Improving the genetic diagnosis of mitochondrial disease using custom next-generation sequencing strategies.

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Proposal summary

Mitochondrial disease represents one of the most common inborn errors of metabolism with a minimum disease prevalence of ~12.5 per 100,000 (Gorman *et al.*, 2015) in adults and ~4.7 per 100,000 in children (Skladal *et al.*, 2003). Mitochondrial disease affects people of all ages and, although symptoms can sometimes be treated or ameliorated, there is no cure (McFarland *et al.*, 2010). Mitochondrial disease is associated with a diverse spectrum of clinical presentations ranging from isolated symptoms such as seizures or cardiomyopathy, to severe neurological, syndromic presentations such as Leigh syndrome or Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes (MELAS). The underlying genetic defect can be within either the mitochondria's own genome (mtDNA) - a 16.6kb DNA molecule encoding 37 genes (Anderson *et al.*, 1981) - or the nuclear genome, which encodes ~1150 mitochondrial proteins (Calvo *et al.*, 2016). In view of this complexity, the care and diagnostic investigation of mitochondrial patients is part of the NHS Highly Specialised Services for Mitochondrial Diseases of Adults and Children within the UK.

The vast genotypic and phenotypic heterogeneity means that the diagnostic algorithm is often complicated; for a large number of patients, the underlying genetic defect remains unknown often in spite of an established respiratory chain enzyme defect. For example, half of all paediatric patients with an isolated complex I deficiency, the most common respiratory chain defect, lack a genetic diagnosis (Swalwell *et al.*, 2011). There is little that can be offered to these families in terms of counselling and recurrence risk prediction for future pregnancies. High-throughput sequencing offers real hope for families affected by massively heterogeneous conditions; the application of these emerging technologies to "undiagnosed" patients maximises the chance of elucidating the underlying genetic defect.

My research proposal focuses on improving the genetic diagnostic pathway for predominantly paediatric patients with suspected mitochondrial disease with a view to enabling their parents' access to reproductive options for future pregnancies. I plan to expand the scope of genetic testing in our Highly Specialised Mitochondrial Diagnostic Service Laboratory by employing three approaches - Sanger sequencing of new disease genes where there are clear genotype-phenotype correlations, targeted next-generation sequencing of panels of candidate genes and unbiased whole exome sequencing. Our current strategy permits analysis of <10% of all candidate genes, whereas the system detailed in my proposal will raise this percentage significantly, with scope to incorporate new disease genes as they are described.

Collaborative research projects with international academic research institutes have meant that a number of NHS patients have already undergone exome sequencing and a molecular diagnosis has been made via this research-reliant pathway. I believe it is vital that we can provide a similar service within the NHS diagnostic framework and my proposal will move conventional diagnostics using Sanger sequencing of single genes to a futureproof high-throughput strategy.

I have access to a paediatric patient cohort (n=60) with a proven biochemical defect in muscle and many more with suspected mitochondrial disease for whom our current strategy has failed to identify the causative genetic defect; this proposal aims to obtain a genetic diagnosis for these patients. There will also be a prospective element as the new high-throughput screening strategy will be applied to each new paediatric patient with a biochemical diagnosis of isolated complex I deficiency (n=30/year).

Where novel mutations in known disease genes, or novel mutations in unreported candidate genes are identified, functional investigations will be undertaken to establish their pathogenicity. In the absence of a cure for mitochondrial disease, the provision of reproductive counselling is a vital resource for families affected by inherited mitochondrial disease and the current repertoire includes prenatal genetic diagnosis (chorionic villus biopsy; amniocentesis; non-invasive screening) or pre-implantation genetic diagnosis during in vitro fertilisation procedures. Moreover, determining the phenotypic effect of these genetic mutations on mitochondrial function will be vital to understanding the underlying mechanisms and pathways involved. In addition to providing a genetic diagnosis for patients (and their families), it is hoped that advances in knowledge relating to mitochondrial pathology may lead to new treatments.

Author's Declaration

This doctoral project was undertaken with the financial support of a National Institute for Health Research (NIHR) doctoral fellowship (NIHR-HCS-D12-03-04). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy. The research detailed within was conducted in the Wellcome Trust Centre for Mitochondrial Research, Institute of Neurosciences, under the supervision of Professor Robert W Taylor, Professor Zofia MA Chrzanowska-Lightowlers and Professor Douglass M Turnbull.

I certify that none of the material offered in this thesis has been previously submitted by me for a degree or other qualification at this or any other university. Part of my introduction has been accepted for publication in the *Journal of Pathology* (Alston *et al.*, 2017).

Abbreviations

ΔΨ	Electrochemical gradient
100KGP	One hundred thousand genomes project
3-MGA	3-methylglutaconic aciduria
AARS2	Mitochondrial alanyl-tRNA synthetase
ACAD9	Acyl-CoA dehydrogenase family, member 9 protein
ACO2	Mitochondrial aconitase
ADCK3	AarF domain containing kinase 3
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
ALA	5'-aminolevulinic acid
ALAD	ALA dehydratase
ALAS	5'-aminolevulinate synthase
APAF1	Apoptotic protease-activating factor 1
ATAD3A	ATPase family AAA domain-containing protein 3A
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BER	Base excision repair
bp	Base pair
BTBGD	Biotin or thiamine-responsive basal ganglia disease
Ca^{2+}	Calcium ion
CARS2	Mitochondrial cysteinyl-tRNA synthetase
CASP	Caspase
CCD	Charge-coupled device
cffDNA	Cell free fetal DNA
CHCHD4	Coiled-coil-helix-coiled-coil-helix domain containing protein 4
CHiP	Chromatin immunoprecipitation
CMT	Charcot Marie Tooth
CMT1B	Charcot Marie Tooth disease, demyelinating type 1B
CNS	Central nervous system
CoQ	Ubiquinone
COX	Cytochrome *c* oxidase
CPEO	Chronic progressive external ophthalmoplegia
CPIII	Coproporphyrinogen III
CPO	Coproporphyrinogen III oxidase

CRISPR	Clustered regularly interspaced short palindromic repeats
CVB	Chorionic villus biopsy
Cyt *c*	Cytochrome *c*
DARS2	Mitochondrial aspartyl-tRNA synthetase
DDD	Deciphering developmental disorders
ddNTP	Di-deoxyribonucleotide
Del	Deletion
DIABLO	Direct IAP-binding protein with low pI
D-Loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DSBR	Double-strand break repair
dsDNA	Double-stranded DNA
e-	Electron
EARS2	Mitochondrial glutamyl-tRNA synthetase
ECG	Electrocardiogram
ECSIT	Evolutionarily conserved signalling intermediate in toll pathway protein
EM	Electron microscopy
EMRE	Essential MCU regulator
ERV1	Flavin adenine dinucleotide-linked sulfhydryl oxidase
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FARS2	Mitochondrial phenylalanyl-tRNA synthetase
FC	Ferrochelatase
FDX	Ferreredoxin
FDXR	Ferredoxin reductase
Fe-S	Iron sulfur (Fe-S)
FILA	Fatal infantile lactic acidosis
FLAD1	Flavin adenine dinucleotide synthetase 1
FMN	Flavin mononucleotide
FMNH2	Reduced flavin mononucleotide
FOXRED1	FAD-dependent oxidoreductase domain containing 1 protein
FXN	Frataxin
GARS	Glycyl-tRNA synthetase
GFER	Growth factor, augmenter of liver regeneration

GI	Gastrointestinal
GRACILE	Growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death
GTP	Guanosine-5'-triphosphate
H	Heavy
H+	Proton
H_2O	Water
HARS2	Mitochondrial histidyl tRNA synthetase
HCCS	Holocytochrome *c* synthase
HCPC	Health and Care Professions Council
HDR	Homology-directed recombination
HFEA	Human Fertilisation and Embryology Authority
HMG-CoA	Hydroxymethylglutaryl-coenzyme A
HNPGL	Head and neck paragangliomas
HR	Homologous recombination
HSC20	Mitochondrial iron-sulfur cluster co-chaperone
HSP	Heat shock protein
HSP1/HSP2	Initiators of transcription of the Heavy strand
IARS2	Mitochondrial isoleucyl-tRNA synthetase
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
InDel	Insertion and deletion
ISC	Iron sulfur clusters
ISCU	Iron sulfur cluster assembly enzyme
IVF	*In vitro* fertilisation
KARS	Lysyl-tRNA synthetase
kb	Kilobases
kDa	Kilo Dalton
L	Light
LARS2	Mitochondrial leucyl-tRNA synthetase
LBSL	Leukoencephalopathy with brain stem and spinal cord involvement and elevated lactate
LDL	Low-density lipoprotein
LETM1	Leucine zipper/EF-hand-containing transmembrane protein 1
LPPVK	Leu-Pro-Pro-Val-Lys substrate binding motif

LS	Leigh syndrome
LSP	Initiators of transcription of the light mtDNA strand
LYR	Leucine-Tyrosine-Argine
LYRM4	LYR motif containing 4 / ISD11 protein
m.	Mitochondrial DNA base pair identifier
MAF	Minor allele frequency
MARS2	Mitochondrial methionyl-tRNA synthetase
MCU	Mitochondrial calcium uniporter
MDa	Mega Dalton
MECP2	Methyl-CpG binding protein 2
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes
Mg^{2+}	Magnesium ion
MIA40	Mitochondrial intermembrane space import and assembly protein 40
MICU	Mitochondrial calcium uptake protein
MLASA	Myopathy, lactic acidosis and sideroblastic anaemia
mM	Millimolar
MMEJ	Microhomology-mediated homologous end-joining
MPP	Mitochondrial processing peptidase
MPZ	Myelin protein zero
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRO	Mitochondrion-related organelles
MRPL	Mitoribosomal protein - large subunit component
MRPP	Mitochondrial RNase P protein
MRPS	Mitoribosomal protein - small subunit component
MRRF	Mitochondrial ribosome recycling factor
mt	Mitochondrial
mt aaRSs	Mitochondrial aminoacyl tRNA-synthetases
MTCYB	mtDNA-encoded cytochrome *b*
mtDNA	Mitochondrial DNA
MTERF	Mitochondrial transcription termination factor
MTFMT	Mitochondrial methionyl-tRNA formyltransferase
mtIF	Mitochondrial translational initiation factor
MTND	Mitochondrial NADH dehydrogenase
MTO1	Mitochondrial tRNA translation optimization 1

MTRF	Mitochondrial translational release factor
MTS	Mitochondrial targeting sequence
mtSSB	Mitochondrial single-stranded DNA binding protein
mt-SSU	Small mitoribosomal subunit
mtWGS	Whole mitochondrial genome sequencing
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NARP	Neurogenic ataxia and retinitis pigmentosa
NARS2	Mitochondrial asparaginyl-tRNA synthetase
NCLX	Sodium calcium exchange protein
NCS	Nerve conduction studies
NDUFA	NADH dehydrogenase (ubiquinone) 1 α subcomplex
NDUFAB	NADH dehydrogenase (ubiquinone) 1, α/β subcomplex
NDUFAF	NADH dehydrogenase (ubiquinone) assembly factor
NDUFB	NADH dehydrogenase (ubiquinone) 1 β subcomplex
NDUFS	NADH dehydrogenase (ubiquinone) Fe-S protein
NDUFV	NADH dehydrogenase (ubiquinone) flavoprotein
NFS1	Cysteine desulfurase, mitochondrial
NFκB	Nuclear factor kappa beta subunit
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NHS	National Health Service
NIHR	National Institute for Health Research
NIPT	Non-invasive prenatal testing
NUBPL	Nucleotide binding protein-like protein
OH	Heavy strand origin of replication
OL	Light strand origin of replication
OPA1	Optic atrophy 1, mitochondrial dynamin-like GTPase
OriZ	Origin of replication
OXPHOS	Oxidative phosphorylation
PAM	Presequence translocase-associated motor
PAM	Protospacer adjacent motif
PARS2	Mitochondrial prolyl-tRNA synthetase
PBG	Porphobilinogen
PBGD	Porphobilinogen dehydratase

PBR	Peripheral-type benzodiazepine receptor
PCH6	Pontocerebellar hypoplasia type 6
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha
PEO	Progressive external ophthalmoplegia
PEO1	Gene encoding the Twinkle helicase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGD	Preimplantation genetic diagnosis
Pi	Inorganic phosphate
PND	Prenatal diagnosis
POLG	Mitochondrial DNA polymerase γ
POLG2	Mitochondrial DNA polymerase γ subunit 2
POLRMT	Polymerase (RNA) mitochondrial
PPA2	Inorganic pyrophosphatase 2
PPARα	Peroxisome proliferator-activated receptor alpha
PPO	Protoporphyrinogen IX oxidase
PTC	Peptidyl transferase centre
PUS1	Pseudouridylate synthase 1
Q	Ubiquinone
$Q^{-\cdot}$	Semiquinone radical species
QH_2	Ubiquinol
RARS2	Mitochondrial arginyl-tRNA synthetase
RITOLS	Ribonucleotides are incorporated throughout the lagging strand
RNA	Ribonucleic acid
RNASEH1	RNASEH1 ribonuclease H1
RNAseP	Ribonuclease P
RNAseZ	Ribonuclease Z
ROS	Reactive oxidative species
RRM2B	Ribonucleoside-diphosphate reductase subunit M2 B
rRNA	Ribosomal RNA
S	Svedberg units
SARS2	Mitochondrial seryl-tRNA synthethase
SDH	Succinate dehydrogenase
SDHA	Succinate dehydrogenase complex flavoprotein subunit A

SDHAF	Succinate dehydrogenase assembly factor
SDHB	Succinate dehydrogenase complex iron sulfur subunit B
SDHC	Succinate dehydrogenase complex subunit C
SDHD	Succinate dehydrogenase complex subunit D
sgRNA	Single guide RNA
SLC	Solute carrier protein
SMAC	Second mitochondrial-derived activator of caspase
SNP	Single nucleotide polymorphism
SOLiD	Sequencing by oligonucleotide ligation and detection
SSBP	Single stranded DNA binding protein
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
TARS2	Mitochondrial threonyl-tRNA synthetase
TCA	Tricarboxylic acid
TEFM	Transcription elongation factor
TFAM	Transcription factor A, mitochondrial
TFB1M	Transcription factor B1, mitochondrial
TFB2M	Transcription factor B2, mitochondrial
TIM	Mitochondrial inner membrane translocase subunit
TIMM	Gene encoding the mitochondrial inner membrane translocase subunit
TMEM	Transmembrane protein
TOM	Mitochondrial outer membrane translocase subunit
TOMM	Gene encoding the mitochondrial outer membrane translocase subunit
TOP1MT	Mitochondrial topoisomerase I
tRNA	Transfer RNA
TSFM	Mitochondrial Ts translation elongation factor
TUFM	Mitochondrial elongation factor Tu
UQCR	Ubiquinol-Cytochrome *c* reductase subunit
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen III synthase
UV	Ultraviolet
VARS2	Mitochondrial valyl-tRNA synthetase
WARS2	Mitochondrial tryptophanyl-tRNA synthetase
WES	Whole exome sequencing
WGS	Whole genome sequencing

XIAP	X-linked inhibitor of apoptosis protein
YARS2	Mitochondrial tyrosyl-tRNA synthetase
ZFN	Zinc finger nuclease

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To you all, I am eternally grateful and I will forever endeavour to make you proud. I hope that this thesis is testament to your faith in me. Despite the overwhelming odds, tomorrow came.

Somewhere over the rainbow…

For Spencer and “my” patients.

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CHAPTER ONE

Introduction to the mitochondrion, mitochondrial disease and its diagnosis

Mitochondria are intriguing organelles. They are present in almost all nucleated eukaryotic cells and are involved in a plethora of biological pathways. They are the only organelles to possess extra-nuclear DNA and their dysfunction can have devastating consequences on human health. This introduction describes aspects of mitochondrial biology, genetics and disease that form the foundations of the published works within this thesis.

1.1 THE BIOLOGY OF MITOCHONDRIA

1.1.1 The origins of mitochondria

Mitochondria were first identified in the 1800s as intracellular structures, "bioblasts", in the cytoplasm of nucleated cells (Altmann, 1890). The term "mitochondrion" was subsequently ascribed in relation to their physical appearance from the Greek "mitos" and "chondros" which literally translates to thread-granule (Benda, 1898). Mitochondria evolved, according to the endosymbiont theory (Margulis, 1970), through phagocytosis of a free-living α-proteobacterial ancestor by a prokaryotic host cell approximately 1.5 billion years ago (Yang *et al.*, 1985; Gray, 1999). This event resulted in metabolic symbiosis with the hydrogen produced by the α-proteobacteria providing a source of electrons for the host metabolism, driving the evolution of the symbiont into eukaryota (Ochman and Moran, 2001). Prokaryotic and eukaryotic genome sequences support α-proteobacterial origins for just 10-20% of mitochondrial proteins, prompting the re-evaluation of Margulis' endosymbiont hypothesis. The revised "pre-endosymbiont hypothesis" proposes the existence of a "premitochondrion" – a host that was already able to perform certain mitochondrial functions, such as iron sulfur cluster biogenesis, but lacked the machinery required for energy metabolism that was conferred following α-proteobacterial phagocytosis (Gray, 2014).

Genome sequencing supports a stepwise loss of the endosymbiont's genome with many crucial genes having been transferred to the host genome whilst redundant genes have been entirely lost (Gray, 1992; Andersson *et al.*, 1998; Gray, 1999). The conserved functions of the classical mitochondrion are iron sulfur (Fe-S) cluster biogenesis (Stehling and Lill, 2013) and production of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) although mitochondrion-related organelles (MROs) - hydrogenosomes and mitosomes - exist that have lost one or more of the conserved mitochondrial functions (**Figure 1**) (Karnkowska *et al.*, 2016). Hydrogenosomes, such as those of the parasite *Trichomonas vaginalis* (Bui and Johnson, 1996), can synthesise iron-sulfur clusters but depend upon anaerobic respiration for ATP

metabolism, having lost much of their electron transport chain (Lindmark and Muller, 1973). Mitosomes, such as those harboured by *Giardia intestina* (Jedelsky *et al.*, 2011), also have a preserved capacity for iron cluster biogenesis but play no role in ATP production (Tovar *et al.*, 1999). The anaerobic bacteria *monocercomonoides* is the only eukaryote identified thus far that lacks mitochondria, hydrogenosomes and mitosomes (Karnkowska *et al.*, 2016).

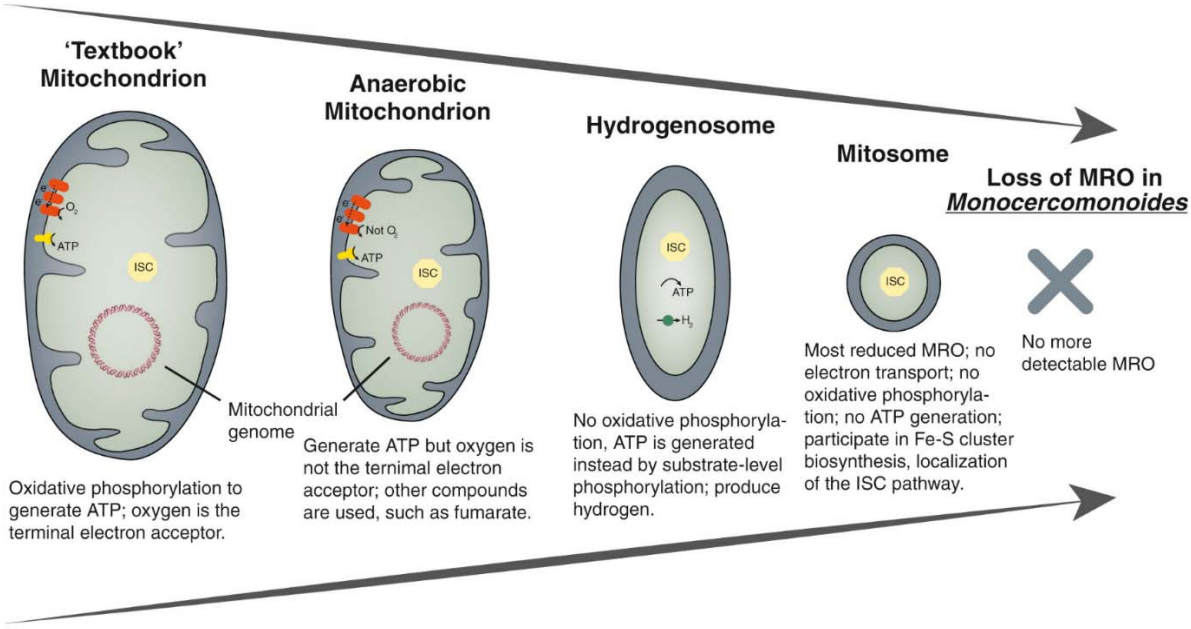

Figure 1. Evolution of mitochondrial reduction. Anaerobic and aerobic mitochondria harbour an electron transfer chain and their own genome and are able to produce ATP and Fe-S clusters (ISC). Hydrogenosomes lack a respiratory chain, but can produce ATP and synthesise ISCs. Mitosomes cannot synthesise ATP but can produce ISCs. *Monocercomonoides* lack machinery for both ISC and ATP biogenesis (Burki, 2016).

1.1.2 Mitochondrial structure

Following the advent of electron microscopy, high resolution imaging of mitochondria revealed a double phospholipid bilayer membrane, reminiscent of prokaryotic ultrastructure which comprises a smooth outer membrane with a highly convoluted inner membrane (Sjostrand, 1953). The presence of porin embedded in the outer membrane permits passive transport of proteins and molecules up to 5kDa in size. The inner mitochondrial membrane (IMM) is passively impermeable and is folded into cristae to create a vast surface area, in which are embedded the complexes of the OXPHOS system. The intermembrane space (IMS) exists between the outer and inner mitochondrial membrane, whilst the mitochondrial matrix is located at the core of the mitochondrion (**Figure 2**).

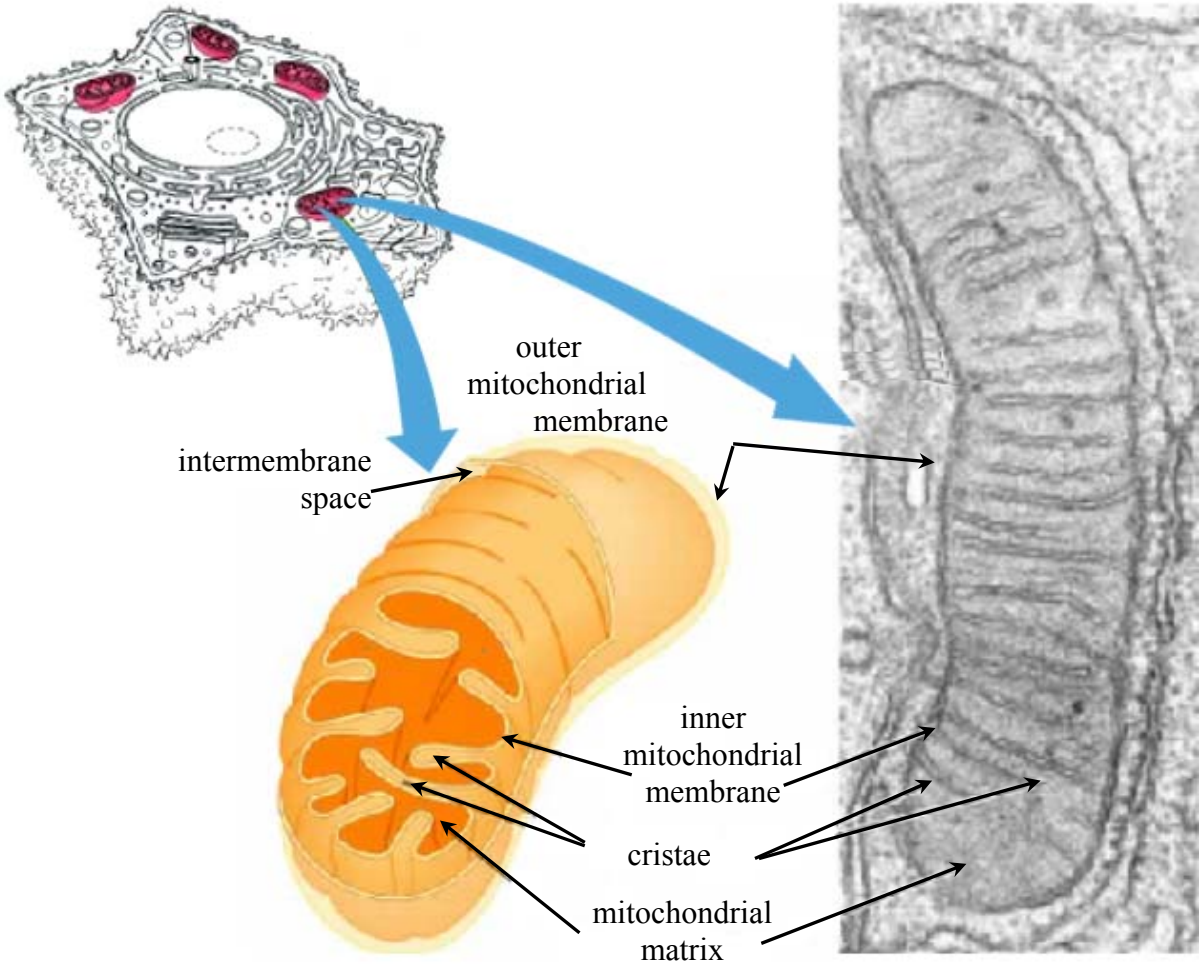

Figure 2. Electron micrograph and cartoon schematic highlighting the subcellular mitochondrial location and ultrastructure. Amended from (Campbell *et al.*, 1999; Cooper, 2000)

Many mitochondrial biochemical reactions occur within the matrix, including the tricarboxylic acid (TCA) cycle and fatty acid oxidation, whilst oxidative phosphorylation takes place across the IMM and IMS. Through the processes of fission and fusion mitochondria are able to exist as a single extensive, trabecular network or as discrete puncta. Continual division and fusion is aided by movement along cytoskeletal tracks and reflects their dynamic state that responds to cell-cycle status and the cell's functional requirements (Bereiter-Hahn and Voth, 1994; Mishra and Chan, 2014). The key regulators of mitochondrial fusion and fission are members of the dynamin family. Mitofusins 1 and 2 (*MFN1* and *MFN2* respectively) drive mitochondrial outer membrane fusion whereas OPA1 (encoded by *OPA1*) regulates mitochondrial inner membrane fusion; mitochondrial fission is regulated by DRP1 *(DNM1L*) (Smirnova *et al.*, 2001; Legros *et al.*, 2002; van der Bliek *et al.*, 2013).

1.1.3 The mitoproteome

The constituents of the mitoproteome perform all mitochondrial functions including iron-sulfur cluster biogenesis, calcium homeostasis, mitochondrial DNA replication, mitochondrial messenger ribonucleic acid (mt-mRNA) transcription, mt-protein translation and oxidative phosphorylation (Calvo *et al.*, 2016). The mitochondrion's own genetic material (mtDNA) reflects its bacterial ancestry, being circular and polyploid in nature. Human mtDNA encodes just 13 polypeptides of the mitoproteome (all of which are OXPHOS components) with the remaining mitoproteome constituents being nuclear-encoded and are therefore synthesised by cytosolic ribosomes prior to mitochondrial import. Early estimations of the mitoproteome size appear to have been over-approximated by ~25%, with the latest build of MitoCarta 2.0 reducing it from 1500 to 1158 proteins (Lopez *et al.*, 2000; Calvo *et al.*, 2016). The MitoCarta 2.0 false positive rate is 5%, and it is likely that additional genes have eluded identification and inclusion due to the limitations of the system; the detection pipeline focused on proteins localized within the mitochondrion, so may not include proteins with extra-mitochondrial localisation and those that transiently localise to the mitochondrion. This is epitomised by the recent identification of DMAC1 (encoded by *TMEM261*) - a novel inner membrane protein functioning as a complex I assembly factor that was identified through proteomic profiling yet is absent from MitoCarta 2.0 (Stroud *et al.*, 2016).

1.1.4 Mitochondrial import of nuclear-encoded proteins

Targeting of nuclear-encoded proteins to the mitochondria typically involves a mainly basic presequence involving the 10-90 most N-terminal residues. These are predicted to form an α-helical domain that is recognised by the mitochondrial import machinery (von Heijne, 1986), reviewed by Kunze and Berger (Kunze and Berger, 2015). Nuclear-encoded mitochondrial proteins are translated within the cytoplasm on cytosolic ribosomes and remain unfolded until they are successfully imported to the appropriate mitochondrial compartment via chaperone (Hsp70) interactions. The protein's mitochondrial targeting sequence (MTS) is recognised by TOM20 and TOM22 precipitating transfer through the outer mitochondrial membrane via the pore in TOM40. Proteins targeted for the matrix must also traverse the inner mitochondrial membrane; the electrochemical gradient ($\Delta\Psi$) attracts the charged presequence and the protein crosses the IMM via the TIM23 pore. The presequence translocase-associated motor (PAM)-complex drives release of the nascent polypeptide into the matrix and the mitochondrial targeting sequence is cleaved by mitochondrial processing peptidase (MPP).

Finally, chaperone (HSP60) proteins drive protein folding into its native state (**Figure 3**) (Kunze and Berger, 2015).

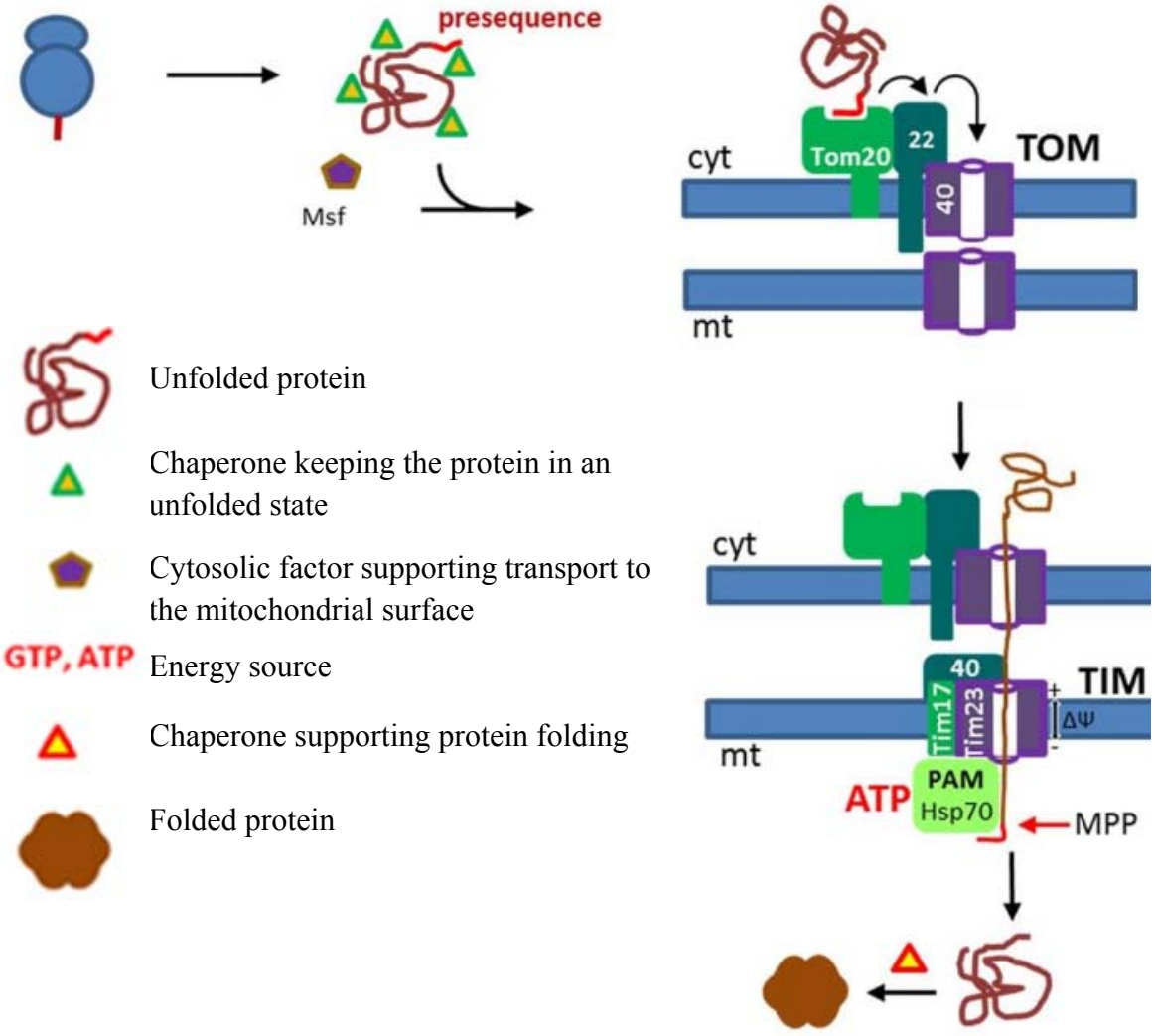

Figure 3. Mitochondrial targeting of nuclear-encoded soluble proteins. Following translation in the cytosol, translated proteins remain unfolded for mitochondrial targeting. Transport through the outer mitochondrial membrane occurs through the action of translocon of the outer mitochondrial membrane (TOM) proteins (TOM22, TOM22, TOM40). Proteins targeted to the matrix must also be transported across the inner mitochondrial membrane by the translocon of the inner mitochondrial membrane (TIM) machinery (TIM17, TIM40, TIM23) facilitated by the membrane potential (ΔΨ) that attracts the charged mitochondrial targeting presequence. Following import, the targeting sequence is cleaved by mitochondrial processing peptidase (MPP) and chaperone (HSP60) proteins direct folding into its native state. Adapted from (Kunze and Berger, 2015).

Of the proteins that remain within the IMS, many nascent proteins have cysteine residues typically arranged as Cx_3C or Cx_9C that are oxidised by MIA40 (encoded by *CHCHD4* in human) during the mitochondrial disulfide relay. Upon complete oxidation, the native protein is released from MIA40 with disulfide bonds that convey tertiary structure and prevents their export from the IMS (**Figure 4**) (Hofmann *et al.*, 2005; Koch and Schmid, 2014; Mordas and Tokatlidis, 2015). MIA40 is subsequently re-oxidised by sulfhydryl oxidase (ERV1, encoded by *GFER*) (Allen *et al.*, 2005), with electron transfer to the electron carrier cytochrome *c* (cyt *c*) (**Chapter 1.2.10**) or directly to molecular oxygen to form water (**Figure 4**).

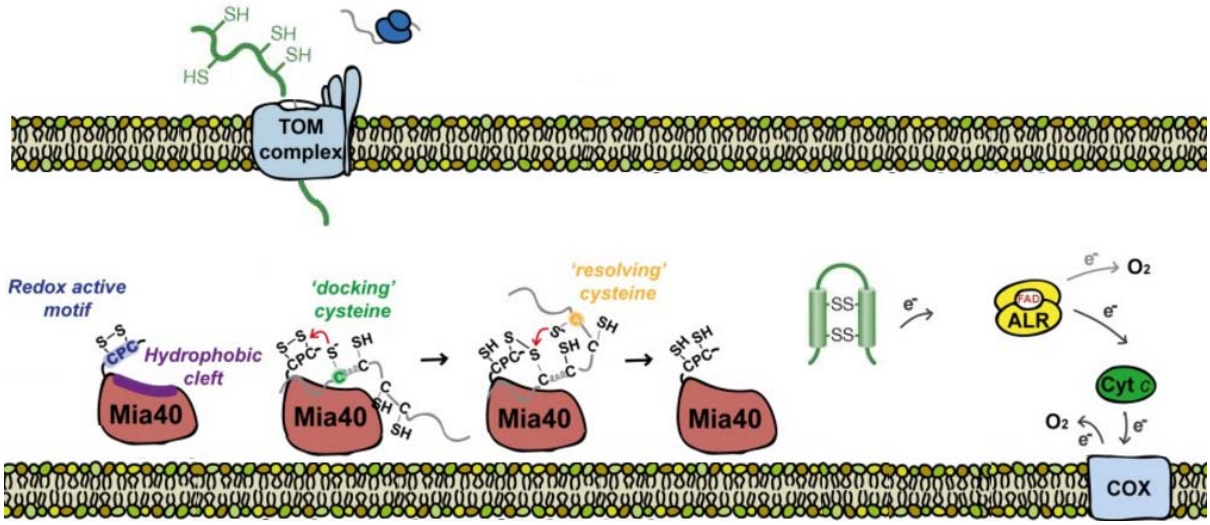

Figure 4. The mitochondrial disulphide relay. Reduced, nascent polypeptides enter the IMS via the TOM import pathway. Substrates containing twin CX_3C and CX_9C motifs are recognised by MIA40 and bind to the hydrophobic binding cleft, creating interactions between the cysteine of the MIA40 CPC motif and the nascent polypeptide. MIA40 oxidation creates disulfide bonds between the "resolving" and "docking" cysteines and the oxidised protein is released. Electrons produced through MIA40 regeneration by ERV1 pass either to molecular oxygen or contribute to ATP production via cyt *c*. Adapted from (Mordas and Tokatlidis, 2015).

