggcgacaga R-gcccagccaagattctgtat	467	Chr6:74201792- 74202258	HS PCR

Primer	Sequence	Fragment size (bp)	Position of primer	Optimal conditions
MTO1_Ex13	F-tgaaatgagtgttgaatgagatga R-cccatgctggaagtttcttattac	417	Chr6:74207332-74207748	HS PCR
MTO1_Ex14	F-ttcccatttgtaaaatgaagacat R-ttaatggcaaacccatagtaacct	487	Chr6:74210097-74210583	HS PCR
OXR1_Ex4	F-ttttcaaagatgacaataccagttg R-tttctgcctttctgtttaatgtttc	296	Chr8:107695296- 107695591	
PDHA1_Ex12	F-ttacaccagcaacaggtcctc R-cactcaataattcatcttttaatgcac	447	ChrX:19377516-19377962	
POLRMT_Ex11	F-catccgtggctatggagaac R-cacctccagagaataccacga	409	Chr19:620258-620666	HS PCR, 62^{O}C, DMSO
PPIF_Ex4	F-ttggatgtttattgacccctttt R-caacaagccagaaatgctga	394	Chr10:81111107- 81111500	
PTPIP51_Ex12	F-caggattttccaaagcagga R-ggattcaagcgattctccag	379	Chr15:41029145-41029523	
RNASEH1_Ex1	F-ccagacgtgcgtcatcttc R-gccgagcaggaaaacgag	398	Chr2:3605566-3605963	DMSO
RNASEH1_Ex2	F-cctggaatcagtctccctca R-acacgaggttccctacatgc	346	Chr2:3604269-3604614	
RNASEH1_Ex3	F-attccctcgtttccctcgt R-ccatcaagtgggaaacacct	397	Chr2:3599654-3600050	
RNASEH1_Ex4	F-ctgcagcgttgtaaaataccc R-aacacgaaccaaagctgctc	367	Chr2:3597815-3598181	
RNASEH1_Ex5	F-ctgctctctgctgggagtct R-cgaaattgaaacatcccctct	468	Chr2:3596388-3596855	
RNASEH1_Ex6	F-gaggggatgtttcaatttcg R-cctgccctgttacaacacct	258	Chr2:3596150-3596407	
RNASEH1_Ex7	F-ccaaaggccaaagcaactaa R-actacgcccagcctcaaata	437	Chr2:3595364-3595800	
RNASEH1_Ex8	F-catgtgttgcttatgtaaagatga R-acatgtcacagaaggccact	300	Chr2:3593219-3593518	
RRM1_Ex12	F-ctgcctcattttcccctatg R-ttccttcctttgctctctgc	541	Chr11:4144230-4144770	
SCO1_Ex6	F-gacaatttttgttaggaatttgc R-aattttggttgaacgcaagg	483	Chr17:10584264- 10584746	
SEPT2_Ex11	F-tgattttctctttagctgttgcttt R-gcttctgtttttcaggagcaa	469	Chr2:242289311- 242289779	
SETX_Ex23	F-agcctcttggagtcactgct R-tgtctacccaggagccacac	496	Chr9:135150464- 135150959	
SLC3A1_Ex8	F-ctgtgtatacagctgtgttc R-atagctgtgatgaatagtc	246	Chr2:44539684-44539929	
THNSL1_Ex1b	F-ggatgttggtaatgagcaatttt R-tgaatgtttccgagtccaca	383	Chr10:25312464- 25312846	
TK2_Ex5	F-tcctgcagatgccactttg R-ccccaagtctgaagaaaacg	194	Chr16:66565228-66565421	
TOP3A_Ex3	F-tcaacatgcctcaaaattcct R-gaagctcctccctctcatttt	237	Chr17:18211587- 18211823	58^{O}C
TOP3A_Ex5	F-gcagcacttaccagccttaca R-gtcacactccaagagcagca	232	Chr17:18208415- 18208646	
TOP3B_Ex6	F-aggtgcaggaaaggggatag R-ccttcttcagggttggtgaa	496	Chr22:22324440- 22324935	
TRMU_Ex6+7	F-gaagcaaagtgtggggtgag R-gccccacagagccttctac	454	Chr22:46747904- 46748357	
TRMU_Ex8	F-tttggtggttggagaatcgt R-tgctggaggccttctcaag	342	Chr22:46749513- 46749854	

Primer	Sequence	Fragment size (bp)	Position of primer	Optimal conditions
USP8_Ex2	F-accactcatcgtctgctgtg R-tgtacgagttcacacattatacacatt	510	Chr15:5073337 4- 50733883	
VDAC1_Ex3	F-cattcagggtcttgcctctt R-ccacctgcaacatcagagaa	460	Chr5:13332649 3- 133326952	

List of custom or diagnostic forward and reverse primers used in this thesis for Sanger Sequencing. Forward and reverse primer sequences, fragment size (bp), amplified region and optimal PCR conditions are given. HS PCR – Hot Start PCR

Appendix B – Scopus Electronic Search Term Strategy

	Search Terms	# Entries
#1	progressive external ophthalmoplegia OR cpeo OR peo	974
#2	#1 AND (adult-onset OR adult)	608
#3	#1 AND (polg OR polg1)	107
#4	#2 AND (polg OR polg1)	67
#5	#1 AND (polg2)	13
#6	#1 AND (rrm2b)	10
#7	#1 AND (tymp OR thymidine phosphorylase)	20
#8	#7 OR (mngie)	56
#9	#1 AND (peo1 OR twinkle OR c10orf2)	81
#10	#1 AND (slc25a4 OR ANT1)	46
#11	#1 AND (dguok OR deoxyguanosine kinase)	8
#12	#1 AND (tk2 OR thymidine kinase)	11
#13	#1 AND opa1	13
#14	#1 AND (mgme1 OR c20orf72)	0
#15	mgme1 OR c20orf72	2
#16	#1 AND dna2	1
#17	#1 AND mpv17	4
#18	mpv17 AND ophthalmoplegia	5

Scopus electronic search terms used and the number of relevant articles identified. '#' – number.

Appendix C – Medline via PubMed Electronic Search Term Strategy

	Search Terms	# Entries
#1	(progressive external ophthalmoplegia[MeSH Terms]) AND adult[MeSH Terms]	434
#2	progressive external ophthalmoplegia[Text Word] OR PEO[Text Word] OR CPEO[Text Word]	1131
#3	#2 AND adult*[Text Word]	624
#4	progressive external ophthalmoplegia OR ophthalmoplegia OR ophthalmoparesis OR PEO OR CPEO	8853
#5	#1 AND polg[Text Word] OR polg1[Text Word]	139
#6	#4 AND (polg OR polg1)	194
#7	#1 AND polg2[Text Word]	5
#8	#4 AND polg2[Text Word]	14
#9	#1 AND rrm2b[Text Word]	2
#10	#4 AND rrm2b[Text Word]	9
#11	#2 AND (tymp[Text Word] OR thymidine phosphorylase[Text Word])	17
#12	#2 AND mngie[Text Word]	20
#13	mngie[Text Word]	115
#14	#1 AND peo1[Text Word] OR twinkle[Text Word] OR c10orf2[Text Word]	140
#15	#4 AND (slc25a4[Text Word] OR ant1[Text Word])	118
#16	#2 AND (dguok[Text Word] OR deoxyguanosine kinase[Text Word])	7
#17	#2 AND (tk2[Text Word] OR thymidine kinase[Text Word])	14
#18	#1 AND opa1[Text Word]	4
#19	ophthalmop*[Text Word] AND opa1[Text Word]	14

#20	(mgme1[Text Word] OR c20orf72[Text Word])	1
#21	ophthalmop* AND dna2[Text Word]	1
#22	ophthalmop*[Text Word] AND mpv17[Text Word]	3

Medline via PubMed electronic search terms used and the number of relevant articles identified. '#' – number.

Appendix D – Genetics Abstracts Electronic Search Term Strategy

	Search Terms	# Entries
#1	ophthalmoplegia	147
#2	#1 AND (polg1 OR polg)	31
#3	#1 AND (slc25a4 OR ant1)	17
#4	#1 AND (peo1 OR twinkle OR c10orf2)	29
#5	#1 AND polg2	6
#6	#1 AND rrm2b	3
#7	#1 AND opa1	4
#8	#1 AND dguok	2
#9	#1 AND (tk2 OR thymidine kinase)	4
#10	#1 AND dna2	1
#11	#1 AND mpv17	1
#12	#1 AND (tymp OR thymidine phosphorylase)	4
#13	#12 AND mngie	4
#14	mngie	26
#15	#1 AND mgme1	1
#16	#1 AND #16	10

Medline via PubMed electronic search terms used and the number of relevant articles identified. '#' – number.

Appendix E – UniProt Electronic Search Term Strategy

	Search Terms	# Entries
#1	peo1	16
#2	polg	26
#3	polg2	2
#4	rrm2b	4
#5	tymp	2
#6	slc25a4	6
#7	dguok	2
#8	tk2	4
#9	opa1	17
#10	mgme1	1
#11	dna2	1
#12	mpv17	3
#13	afg3l2	4
#14	spg7	4

UniProt electronic search terms used and the number of relevant articles identified. '#' – number.

Appendix F – Clinical, Molecular and Genetic Data of *RRM2B*, *POLG2*, *SLC25A4*, *DGUOK*, *TK2*, *OPA1*, *MPV17*, *MGME1*, *DNA2*, *SPG7*, *AFG3L2*, *DNM2* and *RNASEH1* Mutations Associated with Adult-Onset PEO and Multiple mtDNA Deletions

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
RRM2B						
	c.48G>A	p.(Glu16=)	PEO, ptosis, proximal muscle weakness, ataxia, fatigue, sensorineural hearing loss, tinnitus, hypothyroidism, stroke-like episodes, renal disturbance (colics)	Multiple deletions	3	(Pitceathly *et al.*, 2012)
	c.97C>T	p.(Pro33Ser)	PEO, ptosis, hearing loss, muscle weakness, depression, anxiety, gonadal atrophy, pigmentary retinopathy	Multiple deletions	1	(Takata *et al.*, 2011)
	c.121C>T	p.(Arg41Trp)	PEO, ptosis, migraine, mild neck flexion weakness, proximal muscle weakness	Multiple deletions	1	(Pitceathly *et al.*, 2012)
	c.122G>A	p.(Arg41Gln)	PEO, encephalopathy, stroke-like episodes, hearing loss, cognitive impairment	Multiple deletions	1	
	c.208G>A	p.(Asp70Asn)	PEO, ptosis, ataxia, proximal muscle weakness, bulbar involvement, facial muscle weakness, ischaemic heart disease, sleep disorder	Multiple deletions	1	
	c.253_255del	p.(Glu85del)	PEO, ptosis, mild proximal muscle wasting, fatigue	Multiple deletions	1	(Pitceathly *et al.*, 2011)
	c.329G>A	p.(Arg110His)	PEO, ptosis, sensorineural hearing loss,	Depletion	1	(Shaibani *et al.*, 2009)

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
	c.362G>A	p.(Arg121His)	peripheral neuropathy, ataxia, dysarthria, gastrointestinal dysmotility, cachexia			
	c.583G>A	p.(Gly195Arg)	PEO, ptosis, proximal muscle weakness, hearing loss, dysphonia, ataxia, glaucoma, cataracts	Multiple deletions	2	(Fratter *et al.*, 2011; Pitceathly *et al.*, 2012)
	c.632G>A	p.(Arg211Lys)	PEO, ptosis	Multiple deletions	1	(Pitceathly *et al.*, 2012)
	c.671T>G	p.(Ile224Ser)	PEO, diplopia, proximal muscle weakness, cataracts, migraine	Multiple deletions	1	
	c.950del	p.(Leu317*)	PEO, ptosis, dysphagia, dysarthria, ataxia, proximal myopathy, fatigue, glaucoma	Multiple deletions	3	(Fratter *et al.*, 2011)
	c.952G>T	p.(Glu318*)	PEO, ptosis, dysarthria, dysphagia, fatigue	Multiple deletions	1	
	c.965dup	p.(Asn322Lysfs*4)	PEO, ptosis, dysphagia, ataxia, proximal myopathy, fatigue, dysphonia, gastrointestinal dysmotility, irritable bowel syndrome, respiratory failure	Multiple deletions	5	(Fratter *et al.*, 2011; Pitceathly *et al.*, 2012)
	c.979C>T	p.(Arg327*)	PEO, ptosis, exercise intolerance, fatigue, reduced reflexes, ataxia, hypoacusis, mood disturbance, cognitive dysfunction, diplopia	Multiple deletions	19	(Tyynismaa *et al.*, 2009a; Pitceathly *et al.*, 2012)
	c.1046C>G	p.(Ala349Gly)	PEO, ptosis, proximal muscle weakness, fatigue, tremor, impaired memory, breast carcinoma	Multiple deletions	1	(Pitceathly *et al.*, 2012)
POLG2						
	c.1105A>G	p.(Arg369Gly)	PEO, ptosis, proximal muscle weakness, absent	Multiple deletions	2	(Young *et al.*, 2011; Craig

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
			knee jerks, slurred speech, ataxia, exercise intolerance, fatigue, gastrointestinal reflux, delayed gastric emptying, respiratory deficiency/failure, failure to thrive, apnea, lactic acidosis			*et al.*, 2012)
	c.1352G>A	p.(Gly451Glu)	PEO, exercise intolerance, muscle pain, ptosis, facial and limb muscle atrophy	Multiple deletions	1	(Longley *et al.*, 2006)
SLC25A4						
	c.269C>A	p.(Ala90Asp)	PEO, ptosis, exercise intolerance, limb muscle weakness, retinopathy, schizoaffective disorder	Multiple deletions	3	(Deschauer *et al.*, 2005)
	c.293T>C	p.(Leu98Pro)	PEO, ptosis, bipolar affective disorder, facial hypokinesia, temporal wasting, ataxic gait, peripheral neuropathy, hearing loss, migraines, memory lapses, temporoparietal cortex atrophy, diabetes mellitus	Multiple deletions	4	(Napoli *et al.*, 2001; Siciliano *et al.*, 2003)
	c.311A>G	p.(Asp104Gly)	PEO, ptosis, generalised muscle weakness and wasting	Multiple deletions	4	(Komaki *et al.*, 2002)
	c.340G>C	p.(Ala114Pro)	PEO, ptosis, sensorineural hearing loss, generalised muscle weakness, hypo- or hyperthyroidism, dementia	Multiple deletions	27	(Kaukonen *et al.*, 2000)
	c.865G>A	p.(Val289Met)	PEO, neck and proximal limb muscle weakness,	Multiple deletions	1	(Galassi *et al.*, 2008)

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
			bradykinesia, cogwheel rigidity, slurred speech, dysphagia, dysmetria, Romberg sign, postural tremor, absent deep tendon reflexes, severe depression, insomnia, nocturnal panic attacks			
DGUOK						
	c.130G>A	p.(Glu44Lys)	PEO, ptosis, limb girdle weakness, dysphagia, cramps	Multiple deletions	1	(Ronchi *et al.*, 2012c)
	c.462T>A	p.(Asn154Lys)		Multiple deletions	1	
	c.605_606del	p.(Arg202Tyrfs*12)	PEO	Multiple deletions	1	
TK2						
	c.103C>T	p.(Gln32*)	PEO, ptosis, sensorineural hearing loss, facial muscle weakness, proximal muscle wasting, respiratory failure	Multiple deletions	1	(Alston *et al.*, 2013)
	c.156+6T>G	Splice site	PEO, ptosis, dysphagia, facial diplegia, neck muscle weakness, EMG myopathic	Multiple deletions	1	(Garone *et al.*, submitted)
	c.323C>T	p.Thr108Met	PEO, dysarthria, dysphagia, respiratory insufficiency, EMG myopathic	Multiple deletions	1	(Garone *et al.*, submitted)
	c.415G>A	p.(Ala139Thr)	PEO, ptosis, respiratory insufficiency	Multiple deletions	1	Garone *et al.*, submitted)
	u.k.	p.(Asp157Valfs*11)	PEO, ptosis, dysphagia, facial diplegia, neck muscle weakness, EMG myopathic	Multiple deletions	1	(Garone *et al.*, submitted)
	c.547C>T	p.(Arg183Trp)	PEO, progressive proximal muscle weakness and	Multiple deletions	3	(Tyynismaa *et al.*, 2012),

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
			atrophy, dysphagia, dysarthria, respiratory insufficiency			Garone *et al.*, submitted)
	c.562A>G	p.(Thr188Ala)	PEO, progressive proximal muscle weakness and atrophy, dysphagia, dysarthria	Multiple deletions	2	(Tyynismaa *et al.*, 2012)
	c.582G>T	p.(Lys194Asn)	PEO, ptosis, sensorineural hearing loss, facial muscle weakness, proximal muscle wasting, respiratory failure	Multiple deletions	1	(Alston *et al.*, 2013)
	c.604_606del	p.(Lys202del)	PEO, severe ptosis, limb girdle muscle weakness, dysphagia, facial diplegia, dysarthria, respiratory insufficiency	Multiple deletions	2	(Camara *et al.*, 2015), Garone *et al.*, submitted)
OPA1						
	c.113_130del	p.(Arg38_Ser43del)	PEO, ptosis, proximal and facial muscle weakness, sensorineural hearing loss, migraines, anxiety, depression, gastrointestinal dysmobility	Multiple deletions	1	(Milone *et al.*, 2009)
	c.610+364G>A	Splice acceptor site	PEO, optic atrophy, ptosis, cerebellar ataxia, muscle atrophy, spasticity, peripheral axonal neuropathy, decreased vibratory sense	n.d.	3	(Bonifert *et al.*, 2014)
	c.1053T>A	p.(Asp351Glu)	PEO, ptosis, visual loss, sensorimotor neuropathy, proximal muscle weakness, ataxia, absent deep tendon reflexes, wide based gait	n.d.	5	(Ahmad *et al.*, 2015)
	c.1069G>A	p.(Ala357Thr)	PEO, optic atrophy, ptosis, sensorineural hearing	Multiple deletions	1	(Amati-Bonneau *et al.*,

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
			loss, muscle weakness, peripheral axonal neuropathy			2008)
	c.1198C>T	p.(Pro400Ser)	PEO, optic atrophy, sensorineural hearing loss	Multiple deletions	1	(Yu-Wai-Man *et al.*, 2010a)
	c.1294A>G	p.(Ile432Val)	PEO, optic atrophy, ptosis, fatigue, muscle pain, cerebellar ataxia, peripheral axonal neuropathy	Multiple deletions	1	(Stewart *et al.*, 2008)
	c.1316G>T	p.(Gly439Val)	PEO, optic atrophy, sensorineural hearing loss, ataxia, Romberg sign, peripheral axonal neuropathy	Multiple deletions	1	(Amati-Bonneau *et al.*, 2008)
	c.1334G>A	p.(Arg455His)	PEO, optic atrophy, ptosis, sensorineural hearing loss, exercise intolerance, peripheral axonal neuropathy	Multiple deletions	12	(Payne *et al.*, 2004; Amati-Bonneau *et al.*, 2008; Stewart *et al.*, 2008)
	c.1345A>C	p.(Thr449Pro)	PEO, optic atrophy, ataxia, peripheral axonal neuropathy, sensorineural hearing loss, migraines, nystagmus, pes cavus	n.d.	4	(Liskova *et al.*, 2013)
	c.1462G>A	p.(Gly488Arg)	PEO, optic atrophy, sensorineural hearing loss, muscle weakness, gait unsteadiness, peripheral axonal neuropathy, ataxia, dementia, migraines, epilepsy, Romberg sign, reduced vibration sense, weak/absent deep tendon reflexes, bradykinesia, rigidity, tremor, cognitive impairment, apnoea, Parkinsonism, cardiomyopathy	Multiple deletions	9	(Yu-Wai-Man *et al.*, 2010a; Carelli *et al.*, 2015)

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
	c.1484C>T	p.(Ala495Val)	PEO, optic atrophy, sensorineural hearing loss, muscle weakness, peripheral axonal neuropathy, gait unsteadiness, ataxia, Romberg sign, reduced vibration sense, weak/absent deep tendon reflexes, bradykinesia, tremor, Parkinsonism	Multiple deletions	5	(Yu-Wai-Man *et al.*, 2010a; Carelli *et al.*, 2015)
	c.1635C>G	p.(Ser545Arg)	PEO, optic atrophy, ptosis, sensorineural hearing loss, ataxia, Romberg sign, diffuse myalgia, peripheral axonal neuropathy, gait disturbance facial muscle weakness, proximal muscle weakness, distal muscle weakness and atrophy, spasticity	Multiple deletions	7	(Amati-Bonneau *et al.*, 2008; Hudson *et al.*, 2008; Yu-Wai-Man *et al.*, 2010a)
	c.1741A>G	p.(Tyr582Cys)	PEO, sensorineural hearing loss, macrocytic anemia, hypogonadism, central vision loss, mild ataxia	Multiple deletions	1	(Ferraris *et al.*, 2008)
	c.2708_2711del	p.(Val903fs*3)	PEO, optic atrophy, sensorineural hearing loss	Multiple deletions	1	(Yu-Wai-Man *et al.*, 2010a)
	c.2819-1_2821del	p.(Lys940_Val942delinsI)	PEO, optic atrophy, ptosis, fatigue, pes cavus	Multiple deletions	1	(Ranieri *et al.*, 2012)
MPV17						
	c.263A>T	p.(Lys88Met)	PEO, ptosis, progressive distal and proximal muscle weakness, exercise intolerance, axonal	Multiple deletions	1	(Garone *et al.*, 2012)
	c.265A>T	p.(Met89Leu)			1	

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
	c.428T>G	p.(Leu143*)	sensorimotor peripheral neuropathy, hearing loss, gastrointestinal dysmobility, depression, parkinsonism, diabetes mellitus, steatohepatitis		1	
MGME1						
	c.456G>A	p.(Trp152*)	PEO, proximal muscle weakness, generalised muscle atrophy, emaciation, respiratory failure, mild spinal deformities, renal colics	Multiple deletions	2	(Kornblum *et al.*, 2013)
	c.698A>G	p.(Tyr233Cys)	PEO, proximal muscle weakness, respiratory failure, emaciation, cardiac arrhythmias, depression, memory loss		1	
DNA2						
	c.2167G>A	p.(Val723Ile)	PEO, proximal muscle weakness in lower limbs, diplopia, myalgia	Multiple deletions	1	(Ronchi *et al.*, 2013)
SPG7						
	c.233T>A	p.(Leu78*)	PEO, ptosis, ataxia, spasticity, proximal myopathy, moderate dysarthria, bladder symptoms	n.d	2	(Pfeffer *et al.*, 2014)
	c.861dup	p.(Asn288*)	PEO, ptosis, proximal myopathy, mild dysphagia, ataxia, spasticity	Multiple deletions	1	