1.1.5 The mitochondrial genome

The human mitochondrial DNA molecule is a 16,569 base pair (bp), double stranded circular mtDNA molecule that is exclusively maternally transmitted (Giles *et al.*, 1980). It encodes 13 of the OXPHOS subunits and the 22 mitochondrial transfer RNAs (tRNAs) and 2 mitoribosome ribosomal RNAs (rRNAs) that are required for their synthesis (Anderson *et al.*, 1981). Although each cell only has one nucleus with a diploid nuclear genome, a cell harbours many mitochondria, each with multiple copies of mtDNA within its matrix; the mtDNA copy number varies greatly between individuals and across different tissues from the same individual which has important consequences for mtDNA-associated pathology (discussed further in **Chapter 1.3.4**). The mutation rate of mtDNA is approximately 10 fold that of nuclear DNA in humans

(Brown *et al.*, 1979; Wallace and Chalkia, 2013), at least partly due to the proximity of mtDNA molecules to damaging free radical species, particularly reactive oxidative species (ROS) within the matrix (Lagouge and Larsson, 2013). Whilst nuclear DNA is compacted into chromatin-derived nucleosomes through histone binding (Kornberg, 1974), mtDNA compacts into nucleoid structures without histone involvement, driven by TFAM binding to the mtDNA duplex (Bogenhagen, 2012; Lagouge and Larsson, 2013). Unlike most nuclear counterparts, mtDNA-encoded genes are devoid of intronic sequence; the majority of non-coding sequence is located within a single contiguous region that includes the D-loop, which possesses a rare triple-stranded domain (Kasamatsu *et al.*, 1971). The strands of the mitochondrial DNA molecule are named according to their density, reflecting their nucleotide composition and their differential distribution following centrifugation in cesium-chloride. The "heavy" (H) strand is named due to its guanine nucleotide bias, whilst the complementary strand is denoted as "light" (L).

1.1.6 Mitochondrial DNA replication

mtDNA replication occurs in the matrix and creates daughter mitochondria for populating new cells following cell division and enables the cell to increase its metabolic output. There are a number of models of mtDNA replication, either asymmetric or symmetric, demonstrated by the "strand-displacement" model, the "bootlace" or "RITOLS" model and the "strand-coupled bidirectional" model, reviewed in (Young and Copeland, 2016). All models have supportive *in vitro* experimental evidence, and it's possible that they may not be mutually exclusive *in vivo*. *In vivo* CHiP data was most supportive of the asynchronous model of mtDNA replication but a unanimous model of mtDNA replication has yet to be elucidated (Miralles Fuste *et al.*, 2014).

1.1.7 Strand-displacement model of mtDNA replication

Replication commences in an asynchronous manner from two sites on the mitochondrial genome, firstly at the heavy strand origin of replication (O_H), and proceeds unidirectionally and uninterrupted, displacing the opposite strand as it progresses, until two-thirds of the mtDNA is replicated (Clayton, 1982). At this point, a stem-loop structure forms at the light strand origin of replication (O_L) directing POLRMT binding and synthesis of the RNA primer; light strand replication then commences in the opposite direction (Young and Copeland, 2016). (**Figure 5**).

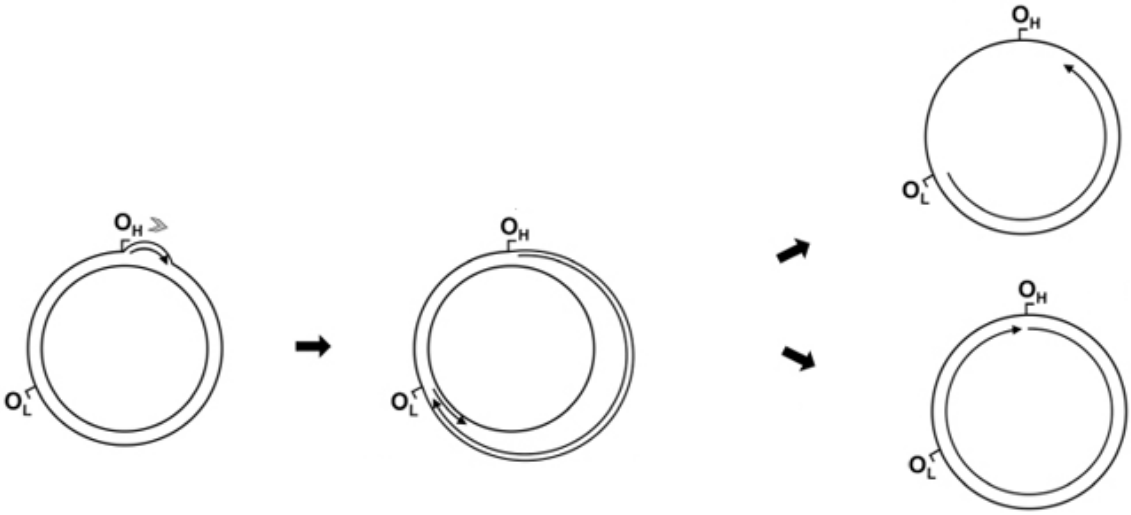

Figure 5. Asynchronous model of mtDNA replication. mtDNA replication commences at O_H, separating the heavy and light strands as the daughter strand is produced. The light strand remains single stranded until O_L is accessible. Light strand replication commences until both strands are replicated. Adapted from (McKinney and Oliveira, 2013).

1.1.8 Bootlace and RITOLS models of mtDNA replication

The bootlace model posits a single origin of replication (OriZ) from which both strands replicate synchronously (**Figure 6A**). The **R**ibonucleotides are **I**ncorporated **T**hrough**O**ut the **L**agging **S**trand (RITOLS) model posits similar origins, but as replication processes and the replication fork advances, complementary tRNA and mRNA transcripts hybridise to the lagging strand template (**Figure 6B-D**) (Holt *et al.*, 2000; Reyes *et al.*, 2013).

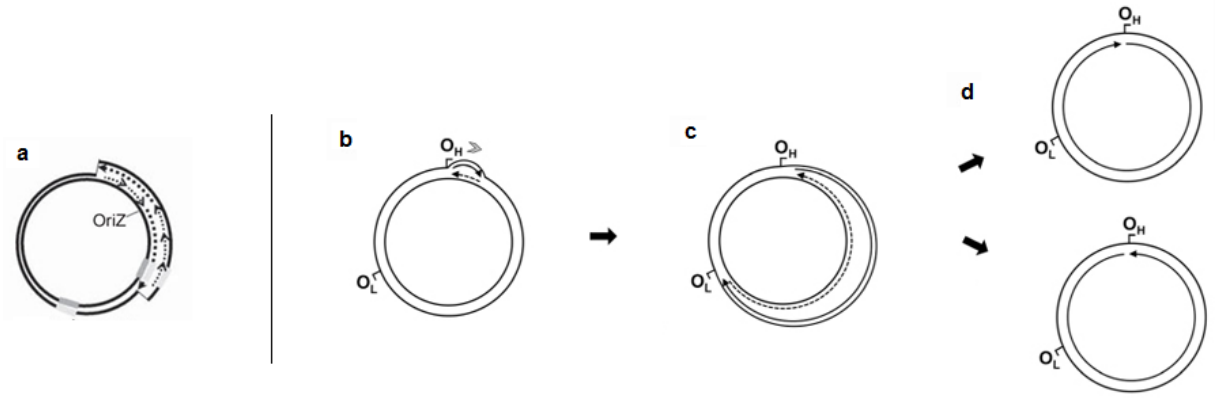

Figure 6. Synchronous and RITOLS replication models of mtDNA replication. A Synchronous replication model of mtDNA replication. Replication initiates at OriZ and proceeds bidirectionally employing conventional leading and lagging strand synthesis. (B–D) RITOLS model of mtDNA replication. Replication commences in a similar manner to the asynchronous model, initiating at OH and separating the light and heavy strands but RNA (dashed lines) hybridise to the light strand, thereby protecting it whilst it would otherwise be single stranded. Adapted from (Krishnan et al., 2008; McKinney and Oliveira, 2013).

1.1.9 Strand Coupled Bidirectional Model of mtDNA replication

The bidirectional model of replication suggests that both strands are initiated at multiple origins of replication across a ~4kb region near O_H, and replication progresses using dual replication forks around the mtDNA molecule (**Figure 7**) (Bowmaker *et al.*, 2003). The displacement (D) loop's triple-stranded nature stalls replication and moves the replication fork downstream (Bowmaker *et al.*, 2003).

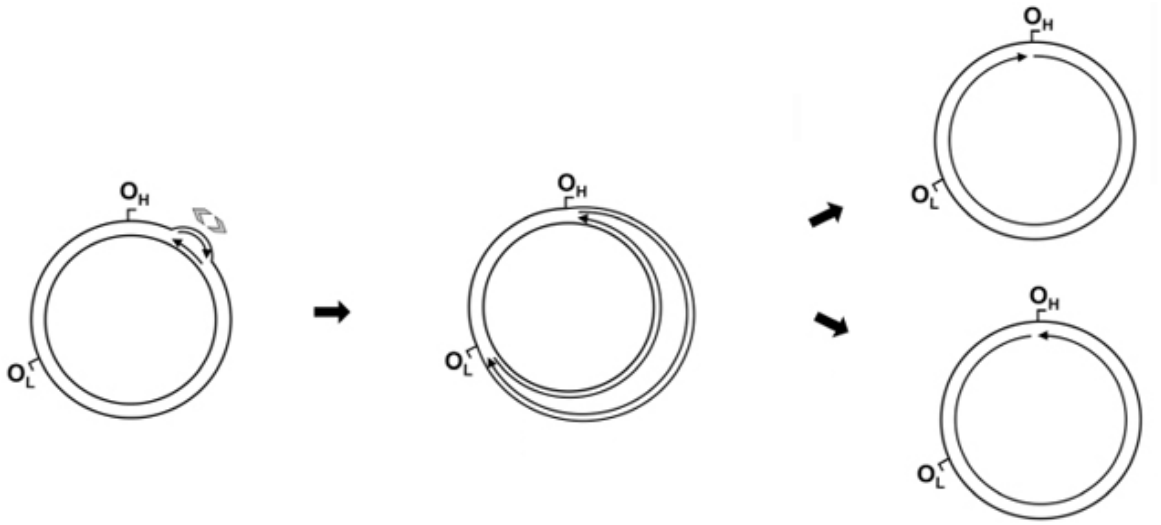

Figure 7. Strand coupled bidirectional model of mtDNA replication. Replication initiates at a broad initiation zone and proceeds bi-directionally, employing conventional lagging strand synthesis, until arrest at the D-loop. Adapted from (McKinney and Oliveira, 2013).

Faithful replication is under the control of key mitochondrial proteins including a topoisomerase (encoded by *TOP1MT*), helicase (twinkle, encoded by *PEO1*), polymerase (γ, encoded by *POLG* and *POLG2*), RNA polymerase (encoded by *POLRMT*) that generates RNA primers thereby initiating DNA synthesis, ribonuclease (encoded by *RNASEH1*), mitochondrial DNA ligase (encoded by *LIG3*) and single stranded RNA binding protein, mtSSB (encoded by *SSBP1*) (Young and Copeland, 2016). Additionally, proteins are also required to maintain appropriate levels of free deoxyribonucleoside 5'-triphosphates for the creation of daughter molecules (e.g. deoxyguanosine kinase (*DGOUK*), thymidine phosphorylase (*TYMP)*, ribonucleotide reductase (*RRM2B*) and thymidine kinase (*TK2*) (Copeland, 2012).

1.1.10 mtDNA repair

Some replication errors are identified and repaired by the 3′-5′ exonuclease activity of polymerase γ, resulting in an error rate of $<1\times10^{-6}$ bp; additionally, mitochondrial genome maintenance exonuclease 1 (*MGME1)* cleaves single-stranded DNA and DNA with free ends, thereby removing damaged mtDNA molecules and products of incomplete replication

(Kornblum *et al.*, 2013; Uhler *et al.*, 2016). Additionally, endogenous (e.g. ROS) and exogenous (e.g. UV) sources of mtDNA damage require base excision repair (BER) and double-strand break repair (DSBR) (Kazak *et al.*, 2012). mtDNA damage often arises through exposure to endogenous oxidative radicals and its repair is mediated through the BER pathway involving excision of the damaged nucleotide, DNA end-repair, gap filling and ligation (Alexeyev *et al.*, 2013). Although somatic double-stranded (ds) mtDNA breaks have a reduced impact on cell viability compared to nuclear dsDNA breaks, there is evidence that mtDNA employs microhomology-mediated homologous end-joining (MMEJ) repair to avoid deleterious consequences to the cell following clonal expansion (Tadi *et al.*, 2016).

1.1.11 Mitochondrial transcription

Transcription of human mtDNA-encoded genes occurs in a polycistronic manner (**Figure 8**), precipitated by TFAM recruitment of POLRMT, TFB1M and TFB2M at either of the two promoters - HSP1 and HSP2 - that are historically referenced as initiators of transcription of the Heavy strand, or at LSP which initiates transcription of the Light strand (Montoya *et al.*, 1982; Mazunin *et al.*, 2015). The transcript associated with the HSP1 promoter is a short mRNA including *MTRNR1* and *MTRNR2* (encoding the 12S and 16S rRNAs), *MTTV* and *MTTF* (mitochondrial valine and phenylalanine tRNAs). The HSP2-associated transcript is a considerably longer polycistronic mRNA that spans the remaining genes (12 mRNAs and 11 tRNAs). More recent *in vivo* studies have failed to substantiate the involvement of HSP2 in transcription initiation, and it is speculated that HSP1 alone is responsible for transcription of the Heavy strand (Litonin *et al.*, 2010). Transcription of the light strand results in a polycistronic entity of near genome-length, comprising one polypeptide mRNA (*MTND6*) plus eight tRNAs (Montoya *et al.*, 1983). In addition to TFAM, POLRMT, TFB1M and TFB2M, other important mitochondrial transcription proteins include TEFM, the transcription elongation factor, MRPL12, a component of the large mitoribosome subunit and MTERF1, a DNA binding protein involved in transcription termination of HSP1-derived transcripts (Minczuk *et al.*, 2011).

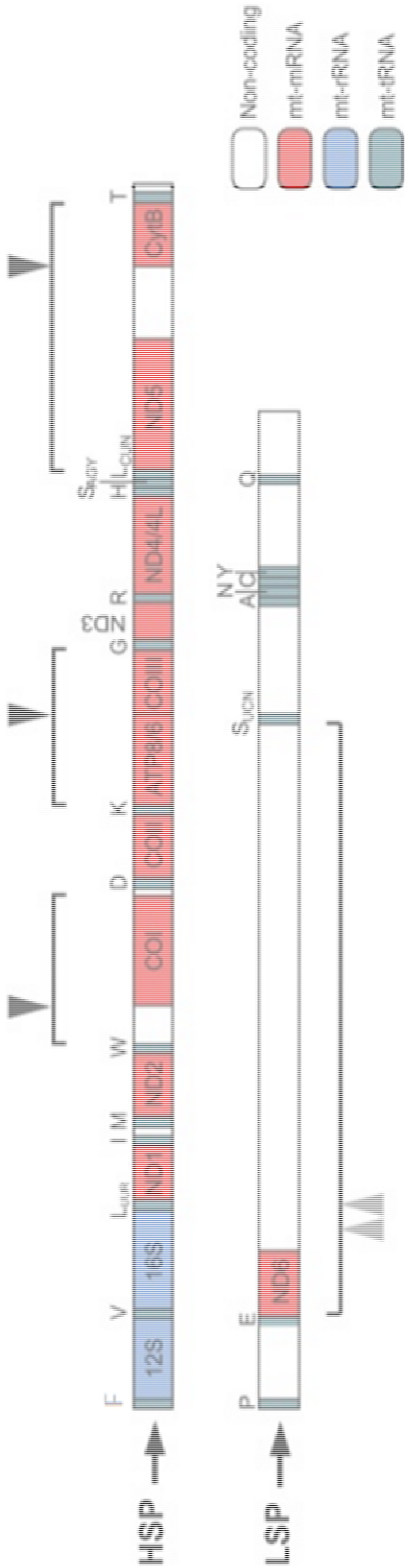

Figure 8. HSP and LSP-derived polycistronic mRNA transcripts. The LSP-derived transcript contains the coding sequences for ND6, the mt-tRNAs for proline, glutamate, serine^{UCN}, alanine, asparagine, cysteine, tyrosine and glutamine. All other coding sequences are produced by the HSP-derived transcript. Post-transcriptional processing splices the mt-tRNAs from the majority of mt-rRNAs and mt-mRNAs; arrows denote the transcripts that are not punctuated by mt-tRNA molecules. Adapted from (Van Haute *et al.*, 2015).

The mt-tRNA molecules are situated immediately 3' and 5' to most polypeptide and rRNA-encoding genes, and their post-transcriptional cleavage from the polycistronic transcripts gives rise to the mature polypeptide mRNA molecules; the 3' end is excised through the action of RNAseZ (encoded by *ELAC2*) whilst the 5' tail of the tRNA is cleaved by the RNAseP complex consisting of MRPP1, MRPP2 and MRPP3 (encoded by *TRMT10C, HSD17B10* and *MRPP3,* respectively) (**Figure 9**) (Van Haute *et al.*, 2015). There is little understanding of the enzymatic machinery involved in processing those transcripts that lack a canonical mt-tRNA "punctuation mark" (Van Haute *et al.*, 2015).

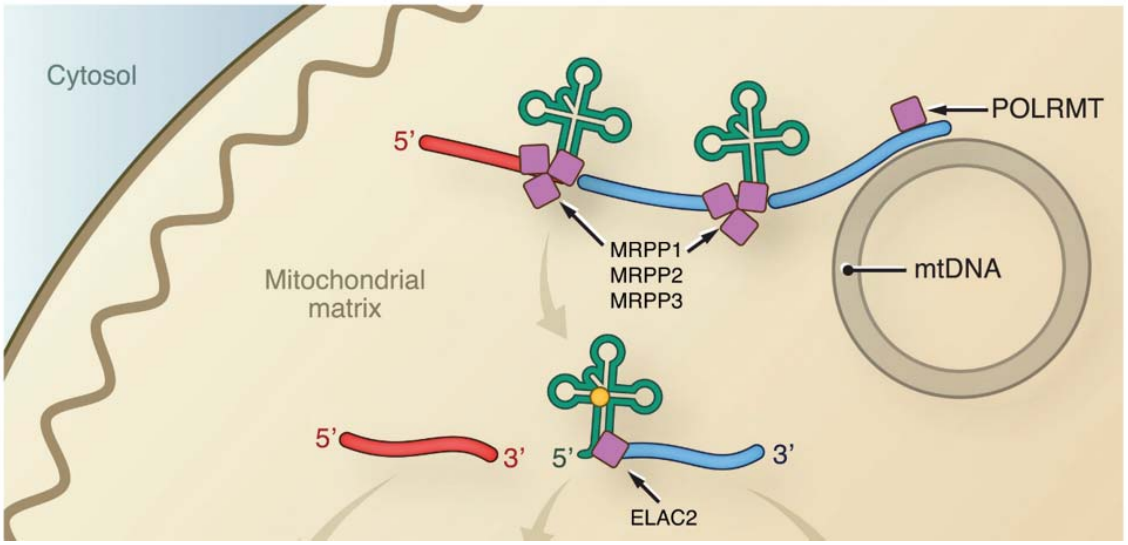

Figure 9. Post-transcriptional cleavage of mt-tRNA from polycistronic transcripts. Following transcription by POLRMT**,** processing by mitochondrial RNaseP releases the individual mt-mRNA, mt-tRNA and mt-rRNA molecules. MRPP1, MRPP2 and MRPP3 cleave the 5′ end of tRNAs whilst RNaseZ cleaves the 3′ ends. Adapted from (Hallberg and Larsson, 2014).

1.1.12 Mitochondrial translation

The main component of the mitochondrial translation machinery is the ribonucleoprotein mitoribosome (Schmeing and Ramakrishnan, 2009). Mitoribosomes are comprised of two subunits, termed the large and small mitochondrial subunits, each in turn consisting of numerous protein components and RNA entities. Given the bacterial origin of mitochondria, it is unsurprising that the mitoribosome has similar origins; despite this, the composition of eukaryotic mitoribosomes differs markedly from that of the bacterial ribosome (O'Brien, 2002). During evolution, eukaryotic mitoribosomes have lost many of the major RNA stem structures possessed by bacterial ribosomes but have a significantly higher protein content, apparent from the resolved crystal structure of the human mitoribosome (Amunts *et al.*, 2015; Greber *et al.*,

2015) and consistent with the philosophy that RNA entities have been gradually replaced by proteins (O'Brien, 2002). These additional proteins are specific to mitoribosomes, lacking homologues in both bacterial and cytoplasmic ribosomes (Amunts *et al.*, 2015). In fact, the protein to RNA ratio in cytosolic 80S particles (70% RNA to 30% protein) is reversed in the corresponding (55S) subunit of the mitoribosome (30% RNA to 70% protein). The small, 28S, subunit of the human mitoribosome comprises the 12S rRNA and 30 polypeptides, 14 of which are mitochondria-specific, whilst the large 39S subunit consists the mtDNA-encoded 16S rRNA plus tRNA-valine ribonucleic acid components together with approximately 53 nuclear-encoded polypeptides, of which ~23 are mitochondria-specific (Mai *et al.*, 2017).

Translation of mitochondrial mRNA proteins occurs in the mitochondrial matrix, with four conserved key stages (**Figure 10**):

1) **Initiation.** The mRNA binds to the mitoribosome and the appropriate start codon is identified by an initiation complex composed of the small mitoribosomal subunit (mt-SSU) and initiation factor mtIF3. The mitochondrial initiation factor mtIF2 promotes binding of a post-translationally modified methionine tRNA; this methionine amino acid is formylated by the methionyl-tRNA formyltransferase (encoded by *MTFMT*) (Takeuchi *et al.*, 1998) and then aminoacylated to the methionine tRNA by the methionine-specific mt-tRNA synthetase (encoded by *MARS2*). Formylation increases its affinity for mtIF2, and these two units form a stable initiation complex with the mt-SSU and mRNA from which translation commences and the polypeptide extends during elongation (Spremulli *et al.*, 2004; Christian and Spremulli, 2012).

2) **Elongation**. During this stage, amino acids are incorporated into the growing polypeptide chain according to the mRNA codon sequence by a mitochondrial tRNA molecule specifically charged with its cognate amino acid. Three fundamental elongation factors are known - the mitochondrial translation elongation factor Tu (TUFM/EF-Tu_{mt}) that presents the aminoacyl-tRNA to the A-site of the mitoribosome, the guanine nucleotide exchange factor Ts (TSFM/EF-Ts_{mt}) that regenerates EF-Tu_{mt} after delivery of the aminoacyl-mt-tRNA, and the mitochondrial elongation factor G1 (GFM1/EF-$G1_{mt}$) that coordinates mRNA and tRNA movement during translocation (Christian and Spremulli, 2012).

3) **Termination.** – Termination occurs when a translating mitoribosome encounters one of the stop codons (UAA, UAG, AGA or AGG) at the A-site, at which point the mitochondrial release factor (mtRF1a) binds to the mitoribosome and releases the mature protein. Other important proteins such as C12orf65 and ICT1 are also involved in translation termination (Mai *et al.*, 2017).

4) **Recycling**. The final step of translation involves the mitochondrial ribosome recycling factors (MTRF1/RF1mt and MRRF/RRF1mt) that induce disassembly of the ribosome whilst mitochondrial elongation factor G2 (GFM2/EFG2mt) mediates release of the mRNA and tRNA to enable the mitoribosome to translate the next mitochondrial mRNA molecule (Zhang and Spremulli, 1998; Rorbach *et al.*, 2008; Tsuboi *et al.*, 2009).

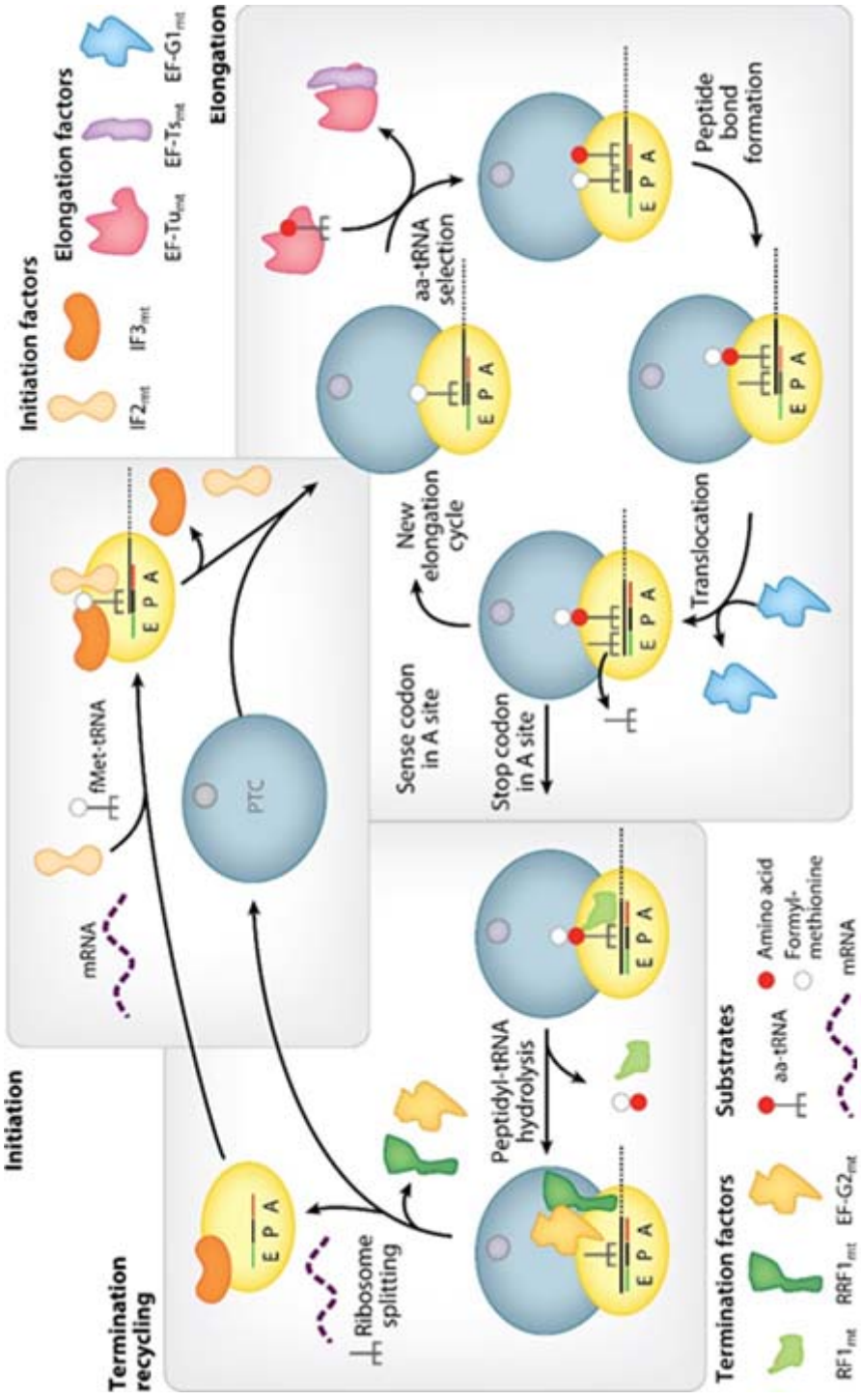

Figure 10. Overview of mammalian mitochondrial protein synthesis. Initiation factors recognise the start codon at the P site of the small ribosomal subunit and bind the formylated methionine (white circle). Upon identification of the start codon, the large subunit associates and initiation factors dissociate. Elongation factors deliver aminoacylated mt-tRNAs (red circle) to the A site of the ribosome; peptidyl bonds form to incorporate the new amino acid, and the mRNA-tRNA complex is translocated ready for the cycle to repeat with the consecutive aminoacylated mt-tRNA. Upon a stop codon entering the A site, release factors and recycling factors terminate translation of the nascent polypeptide, releasing it from the P-site. PTC: peptidyl transferase centre. Taken from (Ott *et al.*, 2016).

1.2 MITOCHONDRIAL FUNCTION

Whilst the synthesis of ATP by oxidative phosphorylation is arguably their most recognised role, mitochondria are fundamental for numerous processes including calcium homeostasis, heme and iron-sulfur cluster biogenesis and apoptosis (Duchen, 2000; Stehling and Lill, 2013; Barupala *et al.*, 2016).

1.2.1 The role of mitochondria in calcium homeostasis

The permeability of the outer mitochondrial membrane to calcium means that cytoplasmic increases in calcium are associated with concomitant increase in calcium concentration within the mitochondrial intermembrane space. The selective permeability of the inner mitochondrial membrane necessitates a system for transporting calcium into the matrix to avoid calcium overload and the IMM-embedded mitochondrial calcium uniporter (MCU) serves this function. The MCU is a dimerised protein that forms a pore, the open state of which is negatively regulated by MICU1, MICU2 and EMRE (Essential MCU Regulator) thereby inhibiting calcium uptake at low calcium levels. Calcium binding to the dimerised MICU1-MICU2 complex under elevated calcium concentrations in the cytoplasm/IMS stimulates opening of the MCU pore (**Figure 11**). The voltage gradient between the IMS and the matrix is ~200mA, meaning that positively charged calcium ions flood into the negatively charged matrix, equilibrating the Ca^{2+} concentration (Kamer and Mootha, 2015). The mechanism by which calcium is effused from the mitochondrial matrix involves active transport, either by the sodium–calcium exchange protein, NCLX (encoded by *SLC24A6*) (Palty *et al.*, 2010) or the Ca^{2+}/H^{+} exchanger exchange protein, HCLX (Leucine zipper/EF-hand-containing TransMembrane protein 1, encoded by *LETM1*) (Jiang *et al.*, 2009).

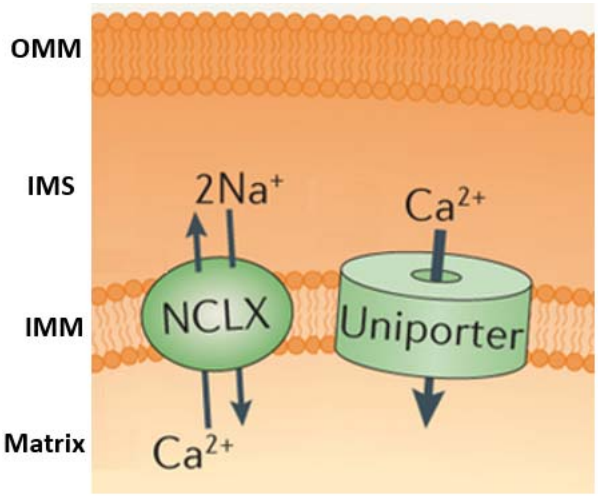

Figure 11. Schematic of the sodium-calcium exchanger and calcium uniporter. Calcium homeostasis is achieved by the dynamic action of NCLX (and HCLX, not shown) and the calcium uniporter. Adapted from (Kamer and Mootha, 2015).

1.2.2 The role of mitochondria in iron-sulfur (Fe-S) cluster biogenesis

Fe-S cluster biogenesis is a fundamental and evolutionarily conserved mitochondrial function, performed by even the most degenerate forms of mitochondria (Shiflett and Johnson, 2010). Mammalian Fe–S clusters are inorganic cofactors composed of iron and inorganic sulfur that typically bind to cysteinyl ligands of an Fe–S protein and confer various abilities to the proteins that contain them (Gakh *et al.*, 2016) including their ability to undergo redox reactions that underpins their utility within the mitochondrial respiratory chain (see **Chapter 1.2.7**) (Beinert, 2000). There are 12 mitochondrial Fe-S clusters within respiratory chain complexes I, II and III and aconitase (Lill *et al.*, 1999; Rouault, 2015), and additional Fe-S cluster proteins in the nucleus and cytosol (Sharma *et al.*, 2010). The mitochondrial Fe-S cluster biosynthesis apparatus resembles that of prokaryotic cells and initial steps involve an iron donor, frataxin (encoded by *FXN*), a sulfur donor (cysteine desulfurase [encoded by *NFS1*], complexed with the LYR-motif containing ISD11 [encoded by *LYRM4*]) and an assembly scaffold, ISCU (**Figure 12A**). Electrons required for the Fe-S cluster configurations are provided by ferredoxins (encoded by *FDX1*/*FDX2*) and ferredoxin reductase (encoded by *FDXR*) (Maio and Rouault, 2016). Upon assembly, Fe-S clusters are inserted either directly, or via an intermediate chaperone system, into recipient proteins (**Figure 12B**).

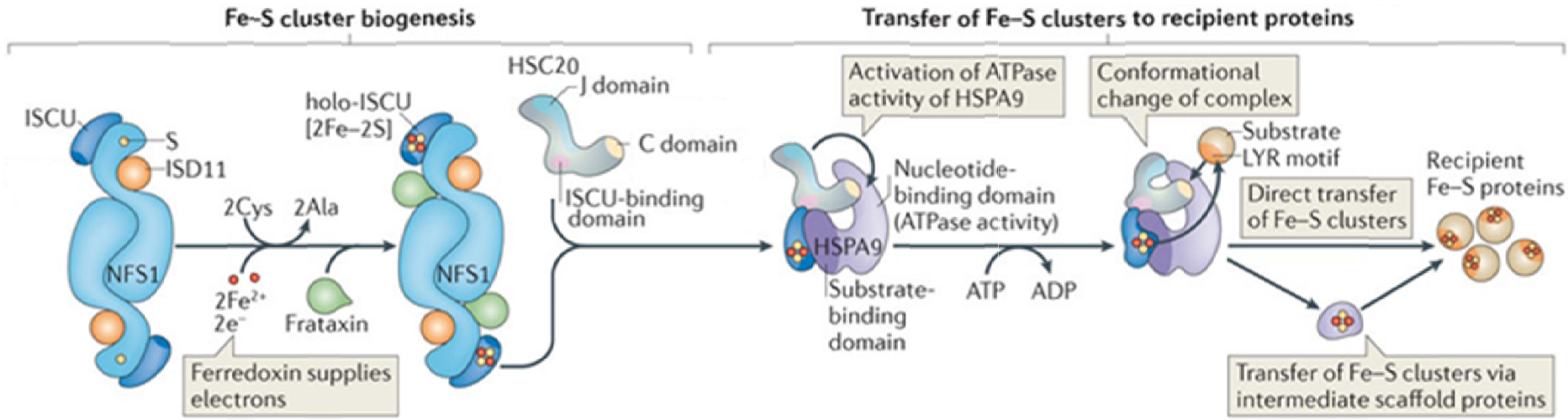

Figure 12. Iron sulfur cluster biogenesis and Fe-S cluster transfer to recipient proteins. A. NFS1 binds to the Leu-Tyr-Arg (LYR) motif-containing protein ISD11. Its cysteine desulfurase activity is allosterically activated by Frataxin which converts cysteine to alanine to form a persulfide intermediate (yellow circle). Recent studies have shown that frataxin forms a complex with NFS1, ISD11 and ISCU to donate iron during *de novo* synthesis of Fe-S clusters (Gakh *et al.*, 2016). B. In mammalian cells, transfer of the Fe-S clusters to acceptor proteins occurs in an ATP-dependent process involving HSPA9, the co-chaperone HSC20 and scaffold protein ISCU. ISCU contains a LPPVK substrate binding motif (Leu-Pro-Pro-Val-Lys) that binds chaperone protein HSPA9; HSC20s C domain recognises and binds to LYR motifs within recipient proteins thereby holding the target in proximity to the Fe–S cluster in readiness for transfer whilst conferring Fe-S protection in the interim. HSPA9-mediated ATP hydrolysis confers a conformational change that is postulated to transfer Fe-S clusters from ISCU to their recipient proteins (Maio and Rouault, 2016). Adapted from (Rouault, 2015).

1.2.3 Coenzyme Q biosynthesis

The majority of CoQ is synthesised in the mitochondria, composed of a benzoquinone ring with a polyisoprenoid side chain that confers stabilisation of the CoQ molecule within the phospholipid bilayer; in the case of humans, CoQ has ten isoprenoid units (CoQ_{10}) that are synthesized by a complex of COQx enzymes via the mevalonate pathway within the inner mitochondrial membrane (**Figure 13**) (Rashid *et al.*, 2015; Alcazar-Fabra *et al.*, 2016).