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
	c.1046insC	p.(Gly352Argfs*44)	PEO, ptosis, ataxia, spastic ataxia, optic atrophy, mild myopathy, cerebellar atrophy	Multiple deletions	1	
	c.1192C>T	p.(Arg398*)	PEO, ptosis, ataxia, spasticity, dysphagia, bladder symptoms, cerebellar atrophy	Multiple deletions	2	
	c.1454_1462del	p.(Arg485_Glu487del)	PEO, ptosis, ataxia, spasticity, dysarthria, upper and lower limb hyperreflexia, upper and lower limb weakness, bladder symptoms, decreased vibratory sense, nystagmus, cognitive impairment	Multiple deletions	2	(Pfeffer *et al.*, 2014; Wedding *et al.*, 2014)
	c.1529C>T	p.(Ala510Val)	PEO, ptosis, proximal myopathy, mild dysphagia, ataxia, spasticity, bladder symptoms, cerebellar atrophy, lower limb proximal weakness, mild cognitive impairment, optic atrophy	Multiple deletions	7	(Pfeffer *et al.*, 2014)
	c.1672A>T	p.(Lys558*)	PEO, ptosis, proximal myopathy, mild dysphagia, ataxia, spasticity, bladder symptoms, cerebellar atrophy	Multiple deletions	2	(Pfeffer *et al.*, 2014)
	c.2102A>C	p.(His701Pro)	PEO, ptosis, dysarthria, upper and lower limb hyperreflexia, upper and lower limb weakness, spasticity, ataxia, bladder symptoms, decreased vibratory sense, nystagmus, cognitive impairment	n.d.	1	(Wedding *et al.*, 2014)
	c.2221G>A	p.(Glu741Lys)	PEO, ptosis, proximal myopathy, ataxia, spasticity, dysphagia, dysphonia, dysarthria, optic atrophy, cerebellar atrophy	Multiple deletions	1	(Pfeffer *et al.*, 2014)
	c.2224G>A	p.(Asp742Asn)			1	

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
	c.2228T>C	p.(Ile743Thr)	PEO, ptosis, ataxia, spasticity, dysarthria	Multiple deletions	1	
AFG3L2						
	c.2011G>T	p.(Gly671Trp)	PEO, ptosis, lower limb proximal muscle weakness, brisk tendon reflexes, flexor plantar reflexes, mild dysarthria, dysmetria, ataxic gait, slurred speech	Multiple deletions	1	(Gorman *et al.*, 2015a)
	c.2065T>C	p.(Tyr689His)	PEO, ptosis, lower limb proximal weakness, brisk tendon reflexes, flexor plantar reflexes, mild dysmetria, ataxic gait, cerebellar atrophy	Multiple deletions	1	
DNM2						
	c.1105C>T	p.(Arg369Trp)	PEO, ptosis, left bundle branch block, blurred vision, diplopia, proximal and distal muscle weakness, decreased deep tendon reflexes, pallhypesthesia, axonal neuropathy, depression, paranoia, cognitive decline, EMG myopathic	Multiple deletions	1	(Gal *et al.*, 2015)
RNASEH1						
	c.424G>A	p.(Val142Ile)	PEO, ptosis, ataxia, muscle pain, dysphagia, speech difficulties, dysarthria, dysphonia, wide-based gait, exercise intolerance, muscle cramps,	Multiple deletions	10	(Reyes *et al.*, 2015; Akman *et al.*, 2016)

Gene	cDNA Change	Amino Acid Change	Associated Phenotypes/Symptoms	Effect on mtDNA	# Patients	Reference(s)
			lower muscle weakness, respiratory insufficiency, cerebellar and brainstem atrophy, mild motor demyelinating neuropathy, distal clonus, fatigue, right bundle branch block			
	c.469C>T	p.(Arg157*)	PEO, ptosis, muscle pain, dysphagia, speech difficulties, dysarthria, dysphonia, wide-based gait, exercise intolerance, muscle cramps, lower muscle weakness, respiratory insufficiency, cerebellar and brainstem atrophy, mild motor demyelinating neuropathy	Multiple deletions	1	(Reyes *et al.*, 2015)
	c.554C>T	p.(Ala185Val)	PEO, ptosis, distal clonus, fatigue, right bundle branch block	Multiple deletions	1	

n.d. – not determined; u.k. – unknown. '#' – number.

Appendix G – *TYMP* Mutations Associated with Adult-Onset PEO and Multiple mtDNA Deletions

cDNA Change	Amino Acid change	Effect on mtDNA	Reference(s)
c.131G>A	p.(Arg44Gln)	n.d.	(Gamez *et al.*, 2002)
c.261G>T	p.(Glu87Asp)	n.d.	(Zimmer *et al.*, 2009)
c.317G>C	p.(Glu106Gln)	n.d.	(Nakhro *et al.*, 2011)
c.340G>A	p.(Asp114Asn)	n.d.	(Zimmer *et al.*, 2009)
u.k.	p.(Asp123*)	n.d.	(Kintarak *et al.*, 2007)
c.433G>A	p.(Gly145Arg)	Multiple deletions and depletion	(Giordano *et al.*, 2008)
c.457G>A	p.(Gly153Ser)	n.d.	(Marti *et al.*, 2005)
c.467A>G	p.(Asn156Gly)	n.d.	(Said *et al.*, 2005)
c.530T>C	p.(Leu177Pro)	n.d.	(Said *et al.*, 2005)
c.605G>A	p.(Arg202Lys)	n.d.	(Feddersen *et al.*, 2009)
c.605G>C	p.(Arg202Thr)	n.d.	(Marti *et al.*, 2005)
c.622G>A	p.(Val208Met)	n.d.	(Marti *et al.*, 2005)
c.766G>T	p.(Val256Phe)	n.d.	(Etienne *et al.*, 2012)
c.854T>C	p.(Leu285Pro)	n.d.	(Marti *et al.*, 2005; Hirano *et al.*, 2006)
c.856A>C	p.(Glu286Lys)	n.d.	(Amiot *et al.*, 2009)
c.866A>C	p.(Glu289Ala)	Multiple deletions and depletion	(Blondon *et al.*, 2005b; Said *et al.*, 2005; Giordano *et al.*, 2008; Amiot *et al.*, 2009; Filosto *et al.*, 2011; Scarpelli *et al.*, 2013)
c.928+1G>A	Splice mutation	n.d.	(Amiot *et al.*, 2009)
c.931G>C	p.(Gly311Arg)	n.d.	(Marti *et al.*, 2005)
c.1112T>C	p.(Leu371Pro)	Depletion	(Kocaefe *et al.*, 2003; Oztas *et al.*, 2010)

cDNA Change	Amino Acid change	Effect on mtDNA	Reference(s)
c.1135G>A	p.(Glu379Lys)	Depletion	(Massa *et al.*, 2009)
c.1142T>G	p.(Leu381Arg)	n.d.	(Etienne *et al.*, 2012)
c.1160-1G>A	Splice mutation	n.d.	(Scarpelli *et al.*, 2013)
c.1160G>A	p.(Gly387Asn)	n.d.	(Said *et al.*, 2005)
c.1160-1G>A	Splice mutation	Depletion	(Amiot *et al.*, 2009; Massa *et al.*, 2009; Filosto *et al.*, 2011)
c.1231_1243del	u.k.	n.d.	(Filosto *et al.*, 2011; Scarpelli *et al.*, 2013)
c.1412C>T	p.(Ala474Val)	n.d.	(Tan *et al.*, 2012)
c.1460_1479del	u.k.	Multiple deletions	(Blazquez *et al.*, 2005)
u.k.	u.k.	Depletion	(Kocaefe *et al.*, 2003)
u.k.	u.k.	n.d.	(Bedlack *et al.*, 2004)
u.k.	Splice mutation	Depletion	(Kocaefe *et al.*, 2003)
u.k.	Splice mutation	Depletion	(Kocaefe *et al.*, 2003)
IVS1-1G>C	Splice mutation	n.d.	(Szigeti *et al.*, 2004)
IVS9-1G>C	Splice mutation	Multiple deletions and depletion	(Giordano *et al.*, 2008; Kalkan *et al.*, 2012)
IVS5+1G>A	Splice mutation	n.d.	(Laforce *et al.*, 2009)
u.k.	u.k.	n.d.	(Tan *et al.*, 2012)

n.d. – not determined; u.k. – unknown.

Appendix H – *POLG* Clinical Manifestations in Adult-Onset PEO and Multiple mtDNA Deletion Patients from the Literature

Finding	Manifestation	Reference(s)
Psychiatric Illness	Depression	(Lamantea *et al.*, 2002; Van Goethem *et al.*, 2003a; Luoma *et al.*, 2004; Mancuso *et al.*, 2004a; Kollberg *et al.*, 2005; Hudson *et al.*, 2007; Galassi *et al.*, 2008; Schulte *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Synofzik *et al.*, 2010a; Gurgel-Giannetti *et al.*, 2012; Palin *et al.*, 2012; Dolhun *et al.*, 2013)
	Psychosis	(Hakonen *et al.*, 2005; Horvath *et al.*, 2006a; Schulte *et al.*, 2009; Kinghorn *et al.*, 2013)
	Cognitive Decline	(Mancuso *et al.*, 2004a; Van Goethem *et al.*, 2004; Hakonen *et al.*, 2005; Luoma *et al.*, 2005; Naimi *et al.*, 2006; Invernizzi *et al.*, 2008; Milone *et al.*, 2008; Paus *et al.*, 2008; Betts-Henderson *et al.*, 2009; Schulte *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Martikainen *et al.*, 2010; McHugh *et al.*, 2010; Synofzik *et al.*, 2010b; Smits *et al.*, 2011a; Gurgel-Giannetti *et al.*, 2012; Hansen *et al.*, 2012; Tang *et al.*, 2012; Roos *et al.*, 2013b; Yu-Wai-Man *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Delgado-Alvarado *et al.*, 2015)
	Dementia	(Van Goethem *et al.*, 2004; Horvath *et al.*, 2006a; Komulainen *et al.*, 2010; Martikainen *et al.*, 2010; Dolhun *et al.*, 2013; Roos *et al.*, 2013b)
	Intellectual Disability	(Kollberg *et al.*, 2005; Blok *et al.*, 2009)
Seizure Disorder	Status Epilepticus	(Van Goethem *et al.*, 2004; Hakonen *et al.*, 2005; Winterthun *et al.*, 2005; Naimi *et al.*, 2006; Tzoulis *et al.*, 2006; Paus *et al.*, 2008; Schulte *et al.*, 2009; Hansen *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Palin *et al.*, 2012; Woodbridge *et al.*, 2013; Yu-Wai-Man *et al.*, 2013)
	Grand Mal Epilepsy	(Neeve *et al.*, 2012)
	Myoclonus	(Van Goethem *et al.*, 2004; Hakonen *et al.*, 2005; Winterthun *et al.*, 2005; Horvath *et al.*, 2006a; Tzoulis *et al.*, 2006; Johansen *et al.*, 2008; Remes *et al.*, 2008; Neeve *et al.*, 2012; Horga *et al.*, 2014)
	Focal Motor Seizures	(Tzoulis *et al.*, 2006; Neeve *et al.*, 2012)

Finding	Manifestation	Reference(s)
	Generalised Seizure	(Winterthun *et al.*, 2005; Stewart *et al.*, 2009; Synofzik *et al.*, 2010b; Kinghorn *et al.*, 2013; Hanisch *et al.*, 2014; Rajakulendran *et al.*, 2016)
Extrapyramidal Movement Disorder	Parkinsonism	(Lamantea *et al.*, 2002; Van Goethem *et al.*, 2003a; Luoma *et al.*, 2004; Mancuso *et al.*, 2004b; Hisama *et al.*, 2005; Pagnamenta *et al.*, 2006; Hudson *et al.*, 2007; Galassi *et al.*, 2008; Invernizzi *et al.*, 2008; Johansen *et al.*, 2008; Remes *et al.*, 2008; Betts-Henderson *et al.*, 2009; Synofzik *et al.*, 2010a; Ferreira *et al.*, 2011a; Milone *et al.*, 2011; Sato *et al.*, 2011; Gurgel-Giannetti *et al.*, 2012; Brandon *et al.*, 2013; Dolhun *et al.*, 2013; Mukai *et al.*, 2013; Horga *et al.*, 2014; Miguel *et al.*, 2014; Delgado-Alvarado *et al.*, 2015; Martikainen *et al.*, 2016)
	Chorea	(Hakonen *et al.*, 2005)
	Dystonia	(Paus *et al.*, 2008; Remes *et al.*, 2008; Horga *et al.*, 2014; Rajakulendran *et al.*, 2016)
Cerebellar Involvement	Ataxia	(Lamantea *et al.*, 2002; Filosto *et al.*, 2003; Van Goethem *et al.*, 2003b; Mancuso *et al.*, 2004a; Hakonen *et al.*, 2005; Hisama *et al.*, 2005; Kollberg *et al.*, 2005; Winterthun *et al.*, 2005; Horvath *et al.*, 2006a; Hudson *et al.*, 2006; Naimi *et al.*, 2006; Tzoulis *et al.*, 2006; Hudson *et al.*, 2007; Johansen *et al.*, 2008; Milone *et al.*, 2008; Paus *et al.*, 2008; Remes *et al.*, 2008; Wong *et al.*, 2008; Blok *et al.*, 2009; Schulte *et al.*, 2009; Stewart *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; McHugh *et al.*, 2010; Synofzik *et al.*, 2010b; Gati *et al.*, 2011; Smits *et al.*, 2011a; Bostan *et al.*, 2012; Gurgel-Giannetti *et al.*, 2012; Hansen *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Palin *et al.*, 2012; Tang *et al.*, 2012; Brandon *et al.*, 2013; Degos *et al.*, 2013; Kinghorn *et al.*, 2013; Mukai *et al.*, 2013; Woodbridge *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Henao *et al.*, 2016; Martikainen *et al.*, 2016; Mongin *et al.*, 2016; Rajakulendran *et al.*, 2016)
“Cerebrovascular” Involvement	Migraine	(Winterthun *et al.*, 2005; Tzoulis *et al.*, 2006; Paus *et al.*, 2008; Tang *et al.*, 2012; Brandon *et al.*, 2013; Woodbridge *et al.*, 2013)

Finding	Manifestation	Reference(s)
	Relapsing/Remitting Neurological Events	(Degos *et al.*, 2013)
	Stroke-like Episodes	(Neeve *et al.*, 2012; Rajakulendran *et al.*, 2016)
	Hypertrophic Olivary Degeneration	(Kinghorn *et al.*, 2013)
Special Sensory	Sensorineural Hearing Loss	(Di Fonzo *et al.*, 2003; Van Goethem *et al.*, 2003b; Mancuso *et al.*, 2004a; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Hudson *et al.*, 2007; Milone *et al.*, 2008; Wong *et al.*, 2008; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Komulainen *et al.*, 2010; Milone *et al.*, 2011; Tang *et al.*, 2012; Brandon *et al.*, 2013; Cheldi *et al.*, 2013; Kinghorn *et al.*, 2013; Woodbridge *et al.*, 2013; Martikainen *et al.*, 2016)
	Retinitis Pigmentosa	(Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003; Blok *et al.*, 2009)
Myopathy	Proximal Myopathy	(Lamantea *et al.*, 2002; Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003; Van Goethem *et al.*, 2003a; Luoma *et al.*, 2004; Mancuso *et al.*, 2004b; Luoma *et al.*, 2005; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Hudson *et al.*, 2006; Santoro *et al.*, 2006; Hudson *et al.*, 2007; Galassi *et al.*, 2008; Invernizzi *et al.*, 2008; Milone *et al.*, 2008; Betts-Henderson *et al.*, 2009; Blok *et al.*, 2009; Stewart *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Martikainen *et al.*, 2010; McHugh *et al.*, 2010; Schicks *et al.*, 2010; Baruffini *et al.*, 2011; Ferreira *et al.*, 2011a; Smits *et al.*, 2011a; Bostan *et al.*, 2012; Gurgel-Giannetti *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Tang *et al.*, 2012; Roos *et al.*, 2013b; Woodbridge *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Horga *et al.*, 2014; Martikainen *et al.*, 2016)
	Distal Myopathy	(Lamantea *et al.*, 2002; Di Fonzo *et al.*, 2003; Mancuso *et al.*, 2004a; Gago *et al.*, 2006; Hudson *et al.*, 2006; Santoro *et al.*, 2006; Schulte *et al.*, 2009; McHugh *et al.*, 2010; Mukai *et al.*, 2013; Horga *et al.*, 2014; Miguel *et al.*, 2014; Rajakulendran *et al.*, 2016)

Finding	Manifestation	Reference(s)
	Exercise Intolerance	(Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003; Luoma *et al.*, 2004; Mancuso *et al.*, 2004a; Mancuso *et al.*, 2004b; Hakonen *et al.*, 2005; Milone *et al.*, 2008; Wong *et al.*, 2008; Blok *et al.*, 2009; Milone *et al.*, 2011; Tang *et al.*, 2012; Woodbridge *et al.*, 2013)
Peripheral Neuropathy	Sensory Neuronopathy/Ganglionopathy	(Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003; Van Goethem *et al.*, 2003b; Mancuso *et al.*, 2004a; Mancuso *et al.*, 2004b; Hakonen *et al.*, 2005; Hisama *et al.*, 2005; Luoma *et al.*, 2005; Gonzalez-Vioque *et al.*, 2006; Santoro *et al.*, 2006; Johansen *et al.*, 2008; Milone *et al.*, 2008; Schulte *et al.*, 2009; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Komulainen *et al.*, 2010; McHugh *et al.*, 2010; Schicks *et al.*, 2010; Synofzik *et al.*, 2010b; Gati *et al.*, 2011; Smits *et al.*, 2011a; Bostan *et al.*, 2012; Gurgel-Giannetti *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Tang *et al.*, 2012; Dolhun *et al.*, 2013; Kinghorn *et al.*, 2013; Woodbridge *et al.*, 2013; Mongin *et al.*, 2016)
	Axonal Sensorimotor Neuropathy	(Di Fonzo *et al.*, 2003; Mancuso *et al.*, 2004a; Mancuso *et al.*, 2004b; Hisama *et al.*, 2005; Horvath *et al.*, 2006a; Milone *et al.*, 2008; Remes *et al.*, 2008; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Gati *et al.*, 2011; Bostan *et al.*, 2012; Hansen *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Tang *et al.*, 2012; Degos *et al.*, 2013; Mukai *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Miguel *et al.*, 2014; Bindu *et al.*, 2016; Henao *et al.*, 2016; Rajakulendran *et al.*, 2016)
Endocrine/Gonadal system	Diabetes Mellitus	(Horvath *et al.*, 2006a; Blok *et al.*, 2009; Komulainen *et al.*, 2010; Roos *et al.*, 2013b; Hanisch *et al.*, 2014)
	Primary Ovarian Failure	(Pagnamenta *et al.*, 2006; Hudson *et al.*, 2007; Blok *et al.*, 2009; Baruffini *et al.*, 2011)
	Hypothyroidism	(Tzoulis *et al.*, 2009; Gurgel-Giannetti *et al.*, 2012)
	Hypogonadism	(Kollberg *et al.*, 2005; Milone *et al.*, 2008)
Gastrointestinal System	Gastrointestinal Dysmotility; Constipation/Diarrhoea;	(Filosto *et al.*, 2003; Van Goethem *et al.*, 2003a; Bostan *et al.*, 2012; Tang *et al.*, 2012; Woodbridge *et al.*, 2013; Horga *et al.*, 2014)

Finding	Manifestation	Reference(s)
	Gastroparesis; Bowel Obstruction; Cachexia	
	Liver Failure	(Neeve *et al.*, 2012)
Heart	Cardiomyopathy	(Van Goethem *et al.*, 2003b; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Komulainen *et al.*, 2010; Palin *et al.*, 2012; Brandon *et al.*, 2013; Mukai *et al.*, 2013)
Ocular	Cataract	(Filosto *et al.*, 2003; Kollberg *et al.*, 2005; Milone *et al.*, 2011; Gurgel-Giannetti *et al.*, 2012; Brandon *et al.*, 2013)
	Diplopia	(Mancuso *et al.*, 2004b; Gago *et al.*, 2006; Tzoulis *et al.*, 2009; Ferreira *et al.*, 2011a; Sato *et al.*, 2011; Palin *et al.*, 2012; Degos *et al.*, 2013; Kinghorn *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Miguel *et al.*, 2014; Rajakulendran *et al.*, 2016)
Other	Dysarthria	(Van Goethem *et al.*, 2003b; Mancuso *et al.*, 2004a; Hakonen *et al.*, 2005; Luoma *et al.*, 2005; Gago *et al.*, 2006; Johansen *et al.*, 2008; Milone *et al.*, 2008; Paus *et al.*, 2008; Betts-Henderson *et al.*, 2009; Blok *et al.*, 2009; Schulte *et al.*, 2009; McHugh *et al.*, 2010; Schicks *et al.*, 2010; Gati *et al.*, 2011; Bostan *et al.*, 2012; Hansen *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Palin *et al.*, 2012; Brandon *et al.*, 2013; Cheldi *et al.*, 2013; Kinghorn *et al.*, 2013; Roos *et al.*, 2013b; Woodbridge *et al.*, 2013; Horga *et al.*, 2014; Miguel *et al.*, 2014; Mongin *et al.*, 2016; Rajakulendran *et al.*, 2016)
	Dysphagia	(Lamantea *et al.*, 2002; Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003; Hakonen *et al.*, 2005; Gago *et al.*, 2006; Horvath *et al.*, 2006a; Hudson *et al.*, 2006; Santoro *et al.*, 2006; Invernizzi *et al.*, 2008; Milone *et al.*, 2008; Betts-Henderson *et al.*, 2009; Schulte *et al.*, 2009; Stewart *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; Komulainen *et al.*, 2010; Ferreira *et al.*, 2011a; Gati *et al.*, 2011; Milone *et al.*, 2011; Brandon *et al.*, 2013; Cheldi *et al.*, 2013; Roos *et al.*, 2013b; Horga *et al.*, 2014; Miguel *et al.*, 2014; Da Pozzo *et al.*, 2015; Rajakulendran *et al.*, 2016)

Finding	Manifestation	Reference(s)
	Dysphonia	(Lamantea *et al.*, 2002; Santoro *et al.*, 2006; Invernizzi *et al.*, 2008; Betts-Henderson *et al.*, 2009; Ferreira *et al.*, 2011a; Delgado-Alvarado *et al.*, 2015)
	Hypoacusis	(Di Fonzo *et al.*, 2003; Schicks *et al.*, 2010)
	Asthma	(Gurgel-Giannetti *et al.*, 2012)
	Pes Cavus	(Hakonen *et al.*, 2005; Santoro *et al.*, 2006)
	Developmental Delay	(Wong *et al.*, 2008)
	Short Stature	(Da Pozzo *et al.*, 2015)

Appendix I – *POLG* Mutations Associated with Adult-Onset PEO and Multiple mtDNA Deletions