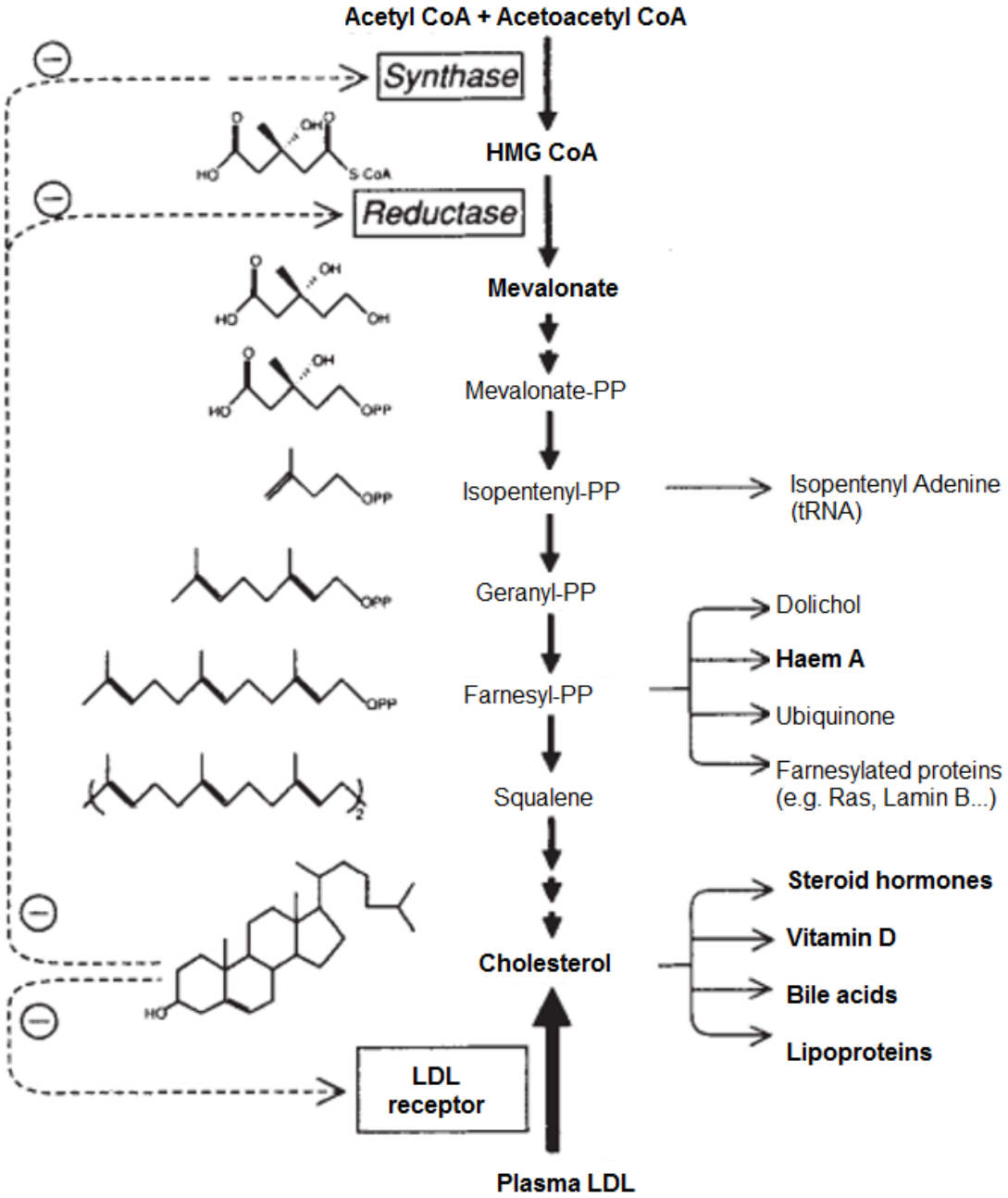

Figure 13. Overview of human mevalonate pathway. *De novo* ubiquinol synthesis occurs within the mitochondria using acetyl Co A (produced following glycolysis) as a substrate. A small exogenous contribution to the cholesterol biogenesis pathway exists, with uptake of circulating dietary Low-density lipoprotein (LDL) via the LDL receptor (Goldstein and Brown, 1990).

CoQ biosynthesis is under regulatory control by many enzymes in the complex, with hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase (involved in conversion of HMG-CoA to mevalonate) being a key regulator of the cholesterol synthesis pathway. Other regulators include COQ7 (*COQ7*), responsible for the penultimate step of CoQ synthesis that is inhibited by PPARα and NFκB under stress conditions (Cascajo *et al.*, 2016) and COQ8 (*ADCK3*) that is hypothesised to regulate CoQ synthesis through phosphorylation of substrates in an ATP-dependent feedback loop (Lagier-Tourenne *et al.*, 2008).

1.2.4 The role of mitochondria in heme biosynthesis

Heme molecules are essential prosthetic groups for several metabolic proteins; they are iron-containing cyclic tetrapyrroles that are vital components in cytochromes, haemoglobin and myoglobin in the form of heme B. Heme biosynthesis involves four stages and eight biosynthetic reactions, requiring the coordination of cytosolic and mitochondrial substrates and enzymes: 1) the initial synthesis of a single pyrrole ring, 2) complete assembly of tetrapyrroles (a porphyrin ring), 3) side chain modifications, and finally 4) iron insertion into the tetrapyrrole ring to form the functional heme (**Figure 14**) (Barupala *et al.*, 2016).

1. The carbon and nitrogen source for pyrroles is 5-aminolevulinic acid (ALA), formed from decarboxylation of glycine and succinyl co A by ALA synthase (ALAS) and cofactor pyridoxal phosphate. There are two isoforms of mammalian ALA synthases, the housekeeping enzyme encoded by *ALAS1* and the erythroid-specific, mitochondrial enzyme *ALAS2* (Bishop, 1990). ALA is then imported into the IMM by the inner membrane glycine exchange protein SLC25A38 (Guernsey *et al.*, 2009). ALA dehydratase (ALAD) forms porphobilinogen (PBG) through condensation of two ALA molecules (Sassa, 1982). This unit represents the single pyrrole ring.
2. Four PBG molecules are condensed by porphobilinogen dehydratase (PBGD) to create the linear 1-hydroxymethylbilane (Battersby *et al.*, 1980). Uroporphyrinogen III synthase (UROS) converts 1-hydroxymethylbilane into the cyclic uroporphyrinogen III (Battersby and Leeper, 1990) which is subsequently converted to coproporphyrinogen III (CPIII) by uroporphyrinogen III decarboxylase.

3. Coproporphyrinogen III is transported back across the outer mitochondrial membrane, likely via the transporter protein (encoded by *PBR*) (Papadopoulos *et al.*, 2006) for the final stages of heme biosynthesis which occur within the IMS. Coproporphyrinogen III oxidase (CPO) converts two of the CPIII propionate groups to vinyl groups to produce protoporphyrinogen IX (Porra and Falk, 1964); this is then converted to protoporphyrin IX by protoporphyrinogen IX oxidase (PPO) (Sano and Granick, 1961).
4. The final stage of heme biosynthesis involves the insertion of iron into the protoporphyrin IX structure by the Fe-S cluster containing protein ferrochelatase (encoded by *FECH*) to create heme B (Granick and Levere, 1964).

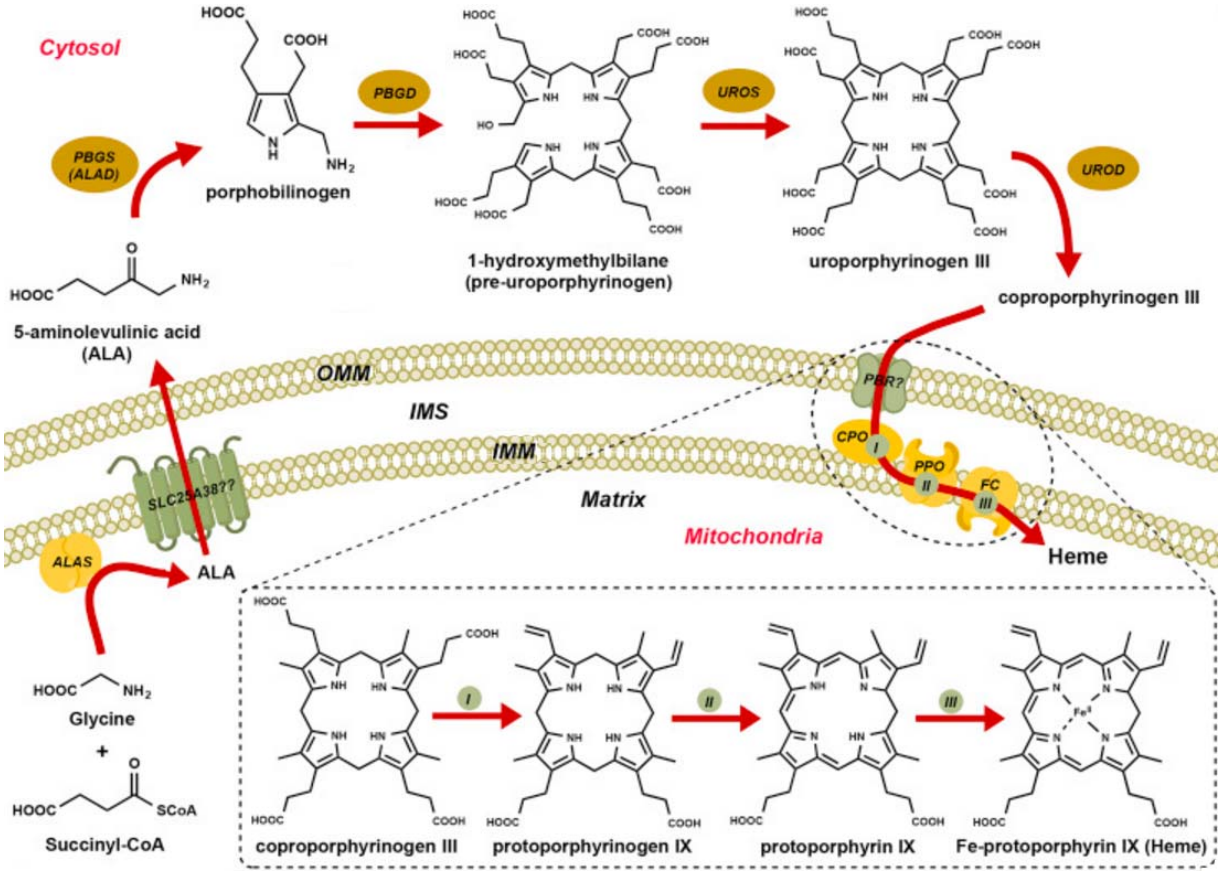

Figure 14. Overview of the heme biosynthesis pathway. ALA is synthesised in the matrix and transported to the cytosol for conversion to coproporphyrinogen III, subsequently transported back into the mitochondrion for final heme conversion. IMM: inner mitochondrial membrane, IMS: intermembrane space, OMM: outer mitochondrial membrane. ALA: 5-aminolevulinic acid, ALAS: ALA synthase, ALAD: ALA dehydratase, PBGD: porphobilinogen deaminase, UROS: uroporphyrinogen synthase, UROD: uroporphyrinogen decarboxylase, PBR: peripheral-type benzodiazepine receptor, CPO: coproporphyrinogen III oxidase, PPO: protoporphyrinogen IX oxidase, FC: Ferrochelatase (Barupala *et al.*, 2016).

1.2.5 The role of mitochondria in apoptosis

Apoptosis is the method by which organisms eradicate dysfunctional cells. Mitochondrial driven apoptosis occurs following BH3-mediated cleavage of Bcl-2 proteins that triggers a cell death signalling cascade. Signalling molecules released from the IMS result in inhibition of apoptosis repressors and activation of pro-apoptotic initiator and effector caspases (**Figure 15**), causing cell shrinkage, chromatin condensation and fragmentation of the nuclear membrane; cell fragments are phagocytosed and recycled (Wang and Youle, 2009).

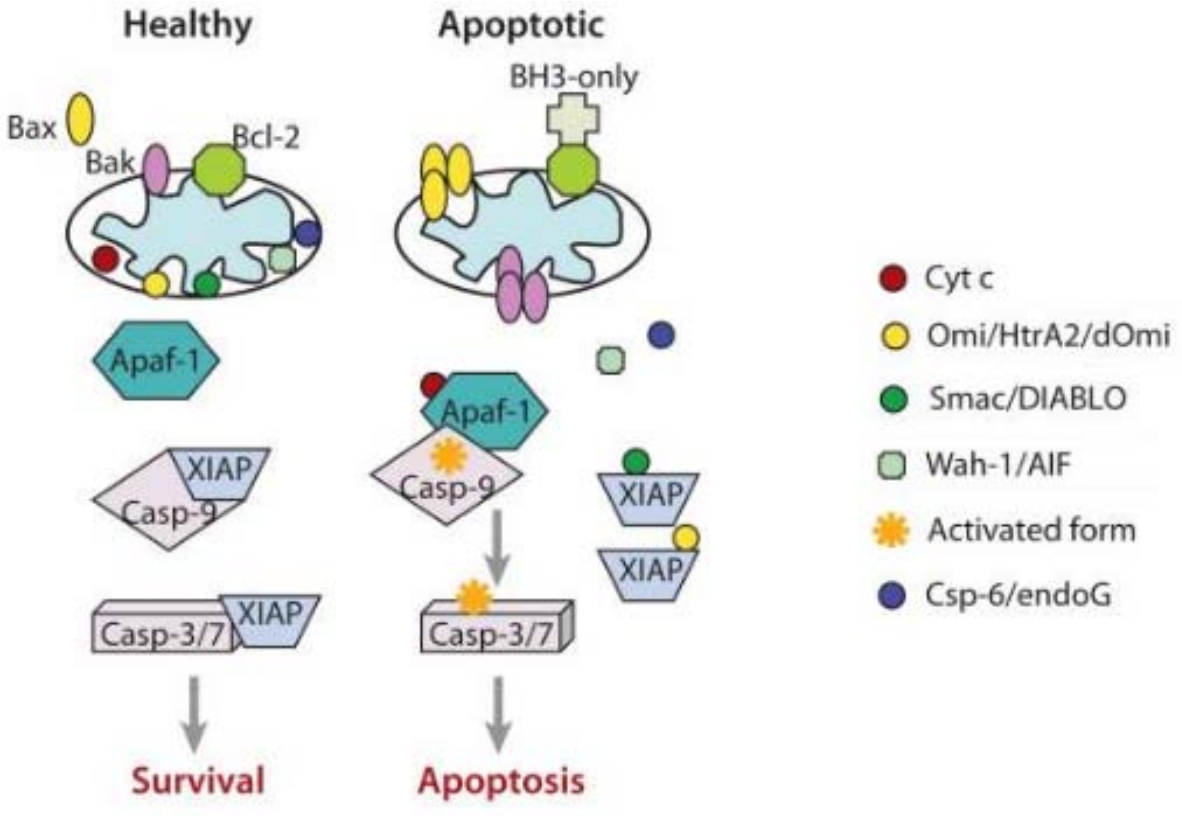

Figure 15. The role of mitochondria in apoptosis. Activation of apoptosis is initiated by the activation or transcriptional upregulation of BH3-only protein. These bind to Bcl-2, prompting relocation of BAX from the cytosol to the mitochondrion. Oligomerisation of pro-apoptotic effectors, BAK and BAX, mediate the formation of VDAC1 mega-pores in the outer mitochondrial membrane and release of smac/DIABLO and cytochrome *c* (Mader *et al.*, 2010). Cytosolic binding of Cyt *c* activates APAF1, which in turn activates the initiator caspase CASP9, whilst smac/DIABLO represses the apoptosis inhibitor XIAP. Downstream activation of pro-apoptotic effector caspases triggers cell death (Czabotar *et al.*, 2014). APAF1: apoptotic protease-activating factor 1, CASP: caspase; AIF: apoptosis-inducing factor; smac/DIABLO: second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI; cyt *c:* cytochrome *c*; XIAP: X-linked inhibitor of apoptosis protein. Adapted from (Wang and Youle, 2009).

1.2.6 The role of mitochondria in ATP production

With the exception of glycolysis, the initial stage of glucose metabolism that occurs in the cytosol, all other processes of ATP production occur within either the matrix or IMS of the mitochondrion (**Figure 16**). The fact that mitochondria are the site of almost all ATP production has earned them the moniker "powerhouse of the cell".

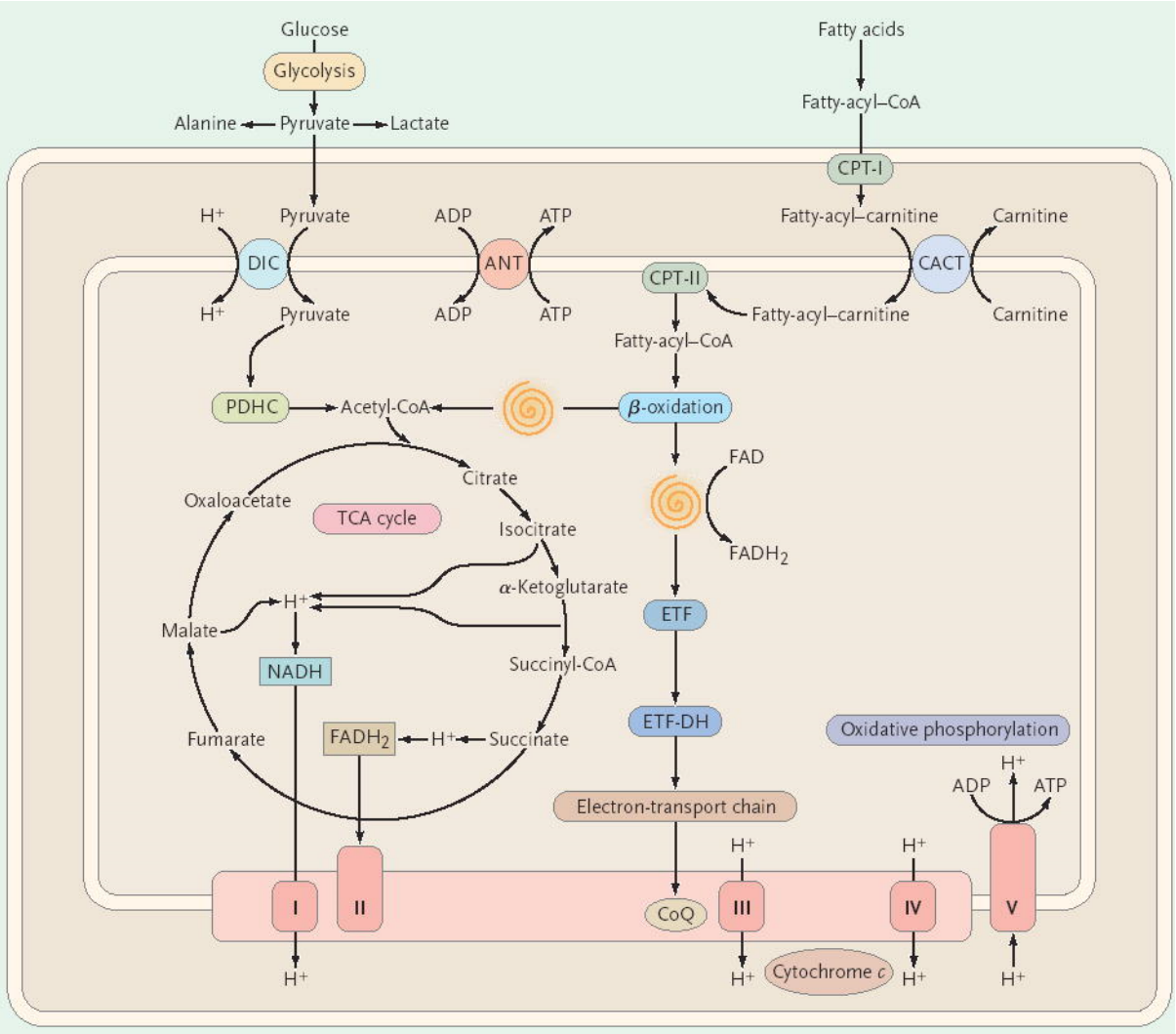

Figure 16. Overview of cellular energy metabolism. During glycolysis, glucose is converted to pyruvate which enters the tricarboxylic acid (TCA) cycle where a series of reactions produces NADH and $FADH_2$. These act as substrates for the mitochondrial respiratory chain. ATP production from dietary fatty acids occurs via the acyl carnitine and β oxidation pathways (DiMauro and Schon, 2003).

1.2.7 Oxidative Phosphorylation

The five multimeric protein complexes, I-V, that comprise the OXPHOS system are responsible for synthesising the vast majority of cellular ATP under aerobic conditions via a series of redox reactions. The substrates, NADH and $FADH_2$, enter the respiratory chain via complexes I and II respectively, and are reduced to NAD/FAD with the electrons passing to the cofactor ubiquinone (Q) for transfer to complex III. The transfer of these high-energy electrons through the complexes results in concomitant pumping of protons by complexes I, III and IV from the matrix across the IMM to the IMS (**Figure 17**). The extrusion of protons from the proton-rich matrix and relocation to the proton-poor IMS creates an electrochemical gradient and proton motive force across the IMM. It is this chemiosmotic attraction that drives ATP synthesis - as protons pass through the half channels of ATP synthase, they drive the molecular motor within to form ATP from ADP and inorganic phosphate. Formulation of the chemiosmotic theory earned Professor Peter Mitchell the Nobel Prize for Chemistry in 1978.

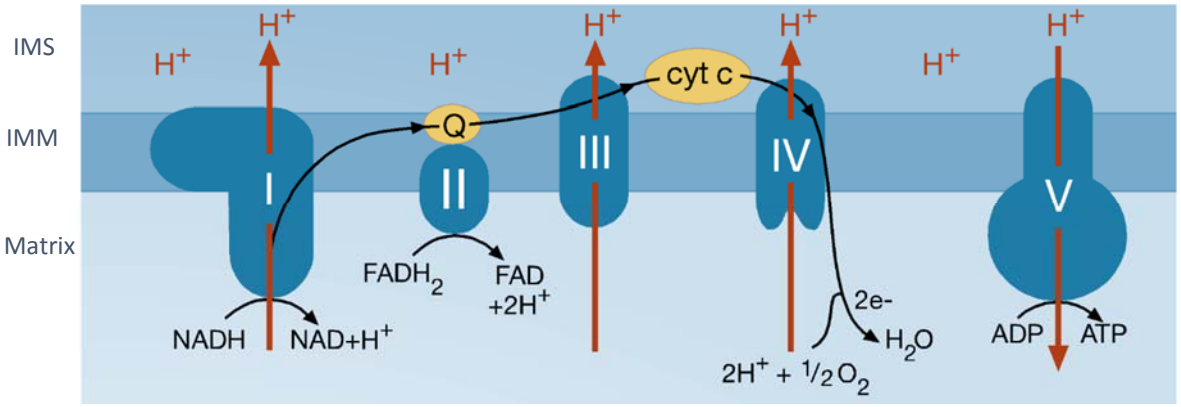

Figure 17. Simple schematic of the OXPHOS complexes, their component subunits and associated ancillary factors. Multimeric protein complexes I-IV shuttle electrons along the respiratory chain, facilitated by the reduction of co-factors Coenzyme Q_{10} (Q) and cytochrome *c* (cyt *c*). Electron transfer is coupled to the transfer of protons (H^+) across the inner mitochondrial membrane to generate a proton motive force which is used by complex V (ATP synthase) to synthesise ATP (adapted from (Alston *et al.*, 2017))**.**

The OXPHOS system has 86 structural subunits, and at least 60 proteins required for either complex assembly or biogenesis (e.g. post-translational modification) (**Figure 18**). The role of the individual complexes in relation to OXPHOS is discussed in the next sections.

	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA structural subunit genes	*MTND1 MTND2 MTND3 MTND4 MTND4L MTND5 MTND6*	-	*MTCYB*	*MTCO1 MTCO2 MTCO3*	*MTATP6 MTATP8*
Nuclear structural subunit genes	*NDUFAB1 NDUFS1 NDUFS2 NDUFS3 NDUFS4 NDUFS5 NDUFS6 NDUFS7 NDUFS8 NDUFA1 NDUFA2 NDUFA3 NDUFA5 NDUFA6 NDUFA7 NDUFA8 NDUFA9 NDUFA10 NDUFA11 NDUFA12 NDUFA13 NDUFB1 NDUFB2 NDUFB3 NDUFB4 NDUFB5 NDUFB6 NDUFB7 NDUFB8 NDUFB9 NDUFB10 NDUFB11 NDUFC1 NDUFC2 NDUFV1 NDUFV2 NDUFV3*	*SDHA SDHB SDHC SDHD*	*UQCRB UQCRC1 CYC1 UQCRC2 UQCRFS1 UQCRH UQCRQ UQCR10 UQCR11*	*COX4 COX5A COX5B COX6A COX6B COX6C COX7A COX7B COX7C COX8*	*ATP5A1 ATP5B ATP5C1 ATP5D ATP5E ATP5F1 ATP5G1 ATP5G2 ATP5G3 ATP5H ATP5I ATP5O ATP5J ATP5J2 ATP5L ATP5L2*
Assembly factor and ancillary protein Genes	*NDUFAF1 NDUFAF2 NDUFAF3 NDUFAF4 NDUFAF5 NDUFAF6 NDUFAF7 FOXRED1 ACAD9 ECSIT NUBPL TMEM126B TIMMDC1 C17orf89 DMAC1 ATP5SL TMEM186*	*SDHAF1 SDHAF2 SDHAF3 SDHAF4*	*BCS1L LYRM7 UQCC1 UQCC2 UQCC3 TTC19 PTCD2*	*COA1 COA3 COA4 COA5 COA6 COA7 COX10 COX11 COX14 COX15 COX16 COX17 COX18 COX19 COX20 SCO1 SCO2 SURF1 PET117 LRPPRC PET100 CEP89 TACO1 OXA1L APOPT1 NDUFA4 FASTKD2*	*ATPAF1 ATPAF2 TMEM70*

Figure 18. Schematic of all known genes encoding OXPHOS subunits and ancillary factors. Characterisation of OXPHOS complexes has identified the constitutive subunits that are either mtDNA or nuclear-encoded, and many of the nuclear-encoded proteins involved in complex assembly, biogenesis or ancillary function. Adapted from (Alston *et al.*, 2017)

1.2.8 Mitochondrial respiratory chain complex I

The ~1MDa complex I (NADH:ubiquinone oxidoreductase) holoenzyme is the first and largest multimeric complex in the respiratory chain. Early electron microscopy (EM) studies revealed that complex I adopts an L-shaped conformation, possessing an inner membrane-embedded arm and a hydrophilic peripheral arm that protrudes into the matrix (Grigorieff, 1998). Complex I comprises three functional modules, denoted N, Q and P, which are assembled in a stepwise fashion from 45 structural subunits. 43 subunits are present in a 1:1 stoichiometry, whilst NDUFAB1 is present in a 2:1 stoichiometry (Stroud *et al.*, 2016). At least sixteen proteins have been implicated in complex I assembly or biogenesis (Baradaran *et al.*, 2013; Alston *et al.*, 2016a; Floyd *et al.*, 2016; Stroud *et al.*, 2016; Zhu *et al.*, 2016) (**Figure 18**). There are 14 'core' subunits that are conserved between human and bacterial complex I and harbour the machinery required for the conserved complex I functions - NADH oxidation, ubiquinone reduction and proton pumping. The seven hydrophobic mtDNA-encoded subunits all represent core subunits, whilst the remaining seven polypeptides are hydrophilic and nuclear-encoded (Wirth *et al.*, 2016) (**Figure 19**).

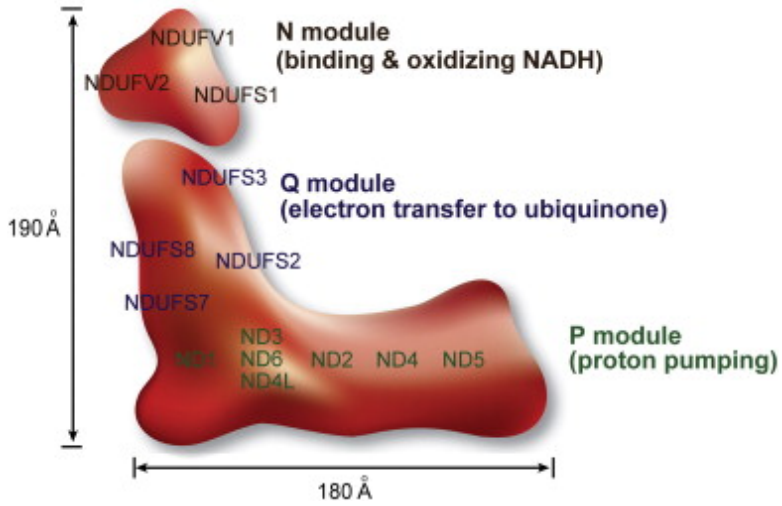

Figure 19. Schematic of complex I structure detailing the three functional modules and the location of the 14 conserved core subunits. The P module is embedded in the inner mitochondrial membrane and contains the seven mtDNA encoded subunits (ND1-6). The Q and N modules protrude into the matrix and perform the functions of ubiquinone reduction and NADH oxidation, respectively. Figure taken from (Mimaki *et al.*, 2012).

The residual 30 mammalian 'supernumerary' or 'accessory' subunits have been evolutionarily acquired and, despite their absence in lower taxa, 25 of these have been shown to be critical for OXPHOS function through CRISPR/Cas9 and TALEN knock out (Stroud *et al.*, 2016). Only NDUFV3, NDUFA7, NDUFA12, NDUFS4 and NDUFS6-deficient cell lines were able to survive when grown on galactose, suggesting their capacity for OXPHOS remained intact (Stroud *et al.*, 2016). These data, together with the resolved crystal structure of mammalian complex I, provide a clear picture of complex I architecture and has highlighted the crucial involvement of the supernumerary subunits in its structure and function.

1.2.8.1 Assembly of complex I

The latest model of complex I assembly harnessed complexome profiling of complex I assembly intermediates, obtained at various time points following chloramphenicol treatment to inhibit mitochondrial protein synthesis. This permitted elucidation of the proteins within each assembly intermediate, and characterisation of the assembly steps from modules Q, proximal P (a)/(b)-, distal P (a)/(b) and N (Guerrero-Castillo *et al.*, 2017) (**Figure 20**).

The Q module is composed of the Fe-S containing subunits NDUFS7 and NDUFS8, two additional 'core' subunits (NDUFS2, NDUFS3) and the NDUFA5 'accessory' subunit. This module is the first to be assembled (Zhu *et al.*, 2016; Guerrero-Castillo *et al.*, 2017). With cooperation of assembly factors TIMMDC1, NDUFAF3 and NDUFAF4 the mtDNA-encoded ND1 protein is inserted as the proximal P(a) module starts to form. NDUFA13, NDUFA8 and NDUFA3 are incorporated next, followed by NDUFA9 and NDUFA1 chaperoned by NDUFAF2 which completes the Q/P(a) subassembly.

The remaining components of the proximal P(b) and distal P(a)/(b) module form separately. Assembly factors NDUFAF1, ECSIT, ACAD9 and COA1 mediate NDUFC1, NDUFC2 and ND2 during the early stages of proximal P(b) module assembly. ND3 chaperoned by TMEM186 and TMEM126B; ND6 and ND4L are incorporated last (Guerrero-Castillo *et al.*, 2017). Early assembly stages of the distal P(a) arm involve NDUFB5, NDUFB10 and NDUFB11 conjugation, followed by NDUFB6. Later, TMEM70, ATP5SL and FOXRED1 modulate incorporation of ND4 and NDUFB1 (Guerrero-Castillo *et al.*, 2017). NDUFB4, NDUFA10 and NDUFS5 assemble onto this distal P arm scaffold.

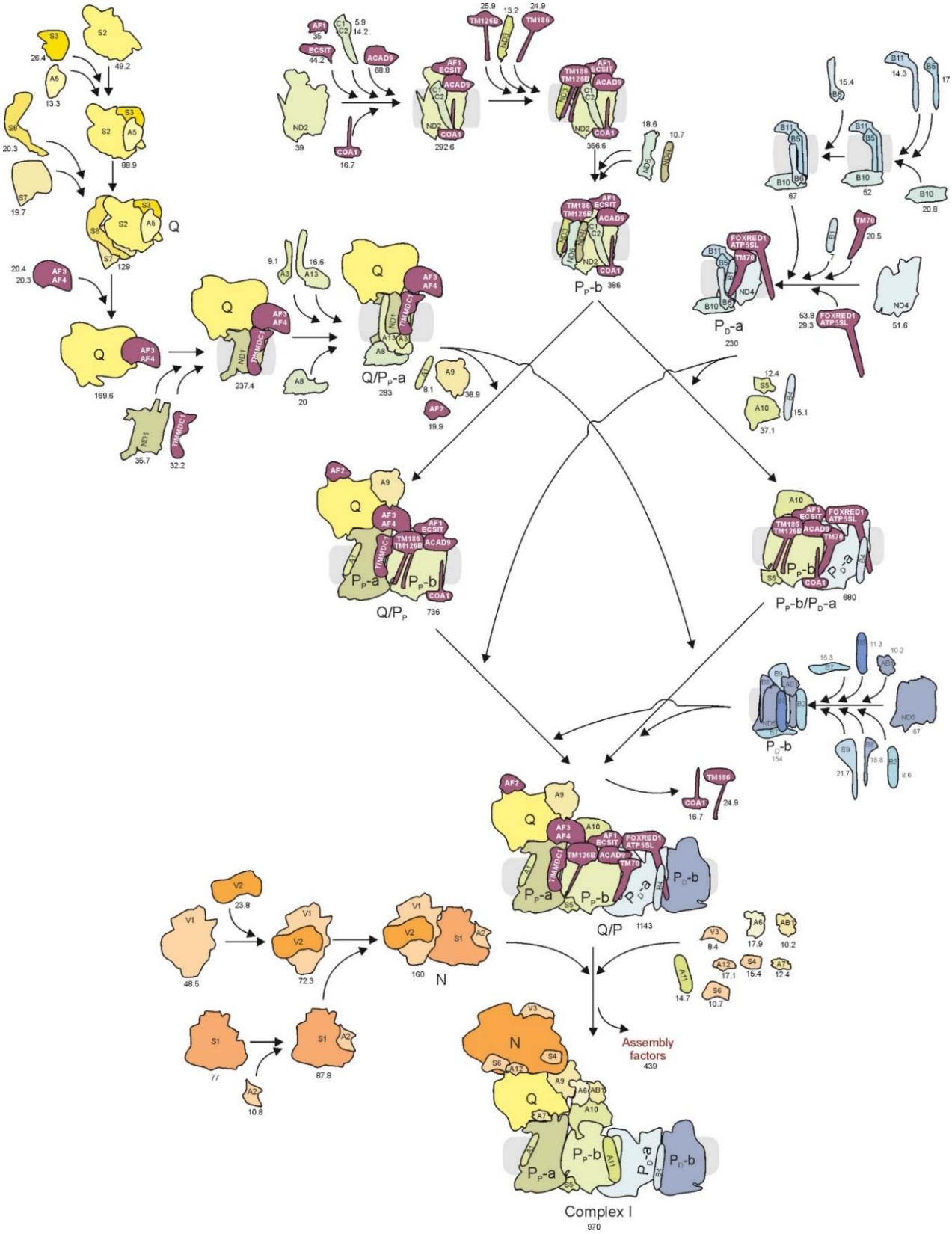

Figure 20. Comprehensive model of complex I assembly. Complexome profiling data supported the assembly of Q module (yellow), then proximal P module (pale green and pale blue), then distal P module (darker blue), with N module (orange) incorporation occurring last. Assembly factors are shaded burgundy. Subunit names have been abbreviated by omission of the leading "NDUF" (Guerrero-Castillo *et al.*, 2017).

The most distal P(b) module subunits NDUFB2, NDUFB3, NDUFB7, NDUFB8, NDUFB9, NDUFAB1 and ND5 form a submodule without clear cooperation from assembly factors (Guerrero-Castillo *et al.*, 2017).

The penultimate stage of complex I assembly involves the formation of a 1.5MDa subassembly from the aforementioned Q- and P- modules. This involves and incorporates all known assembly factors with the exception of COA1 and TMEM186 which are released.

An N module sub-module assembled from NDUFV1, NDUFV2, NDUFS1 and NDUFA2 is incorporated into during the final assembly stage, in conjunction with NDUFV3, NDUFA12, NDUFS6, NDUFS4, NDUFA6, NDUFA7 and the second NDUFAB1 subunit. The final dissociation of the remaining assembly factors gives rise to the mature 1MDa complex I holoenzyme (Guerrero-Castillo *et al.*, 2017).