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.8G>C	p.(Arg3Pro)	Multiple deletions	(Van Goethem *et al.*, 2001)
c.82A>T	p.(Ser28Cys)	n.d.	(Tang *et al.*, 2012)
c.487C>T	p.(Pro163Ser)	n.d.	(Woodbridge *et al.*, 2013)
c.679C>T	p.(Arg227Trp)	Multiple deletions	(Lamantea *et al.*, 2002; Agostino *et al.*, 2003; Horvath *et al.*, 2006a; Horga *et al.*, 2014)
c.752C>T	p.(Thr251Ile)	Multiple deletions and depletion	(Lamantea *et al.*, 2002; Agostino *et al.*, 2003; Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003; Lamantea and Zeviani, 2004; Kollberg *et al.*, 2005; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Blok *et al.*, 2009; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Gati *et al.*, 2011; Cheldi *et al.*, 2013; Hanisch *et al.*, 2014; Miguel *et al.*, 2014)
c.803>C	p.(Gly268Ala)	Multiple deletions	(Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003; Gonzalez-Vioque *et al.*, 2006)
c.823C>T	p.(Arg275*)	n.d.	(Blok *et al.*, 2009)
c.824G>A	p.(Arg275Gln)	Multiple deletions	(Echaniz-Laguna *et al.*, 2010b)
c.830A>T	p.(His277Leu)	n.d.	(Sato *et al.*, 2011)
c.911T>G	p.(Leu304Arg)	Multiple deletions	(Van Goethem *et al.*, 2001; Horvath *et al.*, 2006a; Naimi *et al.*, 2006; Bindu *et al.*, 2016)
c.926G>T	p.(Arg309Leu)	n.d.	(Lamantea *et al.*, 2002)
c.934T>C	p.(Trp312Arg)	Multiple deletions	(Agostino *et al.*, 2003; Del Bo *et al.*, 2003; Horvath *et al.*, 2006a)
c.1139G>A	p.(Gly380Asp)	Multiple deletions	(Naimi *et al.*, 2006)
c.1190C>T	p.(Ala397Val)	Multiple deletions and depletion	(Degos *et al.*, 2013)
c.1250+5G>C	Splice-site variant	n.d.	(Martikainen *et al.*, 2016)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.1270_1271del	p.(Leu424Glyfs*28)	Multiple deletions	(Agostino *et al.*, 2003)
c.1288A>T	p.(Met430Leu)	Multiple deletions	(Invernizzi *et al.*, 2008; Ferreira *et al.*, 2011a)
c.1292G>T	p.(Gly431Val)	Multiple deletions	(Agostino *et al.*, 2003)
c.1298C>G	p.(Ser433Cys)	Multiple deletions	(Horvath *et al.*, 2006a; Hudson *et al.*, 2006)
c.1356T>G	p.(Tyr452*)	Multiple deletions	(Hudson *et al.*, 2006; Echaniz-Laguna *et al.*, 2010b)
c.1389G>T	p.(Leu463Phe)	Multiple deletions	(Hudson *et al.*, 2007)
c.1399G>A	p.(Ala467Thr)	Multiple deletions and depletion	(Van Goethem *et al.*, 2001; Agostino *et al.*, 2003; Di Fonzo *et al.*, 2003; Van Goethem *et al.*, 2003b; Luoma *et al.*, 2005; Winterthun *et al.*, 2005; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Naimi *et al.*, 2006; Tzoulis *et al.*, 2006; Milone *et al.*, 2008; Paus *et al.*, 2008; Blok *et al.*, 2009; Schulte *et al.*, 2009; Stewart *et al.*, 2009; Echaniz-Laguna *et al.*, 2010b; McHugh *et al.*, 2010; Schicks *et al.*, 2010; Gati *et al.*, 2011; Smits *et al.*, 2011a; Hansen *et al.*, 2012; Lax *et al.*, 2012; Neeve *et al.*, 2012; Tang *et al.*, 2012; Kinghorn *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Martikainen *et al.*, 2016; Rajakulendran *et al.*, 2016)
c.1402A>G	p.(Asn468Asp)	Multiple deletions	(Luoma *et al.*, 2004; Gonzalez-Vioque *et al.*, 2006; Schulte *et al.*, 2009)
c.1457G>A	p.(Trp486*)	n.d.	(Miguel *et al.*, 2014)
c.1534G>A	p.(Ser511Asn)	Multiple deletions	(Hudson *et al.*, 2007)
c.1535A>T	p.(Lys512Met)	n.d.	(Dolhun *et al.*, 2013)
c.1550G>T	p.(Gly517Val)	Multiple deletions	(Schulte *et al.*, 2009; Ferreira *et al.*, 2011a; Woodbridge *et al.*, 2013)
c.1676T>C	p.(Leu559Pro)	n.d.	(Horga *et al.*, 2014)
c.1685G>A	p.(Arg562Gln)	Multiple deletions	(Di Fonzo *et al.*, 2003)
c.1720C>T	p.(Arg574Trp)	Multiple deletions	(Horvath *et al.*, 2006a)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.1735C>T	p.(Arg579Trp)	Multiple deletions	(Filosto *et al.*, 2003)
c.1754G>A	p.(Trp585*)	Multiple deletions	(Ferreira *et al.*, 2011a)
c.1760C>T	p.(Pro587Leu)	Multiple deletions and depletion	(Lamantea *et al.*, 2002; Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003; Lamantea and Zeviani, 2004; Kollberg *et al.*, 2005; Gonzalez-Vioque *et al.*, 2006; Horvath *et al.*, 2006a; Blok *et al.*, 2009; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Cheldi *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Miguel *et al.*, 2014)
c.1807A>T	p.(Met603Leu)	Multiple deletions	(Gonzalez-Vioque *et al.*, 2006)
c.1879C>T	p.(Arg627Trp)	Multiple deletions	(Van Goethem *et al.*, 2003b; Horvath *et al.*, 2006a)
c.1880G>A	p.(Arg627Gln)	Multiple deletions	(Schicks *et al.*, 2010) (Luoma *et al.*, 2005; Horvath *et al.*, 2006a; Schulte *et al.*, 2009)
c.1943C>G	p.(Pro648Arg)	Multiple deletions	(Gago *et al.*, 2006; Horvath *et al.*, 2006a; Remes *et al.*, 2008; Miguel *et al.*, 2014)
c.2125C>T	p.(Arg709*)	Multiple deletions	(Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003)
c.2447G>A	p.(Arg722His)	Multiple deletions	(Komulainen *et al.*, 2010)
c.2209G>C	p.(Gly737Arg)	Multiple deletions	(Milone *et al.*, 2008; Tzoulis *et al.*, 2009)
c.2236G>A	p.(Gly746Ser)	Multiple deletions	(Stewart *et al.*, 2009; Lax *et al.*, 2012)
c.2243G>C	p.(Trp748Ser)	Multiple deletions and depletion	(Van Goethem *et al.*, 2004; Hakonen *et al.*, 2005; Winterthun *et al.*, 2005; Horvath *et al.*, 2006a; Naimi *et al.*, 2006; Tzoulis *et al.*, 2006; Johansen *et al.*, 2008; Milone *et al.*, 2008; Paus *et al.*, 2008; Remes *et al.*, 2008; Blok *et al.*, 2009; Schulte *et al.*, 2009; Stewart *et al.*, 2009; Tzoulis *et al.*, 2009; Schicks *et al.*, 2010; Synofzik *et al.*, 2010b; Gati *et al.*, 2011; Smits *et al.*, 2011a; Hansen *et al.*, 2012; Lax *et al.*, 2012; Palin *et al.*, 2012; Tang *et al.*, 2012; Kinghorn *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Hanisch *et al.*, 2014; Horga *et al.*, 2014; Henao *et al.*, 2016; Mongin *et al.*, 2016)
c.2287G>C	p.(Gly763Arg)	Multiple deletions	(Santoro *et al.*, 2006)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.2293C>A	p.(Pro765Thr)	n.d.	(Bostan *et al.*, 2012)
c.2354ins	p.(Gly785fs21*)	n.d.	(Lamantea *et al.*, 2002)
c.2419C>T	p.(Arg807Cys)	Multiple deletions	(Gago *et al.*, 2006; Ferreira *et al.*, 2011a; Miguel *et al.*, 2014)
c.2420G>C	p.(Arg807Pro)	Multiple deletions	(Del Bo *et al.*, 2003; Di Fonzo *et al.*, 2003)
c.2492A>G	p.(Tyr831Cys)	Multiple deletions	(Mancuso *et al.*, 2004b; Wong *et al.*, 2008; Woodbridge *et al.*, 2013)
c.2542G>A	p.(Gly848Ser)	Multiple deletions	(Lamantea *et al.*, 2002; Van Goethem *et al.*, 2003a; Kollberg *et al.*, 2005; Betts-Henderson *et al.*, 2009; Schulte *et al.*, 2009; Schicks *et al.*, 2010; Ferreira *et al.*, 2011a; Gati *et al.*, 2011; Lax *et al.*, 2012)
c.2551A>G	p.(Thr851Ala)	n.d.	(Woodbridge *et al.*, 2013)
c.2557C>T	p.(Arg853Trp)	Multiple deletions	(Gonzalez-Vioque *et al.*, 2006)
c.2564T>C	p.(Val855Ala)	Multiple deletions and depletion	(Degos *et al.*, 2013)
c.2584G>A	p.(Ala862Thr)	Multiple deletions	(Stewart *et al.*, 2009; Lax *et al.*, 2012)
c.2606G>A	p.(Arg869Gln)	Multiple deletions	(Hanisch *et al.*, 2014)
c.2665G>A	p.(Ala889Thr)	Multiple deletions	(Filosto *et al.*, 2003; Hisama *et al.*, 2005)
c.2740A>C	p.(Thr914Pro)	Multiple deletions and depletion	(Wong *et al.*, 2008; Roos *et al.*, 2013b)
c.2740A>G	p.(Thr914Ala)	Multiple deletions	(Echaniz-Laguna *et al.*, 2010b)
c.2752T>C	p.(Trp918Arg)	Multiple deletions	(Invernizzi *et al.*, 2008; Ferreira *et al.*, 2011a)
c.2768G>A	p.(Gly923Asp)	n.d.	(Lamantea *et al.*, 2002)
c.2794C>T	p.(His932Tyr)	Multiple deletions	(Di Fonzo *et al.*, 2003; Mancuso *et al.*, 2004a)
c.2827C>T	p.(Arg943Cys)	n.d.	(Sato *et al.*, 2011)
c.2828G>A	p.(Arg943His)	Multiple deletions	(Lamantea *et al.*, 2002; Blok *et al.*, 2009; Brandon *et al.*, 2013)
c.2831A>G	p.(Glu944Gly)	Multiple deletions	(Da Pozzo *et al.*, 2015)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.2834A>T	p.(His945Leu)	Multiple deletions	(Delgado-Alvarado *et al.*, 2015)
c.2840G>A	p.(Lys947Arg)	n.d.	(Baruffini *et al.*, 2011)
c.2857C>T	p.(Arg953Cys)	Multiple deletions and depletion	(Gurgel-Giannetti *et al.*, 2012; Tang *et al.*, 2012)
c.2864A>G	p.(Tyr955Cys)	Multiple deletions and depletion	(Van Goethem *et al.*, 2001; Lamantea *et al.*, 2002; Luoma *et al.*, 2004; Kollberg *et al.*, 2005; Pagnamenta *et al.*, 2006; Ferreira *et al.*, 2011a; Mukai *et al.*, 2013; Horga *et al.*, 2014; Martikainen *et al.*, 2016)
c.2869G>T	p.(Ala957Ser)	n.d.	(Lamantea *et al.*, 2002)
c.2894T>G	p.(Leu965*)	Multiple deletions	(Horvath *et al.*, 2006a)
c.2993C>T	p.(Ser998Leu)	Multiple deletions	(Martikainen *et al.*, 2010)
c.3104 + 3A>T	Splice mutation	Multiple deletions and depletion	(Milone *et al.*, 2011; Roos *et al.*, 2013b)
c.3139C>T	p.(Arg1047Trp)	Multiple deletions	(Stewart *et al.*, 2009; Lax *et al.*, 2012)
c.3140G>A	p.(Arg1047Gln)	Multiple deletions	(Agostino *et al.*, 2003)
c.3151G>C	p.(Gly1051Arg)	n.d.	(Mancuso *et al.*, 2004a)
c.3235A>C	p.(Ile1079Leu)	n.d.	(Wong *et al.*, 2008)
c.3240_3242dup	p.(Arg1081dup)	Multiple deletions	(Horga *et al.*, 2014)
c.3285C>G	p.(Ser1095Arg)	n.d.	(Wong *et al.*, 2008)
c.3286C>T	p.(Arg1096Cys)	Multiple deletions	(Agostino *et al.*, 2003; Horvath *et al.*, 2006a; Lax *et al.*, 2012; Yu-Wai-Man *et al.*, 2013)
c.3287G>A	p.(Arg1096His)	n.d.	(Schulte *et al.*, 2009; Schicks *et al.*, 2010)
c.3311C>G	p.(Ser1104Cys)	Multiple deletions	(Agostino *et al.*, 2003; Betts-Henderson *et al.*, 2009)
c.3313G>A	p.(Ala1105Thr)	Multiple deletions	(Luoma *et al.*, 2004)
c.3316G>A	p.(Val1106Ile)	n.d.	(Lamantea and Zeviani, 2004)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.3412C>T	p.(Arg1138Cys)	Multiple deletions and depletion	(Milone *et al.*, 2008)
c.3428A>G	p.(Glu1143Gly)	Multiple deletions and depletion	(Di Fonzo *et al.*, 2003; Van Goethem *et al.*, 2004; Hisama *et al.*, 2005; Horvath *et al.*, 2006a; Hudson *et al.*, 2006; Milone *et al.*, 2008; Paus *et al.*, 2008; Remes *et al.*, 2008; Palin *et al.*, 2012)
c.3527C>T	p.(Ser1176Leu)	n.d.	(Lamantea *et al.*, 2002)
c.3550G>A	p.(Asp1184Asn)	Multiple deletions	(Gonzalez-Vioque *et al.*, 2006)
c.3550G>C	p.(Asp1184His)	Multiple deletions	(Martikainen *et al.*, 2010)
c.3556G>C	p.(Asp1186His)	Multiple deletions	(Cheldi *et al.*, 2013)
c.3594ins	p.(Thr1199fs1215*)	n.d.	(Schulte *et al.*, 2009; Schicks *et al.*, 2010)
c.3708G>T	p.(Gln1236His)	Multiple deletions	(Di Fonzo *et al.*, 2003; Luoma *et al.*, 2005; Horvath *et al.*, 2006a)
c.3715T>C	p.(*1240Glnins35)	Multiple deletions	(Lax *et al.*, 2012; Yu-Wai-Man *et al.*, 2013; Horga *et al.*, 2014)