TMEM186 and ATP5SL were recently identified by two independent groups (Stroud *et al.*, 2016; Guerrero-Castillo *et al.*, 2017), along with DMAC1 (TMEM261) which although absent from this model is postulated to interact with ND5 (Stroud *et al.*, 2016). The involvement of COA1 and TMEM70 in complex I assembly are unique to this latest model, but have been implicated in other pathways, COA1 is a complex IV biogenesis factor (Pierrel *et al.*, 2007) whilst TMEM70 is involved in complex V biogenesis (Cizkova *et al.*, 2008). Their identification in alternative pathways is evidence of either a more general function, as is the case for ACAD9 that is also involved in fatty acid β-oxidation (Nouws *et al.*, 2014a). Some recently identified ancillary factors have only partially characterised roles, e.g. *C17orf89* (Floyd *et al.*, 2016), and their absence in this model infers that their role could involve post-translational modification of another subunit, as is the case for NDUFAF7 (also absent from this model) that methylates p.Arg85 of NDUFS2 (Rhein *et al.*, 2013).

1.2.8.2 Function of the complex I domains

The N module is comprised from three "core" subunits - the Fe-S cluster-containing NDUFS1 protein, the flavoproteins NDUFV1 and NDUFV2, 7 "accessory" proteins and one of the two NDUFAB1 subunits (Stroud *et al.*, 2016; Guerrero-Castillo *et al.*, 2017) (**Figure 21** and **Figure 22**). It is the site of NADH oxidation, harbouring a flavin mononucleotide (FMN) moiety that binds within NDUFV1 (Varghese *et al.*, 2015) and acts as the primary electron acceptor to catalyse the reaction:

$$NADH + H^{+} + FMN <=> NAD^{+} + FMNH_2$$

The two electrons from $FMNH_2$ pass down the seven Fe-S clusters within the N module (NDUFS1, NDUFV1, NDUFV2) and Q module (NDUFS7 and NDUFS8) Fe-S proteins, each Fe-S cluster has an increased reduction potential thereby facilitating electron transfer through the complex before transfer to ubiquinone (CoQ_{10}) from the terminal (N2) Fe-S cluster within NDUFS7 at the Q module (**Figure 21**).

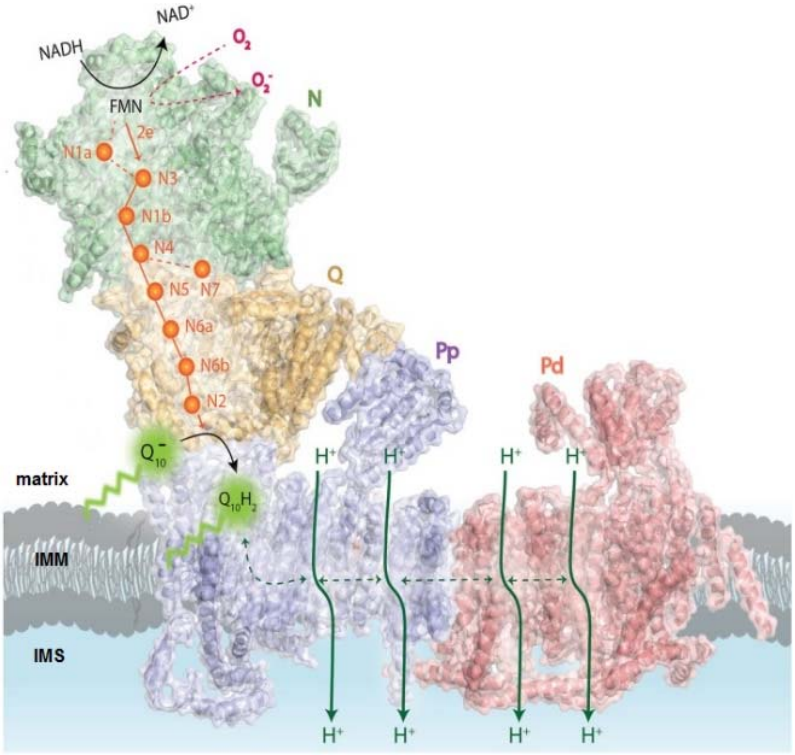

Figure 21. Overall schematic of complex I functional domains. N module (green), Q module (yellow) and P module (proximal: violet and distal: salmon). Green arrows show proposed sites of proton pumping whilst shaded green circles indicate the ubiquinone reduction site. The Fe-S clusters are shown as orange spheres and the site of ROS production is shown in red. Image adapted from (Giachin *et al.*, 2016).

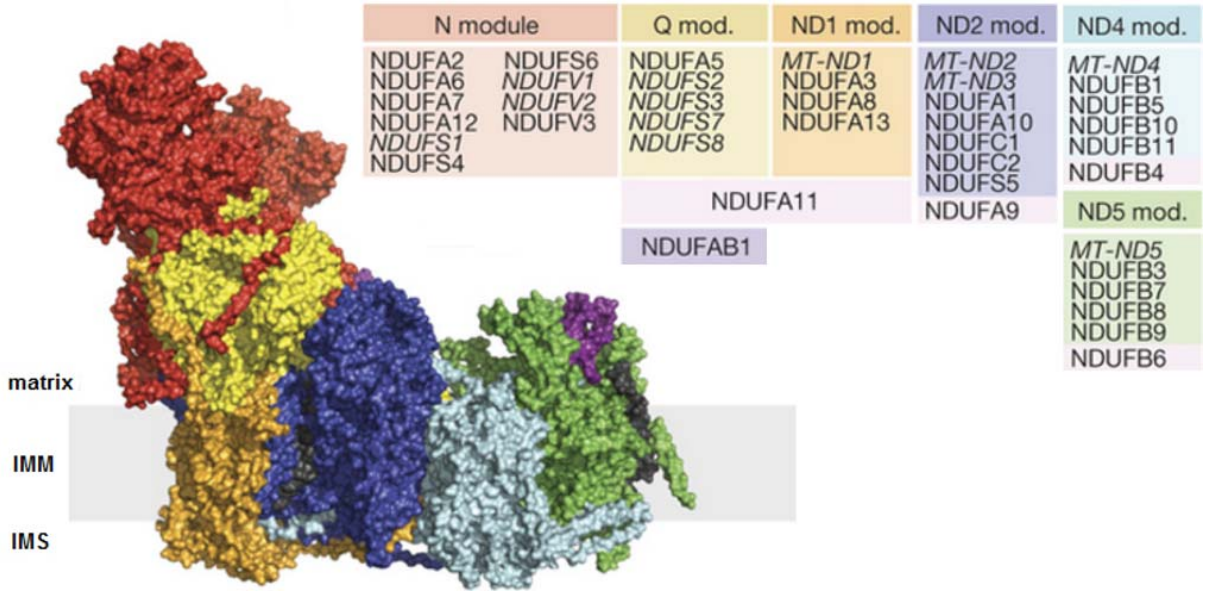

Figure 22. Complex I functional domain constituents. Protein components of the individual complex I functional domains are listed and mapped onto the current crystal structure of complex I; N module (red), Q module (yellow) and proximal P module (ND1/ND2 modules, orange and indigo) and distal P module (ND4/ND5 modules, cyan and green) (Stroud *et al.*, 2016).

A pocket between the NDUFS7 and NDUFS8 subunits of the Q module is the proposed site of ubiquinone binding and its reduction to ubiquinol (Angerer *et al.*, 2012). Once reduced, ubiquinol (QH_2) can diffuse within the IMM thereby facilitating electron transfer to the subsequent electron acceptor within complex III. The reduction of Q to QH_2 is coupled to the translocation of two protons from the mitochondrial matrix into the intermembrane space (Ripple *et al.*, 2013), therefore the two electrons donated per molecule of NADH oxidised contribute four protons to the electrochemical gradient ($\Delta\Psi$) via complex I (**Figure 21**).

The membrane embedded P module harbours 78 transmembrane helices with a central axis of polar residues that run from the Q binding site to the distal part of the P arm and are proposed to be important for proton translocation (Fiedorczuk *et al.*, 2016). The transmembrane helices create conformational shifts within the domain in response to electron transfer from N2 to ubiquinone; this drives proton translocation from the matrix, through the channels within the three antiporter like subunits (ND2, ND4 and ND5) and into the IMS (**Figure 23**) (Baradaran *et al.*, 2013; Fiedorczuk *et al.*, 2016).

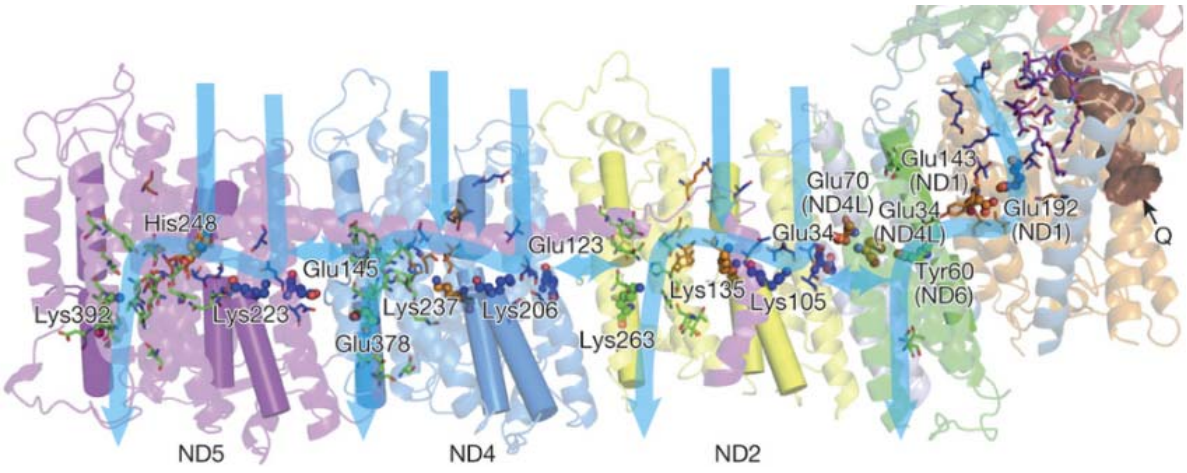

Figure 23. Proposed proton translocation mechanism involving the core subunits of ovine complex I. The polar residues that form the central axis are shown as stick models, whilst blue arrows denote proposed proton translocation pathways. The key residues within transmembrane helices 5, 7, 8 and 12 of antiporter subunits ND2, ND4 and ND5 are labelled. Image taken from (Fiedorczuk *et al.*, 2016).

Complex I is the first of three OXPHOS complexes that contribute to the proton concentration gradient across the inner mitochondrial membrane that creates a proton motive force for ATP synthase function.

1.2.9 Mitochondrial respiratory chain complex II

Complex II (succinate:ubiquinone oxidoreductase) is ~260kDa in size, comprised from 4 nuclear encoded subunits - the soluble catalytic flavoprotein (Fp, SDHA) and iron sulfur (Ip, SDHB) subunits protrude into the mitochondrial matrix and are bound to the inner mitochondrial membrane by anchoring subunits SDHC and SDHD (Sun *et al.*, 2005) (**Figure 24**). Complex II has dual roles in ATP synthesis - the reversible oxidoreduction of succinate and fumarate within the tricarboxylic cycle and the reversible oxidoreduction of ubiquinone and ubiquinol within the respiratory chain (Hagerhall, 1997; Sun *et al.*, 2005). Succinate dehydrogenase catalyses succinate oxidation with the reverse, fumarate reduction to succinate, is catalysed by fumarate reductase; the reversible reaction is therefore:

succinate + FAD <=> fumarate + $FADH_2$

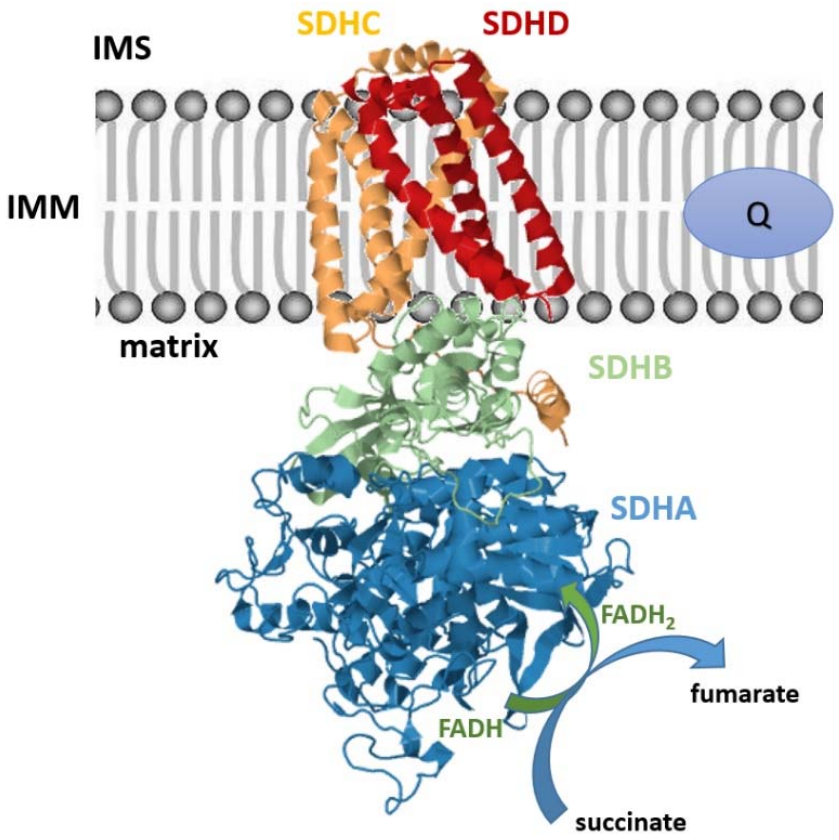

Figure 24. Structure of complex II. SDHA (blue), SDHB (green) SDHC (yellow) and SDHD (red). Electron carrier ubiquinone (Q) accept electrons from $FADH_2$. Schematic created using porcine SDH crystal structure (PDB: 1ZOY).

As the only complex that links the TCA cycle and the respiratory chain, complex II has rate limiting potential and fumarate reduction by succinate dehydrogenase is under the control of the redox potential; a redox potential >-60mV is associated with a rapid rate of fumarate reduction, whilst the catalytic rate slows between -60 and -80 mV (Cecchini, 2003) and is proposed to allow control of the metabolic rate (Sucheta *et al.*, 1992).

SDHA harbours a covalently bound cofactor, flavin adenine dinucleotide (FAD), which is covalently bound to p.His99 within human SDHA (homologous to p.His44 of *E. coli* (Blaut *et al.*, 1989)) via the action of ancillary factor SDHAF2 (Maklashina *et al.*, 2016). FAD acts as the initial acceptor for electrons donated by substrate $FADH_2$, which then pass sequentially down the three Fe-S clusters within SDHB to the Q binding site between the anchoring subunits SDHC and SDHD (Lee *et al.*, 1995) where it reduces Q to form QH_2.

A heme *b* moiety is embedded at the subunit interface between SDHC and SDHD that each harbour a binding histidine residue; this prosthetic group has a low redox potential, attributed in part to the surrounding polar residues and whilst is not involved in electron transfer (Maklashina *et al.*, 2001), it may act as an antioxidant by neutralising reactive oxygen species (Yankovskaya *et al.*, 2003).

1.2.10 Mitochondrial respiratory chain complex III

Complex III (ubiquinol-cytochrome *c* oxidoreductase) is the third enzyme of the mitochondrial respiratory chain. The holoenzyme is ~480kDa in size, comprised from 11 subunits; our current knowledge of the structural subunits and ancillary factors for each complex is summarised in **Figure 18**. The catalytic centres are harboured within cytochrome *b* (*MTCYB*), cytochrome c_1 (*CYC1*) and the Rieske protein (*UQCRFS1*) plus two functional heme moieties - cytochrome b_L (b_{566}) and cytochrome b_H (b_{562}). The crystal structure of complex III has been resolved (**Figure 25**) and its role is to transfer protons from the matrix into the IMS, thereby contributing to the proton motive force, whilst transferring electrons from ubiquinol to cytochrome *c* via the Q cycle in parallel (Mitchell, 1975) (**Figure 26**).

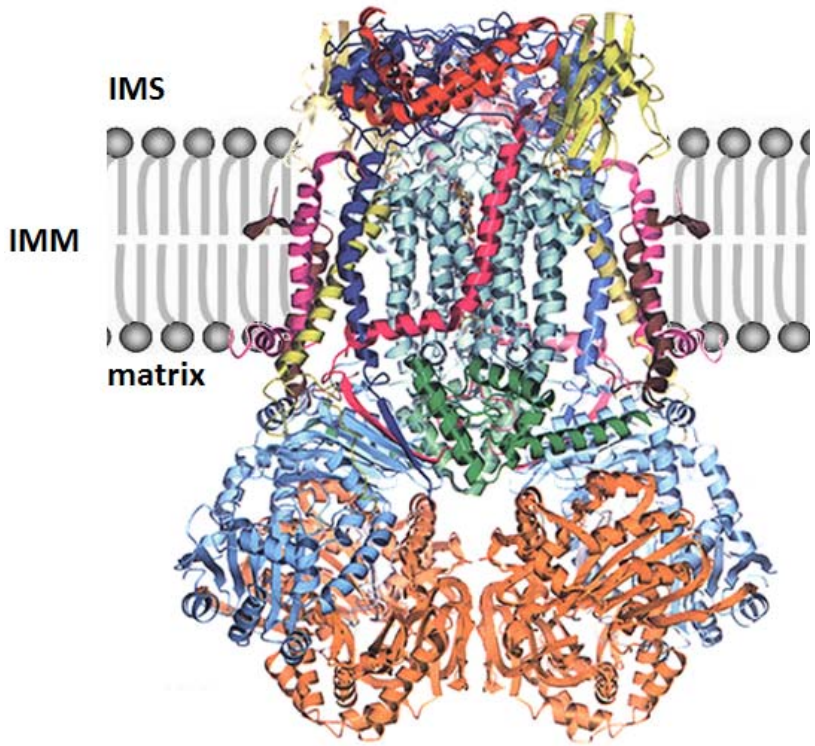

Figure 25. Crystal structure of mammalian complex III. The 11 structural subunits are colour coded: Core 1 (blue), Core 2 (orange), cytochrome *b* (cyan), Cytochrome c1 (dark purple), Rieske iron-sulfur protein (yellow), hinge protein (green), subunit 7 (dark pink), ubiquinone-binding protein (red), subunit 9 (peach), subunit 10 (burgundy), subunit 11(pink). Figure adapted from (Yu *et al.*, 1999).

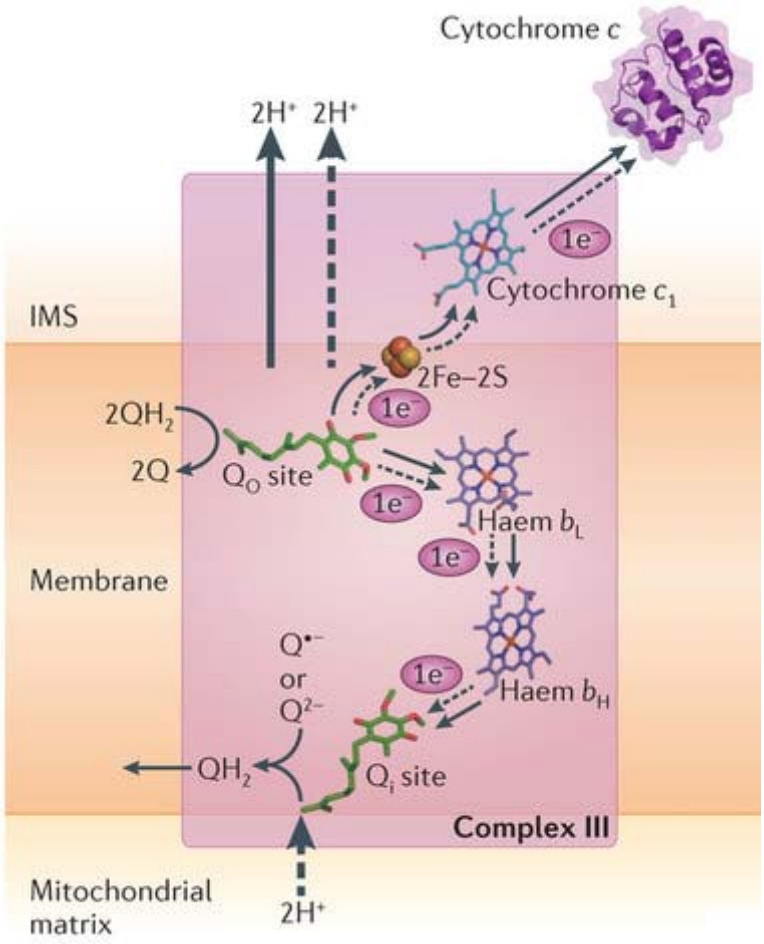

Figure 26. Schematic of the two stages of the Q cycle. A. The first turn of the Q cycle (electron and proton movement shown by solid arrows) pumps two protons to the IMS following oxidation of QH_2. One electron reduces cytochrome *c* via the Fe-S cluster of the Rieske subunit and the heme group within cytochrome c_1. The second electron binds to the haem group within heme b_L and heme b_H, producing a semiquinone radical, $Q^{-\cdot}$. **B.** The second turn of the Q cycle (dashed arrows) again involves binding of QH_2, release of two protons to the IMS, transfer of 1 electron to reduce cytochrome *c* via the Rieske Fe-S cluster and the heme group within cytochrome c_1. The one cycling electron released to the two heme *b* moieties fully reduces the semiquinone radical to form QH_2 and in doing so removes an additional two protons from the matrix. Taken from (Sazanov, 2015).

The reduced form of Q (ubiquinol or QH_2), produced following NADH oxidation by complex I, or $FADH_2$ oxidation by complex II, binds to the Q_o site within complex III via the p.His182 residue of the Rieske subunit and p.Glu272 of cytochrome *b* (Kessl *et al.*, 2003). The original QH_2 is fully oxidised following release of its protons and electrons, and diffuses away from the Q_0 site; the two protons released from QH_2 are pumped into the IMS and its two electrons transfer to prosthetic groups within complex III. One electron is accepted by the Fe-S cluster within the Rieske protein whilst the second cycling electron, is accepted by the heme moiety within cytochrome *b* and transfers to a second Q molecule situated at the Q_1 site to form a semiquinone radical $Q^{-\cdot}$. A second turn of the Q cycle using a second QH_2 bound to the Q_0 site generates an additional reduced cytochrome *c*, translocates a further two protons to the IMS and transfers a second cycling electron to the semiquinone radical $Q^{-\cdot}$ to form a stable, fully-reduced QH_2 molecule. This is released and can be recycled by binding at the Q_0 site, and in addition to preventing loss of an electron, removes two protons from the matrix, thus contributing to the electrochemical gradient ($\Delta\Psi$) across the IMM (Mitchell, 1975).

Cytochrome *c* is a heme-containing protein (encoded by *CYCS*) that acts as the electron carrier between cytochrome *b* within complex III to complex IV in the respiratory chain. Cytochrome *c* biogenesis involves the post-translation modifications by holocytochrome c synthase (HCCS) that covalently bonds a heme moiety to a conserved CXXCH motif within the cytochrome *c* protein (Babbitt *et al.*, 2014). In addition to its role in electron transfer, the release of cytochrome *c* from the mitochondrion acts as the initial step of apoptosis (Manickam *et al.*, 2016).

1.2.11 Mitochondrial respiratory chain complex IV

Complex IV of the respiratory chain, cytochrome *c* oxidase (COX), represents the final enzyme complex of the electron transfer chain. The crystal structure of the ~200kDa holoenzyme has been resolved to 2.9 angstrom (**Figure 27**) (Yoshikawa *et al.*, 1998), revealing the interaction of the 13 structural subunits (Tsukihara *et al.*, 1996); at least 26 additional proteins are involved in complex IV assembly and biogenesis (Rak *et al.*, 2016). Complex IV functions as a dimer embedded within the IMM and the catalytic core is composed of COX1, COX2 and COX3; these harbour the redox prosthetic groups - two copper molecules (Cu_A and Cu_B), two heme groups (a and a_3), one magnesium ion and one zinc ion (Tsukihara *et al.*, 1995).

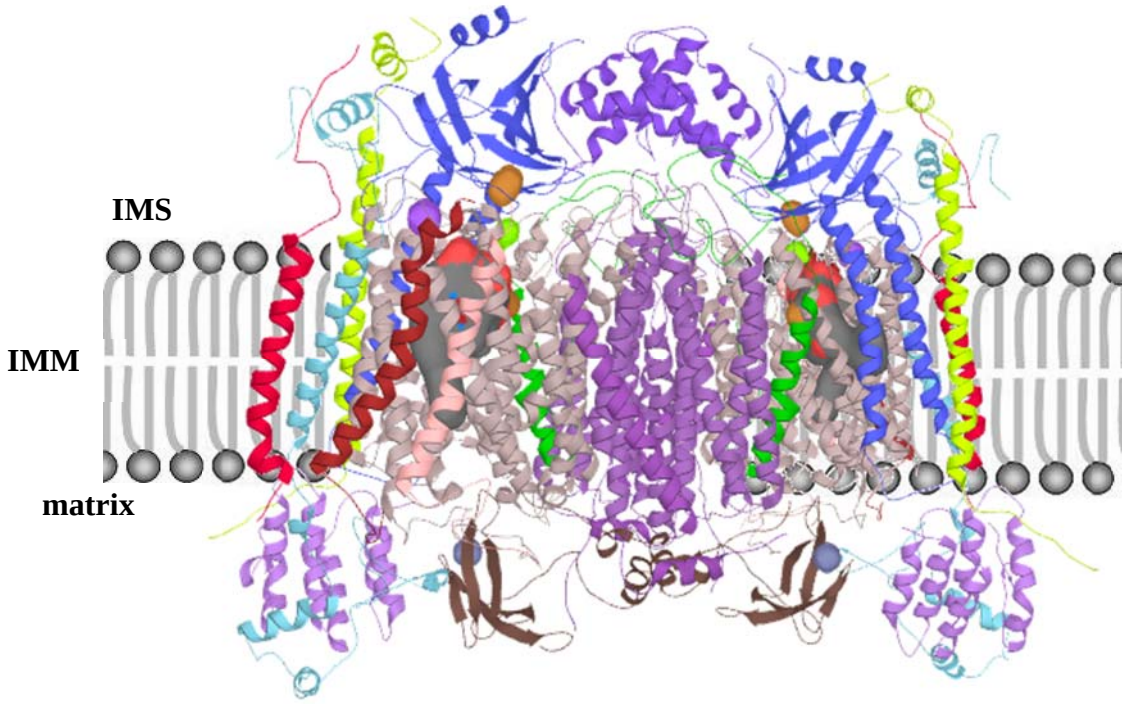

Figure 27. Crystal structure of mammalian complex IV. The 13 subunits are colour coded: Beige: COX1, **dark blue**: COX2, **mid purple**: COX3; **sky blue**: COX4, **lilac:** COX5A; **brown** COX5B; **green** COX6A2; **violet**: COX6B1; **yellow**: COX6C; **dark purple**: COX7A1, **red**: COX7B, **dark red**: COX8B, pink: COX7C. Figure made using PDB: 2OCC.

Following reduction by complex III, the single electron carrier cytochrome *c* binds to complex IV and is oxidised to release its electron which passes to the initial electron acceptor, the copper moiety Cu_A. The electron transfers initially to heme *a*, before transferring to the heme a_2 and Cu_B complex which forms a binuclear centre capable of binding molecular oxygen. Following four repeats of this process to oxidise four cytochrome *c* molecules, this reaction produces two water molecules (**Figure 28**). Recent improvements to the crystal structure resolution has identified a large water cluster, including the Mg^{2+} ion, that provides sufficient capacity to store matrix-derived protons prior to terminal oxygen reduction (Yano *et al.*, 2016). Concomitant to the removal of each proton from the matrix for oxygen reduction, a second proton is proposed to be concurrently extracted from the matrix proton pool; this is translocated into the IMS to contribute to the ΔΨ (Belevich *et al.*, 2006).

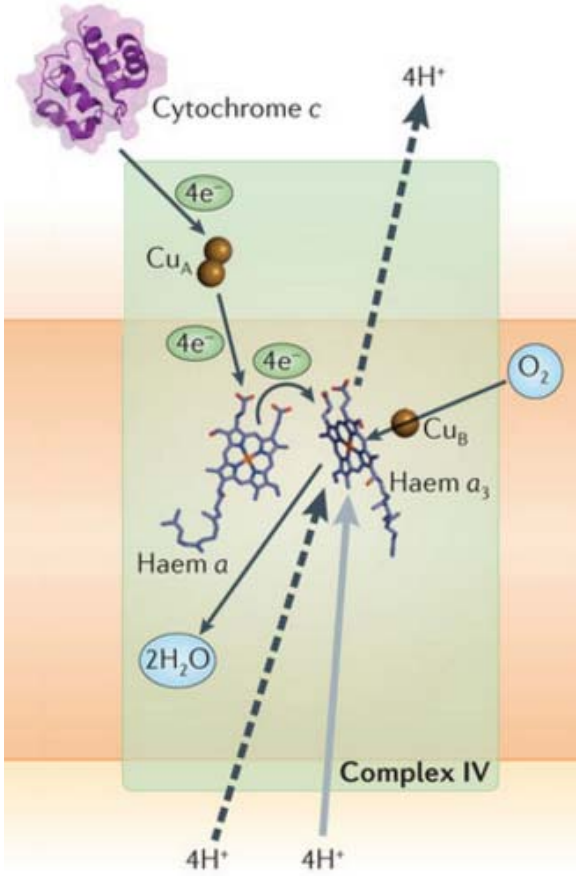

Figure 28. Schematic of cytochrome c oxidase function. The overall reaction (solid arrows) catalyses the production of two water molecules ($2H_2O$) from oxidation of four cytochrome *c* molecules plus protons from the matrix. Protons are removed from the matrix for either oxygen reduction or translocation to the IMS, contributing to the ΔΨ (grey and dashed arrows, respectively). Figure adapted from (Sazanov, 2015).

1.2.12 Mitochondrial respiratory chain complex V

Complex V, ATP synthase, is a ~600kDa complex comprising 13 structural subunits and involves at least 3 additional proteins for biogenesis. The F_1-F_0 holoenzyme consists of the F_0 subunit embedded within the IMM, linked to the F_1 subunit that protrudes into the matrix, the two functional units being joined by central and peripheral stalks (**Figure 29A**). The resolution of its crystal structure (Abrahams *et al.*, 1994; Stock *et al.*, 1999) was critical for the subsequent confirmation of Boyer's proposed mechanism of ATP synthesis which postulated that a structural change within the enzyme drove ADP phosphorylation to form ATP (Kayalar *et al.*, 1977; Gresser *et al.*, 1982).

The crystal structure reinforced Boyer's hypothesis that complex V functions as a molecular motor, and the proton motive force generated by complexes I, III and IV via the redox reactions of the respiratory chain drives ATP synthesis. Protons use the proton motive force to flow down the potential gradient, through the rotational turbine pore within the membrane-embedded F_0 subunit. The F_0 subunit consists of 12 'c' subunits which form the c ring, plus an 'a' subunit which contains two half channels, spanning either from the matrix or the IMS to the midpoint of the IMM (**Figure 29B**). The molecular rotary machine within the matrix-protruding subunit F_1 is composed of subunits $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$; both α and β subunits harbour ADP and Pi binding although only the β subunits have catalytic activity (Abrahams *et al.*, 1994).

The "binding change mechanism" describes the method of ATP synthesis and release (**Figure 30**). The premise is that each of the three β subunits adopts a different structural conformation (open, loose or tight), and cycles through each of these states in turn; the complete 360° rotation catalyses ATP synthesis in a proton-dependent manner (Abrahams *et al.*, 1994).

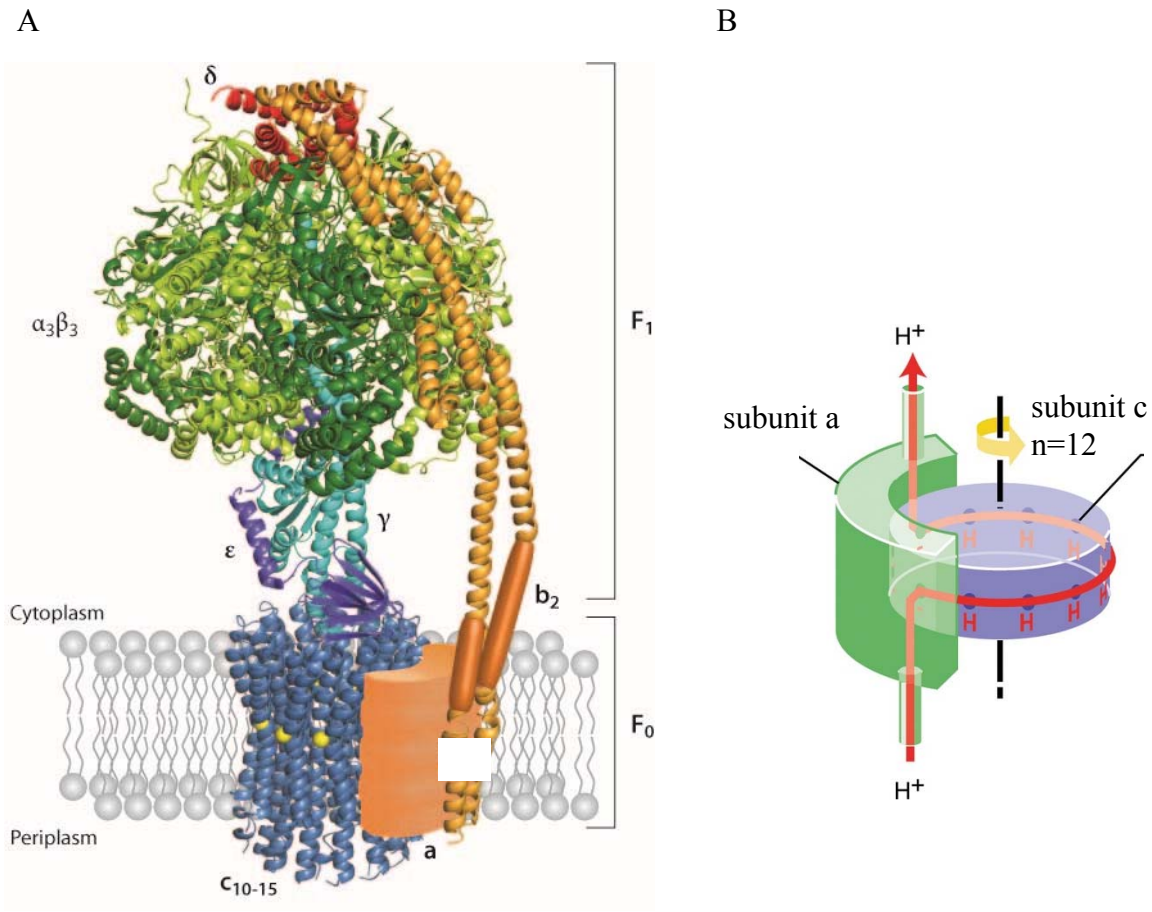

Figure 29. The structure and function of mammalian ATP synthase. A. Schematic incorporating the crystal structure of bovine ATP synthase with subunit 'a' shown as a cartoon for clarity. F_1 subunits: red: δ, green: α/β; turquoise γ, purple: ϵ. F_0 subunits: Peach: a, orange: b; blue: c **B.** The two half channels within subunit 'a' provide the entry point for protons trapped in the IMS. They migrate to the termini and bind to the conserved charged residue at the interface with the 'c' subunit, and upon neutralisation, attempts to migrate by Brownian movement towards the hydrophobic IMM bilayer. This attempted movement (and repetition by subsequent protons entering the system) drives rotation of the c-ring motor, until the proton reaches the second half channel where it is able to enter the matrix upon reionisation of its binding charged residue. Adapted from (Walker, 2013) and (von Ballmoos *et al.*, 2009).

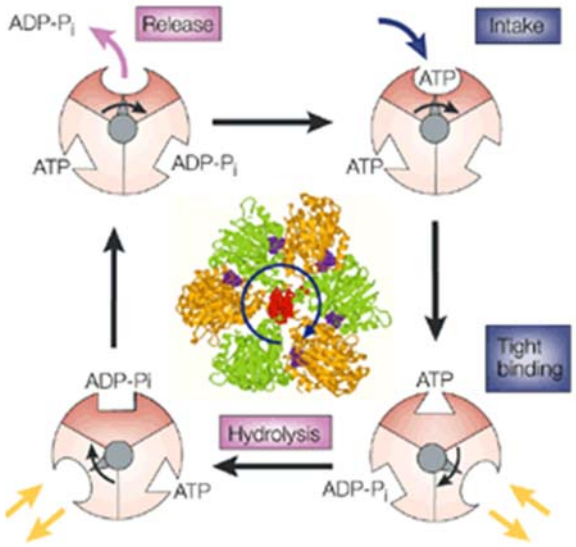

Figure 30. Schematic of the "binding change" mechanism of ATP synthesis by ATP synthase. The asymmetric gamma subunit is shown in red, β subunits are shaded yellow and α subunits are shaded green. The γ subunit is connected to the rotatory F_0 subunit and rotates in turn to confer one of three conformations upon the β subunits, either open, loose or tight. In the first, open conformation, ADP and inorganic phosphate are able to freely move in and out of the accessible β molecule until rotation of the γ subunit 120° anticlockwise causes the β molecule to adopt the loose conformation. In this state, the ADP and Pi is unable to dissipate from the β molecule, but the substrates are insufficiently close to form ATP. A second 120° anticlockwise rotation of the γ subunit causes the β subunit to adopt the tight conformation, during which the ADP undergoes substrate level phosphorylation to form ATP which is released following a third and final 120° anticlockwise rotation which renders the β subunit once again in the open conformation. This permits ATP release into the matrix and entry of ADP and Pi into the β subunit for subsequent cycles of ATP production, according to Boyer's classic model. Adapted from (Yoshida *et al.*, 2001).

1.2.13 Mitochondrial respiratory chain supercomplexes

Although the complexes of the respiratory chain exist and function independently, the electron transport chain requires their cooperation for production of the proton gradient. Moreover, the dynamic nature of mitochondria requires them to be able to respond to external stimuli such as increased energetic demands (Liesa and Shirihai, 2013). There are various theories detailing interactions between the respiratory chain complexes, including the "solid state" theory of mitochondrial complex location within the IMM (Chance and Williams, 1955), suggesting that proximity enhances electron transfer and the polarised "fluid" model that posits that the requirement for proximity is circumvented by mobile electron carriers within the IMM (Hackenbrock *et al.*, 1986). It is apparent that both models hold some merit, but the formation of close proximity respiratory units in the form of the I/III_2 supercomplex and the 1.7MDa I/III_2/IV respirasome (Gu *et al.*, 2016) lends support to the "solid state" and "plasticity" models that facilitate rapid electron transfer and concomitant proton pumping. Evidence from BN-PAGE confirmed a functional respirasome (Acin-Perez *et al.*, 2008), and the crystal structures of mitochondrial supercomplexes have now been reported (**Figure 31**) and reveal vital supercomplex interactions between CI/CIII, CI/CIV and CIII/CIV (Gu *et al.*, 2016; Letts *et al.*, 2016).

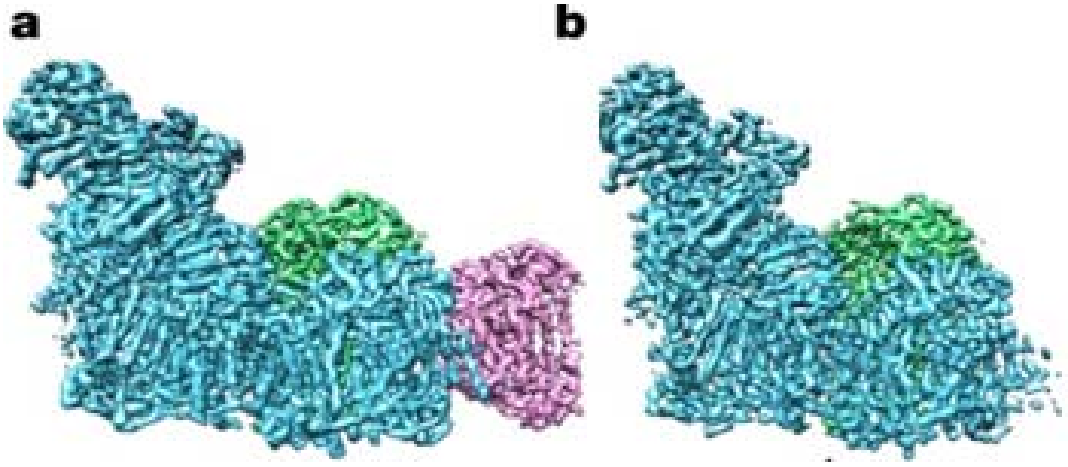

Figure 31. Crystal structures of the ovine supercomplexes. A. The CI/III_2/IV respirasome and **B.** The CI/III_2 supercomplex. Complex I: turquoise; complex IV: pink; complex III: green. Adapted from (Letts *et al.*, 2016).

The reported near-atomic crystal structure of the mitochondrial respiratory chain supercomplexes, together with those of the individual complexes, will be critical for thorough understanding of many mitochondrial disease mechanisms.

1.3 MITOCHONDRIAL DISEASE

1.3.1 Introduction to mitochondrial disease

The umbrella term "mitochondrial disease" refers to a clinically heterogeneous group of primary mitochondrial disorders and the tissues and organs that are most often affected are those with the highest energy demands (**Figure 32**). Symptoms can manifest at any age, and may be isolated or part of a multisystemic syndrome (Lightowlers *et al.*, 2015).

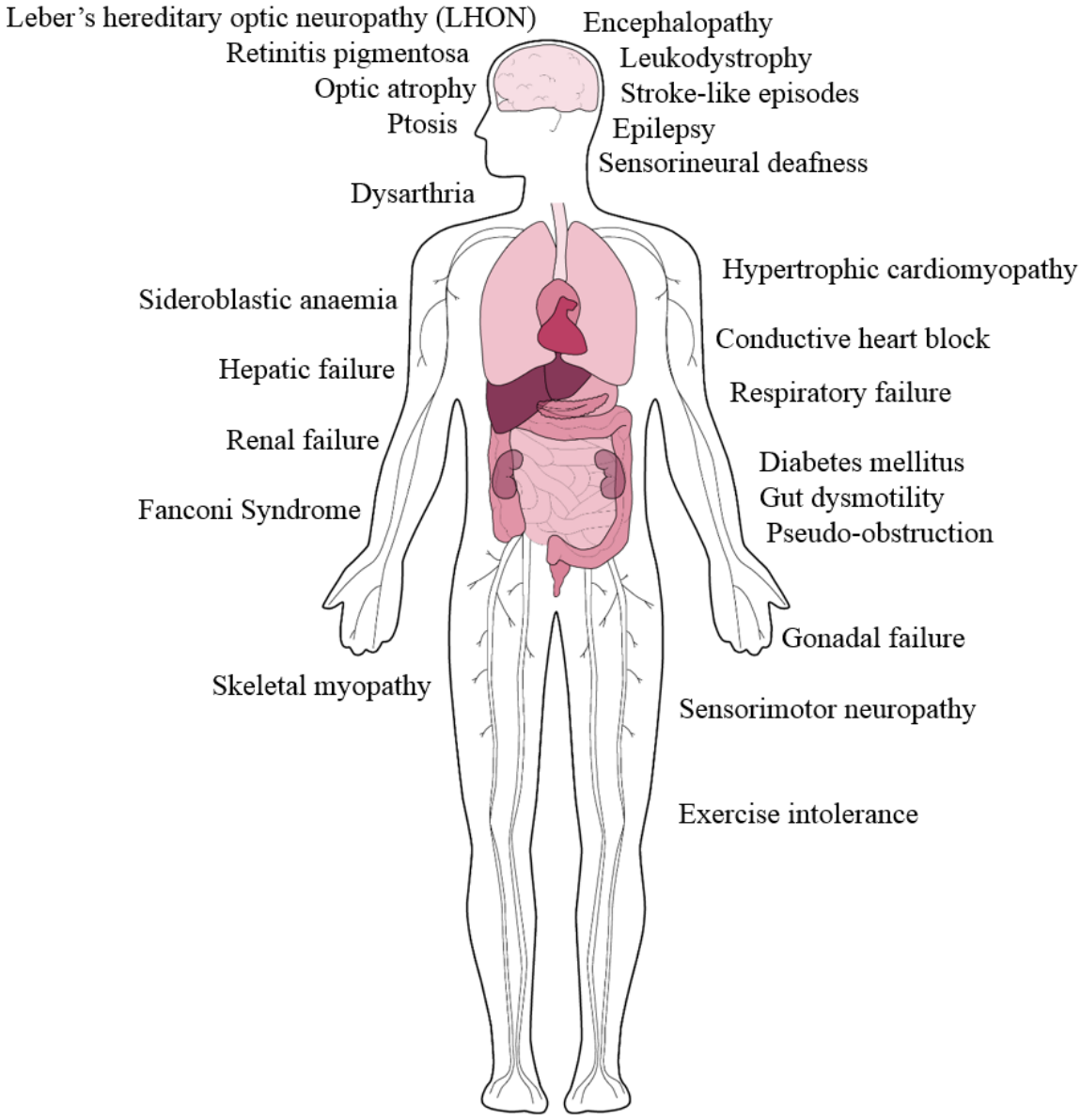

Figure 32. Schematic highlighting the clinical heterogeneity of mitochondrial disease. The commonly affected organs (brain, heart, muscle, liver, kidneys, pancreas and GI tract) and their associated clinical symptoms are shown.

The minimum disease prevalence is approximately 12.5 per 100,000 in adults (Gorman *et al.*, 2015) and 4.7 per 100,000 in children (Skladal *et al.*, 2003). There is no cure for mitochondrial disease, and although some patients have milder presentations than others, the prognosis is often bleak - particularly for clinically-affected children. In the absence of a cure, the provision of reproductive counselling is a vital resource for families affected by inherited mitochondrial disease and the current repertoire includes prenatal genetic diagnosis (chorionic villus biopsy (CVB); amniocentesis; non-invasive screening) or pre-implantation genetic diagnosis during *in vitro* fertilisation procedures. Unfortunately, the general lack of genotype-phenotype correlations in many mitochondrial disorders which together with the genetic heterogeneity associated with the potential candidate gene pool of around 1150 genes means that establishing a genetic diagnosis can be a complicated process and remains elusive for many patients.

1.3.2 The multidisciplinary approach to investigating mitochondrial disease

The laboratory investigation of suspected mitochondrial disease is complex and algorithms employ a multidisciplinary approach which uses clinical and functional studies to guide molecular genetic screening (McFarland *et al.*, 2010). Routine clinical investigations including electrocardiogram (ECG), nerve conduction studies (NCS), blood and urine analysis (Clinical Biochemistry), and magnetic resonance imaging (MRI) can all be suggestive of a mitochondrial aetiology. Individual mitochondrial respiratory chain complex activities can be assessed spectrophotometrically, and identification of an individual complex or combined complex deficiency remains important to highlight relevant candidate genes for molecular analysis (Kirby *et al.*, 2007). Similarly, the histological, histochemical and immunohistochemical analysis of serially-sectioned muscle biopsy samples provides crucial evidence of mitochondrial pathology and can be indicative of a particular pathological mechanism (**Figure 33**) (Rocha *et al.*, 2015). Histochemical and biochemical analysis of patient muscle biopsy has been critical to directing genetic testing but new strategies aim to improve the diagnostic yield. Whilst some symptoms can be treated irrespective of the underlying genetic defect (e.g. surgical correction of ptosis, or seizure control with anticonvulsants), other interventions such as carrier testing of family members and access to reproductive options simply cannot be provided without one.

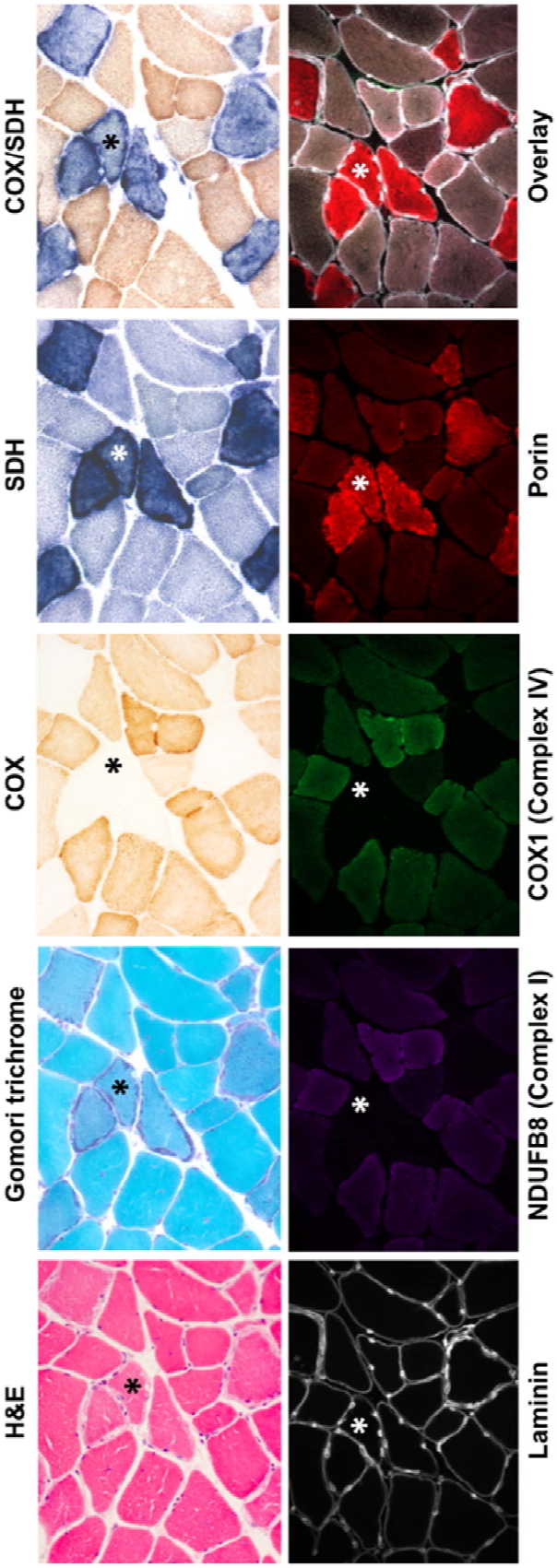

Figure 33. Histochemical and immunohistochemical analysis of serial muscle biopsy sections from a patient with a single, large-scale mtDNA deletion. Histochemical (upper panel) and immunohistochemical (lower panel) assessment of serial muscle biopsy sections reveals abnormal muscle fibres (shown as asterix). Highlighted muscle fibre has normal morphology (H&E/laminin), shows ragged-red fibres (modified Gomori trichrome staining), absence of NDUFB8 (used as a marker of complex I), absence of COX/COX1 (used as a marker for complex IV), increased mitochondrial mass (suggested by increased Porin staining) and increased complex II staining according to histocytochemical SDH activity. The presence of "blue" muscle fibres on serial COX/SDH reacted sections is a hallmark of mitochondrial dysfunction, and together these data suggest a combined respiratory chain enzyme deficiencies involving (at least) complexes I and IV, with preserved complex II activity. Taken from (Alston *et al.*, 2017) and includes data kindly provided by Dr Mariana Rocha.

1.3.3 The genetics of mitochondrial disease

Given the involvement of the mitochondrial and nuclear genomes in the mitoproteome, it follows that mitochondrial dysfunction can result from mtDNA- or nuclear-encoded gene defects. Additionally, they can occur as a primary, congenital condition or a secondary, age-associated effect due to somatic mutation (Greaves *et al.*, 2014).

1.3.4 Mitochondrial-encoded (mtDNA) mitochondrial disease

Unlike nuclear DNA, which is diploid and follows Mendelian laws of inheritance, mitochondrial DNA is exclusively maternally-inherited (Giles *et al.*, 1980). The multicopy nature of mtDNA gives rise to heteroplasmy, a unique aspect of mtDNA-associated genetics, which occurs when there is a mix of mutant and wildtype mtDNA molecules (heteroplasmy) in coexistence. By contrast, homoplasmy occurs when all the mtDNA molecules have the same genotype. Heteroplasmic mutations often have a variable threshold, a level to which the cell can tolerate defective mtDNA molecules (Stewart and Chinnery, 2015). Where the mutation load exceeds this threshold, metabolic dysfunction and associated clinical symptoms occur. Point mutations and large-scale mtDNA deletions represent the two common causes of primary mtDNA disease, both are either maternally inherited or arise *de novo* during embryonic development.

1.3.4.1 mtDNA point mutations

mtDNA point mutations (including small indel mutations) are a significant cause of human disease with estimated population prevalence of 1 in 200 (Chinnery *et al.*, 2012). Over 270 different mtDNA point mutations have been reported, affecting almost every mtDNA-encoded gene and have been associated with clinical symptoms ranging from nonsyndromic sensorineural deafness to MELAS, a devastating syndromic neurological condition whose predominant features **m**itochondrial **e**ncephalopathy, **l**actic **a**cidosis and **s**troke like episodes - give rise to the acronym (Schon *et al.*, 2012). Clinical symptoms can present in child or adulthood, and mutations can be inherited (~75% cases) or occur *de novo* (~25% cases) (Sallevelt *et al.*, 2017). Maternally-transmitted mtDNA defects often involve a clinically-unaffected mother who harbours the familial mtDNA mutation below the threshold required for cellular dysfunction although her oocytes harbour varying mutation loads due to the selection pressures of the mitochondrial bottleneck (Wilson *et al.*, 2016). It is therefore near-impossible

to predict the recurrence risk for subsequent pregnancies although prenatal testing of embryonic tissues using CVB or amniocentesis; this can provide an accurate measure of mtDNA heteroplasmy in the fetus which can inform reproductive choices (Nesbitt *et al.*, 2014). The recurrence risk of *de novo* mtDNA point mutations is very low, but for the risk of germline mosaicism in maternal oocytes (Sallevelt *et al.*, 2017).

1.3.4.2 Single large-scale mtDNA deletions

Single large-scale mtDNA deletions have a population frequency of 1.2/100,000 (Schaefer *et al.*, 2008) with three main associated phenotypes: chronic progressive external ophthalmoplegia (CPEO) (~65% cases), Kearns Sayre Syndrome (~30% cases) and Pearson syndrome (<5% cases) (Mancuso *et al.*, 2015). Pearson syndrome is the most severe presentation associated with single large scale mtDNA deletions; patients present early in life with sideroblastic anaemia and pancreatic dysfunction and is often fatal in infancy (Rotig *et al.*, 1990). Kearns Sayre syndrome patients present before age 20 years with ptosis and/or PEO and pigmentary retinopathy and may have multisystem involvement including myopathy, ataxia or cardiac conduction defects (Mancuso *et al.*, 2015). Progressive external ophthalmoplegia (PEO) is the more benign presentation attributed to single large-scale mtDNA deletions, associated with ophthalmoplegia, ptosis and myopathy (Pitceathly *et al.*, 2012). The differential severity may be partly attributed to the heteroplasmy level and deletion size - Kearns Sayre patients often have larger deletions compared to CPEO patients (Holt *et al.*, 1989). Deletions typically range from 1-10kb in size, with a common 4977bp (m.8470_13446del) deletion caused by mtDNA excision between two 13bp direct repeats at m.8470-8482 and m.13447-m.13459 (Holt *et al.*, 1989; Schon *et al.*, 1989).

Unlike nuclear gene rearrangements, single large-scale mtDNA deletions often arise sporadically during embryonic development and have a low recurrence risk (Chinnery *et al.*, 2004). Clinically-affected women who harbour a large-scale mtDNA deletion have a low (<10%) risk of transmission (Chinnery *et al.*, 2004) and prenatal testing is informative for at-risk pregnancies (Nesbitt *et al.*, 2014).

1.3.4.3 Secondary mtDNA mutations

Whilst large-scale mtDNA deletions and point mutations represent primary mtDNA defects, secondary defects are another common cause of mitochondrial disease. Defective mtDNA maintenance, transcription, protein translation or a defective ancillary process such as mitochondrial import can cause either quantitative (depletion of mtDNA copy number) or qualitative (affecting mtDNA genome integrity, resulting in multiple large mtDNA deletions) effects. These result from mutations affecting nuclear genes and inheritance occurs in a Mendelian (or *de novo*) fashion.

1.3.5 Nuclear mitochondrial disease

The majority of the genes in the mitoproteome are encoded by the nuclear genome (Calvo *et al.*, 2016) and follow Mendelian inheritance patterns. *De novo* (Harel *et al.*, 2016; Thompson *et al.*, 2016), X-linked (Fernandez-Moreira *et al.*, 2007; Sperl *et al.*, 2015), dominant (Tang *et al.*, 2011) and recessive (Tang *et al.*, 2011; Harel *et al.*, 2016) inheritance cases have been reported in the literature. The first nuclear mitochondrial gene mutation was identified in *SDHA*, encoding a structural subunit of complex II in 1995 (Bourgeron *et al.*, 1995), and there has been monumental progress in the discovery of mitochondrial disease candidate genes since. New proteomic and transcriptomic approaches are being applied to models of human disease to uncover new candidates (Heide *et al.*, 2012; Arroyo *et al.*, 2016; Floyd *et al.*, 2016), whilst patient analyses are validating their involvement in human pathology (Alston *et al.*, 2016a). The traditional approach of linkage analysis using multiple affected family members has given way to massively parallel sequencing strategies such as whole exome sequencing (WES), either of affected singletons or proband-parent trios, and new disease genes are still emerging over 20 years on. Of the ~1150 proteins in the mitoproteome, mutations have been reported in over 270 genes (
Figure 34), and not only are new genes being reported but also new mechanisms involving genes already implicated in human disease through alternative pathways (Nouws *et al.*, 2010). It is apparent that more severe clinical phenotypes are often associated with recessive defects, presumably due to varying heteroplasmy levels in clinically-affected tissues and the dichotomous effect of recessive mutations; as such, mtDNA mutations are more common in adults whilst nuclear gene defects are overrepresented in paediatric cases (DiMauro and Schon, 2003).

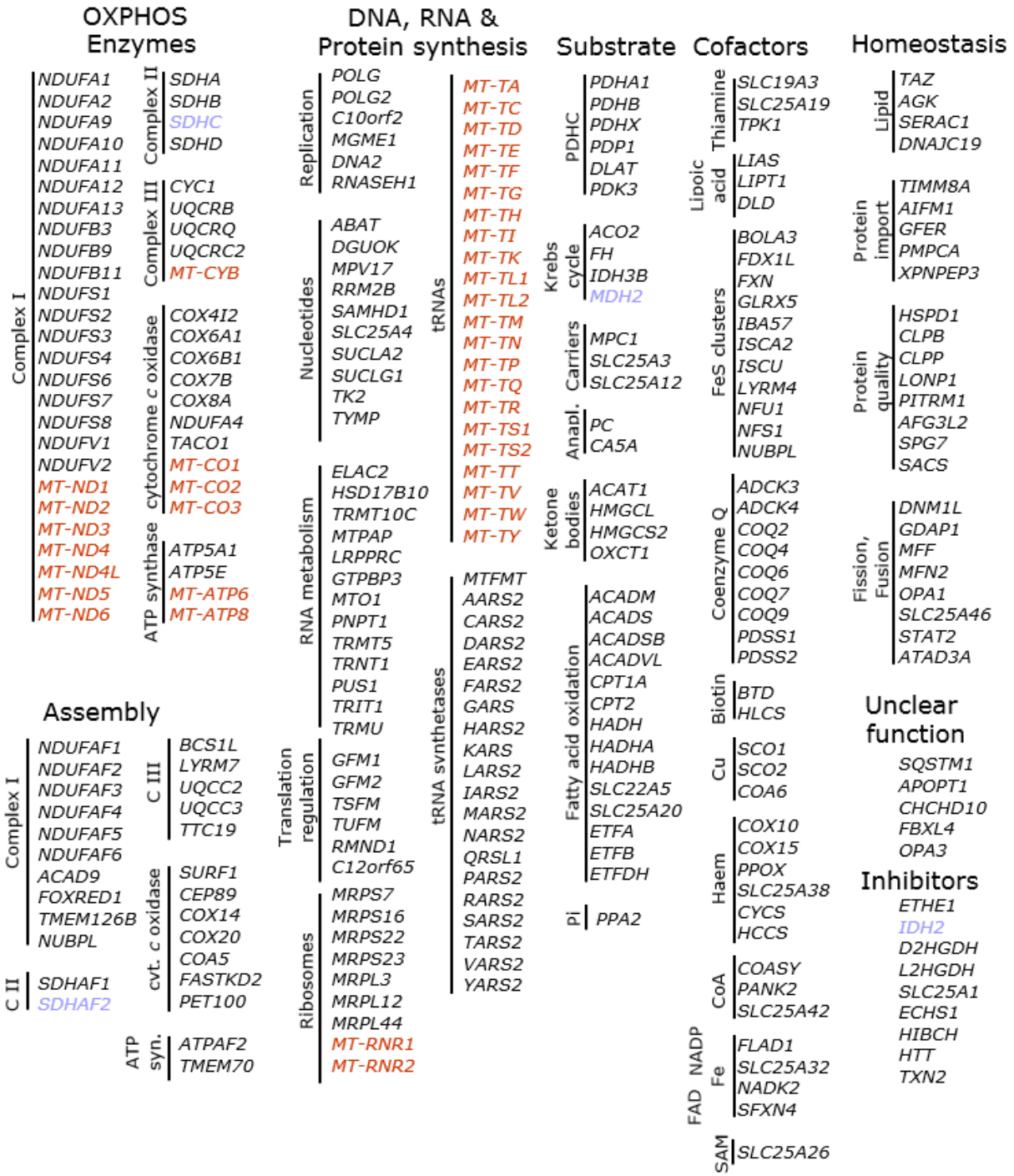

Figure 34. Mitochondrial genes harbouring human disease-associated mutations. Every mitochondrial gene associated with human disease is listed and stratified according to protein function (last updated 27.10.2016). The genes shown in red are mtDNA encoded, whilst purple shaded genes are those implicated in tumorigenesis without metabolic presentations. Adapted from (Mayr *et al.*, 2015).

1.3.6 Isolated respiratory chain complex deficiencies

Histochemical and biochemical evidence of an isolated respiratory chain complex deficiency can be suggestive of a mutation affecting either a structural subunit or an assembly/ancillary factor of one of the five OXPHOS complexes. Our current knowledge of the structural subunits and ancillary factors for each complex is summarized in **Figure 18**.

1.3.6.1 Isolated complex I deficiency

Isolated complex I deficiency represents the biochemical phenotype for approximately 30% of paediatric patients (Kirby *et al.*, 1999), of which 70-80% have a nuclear gene defect (Swalwell *et al.*, 2011). Clinical symptoms associated with complex I deficiency are heterogeneous although the prognosis is typically poor with rapid progression. Lactic acidosis is a common feature, although is often present with another symptoms such as cardiomyopathy or leukodystrophy.

Mutations have been identified in 19/37 structural subunits, and in 10/19 assembly factors (**Table 1**); almost all mutations reported to date are associated with reduced complex I assembly and a comparable reduction in activity however the exceptional p.Asp446Asn *NDUFS2* mutation appears to confer an exclusively catalytic defect (Ngu *et al.*, 2012). This finding is intriguing given that other reported *NDUFS2* variants throughout the gene fail to recapitulate this effect (Loeffen *et al.*, 2001; Tuppen *et al.*, 2010), and moreover it is difficult to postulate the mechanism for loss of NADH activity given its location within the Q domain, opposed to the N domain, without an assembly defect i.e. minimally, the loss of the N module. Nonetheless, functional evidence supports the pathogenicity of the p.Asp446Asn mutation (Ngu *et al.*, 2012).

The most common presentations associated with an isolated complex I deficiency are fatal infantile lactic acidosis (FILA), Leigh syndrome (LS), cardiomyopathy and leukoencephalopathy, although these are not mutually exclusive and can co-occur. For example Ngu *et al.* describe five *NDUFS2*-deficient patients, three of whom presented with MRI changes consistent with Leigh syndrome in conjunction with hypertrophic cardiomyopathy (Ngu *et al.*, 2012). Lactic acidosis is common in paediatric complex I deficient patients, reported in association with biallelic mutations in *NDUFS2*, *NDUFS6*, *NDUFS8*, *NDUFV1*, *NDUFA11*, *NDUFB3*, *NDUFAF3* and *NDUFAF5*. Although there is a general lack of gene-phenotype

correlations associated with FILA, NDUFS6 should be considered the primary candidate gene where it occurs in isolation (Kirby *et al.*, 2004b).

Cardiomyopathy is a common clinical symptom, with *ACAD9* mutations representing the key candidate gene for its pathogenesis (Dewulf *et al.*, 2016) although other genes have been implicated including *NDUFB11* (Shehata *et al.*, 2015), *NDUFAF1* (Fassone *et al.*, 2011) and numerous mtDNA variants. Although 7 complex I structural subunits are mtDNA encoded, cardiomyopathy does not appear to be associated with mutations in these genes (Swalwell *et al.*, 2011).

According to a recent review by Lake *et al.,* mutations in over 75 genes have been associated with Leigh syndrome to date, ascribing it the dubious honour of being the most genetically heterogeneous mitochondrial disease presentation (Lake *et al.*, 2016) and at least 23 of these encode complex I proteins (4 assembly factors and 19 structural subunits: 6 mtDNA/13 nuclear); *MTND1, MTND2, MTND3, MTND4, MTND5, MTND6, NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFA1*, *NDUFA2, NDUFA9, NDUFA10, NDUFA12, NDUFAF2, NDUFAF5, NDUFAF6* and *FOXRED1* (Lake *et al.*, 2016).

Typically, there are few gene-phenotype correlations to guide the genetic diagnosis of complex I deficiency as studies report the majority of complex I deficiency mutations to be private and non-recurrent (Pagniez-Mammeri *et al.*, 2012) although there are a few exceptions, such as the p.Trp22Arg *NDUFB3* (Alston *et al.*, 2016b) and p.Gly212Val *TMEM126B* European founder mutations (Alston *et al.*, 2016a; Sanchez-Caballero *et al.*, 2016) and the p.Cys115Tyr *NDUFS6* Caucasus Jewish founder mutation (Spiegel *et al.*, 2009). Results from the literature suggest that *NDUFS2* and *ACAD9* mutations account for a significant proportion of diagnoses although it is likely that clearer genetic diagnostic trends will emerge from large diagnostic NGS datasets (Haack *et al.*, 2012a). It is crucial that diagnostic centres continue to publish their datasets in order to facilitate epidemiological studies regarding disease incidence stratified by gene.

Table 1. Complex I structural subunit genes and genes encoding complex I assembly, biogenesis or ancillary proteins. Genes in which mutations have been reported are shown in bold. The first disease-causing mutations in specific genes reported in the literature are referenced accordingly.

Core subunits	***MTND1***[1] ***MTND2***[2] ***MTND3***[3] ***MTND4***[4] ***MTND4L***[5] ***MTND5***[6] ***MTND6***[7] ***NDUFS1***[8] ***NDUFS2***[9] ***NDUFS3***[10] ***NDUFS7***[11] ***NDUFS8***[12] ***NDUFV1***[13] ***NDUFV2***[14]
Accessory subunits	***NDUFS4***[15] *NDUFS5* ***NDUFS6***[16] ***NDUFA1***[17] ***NDUFA2***[18] *NDUFA3 NDUFA5 NDUFA6 NDUFA7 NDUFA8* ***NDUFA9***[19] ***NDUFA10***[20] ***NDUFA11***[21] ***NDUFA12***[22] ***NDUFA13***[23] *NDUFAB1 NDUFV3 NDUFB1 NDUFB2* ***NDUFB3***[24] *NDUFB4 NDUFB5 NDUFB6 NDUFB7 NDUFB8* ***NDUFB9***[25] *NDUFB10* ***NDUFB11***[26] *NDUFC1 NDUFC2*
Ancillary proteins	***NDUFAF1***[27] ***NDUFAF2***[28] ***NDUFAF3***[29] ***NDUFAF4***[30] ***NDUFAF5***[31] ***NDUFAF6***[32] *NDUFAF7* ***FOXRED1***[33] ***ACAD9***[34] *ECSIT* ***NUBPL***[35] ***TMEM126B***[36,37] *TIMMDC1 C17orf89 DMAC1 TMEM186 TMEM70* ATP5SL COA1*

* TMEM70 mutations have not been reported in association with complex I deficiency, although it is often mutated in patients with complex V deficiency (**Chapter 1.3.6.5**).

References

1 (Kirby *et al.*, 2004a), 2 (Ugalde *et al.*, 2007), 3 (Crimi *et al.*, 2004), 4 (Singh *et al.*, 1989), 5 (Brown *et al.*, 1995), 6 (Santorelli *et al.*, 1997), 7 (Jun *et al.*, 1994), 8 (Benit *et al.*, 2001), 9 (Loeffen *et al.*, 2001), 10 (Benit *et al.*, 2004), 11 (Triepels *et al.*, 1999), 12 (Loeffen *et al.*, 1998), 13 (Schuelke *et al.*, 1999), 14 (Benit *et al.*, 2003), 15 (Budde *et al.*, 2000), 16 (Kirby *et al.*, 2004b), 17 (Fernandez-Moreira *et al.*, 2007), 18 (Hoefs *et al.*, 2008), 19 (van den Bosch *et al.*, 2012) 20 (Hoefs *et al.*, 2011), 21 (Berger *et al.*, 2008), 22 (Ostergaard *et al.*, 2011), 23 (Angebault *et al.*, 2015), 24 (Calvo *et al.*, 2012), 25 (Haack *et al.*, 2012b), 26 (van Rahden *et al.*, 2015), 27 (Dunning *et al.*, 2007), 28 (Ogilvie *et al.*, 2005), 29 (Saada *et al.*, 2009), 30 (Saada *et al.*, 2008), 31 (Sugiana *et al.*, 2008), 32 (Pagliarini *et al.*, 2008), 33 (Calvo *et al.*, 2010), 34 (Nouws *et al.*, 2010), 35 (Calvo *et al.*, 2010), 36 (Alston *et al.*, 2016a), 37 (Sanchez-Caballero *et al.*, 2016).

1.3.6.2 Isolated complex II deficiency

Complex II deficiency is rare, accounting for 2-8% mitochondrial disease cases (Parfait *et al.*, 2000; Ghezzi *et al.*, 2009) with <50 patients reported in the literature. Biallelic mutations have been associated with congenital metabolic presentations predominantly affecting either the central nervous system (CNS) or heart (hypertrophic cardiomyopathy, leukodystrophy, LS and encephalopathy) (Jain-Ghai *et al.*, 2013) whereas heterozygous mutations are associated with cancer susceptibility, particularly pheochromocytoma and paraganglioma (Timmers *et al.*, 2009). Although initially believed to have distinct gene-phenotype relationships, with *SDHA* and *SDHAF1* linked to mitochondrial disease and *SDHB*/*SDHC*/*SDHD*/*SDHAF2* linked to cancer susceptibility, evidence of genotype-phenotype overlap is emerging, prompting tumour surveillance of unaffected relatives heterozygous for *SDHx* mutations (Hensen *et al.*, 2011; Alston *et al.*, 2015; Renkema *et al.*, 2015).

At present, mutations in *SDHA*, *SDHB*, *SDHD* and *SDHAF1* have been identified in metabolic patients (**Table 2**), whilst mutations in all *SDHx* genes (with the exception of *SDHAF1*) have now been implicated in tumorigenesis (Costa *et al.*, 2015). The overwhelming majority of nuclear OXPHOS subunit mutations occur in a recessive manner; in fact, the only cases of dominantly acting mutations involve *SDHA* with two reports linking dominant *SDHA* mutations with optic atrophy, both with and without cardiac dysfunction (Birch-Machin *et al.*, 2000; Courage *et al.*, 2016).

Table 2. Complex II structural subunit genes and genes encoding complex II assembly, biogenesis or ancillary proteins. Genes in which mutations have been reported in association with primary mitochondrial disease are shown in bold and the first disease-causing mutations in specific genes reported in the literature are referenced accordingly.

Structural subunits	***SDHA***[1], ***SDHB***[2], *SDHC*, ***SDHD***[3]
Ancillary proteins	***SDHAF1***[4], *SDHAF2*, *SDHAF3*, *SDHAF4*

References

1 (Bourgeron *et al.*, 1995), 2 (Alston *et al.*, 2012), 3 (Jackson *et al.*, 2014), 4 (Ghezzi *et al.*, 2009)

1.3.6.3 Isolated complex III deficiency

Exercise intolerance is the predominant clinical phenotype associated with an isolated complex III deficiency and accounts for >50% patients who harbour *MTCYB* mutations, affecting the sole mtDNA-encoded subunit (Lott *et al.*, 2013). Other clinical presentations associated with *MTCYB* mutations include cardiomyopathy and encephalomyopathy, but these are minority reports (Lott *et al.*, 2013).

Pathogenic mutations have been reported in four of the nuclear-encoded structural subunits plus five assembly/ancillary factors (**Table 3**) with presentations including developmental delay, encephalopathy, lactic acidosis liver dysfunction, renal tubulopathy and muscle weakness (de Lonlay *et al.*, 2001; Mordaunt *et al.*, 2015). Mutations within the assembly factor *BCS1L* are the most frequent cause of complex III deficiency after defects within *MTCYB*, with over 25 different mutations reported (Fernández-Vizarra and Zeviani, 2015). Mutations are associated with phenotypic heterogeneity, ranging from isolated encephalopathy to the multisystem GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death).