n.d. – not determined.

Appendix J – *TWNK* Clinical Manifestations in Adult-Onset PEO and Multiple mtDNA Deletion Patients from the Literature

Finding	Manifestation	# Patients	Reference(s)
Psychiatric Illness	Depression	7	(Spelbrink *et al.*, 2001; Van Goethem *et al.*, 2003a; Baloh *et al.*, 2007; Virgilio *et al.*, 2008; Bohlega *et al.*, 2009; Van Hove *et al.*, 2009)
	Cognitive Decline	1	(Van Hove *et al.*, 2009)
	Dementia	4	(Hudson *et al.*, 2005; Echaniz-Laguna *et al.*, 2010a)
Seizure Disorder	Status Epilepticus	1	(Hudson *et al.*, 2005)
Extrapyramidal Movement Disorder	Parkinsonism	6	(Van Goethem *et al.*, 2003a; Baloh *et al.*, 2007; Liu *et al.*, 2008; Vandenberghe *et al.*, 2009; Brandon *et al.*, 2013; Kiferle *et al.*, 2013)
	Orthostatic Tremor	1	(Milone *et al.*, 2013)
Cerebellar Involvement	Ataxia	5	(Hudson *et al.*, 2005; Van Hove *et al.*, 2009; Echaniz-Laguna *et al.*, 2010a)
"Cerebrovascular" Involvement	Migraine	1	(Virgilio *et al.*, 2008)
Special Sensory	Sensorineural Hearing Loss	10	(Kiechl *et al.*, 2004; Houshmand *et al.*, 2006; Van Hove *et al.*, 2009; Echaniz-Laguna *et al.*, 2010a; Fratter *et al.*, 2010; Park *et al.*, 2011)
Myopathy	Proximal Myopathy	56	(Spelbrink *et al.*, 2001; Lewis *et al.*, 2002; Agostino *et al.*, 2003; Van Goethem *et al.*, 2003a; Kiechl *et al.*, 2004; Wanrooij *et al.*, 2004; Hudson *et al.*, 2005; Houshmand *et al.*, 2006; Naimi *et al.*, 2006; Baloh *et al.*, 2007; Rivera *et al.*, 2007; Jeppesen *et al.*, 2008; Virgilio *et al.*, 2008; Massa *et al.*, 2009; Negro *et al.*, 2009; Van Hove *et al.*, 2009; Echaniz-Laguna *et al.*, 2010a; Fratter *et al.*, 2010; Hong *et al.*, 2010; Park *et al.*, 2011; Ronchi *et al.*, 2011; Brandon *et al.*, 2013; Yu-Wai-Man *et al.*, 2013; Ji *et al.*, 2014; Paramasivam *et al.*, 2016)

Finding	Manifestation	# Patients	Reference(s)
	Distal Myopathy	2	(Martin-Negrier *et al.*, 2011; Ji *et al.*, 2014)
	Exercise Intolerance	4	(Kiechl *et al.*, 2004; Jeppesen *et al.*, 2008; Bohlega *et al.*, 2009; Paramasivam *et al.*, 2016)
Peripheral Neuropathy	Sensory Neuronopathy/Ganglionopathy	8	(Hudson *et al.*, 2005; Van Hove *et al.*, 2009; Echaniz-Laguna *et al.*, 2010a; Fratter *et al.*, 2010; Park *et al.*, 2011)
	Axonal Sensorimotor Neuropathy	5	(Kiechl *et al.*, 2004; Baloh *et al.*, 2007; Park *et al.*, 2011; Kiferle *et al.*, 2013; Milone *et al.*, 2013)
Endocrine/Gonadal System	Diabetes Mellitus	5	(Kiechl *et al.*, 2004; Hudson *et al.*, 2005; Houshmand *et al.*, 2006; Bohlega *et al.*, 2009; Van Hove *et al.*, 2009)
	Primary Ovarian Failure	2	(Virgilio *et al.*, 2008; Fratter *et al.*, 2010)
	Hypothyroidism	9	(Houshmand *et al.*, 2006; Virgilio *et al.*, 2008; Bohlega *et al.*, 2009; Milone *et al.*, 2013)
	Thyroid-Resistance Syndrome	1	(Ronchi *et al.*, 2011)
Gastrointestinal system	Gastrointestinal Dysmotility	1	(Nakhro *et al.*, 2011)
	Constipation/Diarrhoea	2	(Houshmand *et al.*, 2006; Fratter *et al.*, 2010)
	Cachexia	2	(Van Goethem *et al.*, 2003a; Nakhro *et al.*, 2011)
Heart	Cardiomyopathy	11	(Spelbrink *et al.*, 2001; Lewis *et al.*, 2002; Houshmand *et al.*, 2006; Bohlega *et al.*, 2009; Negro *et al.*, 2009; Fratter *et al.*, 2010; Hong *et al.*, 2010; Milone *et al.*, 2013)
Ocular	Cataract	5	(Houshmand *et al.*, 2006; Virgilio *et al.*, 2008; Van Hove *et al.*, 2009; Fratter *et al.*, 2010)
	Diplopia	8	(Houshmand *et al.*, 2006; Liu *et al.*, 2008; Virgilio *et al.*, 2008; Ronchi *et al.*, 2011; Kiferle *et al.*, 2013)

Finding	Manifestation	# Patients	Reference(s)
Other	Short Stature	1	(Da Pozzo *et al.*, 2015)

‘#’ – number.

Appendix K – *TWNK* Mutations Associated with Adult-Onset PEO and Multiple mtDNA Deletions

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.907C>T	p.(Arg303Trp)	Multiple deletions	(Agostino *et al.*, 2003; Houshmand *et al.*, 2006; Virgilio *et al.*, 2008; Negro *et al.*, 2009; Fratter *et al.*, 2010; Brandon *et al.*, 2013)
c.908G>A	p.(Arg303Gln)	Multiple deletions	(Van Hove *et al.*, 2009)
c.944G>T	p.(Trp315Leu)	Multiple deletions	(Spelbrink *et al.*, 2001)
c.944G>C	p.(Trp315Ser)	Multiple deletions	(Virgilio *et al.*, 2008)
c.955A>G	p.(Lys319Glu)	Multiple deletions	(Hudson *et al.*, 2005)
c.956A>C	p.(Lys319Thr)	Multiple deletions	(Deschauer *et al.*, 2003)
c.1001G>C	p.(Arg334Pro)	Multiple deletions	(Virgilio *et al.*, 2008)
c.1001G>A	p.(Arg334Gln)	Multiple deletions	(Agostino *et al.*, 2003; Houshmand *et al.*, 2006; Vandenberghe *et al.*, 2009; Fratter *et al.*, 2010; Yu-Wai-Man *et al.*, 2013)
c.1061G>C	p.(Arg354Pro)	Multiple deletions	(Spelbrink *et al.*, 2001; Ji *et al.*, 2014)
c.1071G>C	p.(Arg357Pro)	Multiple deletions	(Rivera *et al.*, 2007)
c.1075G>A	p.(Ala359Thr)	Multiple deletions	(Spelbrink *et al.*, 2001; Kiferle *et al.*, 2013)
c.1078C>G c.1079T>G	p.(Leu360Gly)	Multiple deletions	(Bohlega *et al.*, 2009)
c.1053_1092dup	(p.Asn352_Trp364dup)	Multiple deletions	(Spelbrink *et al.*, 2001; Wanrooij *et al.*, 2004)
c.1088G>T	p.(Trp363Thr)	Multiple deletions	(Fratter *et al.*, 2010)
c.1100T>C	p.(Ile367Thr)	Multiple deletions	(Spelbrink *et al.*, 2001)
c.1105T>C	p.(Ser369Pro)	Multiple deletions	(Spelbrink *et al.*, 2001)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.1106C>A	p.(Ser369Tyr)	Multiple deletions	(Lewis *et al.*, 2002)
c.1110C>A	p.(Phe370Leu)	Multiple deletions and depletion	(Jeppesen *et al.*, 2008)
c.1120C>T	p.(Arg374Trp)	Multiple deletions	(Echaniz-Laguna *et al.*, 2010a)
c.1121G>A	p.(Arg374Gln)	Multiple deletions	(Spelbrink *et al.*, 2001; Naimi *et al.*, 2006; Baloh *et al.*, 2007; Massa *et al.*, 2009; Martin-Negrier *et al.*, 2011; Yu-Wai-Man *et al.*, 2013; Tafakhori *et al.*, 2016)
c.1133T>C	p.(Leu378Pro)	Multiple deletions	(Ronchi *et al.*, 2011)
c.1142T>C	p.(Leu381Pro)	Multiple deletions	(Spelbrink *et al.*, 2001; Da Pozzo *et al.*, 2015)
c.1277G>A	p.(Ser426Asn)	Multiple deletions	(Virgilio *et al.*, 2008)
c.1342A>G	p.(Asn448Asp)	Multiple deletions	(Hong *et al.*, 2010)
c.1364T>C	p.(Met455Thr)	Multiple deletions	(Milone *et al.*, 2013)
c.1374G>T	p.(Gln458His)	Multiple deletions	(Fratter *et al.*, 2010)
c.1378G>C	p.(Ala460Pro)	Multiple deletions	(Fratter *et al.*, 2010)
c.1379C>G	p.(Ala460Gly)	n.d	(Fratter *et al.*, 2010)
c.1421G>C	p.(Trp474Ser)	Multiple deletions	(Virgilio *et al.*, 2008)
c.1422G>T	p.(Trp474Cys)	Multiple deletions	(Spelbrink *et al.*, 2001)
c.1423G>C	p.(Ala475Pro)	Multiple deletions	(Spelbrink *et al.*, 2001; Ji *et al.*, 2014)
c.1423G>A	p.(Ala475Thr)	Multiple deletions	(Liu *et al.*, 2008)
c.1424C>A	p.(Ala475Asp)	Multiple deletions	(Fratter *et al.*, 2010)
c.1432T>A	p.(Phe478Ile)	Multiple deletions	(Virgilio *et al.*, 2008)
c.1435G>A	p.(Glu479Lys)	Multiple deletions	(Virgilio *et al.*, 2008; Park *et al.*, 2011)
c.1640G>A	p.(Phe485Leu)	Multiple deletions	(Kiechl *et al.*, 2004)

cDNA change	Amino Acid change	Effect on mtDNA	Reference(s)
c.1609T>C	p.(Tyr537His)	Multiple deletions	(Ronchi *et al.*, 2011)
c.1964G>A	p.(Gly655Asp)	Multiple deletions	(Paramasivam *et al.*, 2016)

n.d. – not determined.