Twelve *TTC19*-deficient patients have been reported, all presenting with an "ataxia plus" phenotype. Symptoms were either early or late onset (age of onset range: 7 months – 45 years), and rate of deterioration varied between individuals; some patients had a rapidly progressive disease course and died within a few months to years after the onset of symptoms, whilst others lived for decades. Each patient harboured biallelic null alleles due to either frameshift or nonsense mutations (Mordaunt *et al.*, 2015).

Interestingly, many of the clinical presentations associated with biallelic complex III gene mutations are mild; although only very small numbers have been reported (for example, n=2 for *CYC1),* the amino acids involved exhibit high evolutionary conservation, but patients presented with resolving metabolic symptoms (hypoglycaemia, lactic acidosis and liver dysfunction) with onset in response to illness. A similar picture exists for *UQCRB* mutations, with only one case reported, but this time involving a homozygous c.338_341del frameshift mutation. This child also presented following a bout of gastroenteritis with hepatic involvement, hypoglycaemia and lactic acidosis – and despite harbouring homozygous null alleles, she was reported to have recovered without detriment (Haut *et al.*, 2003). Mutations in *UQCRQ* are similarly rare, with only one report of biallelic mutations, although with numerous

affected individuals from a large, multiply consanguineous family – a c.208C>T p.Ser45Phe missense variant accounted for their early onset presentation of developmental delay with Leigh-like deterioration. Unlike many early-onset mitochondrial presentations, the continued neurological deterioration was not associated with rapid demise and some affected individuals remained alive in their thirties (Barel *et al.*, 2008).

Table 3. Complex III structural subunit genes and genes encoding complex III assembly, biogenesis or ancillary proteins. Genes in which mutations have been reported are shown in bold. The first disease-causing mutations in specific genes reported in the literature are referenced accordingly.

Structural subunits	***MTCYB***[1] ***UQCRB***[2] *UQCRC1* ***UQCRC2***[3] *UQCRFS1 UQCRH* ***UQCRQ*****[**4], *UQCR10 UQCR11* ***CYC1***[5]
Ancillary proteins	***BCS1L***[6]**,** ***LYRM7***[7], *UQCC1*, ***UQCC2***[8]**,** ***UQCC3***[9], ***TTC19***[10] *PTCD2*

References

1 (Dumoulin *et al.*, 1996), 2 (Haut *et al.*, 2003), 3 (Miyake *et al.*, 2013), 4 (Barel *et al.*, 2008), 5 (Gaignard *et al.*, 2013), 6 (de Lonlay *et al.*, 2001), 7 (Invernizzi *et al.*, 2013), 8 (Tucker *et al.*, 2013), 9 (Wanschers *et al.*, 2014), 10 (Ghezzi *et al.*, 2011)

1.3.6.4 Isolated complex IV deficiency

Isolated complex IV deficiency is the second most common mitochondrial biochemical presentation after isolated complex I deficiency. It is often associated with early onset and rapidly progressive presentations, particularly Leigh (and Leigh-like) syndrome and severe encephalomyopathy. Pathogenic mutations have been reported in eight structural subunits but the vast majority of defects arise in one of the proteins involved in assembly or biogenesis – to date, seventeen COX biogenesis factors have been associated with mitochondrial disease (**Table 4**). Two common founder mutations have been reported, a founder *LRPPRC* mutation within the French-Canadian population has a carrier frequency of 1 in 28 although additional mutations have been identified out with the Saguenay-Lac-Saint-Jean region of Quebec, Canada (Olahova *et al.*, 2015), and the second founder mutation – c.3G>C within *PET100* that is predicted to abolish the initiator methionine - was identified in the Lebanese population (Lim *et al.*, 2014). Some proteins are linked tightly with specific aspects of COX biogenesis (e.g. *COA6*, involved in copper-dependent *COX2* biogenesis (Stroud *et al.*, 2015)) and others have more diverse roles.

Clinically, presentations are often early onset and devastating, predominantly affecting the heart and CNS. *SURF1* is one of the most common causes of Leigh syndrome and more than 80 different *SURF1* mutations have been associated with the phenotype (Wedatilake *et al.*, 2013). Other phenotypes include cardiomyopathy, attributed to *COA6* mutations (Stroud *et al.*, 2015), and a milder Charcot Marie Tooth (CMT) phenotype has been associated with biallelic *COX6A1* variants (Tamiya *et al.*, 2014).

NDUFA4 was originally described as a complex I subunit but has since been reassigned to complex IV following functional studies (Balsa *et al.*, 2012) supported by the presence of *NDUFA4* defects in a patient with COX deficiency (Pitceathly *et al.*, 2013). Following its reassignment to complex IV, there is some debate as to its role in COX function – current opinion states it may in fact represent a structural complex IV subunit, rather than having a role in COX biogenesis, as was initially postulated (Balsa *et al.*, 2012; Pitceathly *et al.*, 2013; Stroud *et al.*, 2015).

Table 4. Complex IV structural subunit genes and genes encoding complex IV assembly, biogenesis or ancillary proteins. Genes in which mutations have been reported are shown in bold. The first disease-causing mutations in specific genes reported in the literature are referenced accordingly.

Structural subunits	***MTCO1***[1] ***MTCO2***[2], ***MTCO3***[3] ***COX4***[4] *COX5A,* *COX5B,* ***COX6A***[5] ***COX6B***[6] *COX6C COX7A* ***COX7B***[7] *COX7C* ***COX8***[8]
Ancillary proteins	*COA1* ***COA3***[9] *COA4* ***COA5***[10] ***COA6***[11] *COA7* ***COX10***[12] *COX11* ***COX14***[13] ***COX15***[14] *COX16 COX17 COX18 COX19* ***COX20***[15] ***SCO1***[16] ***SCO2***[17] ***SURF1***[18] ***LRPPRC***[19] ***PET100***[20] *PET117* ***CEP89***[21] ***TACO1***[22] ***APOPT1***[23] ***NDUFA4***[24] *OXA1L* ***FASTKD2***[25]

References

1 (Bruno *et al.*, 1999), 2 (Wong *et al.*, 2001), 3 (Hanna *et al.*, 1998), 4 (Shteyer *et al.*, 2009), 5 (Tamiya *et al.*, 2014), 6 (Massa *et al.*, 2008), 7 (Indrieri *et al.*, 2012), 8 (Hallmann *et al.*, 2016), 9 (Ostergaard *et al.*, 2015), 10 (Huigsloot *et al.*, 2011), 11 (Ghosh *et al.*, 2014), 12 (Valnot *et al.*, 2000b), 13 (Weraarpachai *et al.*, 2012), 14 (Antonicka *et al.*, 2003), 15 (Szklarczyk *et al.*, 2013), 16 (Valnot *et al.*, 2000a), 17 (Papadopoulou *et al.*, 1999), 18 (Zhu *et al.*, 1998), 19 (Mootha *et al.*, 2003), 20 (Lim *et al.*, 2014), 21 (van Bon *et al.*, 2013), 22 (Weraarpachai *et al.*, 2009), 23 (Melchionda *et al.*, 2014), 24 (Pitceathly *et al.*, 2013), 25 (Ghezzi *et al.*, 2008).

1.3.6.5 Isolated complex V deficiency

Isolated complex V deficiency is one of the rarer forms of mitochondrial disease; the most common genetic defects involve *MTATP6,* one of the two mtDNA encoded subunits that codes for subunit ‘a’. The m.8993T>C/G and m.9176T>C/G *MTATP6* mutations are amongst the most common mtDNA point mutations and are associated with either LS or milder neurogenic ataxia and retinitis pigmentosa (NARP) syndrome (Pfeffer *et al.*, 2012). The threshold associated with clinical symptoms is high for both the m.8993T>C/G and m.9176T>C/G mutations, with severely affected individuals often harbouring in excess of 97% mutant load. To date, mutations have been reported in just four nuclear complex V genes (**Table 5**), in association with varied clinical phenotypes including ataxia and cardiomyopathy with abnormal metabolites including lactate and 3-methylglutaconic aciduria (3-MGA).

The most common nuclear defects involve *TMEM70* mutations and include a Roma founder mutation causing multiple cases of lactic acidosis and cardiomyopathy (Cizkova *et al.*, 2008). Other *TMEM70* defects have been associated with encephalopathy and cataracts which have been reported in non-Roma populations (Spiegel *et al.*, 2011). Rare causes of complex V deficiency include mutations in *ATP12*, identified in two paediatric patients with lactic acidosis, dysmorphic features and 3-methylglutaconic aciduria (De Meirleir *et al.*, 2004).

Recently, *TIMM50* mutations have been linked to complex V deficiency in association with severe intellectual disability, epilepsy and 3-MGA. TIM50 is a component of TIM23 and is involved in translocation of nuclear-encoded proteins across the IMM (**Chapter 1.1.4**); its specific role in complex V function has yet to be established (Shahrour *et al.*, 2016).

Table 5. Complex V structural subunit genes and genes encoding complex V assembly, biogenesis or ancillary proteins. Genes in which mutations have been reported are shown in bold and the first disease-causing mutations reported in the literature are referenced.

Structural subunits	***MTATP6***[1] ***MTATP8***[2] ***ATP5A1***[3] *ATP5B ATP5C1 ATP5D* ***ATP5E***[4] *ATP5F1 ATP5G1 ATP5G2 ATP5G3 ATP5H ATP5I ATP5O ATP5J ATP5J2 ATP5L ATP5L2*
Ancillary proteins	*ATPAF1,* ***ATPAF2***[5]***,*** ***TMEM70***[6]

References

1 (Holt *et al.*, 1990), 2 (Jonckheere *et al.*, 2008), 3 (Lieber *et al.*, 2013), 4 (Mayr *et al.*, 2010), 5 (De Meirleir *et al.*, 2004), 6 (Cizkova *et al.*, 2008)

1.3.7 Multiple respiratory chain defects

Mitochondrial function is regulated and maintained by around 1150 nuclear encoded genes; these nuclear-encoded genes are translated by cytosolic translational machinery and are directed into the mitochondrion via a mitochondrial targeting sequence (**Chapter 1.1.4**). Defects in over 270 nuclear-encoded mitochondrial genes have been identified in patients with a clinical diagnosis of mitochondrial disease (

Figure 34) (Mayr *et al.*, 2015). Defects in genes involved in Fe-S cluster biosynthesis, mtDNA maintenance, mitochondrial mRNA transcription and protein translation typically cause deficiencies involving multiple respiratory chain complexes (**Figure 35**). Often, these disease genes were identified following whole exome sequencing (WES) of patient DNA samples and our laboratory has successfully employed this strategy to identify mutations in each of the mitochondrial pathways described (**Chapter 5**).

Mutations affecting numerous mitoribosome subunits have been reported in the literature, including *MRPL3* (Galmiche *et al.*, 2011), *MRPL12* (Serre *et al.*, 2013) and *MRPL44* (Carroll *et al.*, 2013) of the large mitoribosomal subunit and *MRPS16* (Miller *et al.*, 2004) and *MRPS22* (Smits *et al.*, 2011) of the small ribosomal subunit. *MRPL3* and *MRPL44* mutations are both associated with early-onset cardiomyopathy and multiple OXPHOS deficiencies, but this was in the context of a multisystem presentation that included central nervous system involvement in the case of *MRPL44* (Galmiche *et al.*, 2011; Carroll *et al.*, 2013; Distelmaier *et al.*, 2015). *MRPL12* mutations are associated with growth retardation and neurological regression (Serre *et al.*, 2013), whilst *MRPS16* mutations have been reported in a patient with agenesis of the corpus callosum and dysmorphic features (Miller *et al.*, 2004). *MRPS22* mutations also cause dysmorphic features, similar to those observed in Cornelia de Lange syndrome, with additional cardiac and renal involvement (Smits *et al.*, 2011; Baertling *et al.*, 2015).

Mutations in nuclear-encoded mitoribosome subunits appear to be a rare cause of mitochondrial disease, with single or few cases reported for each disease gene in the literature. By contrast, mutations affecting other mitochondrial pathways are considerably more common – for example, ten patients from five unrelated families (one of which was multiply consanguineous) have been reported harbouring *ELAC2* mutations (Haack *et al.*, 2013; Akawi *et al.*, 2016). The majority of patients had early onset cardiomyopathy, although the multiply consanguineous family presented primarily with intellectual disability. *ELAC2* encodes the mitochondrial

RNAseZ enzyme that cleaves the 3' end of mitochondrial tRNA molecules to excise them from the polycistronic mRNA transcript (**Chapter 1.1.11**).

Mutations in *MTFMT,* encoding the mitochondrial methionyl-tRNA formyltransferase, are one of the most common causes of multiple OXPHOS deficiencies, reported in 15 patients from 13 families to date (Tucker *et al.*, 2011; Haack *et al.*, 2012a). The prevalence is partly due to a common c.626C>T founder mutation which causes aberrant splicing (p.Arg181Serfs*5) and has MAF=0.0011. MTFMT is responsible for post-translational modification of the initiation methionine, required for mitochondrial translation initiation (**Chapter 1.1.12**). Affected patients present with early onset LS often with developmental delay, hypotonia and/or ataxia (Haack *et al.*, 2012a).

The mitochondrial aminoacyl tRNA-synthetases (mt aaRSs) are a family of enzymes required for conjugating the tRNA molecule with its cognate amino acid and together represent common causes of multiple OXPHOS deficiencies. A total of nineteen different mt aaRSs are required for human mitochondrial protein synthesis, two of these function in both the cytosol and mitochondria (**Table 6**).

Table 6. The mitochondrial aminoacyl tRNA synthetases. Genes in which mutations have been reported are shown in bold and the first disease-causing mutations reported in the literature are referenced.

mt-tRNA only	***AARS2***[1] ***CARS2***[2] ***DARS2***[3] ***EARS2***[4] ***FARS2***[5] ***HARS2***[6] ***IARS2***[7] ***LARS2***[8] ***MARS2***[9] ***NARS2***[10] ***PARS2***[11] ***RARS2***[12] ***SARS2***[13] ***TARS2***[14] ***VARS2***[14] *WARS2* ***YARS2***[15]
dual location	***GARS***[16] ***KARS***[17]

References

1 (Gotz *et al.*, 2011), 2 (Coughlin *et al.*, 2015), 3 (Scheper *et al.*, 2007), 4 (Steenweg *et al.*, 2012), 5 (Elo *et al.*, 2012), 6 (Pierce *et al.*, 2011), 7 (Schwartzentruber *et al.*, 2014), 8 (Pierce *et al.*, 2013), 9 (Bayat *et al.*, 2012), 10 (Vanlander *et al.*, 2015), 11 (Sofou *et al.*, 2015), 12 (Edvardson *et al.*, 2007), 13 (Belostotsky *et al.*, 2011), 14 (Diodato *et al.*, 2014b), 15 (Riley *et al.*, 2010), 16 (Sivakumar *et al.*, 2005), 17 (McLaughlin *et al.*, 2010)

The clinical presentations associated with mt-aa tRNA synthetases are diverse, including Charcot Marie Tooth (*GARS* and *KARS* mutations), Perrault syndrome (*HARS2* and *LARS2* mutations) and pontocerebellar hypoplasia (*RARS2* mutations, **Chapter 3**). *DARS2* mutations are associated with leukoencephalopathy with brain stem and spinal cord involvement and elevated lactate (LBSL) and mutations have been reported in over 30 families (Diodato *et al.*, 2014a).

Although mutations in the genes shown in (**Figure 35**) are most often associated with multiple OXPHOS deficiencies - an anticipated consequence, given their involvement in numerous OXPHOS components - it not unprecedented to find exceptions where isolated complex deficiencies are observed which poses a challenge for diagnostics (Haack *et al.*, 2012a; Taylor *et al.*, 2014) (**Chapter 1.5.4**).

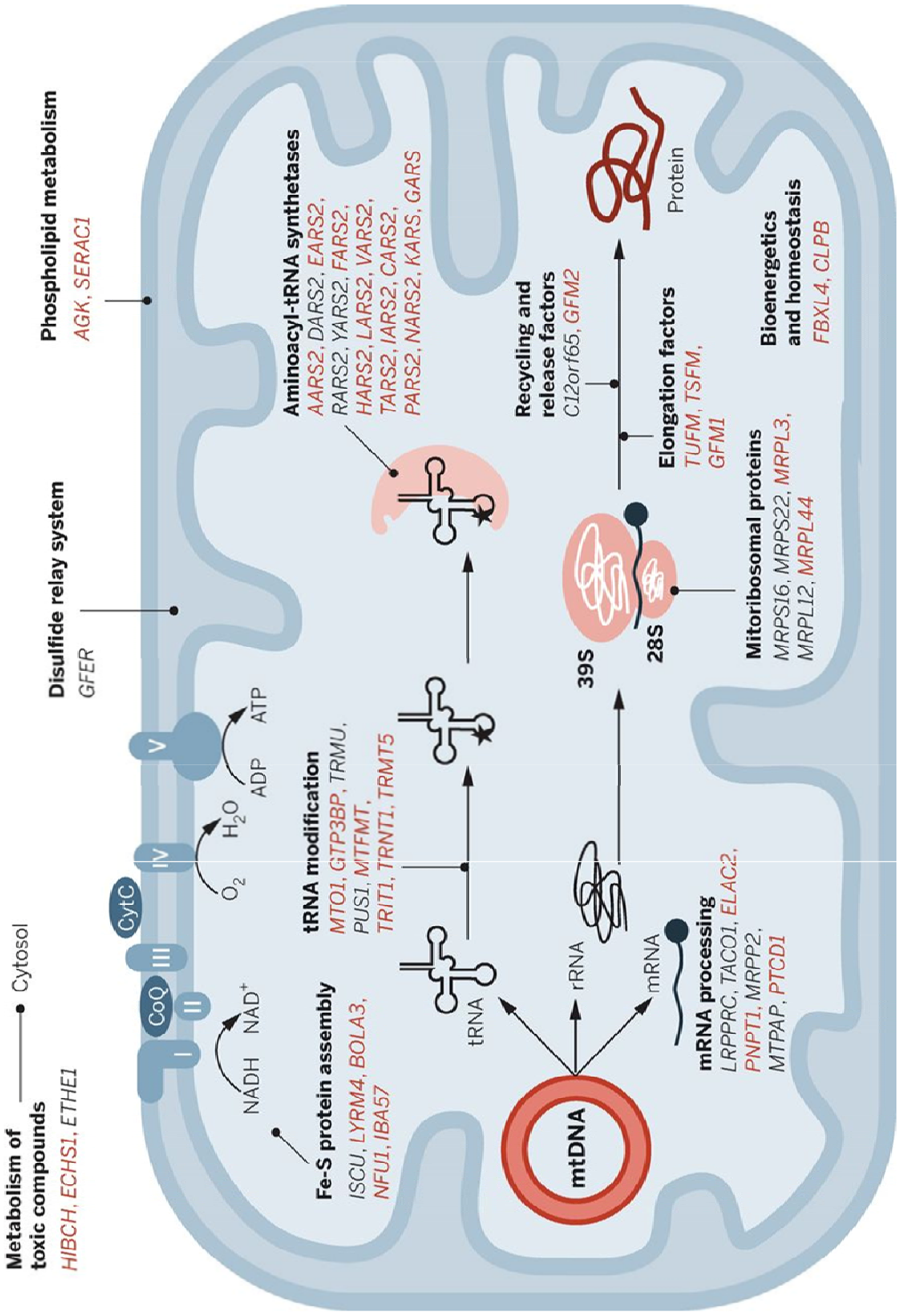

Figure 35. Schematic highlighting the nuclear gene mutations associated with multiple OXPHOS deficiencies. Multiple OXPHOS deficiency genes function are caused by defects in Fe-S cluster biogenesis, mitochondrial import and metabolism, tRNA modification, mRNA processing, and mitochondrial translation. Genes shown in red were initially identified using NGS strategies. (Lightowlers et al., 2015).

1.3.8 Non-OXPHOS mitochondrial disease

Not all mitochondrial disease patients have evidence of OXPHOS dysfunction but have other evidence implicating a diagnosis of mitochondrial disease including persistently-elevated lactates, abnormal urinary organic acid profiles (e.g. 3-MGA), suggestive MRI brain and neuroradiological signatures and multisystem involvement. Genetic causes identified by WES approaches of such mitochondrial disorders include defective enzymes of the tricarboxylic cycle (e.g. *ACO2 (Spiegel et al., 2012)*) or cofactor transport (e.g. *SLC19A3* (Zeng *et al.*, 2005)).

Aconitase (*ACO2*) is a mitochondrial Fe-S protein that functions within the TCA cycle (**Figure 16**) to reversibly catalyse citrate to isocitrate conversion. Biallelic mutations have been reported in seven patients with childhood-onset cerebellar-retinal degeneration syndrome (Spiegel *et al.*, 2012; Metodiev *et al.*, 2014). The patients' metabolic profiles varied, some reported acidosis and hyperglycaemia whilst others were unremarkable; moreover lactates were consistently within the normal range (blood lactate <2.0mM) (Spiegel *et al.*, 2012; Metodiev *et al.*, 2014).

Mutations within *SLC19A3,* encoding the thiamine transporter, cause biotin/thiamine-responsive basal ganglia disease (BTBGD). Associated symptoms cause stepwise regression following episodes of acute and recurrent encephalopathy consistent with brain lesions affecting primarily (but not exclusively) the basal ganglia, akin to Leigh syndrome. Although *SLC19A3* mutations cause defective plasma membrane transportation of thiamine, the consequential loss of mitochondrial thiamine derivatives (thiamine pyrophosphate) cause Leigh-like pathology. *SLC19A3* mutations are relatively common, with over 50 patients reported (Flones *et al.*, 2016; Ygberg *et al.*, 2016) (Zeng *et al.*, 2005; Alfadhel *et al.*, 2013; Gerards *et al.*, 2013; Aljabri *et al.*, 2016; Pronicka *et al.*, 2016) and represents one form of mitochondrial disease for which a therapy exists. Thiamine supplementation has shown consistent amelioration of disease progression and in some cases recovery (Ortigoza-Escobar *et al.*, 2016).

These cases highlight that even in the absence of classical markers of mitochondrial disease, such as elevated lactate and deranged OXPHOS activities, a mitochondrial aetiology may still underlie the clinical presentation. In the case of *SLC19A3* mutations, early recognition of the characteristic MRI brain changes are crucial for rapid genetic diagnosis and prompt commencement of thiamine therapy to maximise the therapeutic potential.

1.4 TREATMENT AND PREVENTION OF MITOCHONDRIAL DISEASES

There are no effective cures for most mitochondrial diseases, although existing surgical and pharmacologic interventions are of benefit to some patients.

1.4.1 First do no harm

In some cases, whilst interventions may not ameliorate symptoms, they can prevent further deterioration and certain life-threatening events can be circumvented through clinicians' understanding of the facets associated with their patient's condition. For example, seizures are genetically heterogeneous and prevalent amongst adult and paediatric mitochondrial patient cohorts (Zsurka and Kunz, 2015). Seizures can be managed with a variety of anti-epileptic agents in the clinical setting, but some impair mitochondrial function. This is particularly true of valproate which is hepatotoxic and particularly detrimental if administered to patients with Alpers-Huttenlocher syndrome presenting with a hepatocerebral mtDNA depletion disorder associated with recessive *POLG* mutations (Bindoff and Engelsen, 2012), and an alternative should therefore be administered to these patients. Statin prescription is another risk factor for individuals with elevated cholesterol and mitochondrial disease; HMG CoA reductase inhibition (the action of statin medication) inhibits coenzyme Q_{10} biosynthesis and therefore poses an additional risk to patients already susceptible to metabolic dysfunction (Schirris *et al.*, 2015; Christensen *et al.*, 2016). Dietary management of cholesterol may prove a more compatible approach. Due to the link between the m.1555A>G mtDNA genotype (MAF=0.0025 (Rahman *et al.*, 2012)) and aminoglycoside-dependent ototoxicity (Prezant *et al.*, 1993; Guan *et al.*, 2001), prospective mutation screening is now commonplace in our centre prior to aminoglycoside antibiotic therapy, thereby preventing cases of avoidable sensorineural hearing loss for at-risk patients (Bitner-Glindzicz *et al.*, 2010; Abusamra and McShane, 2016).

1.4.2 Treat the treatable

Ptosis may be the predominant factor limiting the quality of life for some patients presenting with CPEO and its surgical correction can impact significantly on their wellbeing (Sebastia *et al.*, 2015). Similarly, patients presenting with cardiomyopathy may benefit from cardiac monitoring and pacemaker installation to improve prognosis (Bates *et al.*, 2012; Kennedy *et al.*, 2016).

1.4.3 Interventions to ameliorate symptom severity

Dietary supplementation is common in mitochondrial disease patients, particularly CoQ_{10}, L-carnitine and riboflavin (Parikh *et al.*, 2015; Karaa *et al.*, 2016) that either increase ATP production (via increased substrate pools, upregulating complex activity or by bypassing the rate-limiting, deficient complex) or through antioxidant activity. Whilst there is evidence supporting CoQ_{10} benefit for primary CoQ_{10} deficient patients (Gironi *et al.*, 2004; Quinzii *et al.*, 2007), there is limited evidence of efficacy for patients affected by other forms of mitochondrial disease (Pfeffer *et al.*, 2013). L-carnitine is an acyl carrier, required for transport of long chain fatty acids into the matrix for subsequent β-oxidation; supplementation could increase β-oxidation and production of the TCA substrate, coenzyme A (Ribas *et al.*, 2014; Mamedov *et al.*, 2015) but again, there is scant evidence of efficacy in the clinical trial setting. A similar picture exists for riboflavin which can be a successful therapeutic agent for some patients with mitochondrial disease e.g. some patients with *FLAD1* (Olsen *et al.*, 2016), *SLC25A2* (Schiff *et al.*, 2016) and *ACAD9* mutations (Gerards *et al.*, 2011), whilst other patients show no improvement (Pfeffer *et al.*, 2013; Nouws *et al.*, 2014b; Olsen *et al.*, 2016). Endurance training has been shown to improve skeletal muscle OXPHOS capacity (Taivassalo *et al.*, 2001; Jeppesen *et al.*, 2006); the damage exerted upon muscle fibres following high-effort is repaired by satellite cells harbouring significantly lower mutation load, thereby improving strength and slightly reducing the frequency of COX-deficient fibres (Murphy *et al.*, 2008). Exercise also increases mitochondrial biogenesis through induction of the PGC1α pathway, leading to an overall improvement in ATP production (Taivassalo *et al.*, 2001).

1.4.4 Clinical trials

At present, recruitment is underway for various pharmaceutical therapeutics (http://www.mitoaction.org/study). Our Centre is presently assessing the efficacy and safety of bezafibrate for adult patients with mitochondrial myopathies. Bezafibrate is already licensed for hyperlipidaemia therapeutics, targeting PGC1α to increase mitochondrial metabolism. Bezafibrate has already been repurposed for ameliorating muscle pain in carnitine palmitoyltransferase II deficient patients (Bonnefont *et al.*, 2010) and it is hoped that increasing biogenesis might similarly improve myopathy for mitochondrial patients in spite of their genetic burden.

1.4.5 Gene therapy

The possibility of exploiting molecular biology for treatment of mitochondrial disease has advanced dramatically in the last few years. The three main options offering potential for treatment of mtDNA disease are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeat - CRISPR-associated-9 (CRISPR/Cas9) (**Figure 36**).

TALENs and ZFNs function as heterodimers, binding to mtDNA mutation-specific sequences. Zinc fingers are structural motifs that bind to 3-4bp gDNA; combining multiple zinc fingers creates specificity. Similarly, TALENs are transcription factor-nucleases constructed from specific 33-35 amino acid modules. Upon binding, the *Fok*I nuclease domains within the ZFNs/TALENs dimerize and generate double-strand breaks that target the mutated mtDNA molecules for repair (Bacman *et al.*, 2013; Gammage *et al.*, 2014; Hashimoto *et al.*, 2015; Gammage *et al.*, 2016). ZFNs are small, which is a positive aspect regarding delivery as a therapeutic agent, but they are expensive; TALENs are inexpensive and more flexible, but are more bulky which can hinder efficient delivery.

The CRISPR/Cas9 system uses a specific ~20bp single guide RNA (sgRNA) to direct a Cas9 nuclease to the target mutation where it induces a double-stranded DNA break in the presence of a PAM site. This is subsequently repaired by either non-homologous end joining (NHEJ) or homology-directed recombination (HDR) to introduce either a point mutation or indel, insertion or deletion mutations (Pellagatti *et al.*, 2015). The affordability, flexibility and specificity associated with CRISPR/Cas9 establishes this as the most promising therapeutic system, although off-target effects represent a major hurdle in the application of CRISPR/Cas9 technology to clinical applications (Zhang *et al.*, 2015).

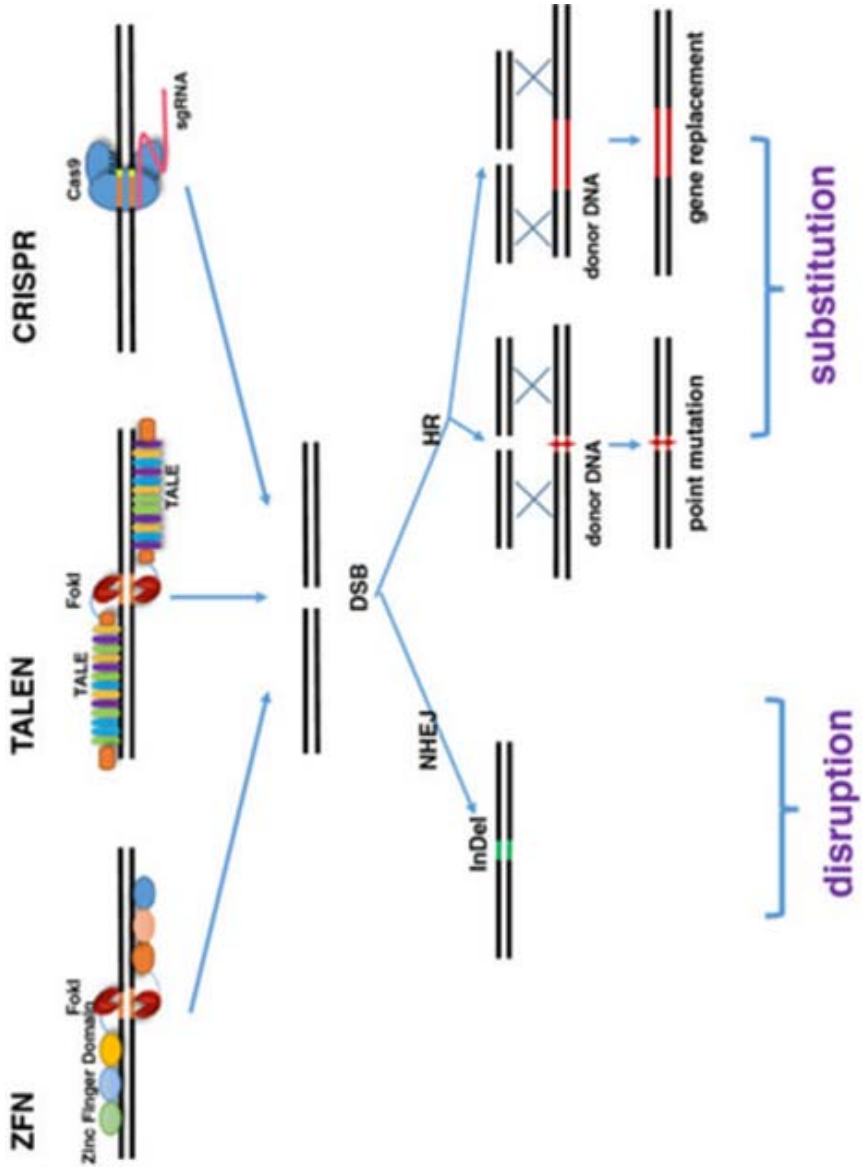

Figure 36. Mechanisms of Zinc Finger nuclease, TALEN and CRISPR/Cas9 genome editing. Induction of double strand breaks by either ZFN, TALEN and CRISPR/Cas9 stimulate repair which induces genome modification; NHEJ-mediated repair introduces variable length insertion or deletion mutations, whereas HR-mediated repair causes point mutations or gene replacement. Adapted from (Chen *et al.*, 2014).

1.4.6 Prevention of mitochondrial disease

In the absence of a cure for mitochondrial disease, reproductive options are a valuable resource to at risk couples. Where a genetic diagnosis exists, Genetic counselling enables calculation of the recurrence risk for inherited nuclear mitochondrial disorders (typically 25% for autosomal recessive conditions or 50% for autosomal dominant mutations), but it is often difficult to confidently predict the recurrence risk for mtDNA mutations. Interventions currently available to at risk-couples are prenatal diagnosis (PND) and preimplantation genetic diagnosis (PGD).

Prenatal testing is typically performed by biopsy of fetal material from an ongoing pregnancy; either chorionic villus biopsy (CVB) at 10-12 weeks gestation or amniotic fluid sampling at 15–22 weeks gestation. Non-invasive prenatal testing (NIPT) is a relatively recent addition to the prenatal testing repertoire. In this procedure, a blood sample is taken from the mother between 9 and 16 weeks gestation. This sample contains cell free fetal DNA (cffDNA) derived from the developing fetus and can be therefore enriched and used for genetic testing. Although currently unsuitable for maternally transmitted gene mutations due to an innate inability to distinguish between maternal cell contamination and maternally-inherited mutations, it represents a method for aneuploidy detection, screening for paternally-transmitted mutations and determination of fetal sex for pregnancies at risk of a sex-linked condition (Ho *et al.*, 2015; Norton *et al.*, 2015; Xiong *et al.*, 2015; De Franco *et al.*, 2016; Dobson *et al.*, 2016).

Annually, approximately 30,000 women in the UK (5% of the pregnant population) are offered invasive prenatal testing; although the vast majority of these will be reactive tests following detection of potential anomalies during routine ultrasonography it includes a number of pregnancies that are at-risk of inherited genetic disease (Nesbitt *et al.*, 2014). Couples who are known carriers of recessive mutations are routinely offered invasive prenatal testing to confirm the genotype of their fetus; where testing yields a negative diagnosis, this alleviates much of the anxiety associated with a possibly-affected pregnancy whilst a positive diagnosis enables couples to make an informed decision as to whether they wish to continue with their pregnancy and prepare for the birth of their child if they do. There are benefits and risks associated with both types of invasive testing - CVB can be performed earlier in pregnancy, but carries a higher risk of procedure associated miscarriage (~1%) and the possibility of confined placental mosaicism could result in inaccurate reporting of risk. Amniocentesis is performed later, but has a lower associated risk of pregnancy loss (0.15-0.5%) (Eddleman *et al.*, 2006; Bakker *et al.*, 2016). Both amniocentesis and CVB carry a risk of maternal cell contamination, and this

must be excluded by (for example) microsatellite analysis. The other main disadvantage to invasive prenatal testing is that there are no cures for affected pregnancies, and termination is the only option to prevent the birth of a clinically-affected child.

Between January 2007 and September 2016, our centre has performed a total of 92 invasive prenatal tests at significant risk of transmitting mitochondrial disease; 32 of these were screening for mtDNA mutations and 60 related to nuclear-encoded recessive mitochondrial disorders.

PGD offers a solution for at-risk couples who do not view termination of an affected pregnancy as a feasible option yet wish to have clinically-unaffected children. PGD is a well-established *in vitro* fertilisation (IVF) -based procedure that genetically diagnoses genetic disorders in 8-cell stage embryos with only unaffected embryos selected for implantation, effectively avoiding transmission. Current evidence suggests that there are no major shifts in the mtDNA mutation heteroplasmy levels during early embryonic development and heteroplasmy levels from a single blastomere biopsied from an eight-cell embryo are representative of the mutation load across the entire embryo (Richardson *et al.*, 2015). PGD therefore represents a feasible reproductive option and has been widely utilised for prevention of both nuclear and mtDNA disorders (Smeets *et al.*, 2015; Girardet *et al.*, 2016; Goldman *et al.*, 2016; Simpson and Rechitsky, 2017). Our centre has established a diagnostic PGD service as part of our Highly Specialised clinical service for NHS patients within the UK, and we have performed 15 cycles of PGD analysis for 11 families at risk of transmitting mtDNA mutations. Patients who wish to pursue PGD for nuclear genes are currently referred to ViaPath at Guy's Hospital, London for treatment although provision of an in-house PGD service for nuclear mitochondrial disease genes is under discussion.

Mitochondrial replacement therapy is another intervention that aims to prevent the transmission of mtDNA mutations from a carrier mother to her children. There are two techniques - pronuclear transfer and spindle transfer – that both require a donor oocyte which is enucleated, either post fertilisation in the case of pronuclear transfer, or pre-fertilisation for spindle transfer (**Figure 37**). The maternal nuclear genetic material is removed from the maternal oocyte/zygote and injected into the perivitelline space of the enucleated donor oocyte/zygote, with fused occurring via electrofusion. The chimeric zygote is highly unlikely to develop clinical symptoms associated with the familial mtDNA mutation given that it's mtDNA is almost

exclusively that of the healthy donor, but will have all the physical characteristics associated with being a "biological child", from the parental nuclear genes.

British Law was amended in 2015 meaning that the UK was the first country to legally approve mitochondrial replacement therapy; despite the law change it is yet to be licenced by the country's independent regulator, the Human Fertilisation and Embryology Authority (HFEA), as a clinical service. Research is continuing to develop and trial the therapy to firmly establish efficacy (Craven *et al.*, 2010; Hyslop *et al.*, 2016). Despite the illegality in many countries, Mexico has no legislation banning its use and in September 2016 it was announced that the first child has been born using mitochondrial replacement therapy (Zhang *et al.*, 2016). It is hoped that the birth of the first, apparently-healthy child using the technique will facilitate licencing of mitochondrial replacement therapy to accredited laboratories to enable its provision to at-risk families although clearly the follow up of children born using this technique is vitally important.

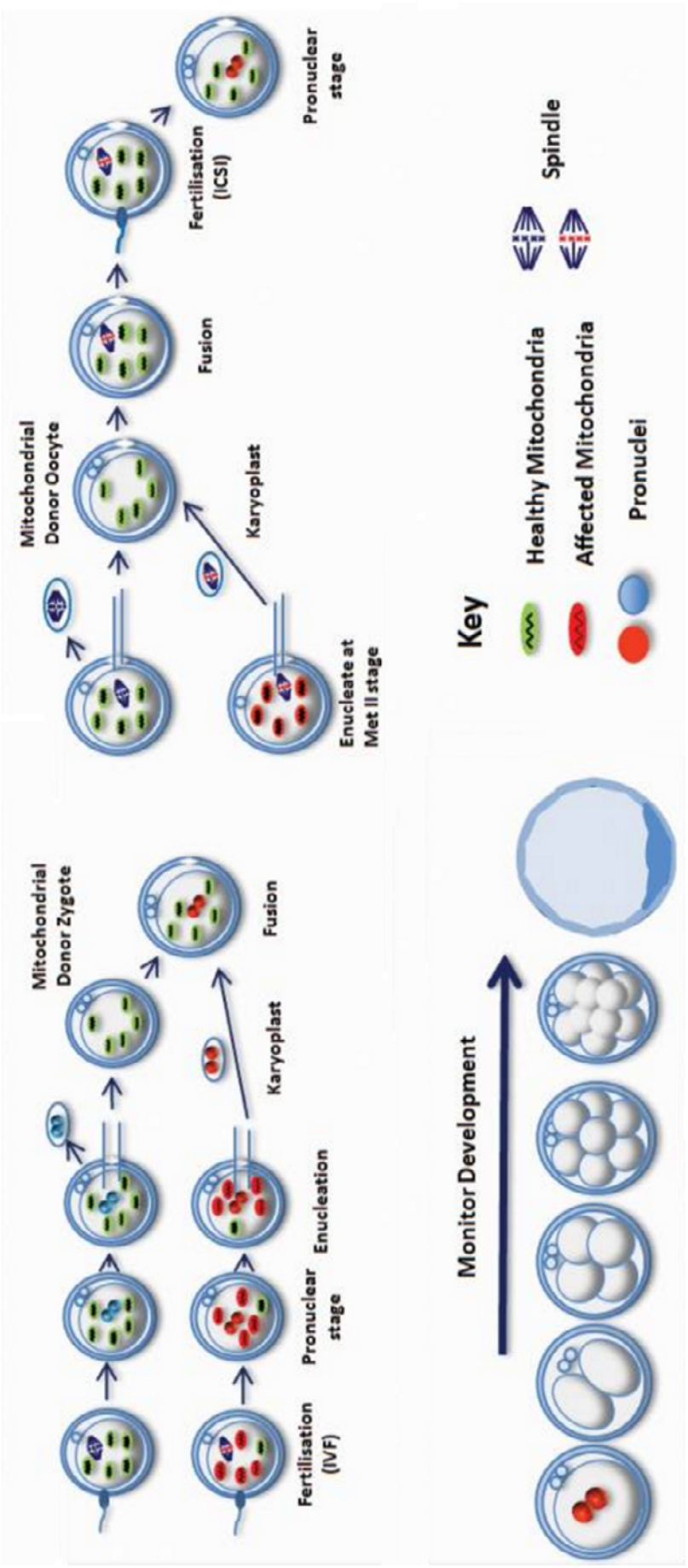

Figure 37. Two approaches for performing mitochondrial replacement. A Pronuclear transfer involves enucleation of fertilised donor and recipient (mtDNA mutation harbouring) zygotes. Recipient karyoplast is fused with the donor enucleated zygote to create a zygote with minimal (carryover-dependent) mtDNA mutation load. **B** Metaphase II spindle transfer involves removal of the karyoplast from donor and recipient oocytes; the maternal karyoplast is fused with the enucleated donor oocyte and fertilization occurs via intracytoplasmic sperm injection. Adapted from (Craven *et al.*, 2011).

1.5 MOLECULAR GENETIC ANALYSIS OF MITOCHONDRIAL DISEASE

The molecular genetic diagnosis of mitochondrial patients has always been a complicated process due to the associated clinical and genetic heterogeneity. In the clinical setting, ascertainment of the genetic diagnosis is often important for clinical management of patients and enables genetic counselling of patients and their families. For families with children affected by mitochondrial disease, a genetic diagnosis allows calculation of recurrence risks and access to reproductive options for subsequent pregnancies. As such, establishing a genetic diagnosis is of utmost importance.

1.5.1 Historical approach to genetic diagnosis

Historically, linkage analysis represented the most successful method for identifying novel candidate disease genes. Based on the premise that physically close genomic loci typically remain close after meiosis, the genotyping of multiple genetic markers across the genome in affected and unaffected family members permits the identification of potential disease-associated loci - those in which marker genotypes segregate with a clinically-affected status. The power of linkage analysis as a technique dramatically increases with the number of samples, and it is not an appropriate technique for investigation of singleton cases. In 1982, the first locus to be linked to human disease was on chr1q, in association with a dominant form of Charcot Marie Tooth syndrome (CMT1B) (Bird *et al.*, 1982); the causative gene *MPZ* was only discovered some ten years later (Hayasaka *et al.*, 1993). In the early days of disease gene characterisation, the genetic investigation of clinically-affected individuals was pivotal to progress, and this holds true over thirty years later although the scope and methodologies have moved on considerably.

1.5.2 Sanger sequencing

The utility of chain terminating nucleotides for DNA sequencing was discovered by Fred Sanger, and was refined to a technique developed in 1977 that bears his name, and is still prominent in genetic analysis today, Sanger (or di-deoxy) sequencing (Sanger *et al.*, 1977). The premise being that a DNA polymerase will bind to a primed DNA strand and form bonds between the free 3’ hydroxyl group of one dNTP and the 5’ phosphate group of the subsequent dNTP, thereby extending the DNA strand until it becomes rate limited, typically by either free dNTP pools, primers or time (Chidgeavadze *et al.*, 1984). In the case of di-deoxy sequencing, a minority proportion of dNTPs are substituted with nucleotide analogues, di-

deoxyribonucleotides (ddNTPs), which lack a free hydroxyl group which is fundamental for extension. Given that normal dNTPs are far in excess of ddNTPs in the reaction mix, incorporation of these modified nucleotides into an extending DNA strand occurs in a random fashion and halting the extension and dictating the resultant DNA fragment size. Sanger's initial protocol incorporated radioactivity to each ddNTP and the extension was performed in quadruplicate, one reaction per nucleotide, which permitted sequence determination when run on a high resolution polyacrylamide gel and subject to autoradiography (**Figure 38**). Modern Sanger sequencing is fundamentally the same, but the use of polyacrylamide gels and autoradiography has been replaced with commercially available capillary electrophoresis equipment capable of automated sequencing, imaging and sequence determination using lasers and charge-coupled device (CCD) cameras to visualise fluorophore tagged dNTPs.

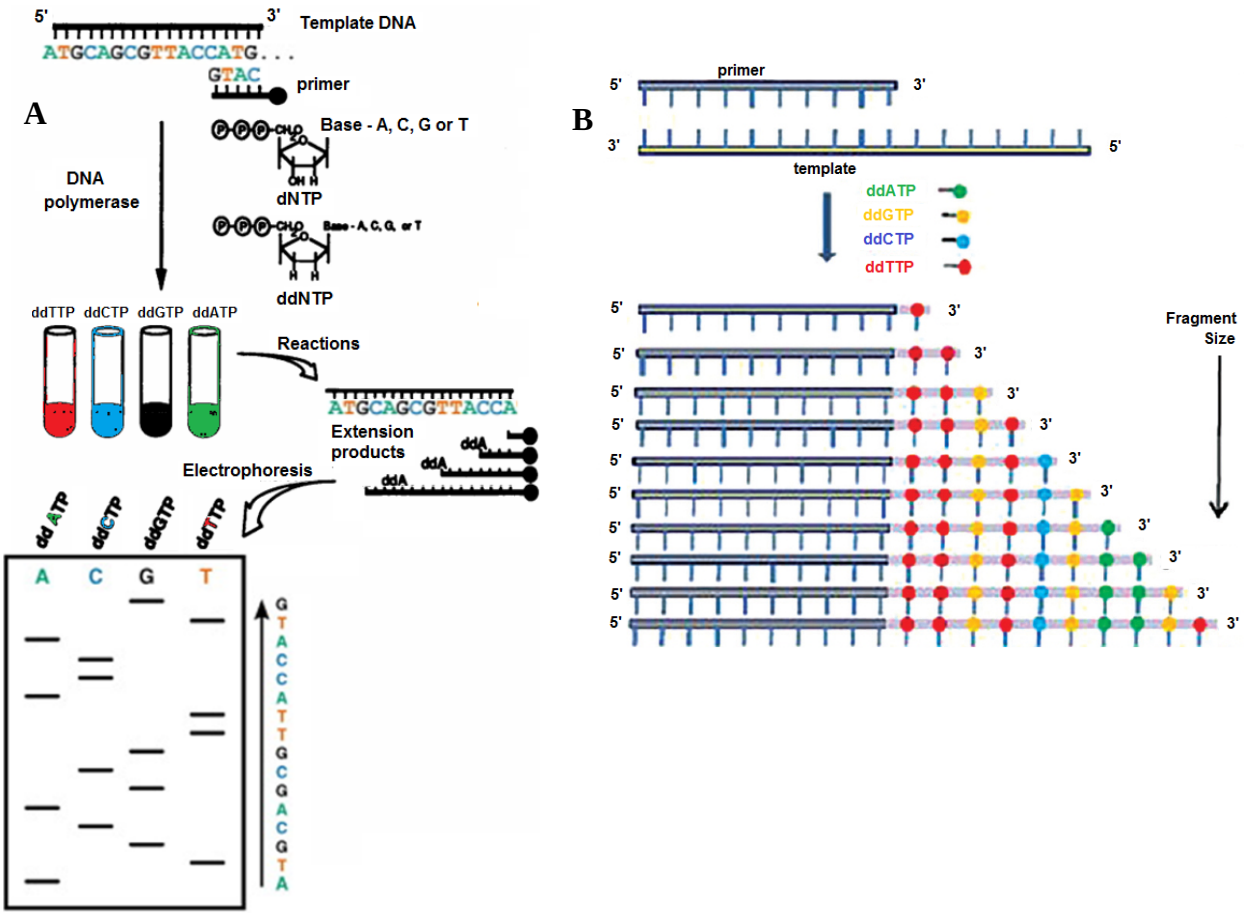

Figure 38. Sanger sequencing methodology. Chain terminated products of random sizes are visualised and sequences are obtained by either (A) autoradiography or (B) capillary electrophoresis. Adapted from (Hunkapiller *et al.*, 1991; Heather and Chain, 2016).

The current capillary electrophoresis platforms are capable of sequencing amplicons 650-1000bp in size, and were the workhorses used for the Human Genome Project that produced the first draft of the Human genome sequence in 2001 (Lander *et al.*, 2001).

1.5.3 Next-generation sequencing

Although Sanger is still often regarded as the 'gold standard' methodology for genetic sequence analysis, the evolution of high throughput technologies are providing increasingly accessible avenues for genetic analysis. Different platforms have harnessed different technologies, including the pyrosequencing-based approach employed by the Roche 454, Life Technologies' sequencing by oligonucleotide ligation and detection (SOLiD) which utilises a DNA ligase instead of a DNA polymerase, the fluorescent sequencing by synthesis adopted by Illumina's series (MiSeq, NextSeq, HiSeq) and the IonTorrent produced by Life Technologies that determines nucleotide incorporation on the basis of pH change. An overview of the technical and financial aspects of each of the main platforms is shown in **Figure 39**.

The two most popular platforms are Illumina's MiSeq/NextSeq/HiSeq series and Life Technologies' IonTorrent PGM/Proton models. Both Illumina and Ion Torrent provide scalable solutions, offering platforms that are designed specifically for targeted gene sequencing (MiSeq/PGM) and those that have the capacity to sequence the entire exome (NextSeq/Proton) or genome (HiSeq/Proton). Moreover, the IonTorrent platforms offer the ability to scale independent of the machine, so selection of a higher capacity chip (with more wells and semiconductors) provides more sequencing data but comes with a compromise at the cost level. This flexibility means that it is an extremely accessible option.

		PCR mode	Platform cost (2016)	Max output	Run time	Read length	Reads/run	Reagent cost £/run	Cost £/Gb	WES	WGS	Error rate	Error type
Applied Biosystems	**ABI3730**	PCR	£150k	62Kb	2h	650bp	96	111	1.75m	N	N	<1%	various
Illumina	**MiSeq**	bridge PCR	£75k	15Gb	56h	300bp	25 x10^6	1,178	80	N	N	0.80%	mis sense
Illumina	**NextSeq 500**		£190k	120Gb	29h	150bp	400 x10^6	3,265	30	Y	Y		
Illumina	**HiSeq 2500**		£530k	1.5Tb	6d	150bp	5 x10^9	11,096	20	Y	Y		
Illumina	**HiSeq X**		£920k	1.8Tb	<3d	150bp	6 x10^9	4,909	5	Y	Y		
IonTorrent	**PGM 314 chip**	em PCR	£38k	50Mb	2.3h	200bp	0.55 x10^6	365	3,300	N	N	1.71%	indel
IonTorrent				100Mb	3.7h	400bp	0.55 x10^6	365	1,650	N	N		
IonTorrent	**PGM 316 chip**			600Mb	3.0h	200bp	3 x10^6	519	860	N	N		
IonTorrent				1Gb	4.9h	400bp	3 x10^6	519	430	N	N		
IonTorrent	**PGM 318 chip**			1Gb	4.4h	200bp	5.5 x10^6	673	710	N	N		
IonTorrent				2Gb	7.3h	400bp	5.5 x10^6	673	305	N	N		
IonTorrent	**S5**		£50k	16Gb	2.5h	200bp	80 x10^6	982	60	Y	Y		

Figure 39. Comparison of capacities and associated costs for the market leading NGS platforms. Data obtained from (*Illumina | Sequencing and array-based solutions for genetic research*; *Life Technologies Ion Torrent*, Glenn, 2016 #546).

1.5.4 The application of NGS to mitochondrial disease

The application of next-generation sequencing (NGS) is particularly effective for heterogeneous conditions such as mitochondrial disease, where a large number of genes can be interrogated simultaneously and in the diagnostic setting, NGS-technologies are revolutionising the genetic testing pipeline with Sanger sequencing of candidate nuclear genes on a sequential basis being replaced with powerful, high throughput analysis.

A variety of options are currently being implemented - targeted panels of candidate genes (Alston *et al.*, 2016b), unbiased WES (Haack *et al.*, 2010) and whole genome sequencing (WGS) (Hartmannova *et al.*, 2016) (**Figure 40**).

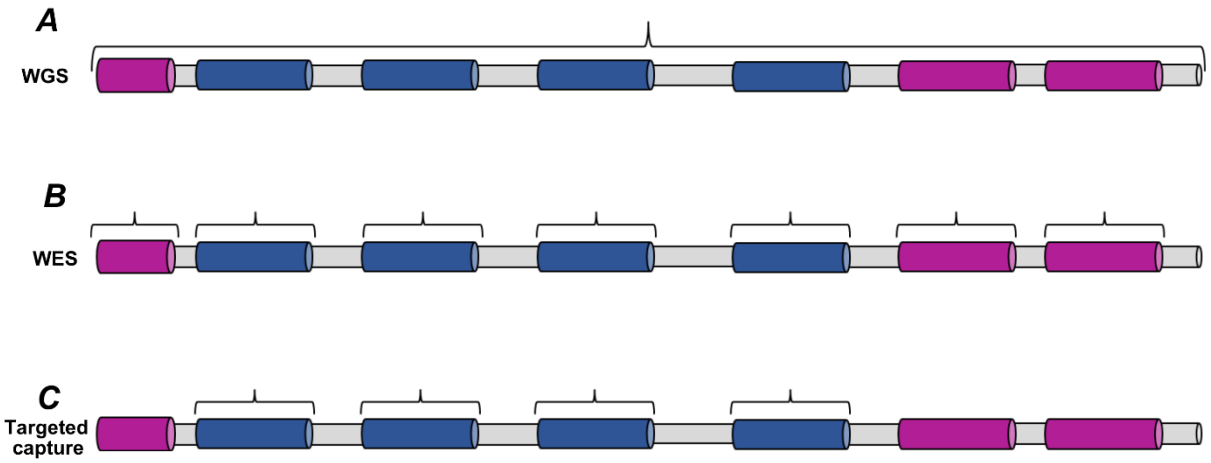

Figure 40. Schematic of NGS strategies employed in the genetic diagnosis of mitochondrial disease. A, Whole genome sequencing (WGS) analyses all coding and non-coding regions of the genome, whilst whole exome sequencing (WES; **B**) only targets the coding exons plus immediate intron-exon boundaries. **C**, Target capture facilitates sequencing of a predetermined genomic region or list of candidate disease genes. Non-coding/intronic regions are shaded grey, exons of candidate genes are shaded blue and exons of non-candidate genes are shaded pink. Adapted from (Alston *et al.*, 2017).

Custom, panel based NGS strategies can be very successful for providing a rapid genetic diagnosis in the clinical setting, but this success depends upon the degree of characterisation to ensure the appropriate candidate genes are targeted. Stratification according to respiratory chain defect can be appropriate for many patients in whom muscle biopsy is available, but even then it may be misleading – a number of patients with an isolated complex I deficiency in fact have a defect of mitochondrial translation (Haack *et al.*, 2012a).This strategy can also be ineffective for genes that exhibit inconsistent biochemical profiles (Tetreault *et al.*, 2015) or patients whose presumed mitochondrial presentation is subsequently established to have a (presumed) non-

mitochondrial aetiology (e.g. *MECP2* mutations causing Rett syndrome (Kohda *et al.*, 2016)). Stratification according to clinical phenotype is similarly complicated by genetic heterogeneity (Blok *et al.*, 2009).

One solution to the stratification dilemma - and one that has been successfully implemented for the analysis of other heterogeneous Mendelian disorders - is a combination of unbiased WES with targeted analysis of "virtual" gene panels (Lieber *et al.*, 2013; Wortmann *et al.*, 2015) which allows informative reporting of negative results and removes the possibility of incidental findings. Further analysis of the WES data for patients lacking a diagnosis following virtual panel analysis could be subsequently undertaken in a research setting. Indeed, most of the candidate genes included on diagnostic virtual panels have their origins in research which has been incredibly fruitful in elucidating genes involved in human pathology, including heterogeneous mitochondrial clinical phenotypes such as cardiomyopathy with mutations identified in *AARS2* (Gotz *et al.*, 2011), *MRPL3* (Galmiche *et al.*, 2011), *MTO1* (Ghezzi *et al.*, 2012) and *ACAD9* (Haack *et al.*, 2010). Recently, the report of both biallelic and *de novo* dominant mutations affecting the novel mitochondrial disease gene *ATAD3A* (Harel *et al.*, 2016) highlights the importance of thorough analysis of single heterozygous variants in presumed recessive aetiologies and the concurrent analysis of parental samples; this is further highlighted by the recent expansion of the *SLC25A4* mutation spectrum, which now includes *de novo* dominant mutations (Thompson *et al.*, 2016), with dominant and recessive mutations having previously been reported. New candidate genes continue to be discovered in a research setting and are subsequently included in diagnostic screening - one success is exemplified by the report of patients harbouring *TMEM126B* mutations, a candidate gene identified by research-based complexome profiling (Heide *et al.*, 2012; Alston *et al.*, 2016a; Sanchez-Caballero *et al.*, 2016). Similarly, characterisation of predicted mitochondrial proteins of unknown function is another critical strategy for identifying potential disease candidate genes (Floyd *et al.*, 2016).

Mitochondrial diseases are clinically and genetically diverse. This diversity results in a complicated diagnostic algorithm that is very much tailored to the specific patient using the referral information alongside all available test results to direct the most appropriate testing pathway. For example, an adult patient who presents with CPEO and in whom numerous COX deficient fibres have been identified on muscle histochemistry would undergo common mtDNA mutation screening (likely focusing on m.3243A>G and long range PCR as a first line of genetic investigation), whereas a paediatric patient who presented with Leigh syndrome and evidence

of complex IV deficiency following muscle histochemistry and biochemistry would undergo whole mtDNA genome analysis and sequencing of the common nuclear Leigh syndrome genes, e.g. *SURF1* (Zhu *et al.*, 1998), *PDHA1* (Sperl *et al.*, 2015).

Given the small size of the mtDNA genome, this is often sequenced in patients with clinical diagnosis of mitochondrial disease to exclude a primary mtDNA defect before the scrutiny of nuclear genes commences. Although Sanger sequencing was previously the preferred method for most diagnostic laboratories, NGS-based testing is becoming more prevalent (Tang *et al.*, 2013) and not only does it identify a primary mtDNA defect but it also provides an accurate measure of heteroplasmy. Indeed, our routine diagnostic pipeline for mtDNA analysis has recently been revised, with a long-range polymerase chain reaction (PCR) and NGS-sequencing on our in-house Ion Torrent PGM sequencer replacing Sanger sequencing as the standard whole mitochondrial genome sequencing (mtWGS) procedure.

Presently, our in-house molecular diagnostics funded by the NHS do not routinely include WES or WGS. Recruitment to sequencing initiatives including Deciphering Developmental Disorders (DDD) (https://www.ddduk.org) and the 100k genome project (100kgp) driven by Genomics England (https://www.genomicsengland.co.uk/) provide access to exome and whole genome sequencing for NHS patients, but each Regional Genetics Laboratory in the UK is deciding how to implement the technology in their own laboratories for their local patients. Despite a proven track record in a research setting and the increasing availability of affordable NGS options to diagnostic laboratories, the case has yet to be made regarding the clinical validity of unrestricted WES within a diagnostic setting. The utility of targeted panels has been well established in other diagnostic labs to facilitate high throughput screening of multiple patients for monogenic disorders or smaller patient cohorts affected with genetically heterogeneous conditions (Nemeth *et al.*, 2013; Rattenberry *et al.*, 2013; Plaskocinska *et al.*, 2016). My doctoral proposal was funded by the National Institute for Health Research (NIHR) to implement NGS technology within our Nationally Commissioned NHS laboratory. Beyond analysis of the entire mitochondrial genome in routine testing, routine genetic testing available as a national service offered 68 genes responsible for various aspects of mitochondrial function including mtDNA maintenance, translation and complex subunits (**Appendix I**), yet this represents just a fraction of the ~1150 genes of the mitoproteome.

CHAPTER TWO

Aims and Scope

2.1 Aims and scope

I joined the Wellcome Trust Centre for Mitochondrial Research in 2008, having recently obtained my Health and Care Professions Council (HCPC) State Registration as a Clinical Scientist at the North of Scotland Medical Genetic Service in Aberdeen. Working as a Clinical Scientist with the Nationally Commissioned *Rare Mitochondrial Disease Service for Adults and Children* in Newcastle, I rapidly appreciated the complexity associated with mitochondrial disease – the dual genome involvement, the clinical variability and the extensive genetic heterogeneity. Since 2009, I have been responsible for the genetic testing associated with isolated complex I deficiency, one of the more common paediatric biochemical profiles and one that is associated with a vast list of candidate genes. Our diagnostic algorithm for a paediatric patient presenting with cardiomyopathy, lactic acidosis and isolated complex I deficiency on muscle biochemistry would commence with sequencing of the mitochondrial genome, followed by analysis of a subset of conserved structural nuclear genes (*NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS5*, *NDUFS6*, *NDUFS7*, *NDUFS8*, *NDUFV1*, *NDUFV2* and *NDUFAB1*) and the assembly factor *ACAD9*. This strategy relies upon muscle biopsy to focus the candidate genes to a manageable number as necessitated by the diagnostic setting. Whilst this strategy obtains a genetic diagnosis for many paediatric patients with complex I deficiency (Tuppen *et al.*, 2010; Swalwell *et al.*, 2011), a genetic diagnosis is not obtained for ~50% of our patients.

The aim of this doctoral research project was to improve the genetic diagnostic yield for patients with a clinical diagnosis of mitochondrial disease through expanding the number of candidate genes investigated and introducing next-generation sequencing into our diagnostic repertoire.

One aspect of the work that I have undertaken has been the design and implementation of a targeted next generation sequencing pipeline for patients with complex I deficiency. Additionally, I have been involved in the examination and validation of variants identified in our patients who lacked a genetic diagnosis upon completion of our diagnostic genetic investigations and were subsequently referred for research-based WES. The third aspect of my doctoral project has been to establish genetic sequencing tests for mitochondrial disease genes that do not warrant a next-generation sequencing strategy for their analysis, either due to their small candidate gene pool (e.g. complex II deficiency) or their involvement in a clearly-correlated clinical phenotype (e.g. mitochondrial dysfunction and pontocerebellar hypoplasia reported in patients with *RARS2* mutations). Throughout my doctoral project I have maintained my role as a Clinical Scientist in the diagnostic laboratory and have had the opportunity to

diagnostically confirm my research findings including validating the pathogenicity of novel variants and carrier testing for relevant family members. I have undertaken the laboratory experiments, analysis and reporting, and prenatal testing for patients and their families diagnosed as part of my project.

CHAPTER THREE

The genetic diagnosis of mitochondrial disease patients presenting with phenotypically suggestive clinical features.

3.1 Candidate gene sequencing of phenotypically suggestive patients.

The analysis strategy within the remit of our Molecular Genetic service permits analysis of a discrete set of candidate genes - the most common causes of mitochondrial disease – and includes mtDNA and nuclear gene analysis (**Appendix I**). The selection of candidate gene(s) pertains to the individual patient's phenotype and is very much a personalised pipeline due to the heterogeneity associated with mitochondrial disease.

A biochemical diagnosis can often be critical in the selection of candidate genes for analysis, particularly in tandem with in-depth patient phenotyping and imaging data. This was epitomised by the first study I present here. Two similarly-affected sisters presented perinatally with a rapidly progressive neurological disorder, dying on day 1 and day 14 of life. MRI brain revealed marked cerebellar hypoplasia and multiple mitochondrial respiratory chain deficiencies (I and IV) in muscle suggestive of mitochondrial disease. The recognition of MRI features and respiratory chain dysfunction prompted clinicians to refer a DNA sample for sequencing of *RARS2* that encodes the mitochondrial arginyl-transfer RNA synthetase; biallelic *RARS2* mutations are a well characterised cause of pontocerebellar hypoplasia type 6 (PCH6, OMIM#611523) (Edvardson *et al.*, 2007). Sanger sequencing of the coding region and intron-exon boundaries revealed a single heterozygous pathogenic mutation (c.1A>G, p.?), predicted to abolish the initiating methionine codon resulting in a null allele. cDNA analysis of patient fibroblast-derived RNA revealed aberrant splicing, with skipping of exons 6-8 due to a deep intronic variant: c.613-3927C>T. Both clinically affected children were compound heterozygous for the *RARS2* variants, and each parents were carriers of one variant *RARS2* allele, thereby supporting recessive inheritance. Splicing mutations are not an uncommon cause of mitochondrial disease, in fact the most common *DARS2* mutations involve an intronic splicing mutation (c.228-20_-21delinsC, p.Arg76Serfs*5 (Scheper *et al.*, 2007), MAF=0.001 [up to 1/95 carrier frequency in Finland (Isohanni *et al.*, 2010)]) and a common *NUBPL* branch site mutation (c.815-27T>C, MAF=0.0035) (Tucker *et al.*, 2012; Wydro and Balk, 2013); our case was unique in that it represented the first report of a deep intronic splicing mutation in a mitochondrial disease presentation. Having identified both pathogenic alleles, we were able to offer and perform prenatal testing of the couple's subsequent pregnancy, leading to the birth of a healthy son. This article was accepted for publication in *Journal of Neuropathology & Experimental Neurology*, and published in July 2015 (included within this thesis, **Chapter 3.2**). The second study I present here describes the second incidence of *SDHD*-related mitochondrial disease, identified by Sanger sequencing of candidate genes following biochemical

characterisation of an isolated complex II respiratory chain deficiency. The proband had an abnormal anomaly scan at 31 weeks gestation, with ultrasound findings suggestive of left ventricular dilation. Subsequent cardiac MRI at 32 weeks gestation confirmed a clinical diagnosis of cardiomyopathy *in utero*, and sadly the proband died on day one of life. Post mortem analysis revealed marked fibrosis, prompting referral of a skeletal muscle biopsy for respiratory chain enzymology and histochemistry. These supported a metabolic aetiology, with evidence of an isolated complex II deficiency.

Given just six proteins are involved in complex II function, they are all encoded by relatively small genes and complex II deficiency represents an extremely rare form of mitochondrial disease, Sanger sequencing was employed to screen the candidate genes, yielding a novel homozygous c.275A>G, p.Asp92Gly variant in *SDHD.* Although *SDHD* defects are linked with cancer susceptibility (Hensen *et al.*, 2011), no *SDHD* mutations had been linked to metabolic presentations at the time of diagnosis, and it therefore represented a novel disease gene requiring functional investigation to confirm pathogenicity. BN-PAGE and Western blotting confirmed a reduction of assembled complex II relative to controls, with preservation of assembled complexes I, III, IV and V. Similarly, reduced steady state levels of complex II subunits SDHA and SDHD were consistent with impaired assembly and subsequent turnover of unincorporated subunits. The specific mutation was modelled in yeast which corroborated the causality of the variant. Of particular interest was the report of the p.(Asp92Tyr) mutation, involving the same p.Asp92 residue, as a recognised founder head and neck paraganglioma (HNPGL) mutation in the Dutch population (Hensen *et al.*, 2011), confirming that mutations affecting at least that residue within SDHD could cause either a recessive metabolic dysfunction or dominant cancer susceptibility.

The first case of *SDHD*-related mitochondrial disease was reported in 2014, including lentiviral rescue to confirm disease causality (Jackson *et al.*, 2014). Our patient represents the second case of *SDHD*-related mitochondrial disease. This article was accepted for publication in *Human Genetics*, and published in May 2015 (included within this thesis, **Chapter 3.3**).

As part of my doctoral project to improve the genetic diagnosis of mitochondrial disease, I established diagnostic sequencing tests for eleven additional genes (**Appendix II)**.

3.2 Neuropathologic Characterization of Pontocerebellar Hypoplasia Type 6 Associated With Cardiomyopathy and Hydrops Fetalis and Severe Multisystem Respiratory Chain Deficiency due to Novel *RARS2* Mutations.

Charlotte L. Alston, Nichola Z. Lax, Katherine Schon, Soo-Mi Park, Deepa Krishnakumar, Langping He , Gavin Falkous, Amanda Ogilvy-Stuart, Christoph Lees, Rosalind H. King, Iain P. Hargreaves, Garry K. Brown, Robert McFarland, Andrew F. Dean, Robert W. Taylor. Neuropathologic Characterization of Pontocerebellar Hypoplasia Type 6 Associated With Cardiomyopathy and Hydrops Fetalis and Severe Multisystem Respiratory Chain Deficiency due to Novel *RARS2* Mutations. *J Neuropathol Exp Neurol.* 2015;74(7):688-703.

3.3 A recessive homozygous p.Asp92Gly *SDHD* mutation causes prenatal cardiomyopathy and a severe mitochondrial complex II deficiency.

Charlotte L. Alston, Camilla Ceccatelli Berti, Emma L. Blakely, Monika Oláhová, Langping He, Colin J. McMahon, Simon E. Olpin, Iain P. Hargreaves, Cecilia Nolli, Robert McFarland, Paola Goffrini, Maureen J. O'Sullivan, Robert W. Taylor. A recessive homozygous p.Asp92Gly *SDHD* mutation causes prenatal cardiomyopathy and a severe mitochondrial complex II deficiency. *Hum Genet.* 2015;134(8):869-79.

CHAPTER FOUR

Application of a custom targeted capture and Ion Torrent PGM to mitochondrial disease patients with isolated complex I deficiency.

4.1 Application of a custom targeted capture and Ion Torrent PGM to mitochondrial disease patients with isolated complex I deficiency

In the diagnostic setting, the overwhelming majority of patient sequencing data is obtained using Sanger sequencing of PCR amplified genomic DNA and capillary electrophoresed using an in-house ABI3130xl genetic analyser. This is time and labour intensive and requires a large amount of genomic DNA. With the acquisition of the Ion Torrent PGM sequencing platform in the Wellcome Trust Centre for Mitochondrial Research came the opportunity to introduce next-generation sequencing into the diagnostic arena. The backbone of my PhD proposal focused on the design and implementation of a targeted NGS strategy to concurrently sequence all the known candidate genes implicated in isolated complex I deficiency, a condition that is prevalent in the paediatric population and is associated with vast clinical and genetic heterogeneity. I designed a custom Ampliseq capture array to target the exonic and intron-exon boundaries of 49 complex I genes (**Appendix III**) which facilitated multiplex PCR-based analysis of ten barcoded patient DNA samples per 316x PGM chip. Using this methodology, analysis of diagnostic patient samples uncovered the first cases of biallelic *TMEM126B* mutations. The proband was of Belgian descent and harboured compound heterozygous *TMEM126B* variants, a c.635G>T p.(Gly212Val) missense variant and a c.401delA p.(Asn134Ilefs*2) frameshift mutation. Following dialogues with other diagnostic centres, our cohort consisted six patients from four unrelated families from Poland, Belgium or USA, yet just two variants accounted for all diagnoses, with evidence of common founders established using a single nucleotide polymorphism (SNP) array.

The clinical phenotypes associated with biallelic *TMEM126B* variants in our cohort were typically those of a late onset, pure myopathy although the youngest member of the cohort presented in acute metabolic crisis in infancy (now age 6).

This manuscript was accepted by the *American Journal of Human Genetics* and published in July 2016 (included within this thesis, **Chapter 4.2**). Another manuscript by was reported concurrently by Sanchez-Caballero *et al.* (Sanchez-Caballero *et al.*, 2016) describing a further two unrelated patients harbouring biallelic *TMEM126B* mutations, one of which was common to both cohorts.

In addition to the identification of *TMEM126B* as a novel complex I deficiency gene, I made 27 diagnoses using the targeted NGS capture (**Appendix IV**); testing of affected relatives

obtained a further four diagnoses. I identified 22 novel variants of likely pathological significance – 18 of these occurred within known disease genes. 16 variants were previously reported pathogenic mutations within known disease genes, including two patients who harboured an identical homozygous c.64T>C, p.Trp22Arg *NDUFB3* mutation. The p.Trp22Arg *NDUFB3* mutation had been previously reported on two independent occasions, once as a homozygote and once in compound with a nonsense mutation, with pathogenicity having been established by lentiviral rescue of the biochemical phenotype in patient cell lines (Calvo *et al.*, 2012; Haack *et al.*, 2012a). Whilst variant's pathogenicity was not in question, there was disparity between the clinical presentation of the reported patients and that of our patients. The previous reports describe severe metabolic presentations and poor prognosis, whereas our patients were in good health, although of short stature.

Discussions with one of the referring paediatric metabolic specialists in Dublin prompted referral of an additional patient with a very similar phenotype – short stature, delicate facial features, smooth philtrum, frontal bossing and complex I deficiency in muscle – who also harboured the same homozygous mutation. The identification of two unrelated patients from the same region, born to non-consanguineous parents and harboured the same homozygous mutation prompted a search of our undiagnosed patient cohort for patients referred from Dublin or Belfast whose referral information detailed intrauterine growth restriction or short stature. Screening for the p.Trp22Arg *NDUFB3* mutation diagnosed a further four homozygotes. Two further homozygous children were diagnosed by endocrinology-actioned WES, presenting with short stature but lacking any obvious signs of metabolic dysfunction.