Appendix L – *RNASEH1* Sanger Sequencing Results for 66 Adult-onset PEO with Multiple mtDNA Deletions Patients

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
I	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
II	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
III	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
IV	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
V	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD (Fw only)	NMD	NMD (Fw only)	NMD (Rv only)

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
VI	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD (Rv only)
VII	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
VIII	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
IX	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402), c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
X	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402),	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)					
XI	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402), c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)	c.[498A>G]+[=],p.Pro16NMD (Rv only)	6Pro (rs10186193)	NMD	NMD	NMD
XII	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402), c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)	c.[498A>G]+[=],p.Pro16NMD	6Pro (rs10186193)	NMD	NMD	NMD
XIII	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402), c[409+34C>A]+[409	c.[498A>G]+[=],p.Pro16NMD	6Pro (rs10186193)	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			+34C>A] (rs75663960) (Rv only due to fs)					
XIV	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402), c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	c.[649+13A>G]+[=] (rs75789502)	c.[774+132A>T]+[=] (rs116083909)	NMD
XV	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XVI	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XVII	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XVIII	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XIX	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XX	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	c.564+16C>T]+[=] (rs375447388)	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
XXI	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXII	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XXIII	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXIV	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	c.[702A>G]+[=],p.Ala234Ala (rs143510329)	NMD
XXV	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XXVI	NMD (Rv only)	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XXVII	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	c.[649+13A>G]+[=] (rs75789502)	c.[774+132A>T]+[=] (rs116083909)	NMD
XXVIII	NMD	NMD	c[409+34C>A]+[409	NMD	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			+34C>A] (rs75663960)					
XXIX	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXX	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXXI	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD (Fw only)	NMD	NMD
XXXII	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXXIII	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXXIV	NMD	NMD	c[409+34C>A]+[409 +34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD (Fw only)	NMD	NMD
XXXV	NMD	NMD	c[409+34C>A]+[409 +34C>A]	NMD	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			(rs75663960)					
XXXVI	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXXVII	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD (Fw only)	NMD	NMD
XXXVIII	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
XXXIX	NMD	NMD	NMD	NMD (Fw only)	NMD	NMD	NMD (Rv only)	NMD
XL	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
XLI	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
XLII	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
XLIII	NMD	NMD	NMD	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
XLIV	NMD	NMD	NMD	c.[474A>G]+[=],p.Ala158Ala (rs61738918)	NMD	NMD	NMD	NMD
XLV	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
XLVI	NMD	NMD	NMD	NMD (Fw only)	NMD (Rv only)	NMD	NMD	NMD (Rv only)
XLVII	NMD	NMD	NMD (Rv only)	c.[498A>G]+[=],p.Pro166	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
				6Pro (rs10186193)				
1	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
2	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
3	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	NMD	NMD	NMD	NMD	NMD
4	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[509+44C>G]+[=]	NMD	NMD	NMD	NMD
5	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
6	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	NMD	NMD
7	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
8	NMD	NMD	c[409+34C>A]+[409+34C>A]	c.[498A>G]+[498A>G], p.Pro166Pro	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			(rs75663960)	(rs10186193)				
9	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
10	c.[128+44G>A]+[=]	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	c.564+16C>T]+[=] (rs375447388)	NMD	NMD	NMD
11	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
12	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
13	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
14	NMD	NMD	c[409+34C>A]+[409+34C>A] (rs75663960)	c.[498A>G]+[498A>G], p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
15	NMD	NMD	c.245-118_245-	c.[498A>G]+[=],p.Pro166Pro	NMD	NMD	NMD	NMD

Patient	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
			115het_delAGAG (rs151141402), c[409+34C>A]+[409+34C>A] (rs75663960) (Rv only due to fs)	6Pro (rs10186193)				
16	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
17	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	NMD	NMD	NMD	c.[702A>G]+[=],p.Ala234Ala (rs143510329)	NMD
18	NMD	NMD	c.245-118_245-115het_delAGAG (rs151141402) (Rv only due to fs)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD
19	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD
20	NMD	NMD	c[409+34C>A]+[=] (rs75663960)	c.[498A>G]+[=],p.Pro166Pro (rs10186193)	NMD	NMD	NMD	NMD

Patients excluded from the WES are listed using Roman numerals. Patient 1-20 who underwent WES are listed at the bottom of the table. Novel variants are highlighted in red. NMD – no mutation detected. Rv – reverse. Fw – forward.

Appendix M – WES Read Coverage and Depth Statistics of the Adult-Onset PEO with Multiple mtDNA Deletions Cohort

Patient	Mean Depth per CCDS (bp)	Min Depth	Max Depth	# CCDS Bases Covered 30-fold	% CCDS Bases Covered 30-fold	% CCDS Bases Covered 20-fold	% CCDS Bases Covered 20-fold	% CCDS Bases Covered 10-fold	% CCDS Bases Covered 10-fold	% CCDS Bases Covered 5-fold	% CCDS Bases Covered 5-fold	% CCDS Bases Covered 1-fold	% CCDS Bases Covered 1-fold
1	46.79	0	1516	17928267	54.41	22039667	66.89	26656034	80.90	29102175	88.33	31095238	94.38
2	80.88	0	5875	27423246	83.23	29026231	88.10	30033915	91.16	30414434	92.31	30903151	93.80
3	46.19	0	2107	20158197	61.18	24042480	72.97	27156273	82.42	28718437	87.16	30566762	92.77
4	47.35	0	1840	18089146	54.90	22162163	67.27	26710156	81.07	29125522	88.40	31093549	94.37
5	43.77	0	1116	17519213	53.17	21873989	66.39	26641180	80.86	29110976	88.36	31097774	94.39
6	46.79	0	1586	20665823	62.72	24447320	74.20	27427183	83.25	28936342	87.83	30672655	93.10
7	67.47	0	5211	26052706	79.07	28357620	86.07	29804518	90.46	30306973	91.99	30848858	93.63
8	51.78	0	2415	21431786	65.05	24682166	74.91	27360019	83.04	28781235	87.35	30571544	92.79
9	75.20	0	5052	26942407	81.77	28779489	87.35	29933682	90.85	30359506	92.15	30864617	93.68
10	47.88	0	1177	18450525	56.00	22495422	68.28	26924631	81.72	29244713	88.76	31096901	94.38
11	60.77	0	4669	22231774	67.48	25665082	77.90	28920959	87.78	30330774	92.06	31299626	95.00
12	70.52	0	4710	26577450	80.67	28628332	86.89	29861392	90.63	30295824	91.95	30818822	93.54
13	70.34	0	4621	26449272	80.28	28558602	86.68	29861501	90.63	30327236	92.05	30849881	93.63
14	52.31	0	1314	19224564	58.35	23016914	69.86	27195573	82.54	29395031	89.22	31151592	94.55
15	77.42	0	5274	27124871	82.33	28865777	87.61	29975917	90.98	30384487	92.22	30876609	93.71
16	50.70	0	2417	21288660	64.61	24693067	74.95	27452272	83.32	28882866	87.66	30636573	92.99
17	54.80	0	2217	22220223	67.44	25207075	76.51	27683737	84.02	29055086	88.19	30716923	93.23

Patient	Mean Depth per CCDS (bp)	Min Depth	Max Depth	# CCDS Bases Covered 30-fold	% CCDS Bases Covered 30-fold	% CCDS Bases Covered 20-fold	% CCDS Bases Covered 20-fold	% CCDS Bases Covered 10-fold	% CCDS Bases Covered 10-fold	% CCDS Bases Covered 5-fold	% CCDS Bases Covered 5-fold	% CCDS Bases Covered 1-fold	% CCDS Bases Covered 1-fold
18	71.20	0	6477	26588827	80.70	28668001	87.01	29903743	90.76	30338801	92.08	30841613	93.61
19	50.99	0	1770	19015765	57.72	22894983	69.49	27150352	82.40	29379418	89.17	31157142	94.57
20	75.61	0	6264	26899440	81.64	28766484	87.31	29933470	90.85	30365108	92.16	30874692	93.71

‘#’ – number. CCDS – exome consensus coding sequence.

Appendix N – VUS Identified by WES in the Adult-Onset PEO with Multiple mtDNA Deletions Cohort

Patient	Gene	Mutations		Minor Allele Frequency			GO-Terms Matched
		cDNA Change	Amino Acid Change	ExAC	NHLBI ESP	1000G	
2	*TICRR*	c.2990T>G/=	p.Val997Gly, Heterozygous	Ø	Ø	Ø	DNA repair, replication
	TOP3B	c.524C>T/=	p.Ala175Val, Heterozygous	Ø	Ø	Ø	Topoisomerase
	MARK2	c.894C>G/=	p.Ile298Met, Heterozygous	Ø	Ø	Ø	n.a.
	POLR2A	c.5789A>C/=	p.Tyr1930Ser, Heterozygous	Ø	Ø	Ø	Transcription, nucleotide, polymerase, DNA repair
	FDXR	c.349G>A/=	p.Val117Met, Heterozygous	0.00000861	Ø	Ø	Mitochondr*
6	*PPIF*	c.382G>A/=	p.Glu128Lys, Heterozygous	0.00001661	Ø	Ø	Mitochondr*
7	*USP8*	c.179A>G/=	p.Tyr60Cys, Heterozygous	Ø	Ø	Ø	n.a.
	SETX	c.6941A>G/=	p.Tyr2314Cys, Heterozygous	0.00000895	Ø	Ø	Polymerase, transcription, helicase
8	*APEX1*	c.467G>A/=	p.Arg156Gln, Heterozygous	0.00003341	Ø	Ø	Mitochondr*, DNA repair, transcription, exonuclease
9	*BCL2L13*	c.658G>T/=	p.Asp220Tyr, Heterozygous	0.00000824	Ø	Ø	Mitochondr*
	THNSL1	c.524G>A/=	p.Gly175Asp, Heterozygous	Ø	Ø	Ø	n.a.
	PTCHD2	c.2264G>A	p.Arg755Gln	0.0001015	Ø	Ø	n.a.
		c.3450_3451insGCT	p.Gln1150delinsGlnAla	Ø	Ø	Ø	
12	*GART*	c.2323delC/=	p.Arg775Valfs*2, Heterozygous	Ø	Ø	Ø	Purine, ligase
	RIF1	c.113C>T	p.Thr38Ile, Heterozygous	0.00001654	Ø	Ø	n.a.
14	*EXD2*^	c.1547C>G/=	p.Pro516Arg, Heterozygous	Ø	Ø	Ø	Exonuclease
16	*DCP2*	c.427A>G/=	p.Arg143Gly, Heterozygous	Ø	Ø	Ø	n.a.

	XRCC6	c.645G>C/=	p.Leu215Phe, Heterozygous	0.00001112	Ø	Ø	DNA repair, transcription
	OAS2	c.2152_2300del/=	p.Asn718_*720del, Heterozygous	Ø	Ø	Ø	Mitochondr*, nucleotide, purine
	OGDH	c.20G>A/=	p.Cys7Tyr, Heterozygous	Ø	Ø	Ø	Mitochondr*
	OXR1	c.328A>G/=	p.Thr110Ala, Heterozygous	0.00000826	Ø	Ø	Mitochondr*
20	*DDHD2*	c.1973G>A/=	p.Arg658His, Heterozygous	0.00000824	Ø	Ø	n.a.
	CLPX	c.1891G>A/=	p.Ala631Thr, Heterozygous	0.00000825	Ø	Ø	Transcription, mitochondr*, nucleotide
	ACSM1	c.915delA/=	p.Thr305fs, Heterozygous	Ø	Ø	Ø	Mitochondr*

Variants of unknown significance (VUS) that were identified by WES in the adult-onset PEO with multiple mtDNA deletions cohort. Variants are listed due based upon the GO-Terms matched or *in silico* predictions. 'Ø' denotes that a variant absent from external databases. '^' denotes variants that were false positives or did not segregate in Sanger sequencing studies.

Appendix O – WES Read Coverage and Depth Statistics of the Mitochondrial RC Deficiency Cohort

Patient	Mean Depth per CCDS (bp)	Min Depth	Max Depth	# CCDS Bases Covered 30-fold	% CCDS Bases Covered 30-fold	# CCDS Bases Covered 20-fold	% CCDS Bases Covered 20-fold	# CCDS Bases Covered 10-fold	% CCDS Bases Covered 10-fold	# CCDS Bases Covered 5-fold	% CCDS Bases Covered 5-fold	# CCDS Bases Covered 1-fold	% CCDS Bases Covered 1-fold
21	73.65	0	6197	26838572	81.46	28720597	87.17	29875411	90.68	30289024	91.93	30760299	93.36
22	72.56	0	5673	26846045	81.48	28716822	87.16	29856967	90.62	30274093	91.89	30745928	93.32
23	142.36	0	4899	29389138	89.20	29978180	90.99	30378846	92.20	30582382	92.82	30993567	94.07
24	52.62	0	2301	21380705	64.89	25489798	77.36	29147855	88.47	30556260	92.74	31411912	95.34
25	49.15	0	1765	19600852	59.49	23212490	70.45	26648976	80.88	28134599	85.39	29165927	88.52
26	61.16	0	3629	25340123	76.91	28018731	85.04	29666439	90.04	30211698	91.70	30735134	93.29
27	183.78	0	7710	29853739	90.61	30191548	91.64	30465693	92.47	30632803	92.97	31016761	94.14
28	66.37	0	5088	26089119	79.18	28367628	86.10	29768244	90.35	30251849	91.82	30752736	93.34
29	43.22	0	1712	18526126	56.23	23482136	71.27	28199914	85.59	30134026	91.46	31342028	95.13
30	50.33	0	2433	20721434	62.89	25120924	76.25	29041531	88.14	30527038	92.65	31415167	95.35
31	33.41	0	861	13639045	41.40	18600490	56.45	24756815	75.14	28244491	85.73	31051275	94.24
32	73.70	0	4358	26831926	81.44	28733560	87.21	29884853	90.70	30292343	91.94	30750505	93.33
33	39.36	0	7208	16901296	51.30	22376077	67.91	27825651	84.45	30035534	91.16	31330079	95.09
34	48.99	0	2936	20462335	62.11	24936914	75.69	28928150	87.80	30468589	92.48	31402486	95.31
35	76.44	0	2775	24193731	73.43	26853761	81.50	29373382	89.15	30482327	92.52	31292064	94.98
36	60.76	0	7390	22068259	66.98	25432908	77.19	28711509	87.14	30194657	91.64	31247149	94.84
37	84.25	0	3709	25091992	76.16	27482271	83.41	29688410	90.11	30642284	93.00	31331222	95.09

Patient	Mean Depth per CCDS (bp)	Min Depth	Max Depth	# CCDS Bases Covered 30-fold	% CCDS Bases Covered 30-fold	# CCDS Bases Covered 20-fold	% CCDS Bases Covered 20-fold	# CCDS Bases Covered 10-fold	% CCDS Bases Covered 10-fold	# CCDS Bases Covered 5-fold	% CCDS Bases Covered 5-fold	# CCDS Bases Covered 1-fold	% CCDS Bases Covered 1-fold
38	68.99	0	2225	23425033	71.10	26330834	79.92	29102469	88.33	30337744	92.08	31240118	94.82
39	65.14	0	7269	22783203	69.15	25930697	78.70	28964979	87.91	30325065	92.04	31272723	94.92
40	34.58	0	7035	15303006	46.45	20569456	62.43	26494514	80.41	29328182	89.01	31161053	94.58

‘#’ – number. CCDS – exome consensus coding sequence.

Appendix P – VUS Identified By WES in the Early-Onset RC Deficiency Cohort

Patient	Gene	Mutations		Minor Allele Frequency			GO-Terms Matched
		cDNA Change	Amino Acid Change	ExAC	NHLBI ESP	1000G	
23	*ALKBH8*	Homozygous c.886T>G	p.Cys296Gly (rs61743188), Homozygous	0.001245	Ø	0.004	tRNA
	NXPE4	c.1456A>G	p.Arg486Gly	0.00004145	Ø	Ø	n.a.
		c.1484A>T	p.Gln495Leu	0.00004145	Ø	Ø	
	POLRMT	c.3541C>T/=	p.Gln1181*, Heterozygous	Ø	Ø	Ø	Mitochondr*
25	*MIEF2*^	c.115G>T	p.Ala39Ser	Ø	Ø	Ø	Mitochondr*
		c.124G>T	p.Val42Leu	0.000009174	Ø	Ø	
	MRPL9^	c.13G>T	p.Val5Phe	Ø	Ø	Ø	Mitochondr*, translation
		c.365G>A	p.Arg122Gln (rs371659600)	0.003148	0.000116279	0.006	
	DNAJB4	c.790G>C/=	p.Gly264Arg, Heterozygous	Ø	Ø	Ø	n.a.
26	*MRPS12*	c.23A>G	p.His8Arg (rs33988199)	0.005288	0.038	0.017	Translation, mitochondr*
		c.244C>T	p.Arg82*	0.000008320	Ø	Ø	
	MRPL14	c.305dupC/=	p.Ser102fs, Heterozygous	Ø	Ø	Ø	Translation, mitochondr*
33	*TEP1*	Homozygous c.3476C>A	p.Pro1159His (rs145944940), Homozygous	0.0001657	0.000153775	Ø	n.a.
	FGFR4	Homozygous c.215G>C	p.Arg72Pro, Homozygous	Ø	Ø	Ø	n.a.
	GNL3L^	Hemizygous c.883C>A	Hemizygous p.Leu295Met	Ø	Ø	Ø	Mitochondr*
	LETM2^	c.496T>C	p.Ser166Pro	0.0001731	Ø	Ø	Mitochondr*
		c.629C>G	p.Ser210Cys (rs147828005)	0.0002891	0.000615	0.0005	

36	*TICRR*	c.2254G>A	p.Asp752Asn	Ø	Ø	Ø	Translation
		c.5354G>A	p.Arg1785His (rs141989878)	0.001172	0.000692414	0.000199681	
37	*NUP210*	Homozygous c.5423A>G	p.Gln1808Arg (rs548431600), Homozygous	0.0005065	Ø	0.001	Translation, tRNA
38	*TRMT61B*	Homozygous c.1109T>C	p.Leu370Ser (rs150890124), Homozygous	0.001921	0.000399361	0.0029	Translation, mitochondr*
	COQ2	c.286C>T/=	p.Pro96Ser, Heterozygous	Ø	Ø	Ø	Mitochondr*
	TMEM126A	c.364G>C/=	p.Ala122Pro, Heterozygous	Ø	Ø	Ø	Mitochondr*
39	*PTCD3*	c.1366T>C/=	p.Phe456Leu (rs200807149), Heterozygous	0.00008263	0.000230663	Ø	Translation, mitochondr*

Variants of unknown significance (VUS) that were identified by WES in the early-onset RC deficiency cohort. Variants are listed due based upon the GO-Terms matched or *in silico* predictions. 'Ø' denotes that a variant absent from external databases. '^' denotes genes or variants that were false positives or did not segregate in Sanger sequencing studies.

Appendix Q – List of mtDNA Polymorphisms of *YARS2* Patients for mtDNA Haplogrouping

Patient 43.1 - H1m1	Patient 44 - G2a3a	Patient 45 - T2e1a	Patient 46.1 - HV13
m.93A>G	m.73A>G	m.41C>T	m.199T>C
m.146T>C	m.152T>C	m.73A>G	m.263A>G
m.263A>G	m.185G>A (het)	m.150C>T	m.309insCC
m.310insTC (het)	m.263A>G	m.263A>G	m.750A>G
m.750A>G	m.310insCC (het)	m.309insC (het)	m.1438A>G
m.1438A>G	m.315insC	m.315insC	m.2706A>G
m.3010G>A	m.489T>C	m.709G>A	m.3106delC
m.3107delC	m.709G>A	m.750A>G	m.4769A>G
m.4171C>T	m.750A>G	m.1438A>G	m.7028C>T
m.4769A>G	m.1438A>G	m.1888G>A	m.7256C>T
m.8860A>G	m.2706A>G	m.2706A>G	m.8860A>G
m.9468A>G	m.3107delC	m.3107delC	m.9305G>A
m.15323G>A	m.3882G>A	m.4216T>C	m.10382A>G
m.15326A>G	m.4769A>G	m.4769A>G	m.12879T>C
m.16519T>C	m.4833A>G	m.4917A>G	m.13708G>A
	m.5108T>C	m.7028C>T	m.15326A>G
	m.5601C>T	m.7828A>G	m.16357T>C
	m.6152T>C	m.8697G>A	
	m.7028C>T	m.8790G>A	
	m.7600G>A	m.8860A>G	
	m.8701A>G	m.10463T>C	
	m.8860A>G	m.11251A>G	
	m.9377A>G	m.11719G>A	
	m.9540T>C	m.11812A>G	
	m.9575G>A	m.13368G>A	
	m.10398A>G	m.14233A>G	
	m.10400C>T	m.14766C>T	
	m.10873T>C	m.14905G>A	
	m.11719G>A	m.15244A>G	
	m.12705C>T	m.15326A>G	
	m.13563A>G	m.15452C>A	
	m.14569G>A	m.15607A>G	

Patient 43.1 - H1m1	Patient 44 - G2a3a	Patient 45 - T2e1a	Patient 46.1 - HV13
	m.14766C>T	m.15928G>A	
	m.14783T>C	m.16093T>C (het)	
	m.15043G>A	m.16126T>C	
	m.15301G>A	m.16153G>A	
	m.15326A>G	m.16294C>T	
	m.16169C>T	m.16301C>T	
	m.16189T>C (low level het)	m.16519T>C	
	m.16223C>T		
	m.16227A>G		
	m.16262C>T		
	m.16278T>C		
	m.16294C>T		
	m.16318A>G		
	m.16526G>A		

D-loop control region polymorphisms are highlighted in red.

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