Clinical photography of the available children showed that distinctive facial features (prominent forehead, low set ears and smooth philtrum) and short stature were common to all members of the cohort. Interestingly, SNP analysis supported multiple independent occurrences of the mutation although a common founder was suggested for some members of the cohort. Our cohort of ten p.Trp22Arg *NDUFB3* homozygote children highlights that – in contrast to the reported cases - the long-term prognosis can be very good, and moreover that recognition of the distinctive facial features could remove the requirement of muscle biopsy to direct molecular genetic investigations, prompting direct screening for the specific mutation to provide a genetic diagnosis. This manuscript was accepted by the *Journal of Medical Genetics*, and published in March 2016 (included within this thesis, **Chapter 4.3**).

4.2 Biallelic Mutations in *TMEM126B* Cause Severe Complex I Deficiency with a Variable Clinical Phenotype

Charlotte L. Alston*, Alison G. Compton*, Luke E. Formosa*, Valentina Strecker, Monika Oláhová, Tobias B. Haack, Joél Smet, Katrien Stouffs, Peter Diakumis, Elżbieta Ciara, David Cassiman, Nadine Romain, John W. Yarham, Langping He, Boel De Paepe, Arnaud V. Vanlander, Sara Seneca, René G. Feichtinger, Rafal Płoski, Dariusz Rokicki, Ewa Pronicka, Ronald G. Haller, Johan L.K. Van Hove, Melanie Bahlo, Johannes A. Mayr, Rudy Van Coster, Holger Prokisch, Ilka Wittig, Michael T. Ryan, David R. Thorburn, Robert W. Taylor. Biallelic Mutations in *TMEM126B* Cause Severe Complex I Deficiency with a Variable Clinical Phenotype. *Am J Hum Genet.* 2016;99(1):217-27.

(* denotes joint first authors)

4.3 A recurrent mitochondrial p.Trp22Arg *NDUFB3* variant causes a distinctive facial appearance, short stature and a mild biochemical and clinical phenotype

Charlotte L. Alston, Caoimhe Howard, Monika Oláhová, Steven A. Hardy, Langping He, Philip G. Murray, Siobhan O'Sullivan, Gary Doherty, Julian P. H. Shield, Iain P. Hargreaves, Ardeshir A. Monavari, Ina Knerr, Peter McCarthy, Andrew A. M. Morris, David R. Thorburn, Holger Prokisch, Peter E. Clayton, Robert McFarland, Joanne Hughes, Ellen Crushell, Robert W. Taylor. A recurrent mitochondrial p.Trp22Arg *NDUFB3* variant causes a distinctive facial appearance, short stature and a mild biochemical and clinical phenotype. *J Med Genet* 2016;53:634-641.

CHAPTER FIVE

Application of unbiased whole exome sequencing (WES) to clinically-characterised mitochondrial disease patients lacking a genetic diagnosis.

5.1 Application of unbiased whole exome sequencing (WES) to clinically-characterised mitochondrial disease patients lacking a genetic diagnosis.

A proportion of patients remain without a genetic diagnosis following completion of diagnostic-based genetic testing, including candidate gene sequencing for patients with good genotype-phenotype indicators (**Chapter 3**) or targeted NGS sequencing on the basis of biochemical evidence of complex I deficiency (**Chapter 4**). A subset of these patients with strong clinical evidence of mitochondrial disease were selected for research-based whole exome sequencing (WES) with the aim to identify their underlying genetic defect. This has proved to be a successful strategy, obtaining a genetic diagnosis for 60% of our undiagnosed cohort (Taylor *et al.*, 2014), a diagnostic yield consistent with those reported by other studies (Wortmann *et al.*, 2015). A proportion of our WES diagnoses have now been reported, including the first diagnoses of inorganic phosphatase (PPA2) deficiency (**Chapter 5.2**) and the identification of a novel founder mutation in *YARS2* (**Chapter 5.3**).

The collaborative manuscript by Kennedy *et al.* represents the fifth study in my doctoral thesis and describes four unrelated families who, between them, have ten clinically-affected individuals presenting with cardiac dysfunction. I have been involved in the diagnostic pathway of one family (family 4 in Kennedy *et al.*) since the referral of proband's skeletal muscle biopsy in 2011. The child presented aged 10 months with seizures, left ventricular hypertrophy and abnormal metabolic markers (including acylcarnitine and urinary organic acid profiles) with progressive deterioration that resulted in his death aged 2 years. Post mortem revealed marked cardiac fibrosis and respiratory chain enzymology of a cardiac biopsy revealed a marked decrease in complex I activity, prompting Sanger sequencing analysis of mtDNA and ten conserved complex I nuclear genes in accordance with the diagnostic testing algorithm at the time. This screening was negative, prompting research-based WES which identified compound heterozygous *PPA2* variants, although this represented a novel disease gene. The manuscript describes three additional families with multiple affected cases, all of whom harbour biallelic *PPA2* variants. Affected individuals from two of these families presented in early life with rapid deterioration and prominent cardiac dysfunction. The third family was diagnosed post mortem, with the patients being in their twenties and had minimal clinical manifestations prior to their sudden death following ingestion of a negligible volume of alcohol. The *PPA2* gene encodes the mitochondrial inorganic pyrophosphatase enzyme that is responsible for pyrophosphate hydrolysis to form orthophosphate (Pi), a substrate for ADP to ATP conversion.

The relationship between PPA2 dysfunction and mitochondrial disease is relatively straightforward whilst the patients' differential presentations are intriguing. Mechanistically, a deficiency of inorganic phosphate substrate could be rate limiting for ATP synthesis, but why one family presented with later onset, alcohol-responsive sudden death (albeit associated with an immense sensitivity) whilst other cases presented with early-onset cardiac fibrosis is puzzling. This again demonstrates that disparate phenotypes are a common feature of nuclear and mtDNA aetiologies alike (Akawi *et al.*, 2016; Anagnostou *et al.*, 2016; Pierce *et al.*, 2016).

Our manuscript describes the first cases of *PPA2* deficient mitochondrial disease, which highlights its role as a candidate gene for early onset cardiac failure/fibrosis and sudden death at any age, even without metabolic indicators such as abnormal lactates. Additionally, it demonstrates the clinical utility of WES application to undiagnosed mitochondrial disease patients. Carrier testing revealed presymptomatic family members who also harbour biallelic familial *PPA2* variants; their positive diagnoses facilitated counselling, cardiac monitoring and pacemaker implantation. This article was accepted by the *American Journal of Human Genetics*, and published in September 2016 (included within this thesis, **Chapter 5.2**).

In addition to the identification of new disease genes, WES has been successful in diagnosing recurrent, founder mutations and in extending the phenotype associated with known disease genes, both of which are demonstrated within the next study which describes a novel Scottish founder mutation within *YARS2*,encoding the mitochondrial tyrosyl-tRNA synthetase. We undertook a literature search to identify other cases of *YARS2*-related mitochondrial disease, revealing nine different autosomal-recessive *YARS2* mutations across 11 patients (Riley *et al.*, 2010; Sasarman *et al.*, 2012; Riley *et al.*, 2013; Shahni *et al.*, 2013; Nakajima *et al.*, 2014; Ardissone *et al.*, 2015), which included two founder mutations - c.156C>G, p.Phe52Leu in four unrelated Lebanese families, and c.137G>A, p.Gly46Asp in three unrelated Middle Eastern families.

Unlike many mitochondrial gene defects, which exhibit vast clinical heterogeneity, many mt-tRNA synthetase mutations correlate with a particular clinical presentation, for example *RARS2* mutations cause pontocerebellar hypoplasia (PCH) (**Chapter 3.2**) and *DARS2* mutations cause leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) (Scheper *et al.*, 2007); similarly, it was considered that *YARS2* mutations cause a myopathy, lactic acidosis and sideroblastic anaemia (MLASA) syndrome phenotype, with reported cases presenting with early-onset MLASA and multiple mitochondrial respiratory chain complex

deficiencies. Just as pontocerebellar hypoplasia is not diagnostic of *RARS2* mutations (reviewed in (Namavar *et al.*, 2011)), a MLASA phenotype has been associated with other aetiologies, including *PUS1* (Shahni *et al.*, 2013) and *MTATP6* mutations (Burrage *et al.*, 2014).

Unlike the previously reported cases, our six patients are all adult patients, although the childhood onset was consistent with the reported cases. Comparison of the clinical features of our cases with those reported revealed that elevated blood lactate with marked exercise intolerance and progressive myopathy were common to 15/16 cases (94%), whilst the MLASA triad was in fact reported in just 12/16 (75%) of cases. Indeed, sideroblastic anaemia was absent in the two *YARS2* cases we identified by WES. Cardiomyopathy and respiratory failure were features not previously associated with *YARS2* deficiency but were prevalent in our cohort, often being pivotal in their demise. The identification of a homozygous c.1175T>C p.Leu392Ser variant in Subject 1 was obtained by Sanger sequencing; *YARS2* represented a strong candidate gene on the basis of their MLASA phenotype and this diagnostic test was established during my doctoral project; two manuscripts describing other aspects of my doctoral candidate sequencing have been discussed previously in **Chapter 3** (also see **Appendix II**).

Our results suggest that *YARS2* is a candidate gene for adults presenting with myopathy and elevated lactate, even in the absence of sideroblastic anaemia, and particularly where cardiomyopathy or respiratory failure co-occur. Analysis of *YARS2* could be performed prior to muscle biopsy, although a much stronger case for *YARS2* can be made for those found to harbour multiple respiratory chain deficiencies in muscle, without mtDNA mutations – both of which would require biopsy to confidently ascertain and highlights that, for adult patients at least, muscle biopsy cannot yet be marginalised in the diagnostic algorithm.

This article was accepted by *Annals of Neurology*, and is currently *in press* (included within this thesis, **Chapter 5.3**).

5.2 Sudden Cardiac Death due to Deficiency of the Mitochondrial Inorganic Pyrophosphatase PPA2

Hannah Kennedy*, Tobias B. Haack*, Verity Hartill*, Lavinija Mataković*, E. Regula Baumgartner, Howard Potter, Richard Mackay, Charlotte L. Alston, Siobhan O'Sullivan, Robert McFarland, Grainne Connolly, Caroline Gannon, Richard King, Scott Mead, Ian Crozier, Wandy Chan, Chris M. Florkowski, Martin Sage, Thomas Höfken, Bader Alhaddad, Laura S. Kremer, Robert Kopajtich, René G. Feichtinger, Wolfgang Sperl, Richard J. Rodenburg, Jean Claude Minet, Angus Dobbie, Tim M. Strom, Thomas Meitinger, Peter M. George, Colin A. Johnson, Robert W. Taylor, Holger Prokisch, Kit Doudney, Johannes A. Mayr. Sudden Cardiac Death due to Deficiency of the Mitochondrial Inorganic Pyrophosphatase *PPA2*. *Am J Hum Genet.* 2016;99(3):674-82.

(* denotes joint first authors)

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

Charlotte L. Alston*, Ewen W. Sommerville*, Yi Shiau Ng*, Cristina Dallabona, Micol Gilberti, Langping He, Charlotte Knowles, Sophie L. Chin, Andrew M. Schaefer, Gavin Falkous, David Murdoch, Cheryl Longman, Marianne de Visser, Laurence A. Bindoff, John M. Rawles, John C.S. Dean, Richard K. Petty, Maria E. Farrugia, Tobias B. Haack, Holger Prokisch, Robert McFarland, Douglass M. Turnbull, Claudia Donnini, Robert W. Taylor, Gráinne S. Gorman. Clinical features, molecular heterogeneity and prognostic implications in *YARS2*-related mitochondrial myopathy. *Ann Neurol.* 2016. [*In press*]

(* denotes joint first authors)

CHAPTER SIX

Provision of informative genetic counselling and reproductive options to clinically-affected patients and their families.

6.1 Provision of informative genetic counselling and reproductive options to clinically-affected patients and their families.

In the absence of a cure for mitochondrial diseases, the provision of a genetic diagnosis is crucial for genetic counselling and access to reproductive options. Predictive genetic testing of family members can lead to medical interventions (i.e. cardiac monitoring), and moreover has the potential to reduce the risk of an affected child being born to another branch of the family. A genetic diagnosis means we can more confidently ascertain the risk for future pregnancies within the family – either generally, according to the genetic aetiology or more specifically, through interventions such as PND and PGD.

The importance and impact of a genetic diagnosis is demonstrated in the seventh manuscript which I present as part of my doctoral thesis which describes an audit of prenatal testing performed across the three specialist mitochondrial disease laboratories within the UK, based in London, Oxford and Newcastle. The audit examined all prenatal procedures (n=62) performed between April 2007 and January 2013. The vast majority were CVB samples (n=59, 95%), with the remaining three cases being amniocentesis samples. The overwhelming reason for PND was the previous birth of an affected child (n=58, 94%). Three PND cases involved a clinically-asymptomatic woman whose mtDNA mutation was identified through cascade testing whilst one PND procedure was performed for a woman whose status was unclear but whose grandmother and brother were severely affected due to the m.3243A>G mutation.

Consistent with the epidemiology of paediatric mitochondrial disease, where autosomal recessive nuclear gene defects more often underlie paediatric mitochondrial disease, there is a bias towards this aetiology and 45/62 PND procedures involved nuclear disease genes. The most common PND request was for recessive *POLG* mutations (n=19); again this is consistent with *POLG* being the most common mitochondrial disease gene in our paediatric cohort, largely due to the three founder mutations in European populations, c.1399G>A p.Ala467Thr, c.2243G>C p. Trp748Ser and c.2542G>A p.Gly848Ser (Horvath *et al.*, 2006); a recent review reported over 84% of *POLG*-deficient cases harbour at least one of these three mutations (Anagnostou *et al.*, 2016).

The outcome of the PND procedures was consistent with the *a priori* risk - 12/45 fetuses (26%) inherited both parental mutations - and 8 pregnancies were terminated on this basis. One fetus

was terminated following diagnosis of a 47XXY karyotype. The remaining 32 pregnancies were predicted to be unaffected on the basis of their screening results.

Additionally, 17 PND procedures were performed for women either known to carry a pathogenic mtDNA mutation or who previously had an affected child, 2/17 PND results showed high levels of heteroplasmy (>70%), predicted in both cases to correlate with a poor prognosis and resulted in termination. 6/17 PND results were consistent with an intermediate level of heteroplasmy (30-70%), and the remaining 9/17 PND results showed a low level of heteroplasmy in the fetal biopsy. It is difficult to confidently predict the outcome of the PND results suggestive of intermediate fetal heteroplasmy levels, and consultation with genetic counsellors and a mitochondrial disease specialist is provided for all PND cases to discuss the individual nuances of the particular mtDNA variant and the possible impacts for their child. Where a low level of heteroplasmy has been identified, a more confident prediction of an associated low risk can be ascribed; follow up data is not always available and we do not routinely request neonatal blood or POC samples to confirm either negative or positive PND results but given that none of these PND children have presented at clinic we assume that their clinically unaffected status remains.

Our data shows that provision of PND has circumvented the birth of 11 affected children, and has provided peace of mind for the remaining families who went on to deliver healthy children. Our results, and those of other centres (Frydman *et al.*, 2011), supports the validity of PND for preventing mitochondrial disease but this does not always represent an acceptable intervention for our patients who may consider PGD a more appropriate option as it circumvents the possible decision between the birth of an affected child or termination of their pregnancy (**Chapter 1.4.6**).

On the other side of the coin – where no genetic diagnosis has been ascertained - counselling is difficult regarding recurrence risks. We are able to confidently exclude a causative mtDNA defect, thereby removing the possibility of an entirely unpredictable recurrence risk, whilst a presumed autosomal dominant aetiology can be excluded on the premise that both parents are clinically unaffected. Of course, for some cases, the recurrence risk will be very low – for example in the case of single, large-scale mtDNA deletions (Chinnery *et al.*, 2004) and *de novo* mutations (Sallevelt *et al.*, 2017). Contrariwise, couples at risk of transmitting autosomal recessive mutations, have an *a priori* recurrence risk of 25%.

Feedback from our patients frequently echoes the fact that this risk is too burdensome, and they are unwilling to have further children without a genetic test being available for subsequent pregnancies. It is this fact that drives us to consistently evolve the genetic diagnostic algorithm, and I am proud of my efforts and the progress I have made throughout my doctoral project in obtaining a genetic diagnosis for those who need it the most – our patients and their families.

This article was accepted by the *European Journal of Human Genetics*, and was published in November 2014 (included within this thesis, **Chapter 6.2**).

The combined approach of mtDNA sequencing, targeted NGS and WES has dramatically improved the diagnostic yield for our undiagnosed patient cohort and provided counselling and reproductive options to families at risk of an otherwise incurable condition.

6.2 A national perspective on prenatal testing for mitochondrial disease

Victoria Nesbitt, Charlotte L. Alston, Emma L. Blakely, Carl Fratter, Catherine L. Feeney, Joanna Poulton, Garry K. Brown, Doug M. Turnbull, Robert W. Taylor and Robert McFarland. A national perspective on prenatal testing for mitochondrial disease. *Eur J Hum Genet*. 2014;22(11):1255-9.

APPENDICES

Table of Appendices

Appendix I

List of all genes offered for diagnostic sequencing by our Centre at the commencement of my doctoral project.

1	*AARS2*		**18**	*MFN2*		**35**	*PEO1*		**52**	*SUCLG1*
2	*ACAD9*		**19**	*MGME1*		**36**	*POLG*		**53**	*SUPV3L1*
3	*ACO2*		**20**	*MPV17*		**37**	*POLG2*		**54**	*TK2*
4	*APEX1*		**21**	*MRPL41*		**38**	*PUS1*		**55**	*TMEM70*
5	*APOPT1*		**22**	*MTFMT*		**39**	*RMND1*		**56**	*TRMT10C*
6	*ATP5E*		**23**	*MTO1*		**40**	*RNASEH1*		**57**	*TRMT5*
7	*ATPAF2*		**24**	*NDUFAB1*		**41**	*RRM2B*		**58**	*TRMU*
8	*BCS1L*		**25**	*NDUFS1*		**42**	*SDHA*		**59**	*TYMP*
9	*BTD*		**26**	*NDUFS2*		**43**	*SDHAF1*		**60**	*UCRC*
10	*CKMT1A*		**27**	*NDUFS3*		**44**	*SDHAF2*		**61**	*UQCC*
11	*CYC1*		**28**	*NDUFS4*		**45**	*SDHB*		**62**	*UQCR*
12	*DGUOK*		**29**	*NDUFS6*		**46**	*SDHC*		**63**	*UQCRB*
13	*EARS2*		**30**	*NDUFS7*		**47**	*SDHD*		**64**	*UQCRC1*
14	*ELAC2*		**31**	*NDUFS8*		**48**	*SERAC1*		**65**	*UQCRC2*
15	*GFM1*		**32**	*NDUFV1*		**49**	*SLC25A4*		**66**	*UQCRC2*
16	*GTPBP3*		**33**	*NDUFV2*		**50**	*SPG7*		**67**	*UQCRFS1*
17	*ISCU*		**34**	*OPA1*		**51**	*SUCLA2*		**68**	*UQCRH*

Appendix II

List of Sanger-based gene tests established as part of my doctoral project to improve genetic diagnosis of mitochondrial disease patients.

1	*DARS2*
2	*RARS2*
3	*ADCK3*
4	*YARS2*
5	*AGK*
6	*TFB2M*
7	*ETHE1*
8	*GFER*
9	*NFU1*
10	*OPA3*
11	*LYRM4*

Appendix III

Genes incorporated within the 'Isolated complex I' Ampliseq capture for IonTorrent PGM sequencing.

	Approved Symbol	Approved Name	Genbank
1	**NDUFA1**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 1, 7.5kDa	NM_004541.3
2	**NDUFA2**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 2, 8kDa	NM_002488.4
3	**NDUFA3**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 3, 9kDa	NM_004542.3
4*	**NDUFA4**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4, 9kDa	NM_002489.3
5	**NDUFA5**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 5, 13kDa	NM_005000.3
6	**NDUFA6**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 6, 14kDa	NM_002490.3
7	**NDUFA7**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 7, 14.5kDa	NM_005001.3
8	**NDUFA8**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 8, 19kDa	NM_014222.2
9	**NDUFA9**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 9, 39kDa	NM_005002.4
10	**NDUFA10**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 10, 42kDa	NM_004544.3
11	**NDUFA11**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 11, 14.7kDa	NM_175614.4
12	**NDUFA12**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 12	NM_018838.4
13	**NDUFA13**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 13	NM_015965.6
14	**NDUFAB1**	NADH dehydrogenase (ubiquinone) 1, α/β subcomplex, 1, 8kDa	NM_005003.2
15	**NDUFB1**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 1, 7kDa	NM_004545.3
16	**NDUFB2**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 2, 8kDa	NM_004546.2
17	**NDUFB3**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 3, 12kDa	NM_002491.2
18	**NDUFB4**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 4, 15kDa	NM_004547.5
19	**NDUFB5**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 5, 16kDa	NM_002492.3
20	**NDUFB6**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 6, 17kDa	NM_002493.4
21	**NDUFB7**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 7, 18kDa	NM_004146.5
22	**NDUFB8**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 8, 19kDa	NM_005004.3
23	**NDUFB9**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 9, 22kDa	NM_005005.2
24	**NDUFB10**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 10, 22kDa	NM_004548.2
25	**NDUFB11**	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 11, 17.3kDa	NM_019056.6
26	**NDUFC1**	NADH dehydrogenase (ubiquinone) 1, 6kDa	NM_002494.3
27	**NDUFC2**	NADH dehydrogenase (ubiquinone) 1, 14.5kDa	NM_001203260.1
28	**NDUFS1**	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	NM_005006.6
29	**NDUFS2**	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa	NM_004550.4
30	**NDUFS3**	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa	NM_004551.2
31	**NDUFS4**	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa	NM_002495.2
32	**NDUFS5**	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa	NM_004552.2
33	**NDUFS6**	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa	NM_004553.4
34	**NDUFS7**	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa	NM_024407.4
35	**NDUFS8**	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa	NM_002496.3
36	**NDUFV1**	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	NM_007103.3
37	**NDUFV2**	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	NM_021074.4
38	**NDUFV3**	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10kDa	NM_001001503.1

	Approved Symbol	Approved Name	Genbank
39	**NDUFAF1**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 1	NM_016013.3
40	**NDUFAF2**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 2	NM_174889.4
41	**NDUFAF3**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 3	NM_199069.1
42	**NDUFAF4**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 4	NM_014165.3
43	**NDUFAF5**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 5	NM_024120.4
44	**NDUFAF6**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 6	NM_152416.3
45	**NDUFAF7**	NADH dehydrogenase (ubiquinone) 1 α subcomplex, assembly factor 7	NM_144736.4
46	**ACAD9**	acyl-CoA dehydrogenase family, member 9	NM_014049.4
47	**NUBPL**	nucleotide binding protein-like	NM_025152.2
48	**FOXRED1**	FAD-dependent oxidoreductase domain containing 1	NM_017547.3
49	**ECSIT**	Evolutionarily Conserved Signalling Intermediate In Toll Pathway	NM_016581.4
50	**TMEM126B**	Transmembrane Protein 126B	NM_018480.4

* *NDUFA4* (NM_002489.3) has been reassigned to complex IV, but was regarded as a subunit of complex I at the time of Ampliseq panel production.

Appendix IV

Molecular genetic diagnoses made for complex I deficient patients during the course of my doctoral studies

Pt	Gene	Mutations detected	Heterozygosity
1	*ACAD9*	c.1204G>A p.Gly402Arg	Homozygous
2	*ACAD9*	c.1552C>T p.Arg518Cys & c.1715G>A p.Cys572Tyr	Heterozygous
3	*ACAD9*	c.976G>C p.Ala326Pro & c.1594C>T p.Arg532Trp	Heterozygous
4	*ACAD9*	c.1150G>A p.Val384Met & c.1168G>A p.Ala390Thr	Heterozygous
4a	*ACAD9*	c.1150G>A p.Val384Met & c.1168G>A p.Ala390Thr	Heterozygous
5	*ACAD9*	c.665T>A p.Ile222Asn & c.1249C>T p.Arg417Cys	Heterozygous
5a	*ACAD9*	c.665T>A p.Ile222Asn & c.1249C>T p.Arg417Cys	Heterozygous
6	*ACAD9*	c.1250G>A p.Arg417His & c.1795_1798del p.Lys600Cysfs*56	Heterozygous
7	*FOXRED1*	c.1261G>A p.Val421Met	Homozygous
8	*FOXRED1*	c.406C>T p.Arg136Trp	Homozygous
9	*NDUFA1*	c.94G>C p.Gly32Arg	Hemizygous
10	*NDUFA6*	c.269G>C p.Arg90Pro & c.343G>T p.Glu115*	Homozygous
11	*NDUFAF2*	c.221G>A p.Trp74*	Homozygous
11a	*NDUFAF2*	c.221G>A p.Trp74*	Homozygous
12	*NDUFAF5*	c.2T>C p.Met1? & exon 1 deletion	Heterozygous
13	*NDUFAF5*	c.826C>T p.Arg276* & c.848C>T p.Ala283Val	Heterozygous
14	*NDUFAF6*	c.805C>T p.His269Tyr & c.581-7A>G	Heterozygous
15	*NDUFAF6*	c.659C>A p.Thr220Lys	Homozygous
15a	*NDUFAF6*	c.659C>A p.Thr220Lys	Homozygous
16	*NDUFB3*	c.64T>C p.Trp22Arg	Homozygous
17	*NDUFB3*	c.64T>C p.Trp22Arg	Homozygous
18	*NDUFB3*	c.64T>C p.Trp22Arg	Homozygous
19	*NDUFS2*	c.1754C>T p.Pro585Leu	Homozygous
20	*NDUFS2*	c.998G>A p.Arg333Gln	Homozygous
20a	*NDUFS2*	c.998G>A p.Arg333Gln	Homozygous
21	*NDUFS3*	c.642_644delTGA p.Asp214del	Homozygous
22	*NDUFS4*	c.290delG p.Trp97* & c.459delA p.Lys154Asnfs*35	Heterozygous
23	*NDUFS4*	exon 3 & 4 deletion	Homozygous
24	*NDUFS5*	c.152G>A p.Arg51Gln	*De novo*
25	*NDUFS6*	c.316_319delGAAA p.Glu106Glnfs*41	Homozygous
26	*NDUFV1*	c.1268C>T p.Thr423Met	Homozygous
27	*TMEM126B*	c.401het_delA p.Asn134Ilefs*2 & c.635G>T p.Gly212Val	Heterozygous
27a	*TMEM126B*	c.401het_delA p.Asn134Ilefs*2 & c.635G>T p.Gly212Val	Heterozygous

Red: novel gene/variant; **a**: sibling of proband; **heterozygous**: compound heterozygous

Appendix V

Manuscripts in press (n=1)

Alston CL* . Sommerville EW*****, Ng YS*****, Dallabona C, Gilberti M, He S, Knowles C, Chin SL, Schaefer AM, Falkous G, Murdoch D, Longman C, de Visser M, Bindoff LA, Rawles JM, Dean JCS, Petty RK, Farrugia ME, Haack TB, Prokisch H, Turnbull DM, McFarland R, Donnini C, Taylor RW and Gorman GS. Evidence of a new founder effect in adults with *YARS2*-related mitochondrial myopathy. 2016 [MS ID#: NEU16-1020; accepted 25th August 2016 for publication in *JAMA Neurol.*]
(* denotes joint first authors)

Appendix VI

Manuscripts published (n=43) during the course of my PhD studies

February 2013 - October 2016.

Mitochondrial complex I deficiency

Silwal A, Morris A, Warren D, Vadlamani G, **Alston CL**, Taylor RW. Cystic leukoencephalopathy due to *NDUFV1* mutation: a report of the phenotype and its rare co-occurrence with primary hyperoxaluria. *J Pediatr Neurol.*, 2016;14(3):126-132.

Alston CL, Howard C, Oláhová M, Hardy SA, He L, Murray PG, O'Sullivan S, Doherty G, Shield JPH, Hargreaves IP, Monavari AA, Knerr I, McCarthy P, Morris AAM, Thorburn DR, Prokisch H, Clayton PE, McFarland R, Hughes J, Crushell E, Taylor RW. A recurrent mitochondrial p.Trp22Arg *NDUFB3* variant causes a distinctive facial appearance, short stature and a mild biochemical and clinical phenotype. *J Med Genet*. 2016;53(9):634-41.

Alston CL*, Compton AG*, Formosa LE*, Strecker V, Oláhová M, Haack TB, Smet J, Stouffs K, Diakumis P, Ciara E, Cassiman D, Romain N, He L, De Paepe B, Vanlander AV, Seneca S, Feichtinger RG, Płoski R, Rokicki D, Pronicka E, Haller RG, Bahlo M, Mayr JA, Van Coster R, Prokisch H, Wittig I, Ryan MT, Thorburn DR, Taylor RW. Biallelic mutations in *TMEM126B* cause severe complex I deficiency with a variable clinical phenotype. *Am J Hum Genet.* 2016;99(1):217-27.
(* denotes joint first authors)

Kennedy H*, Haack TB*, Hartill V*, Matakovic L*, Baumgartner ER, Potter H, Mackay R, **Alston CL**, O'Sullivan S, McFarland R, Connolly G, Gannon C, King R, Mead S, Crozier I, Chan W, Florkowski CM, Sage M, Höfken T, Alhaddad B, Kremer LS, Kopajtich R, Feichtinger RG, Sperl W, Rodenburg RJ, Minet JC, Dobbie A, Strom TM, Meitinger T, George PM, Johnson CA, Taylor RW, Prokisch H, Doudney K, Mayr JA. Sudden Cardiac Death due to Deficiency of the Mitochondrial Inorganic Pyrophosphatase *PPA2*. *Am J Hum Genet*. 2016;99(3):674-82.
(* denotes joint first authors)

Whole exome sequencing

Ng YS, **Alston CL**, Diodato D, Morris AAM, Ulrick N, Kmoch S, Houstek J, Martinelli D, Haghighi A, Atiq M, Gamero MA, Garcia-Martinez E, Kratochvílová H, Santra S, Brown RM, Brown GK, Ragge N, Monavari A, Pysden K, Ravn K, Casey JP, Khan A, Chakrapani A, Vassallo G, Simons C, McKeever K, O'Sullivan S, Childs A, Østergaard E, Vanderver A, Goldstein A, Vogt J, Taylor RW, McFarland R. The clinical, biochemical and genetic features associated with *RMND1*-related mitochondrial disease. *J Med Genet.* 2016, doi: 10.1136/jmedgenet-2016-103910. [Epub ahead of print]

Thompson K*, Majd H*, Dallabona C*, Reinson K*, King MS, **Alston CL**, He L, Lodi T, Jones SA, Fattal-Valevski A, Fraenkel ND, Saada A, Haham A, Isohanni P, Vara R, Barbosa IA, Simpson MA, Deshpande C, Puusepp S, Bonnen PE, Rodenburg RJ, Suomalainen A, Õunap K, Elpeleg O, Ferrero I, McFarland R, Kunji ER, Taylor RW. Recurrent *De Novo* Dominant Mutations in *SLC25A4* Cause Severe Early-Onset Mitochondrial Disease and Loss of Mitochondrial DNA Copy Number. *Am J Hum Genet.* 2016;99(4):860-876.
(* denotes joint first authors)

Metodiev MD*, Thompson K*, **Alston CL**, Morris AAM , He L, Assouline Z, Rio M, Bahi-Buisson N, Pyle A, Griffin H, Siira S, Filipovska A, Munnich A, Chinnery PF, McFarland R, Rötig A, Taylor RW. Recessive mutations in *TRMT10C* cause defects in mitochondrial RNA processing and multiple respiratory chain deficiencies. *Am J Hum Genet.* 2016;98(5):993-1000.
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ORIGINAL ARTICLE

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Neuropathologic Characterization of Pontocerebellar Hypoplasia Type 6 Associated With Cardiomyopathy and Hydrops Fetalis and Severe Multisystem Respiratory Chain Deficiency due to Novel *RARS2* Mutations

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Abstract

Autosomal recessive mutations in the *RARS2* gene encoding the mitochondrial arginyl-transfer RNA synthetase cause infantile-onset myoencephalopathy pontocerebellar hypoplasia type 6 (PCH6). We describe 2 sisters with novel compound heterozygous *RARS2* mutations who presented perinatally with neurologic features typical of PCH6 but with additional features including cardiomyopathy, hydrops, and pulmonary hypoplasia and who died at 1 day and 14 days of age. Magnetic resonance imaging findings included marked cerebellar hypoplasia, gyral immaturity, punctate lesions in cerebral white matter, and unfused deep cerebral grey matter. Enzyme histochemistry of postmortem tissues revealed a near-global cytochrome *c* oxidase-deficiency; assessment of respiratory chain enzyme activities confirmed severe deficiencies involving complexes I, III, and IV. Molecular genetic studies revealed 2 *RARS2* gene mutations: a c.1A>G, p.? variant predicted to abolish the initiator methionine, and a deep intronic c.613-3927C>T variant causing skipping of exons 6–8 in the mature *RARS2* transcript. Neuropathologic investigation included low brain weights, small brainstem and cerebellum, deep cerebral white matter pathology, pontine nucleus neuron loss (in 1 sibling), and peripheral nerve pathology. Mitochondrial respiratory chain immunohistochemistry in brain tissues confirmed an absence of complexes I and IV immunoreactivity with sparing of mitochondrial numbers. These cases expand the clinical spectrum of *RARS2* mutations, including antenatal features and widespread mitochondrial respiratory chain deficiencies in postmortem brain tissues.

Key Words: Mitochondrial disease, Pontocerebellar hypoplasia type 6, *RARS2*, Respiratory chain deficiency.

INTRODUCTION

Mitochondrial respiratory chain diseases represent a clinically and genetically diverse collection of isolated or multiorgan disorders with an incidence estimated at 1 in 5000 births (1). Mutations in either mitochondrial or nuclear DNA can lead to defective protein synthesis resulting in impaired assembly and function of the oxidative phosphorylation system and reduced adenine triphosphate biosynthesis. This can

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result in a wide range of phenotypes affecting both children and adults. The increasing use of targeted exome sequencing and next-generation sequencing has led to the identification of an expanding spectrum of mutations in nuclear encoded mitochondrial proteins (2), including mitochondrial aminoacyl transfer RNA (tRNA) synthetases (mt-aaRSs) (3).

All mt-aaRSs are translated in the cytosol before being imported into the mitochondria where they play a key role in mitochondrial protein translation by catalyzing the attachment of amino acids to their cognate tRNA molecules. This is a 2-step process by which the mt-aaRS activates 1 of the 20 amino acids with adenine triphosphate to form an aminoacyl-adenylate intermediate, which then transfers the aminoacyl part to the cognate tRNA. Once a tRNA is charged with its cognate amino acid, it is delivered to the mitoribosome where the amino acid can become incorporated into the growing polypeptide chain (4).

Defects in mt-aaRSs have emerged as an important cause of perinatal or infantile onset respiratory chain diseases. These are associated with autosomal recessive inheritance and are often fatal in early life. Mutations in *RARS2* (mitochondrial arginyl-tRNA synthetase; 6q15) have been identified in children with pontocerebellar hypoplasia type 6 ([PCH6]; OMIM#611523); these were first described in a consanguineous Sephardic Jewish family in which affected children presented with intractable seizures, hypotonia, profound neurodevelopmental delay, microcephaly, and feeding difficulties (5). Neuroimaging revealed progressive cerebellar atrophy with cerebral involvement. Each affected child was homozygous for an intronic c.110+5A>G mutation leading to aberrant mRNA splicing and associated with respiratory chain defects in muscle and fibroblasts. An additional 16 patients harboring autosomal recessive *RARS2* mutations have since been reported (6–10); detailed postmortem neuropathologic reports are only available for 2 cases (11). These cases demonstrated additional features of PCH subtypes 2 and 4, including severe dystonia, optic atrophy, and thinning of the corpus callosum (9). In addition to the PCH, anterior horn cell disease was also found at autopsy, a finding typically associated with PCH1 (7).

Here we report 2 siblings with a PCH6 presentation in whom, in addition to the characteristic pontocerebellar hypoplasia and microcephaly, there was evidence of cardiomyopathy and peripheral neuropathy. We show that this fatal neurologic disease was due to novel compound heterozygous *RARS2* mutations, including the first deep intronic recessive mutation to cause fatal pediatric mitochondrial disease, and describe the neuropathologic characteristics in postmortem brain tissue from both clinically affected siblings.

MATERIALS AND METHODS

This study had relevant ethical approval from the institutional review board of Newcastle University and complied with the Declaration of Helsinki, and written informed consent was obtained.

Patients

Patient I-1

A female was born at 36 weeks to nonconsanguineous healthy, UK Caucasian British parents following a normal pregnancy and delivery. Birth weight was 2.470 kg (25th–50th centile); head circumference was 32.5 cm (50th–75th centile). On delivery, general condition was immediately and unexpectedly poor, with Apgar scores of 1 at 1 minute and 1 at 5 minutes. She was blue, floppy, and took only 1 breath. She was intubated at birth, and resuscitation was attempted for over an hour but was ultimately unsuccessful. Apart from a dermal pit on the lower back, no dysmorphic features were noted on examination.

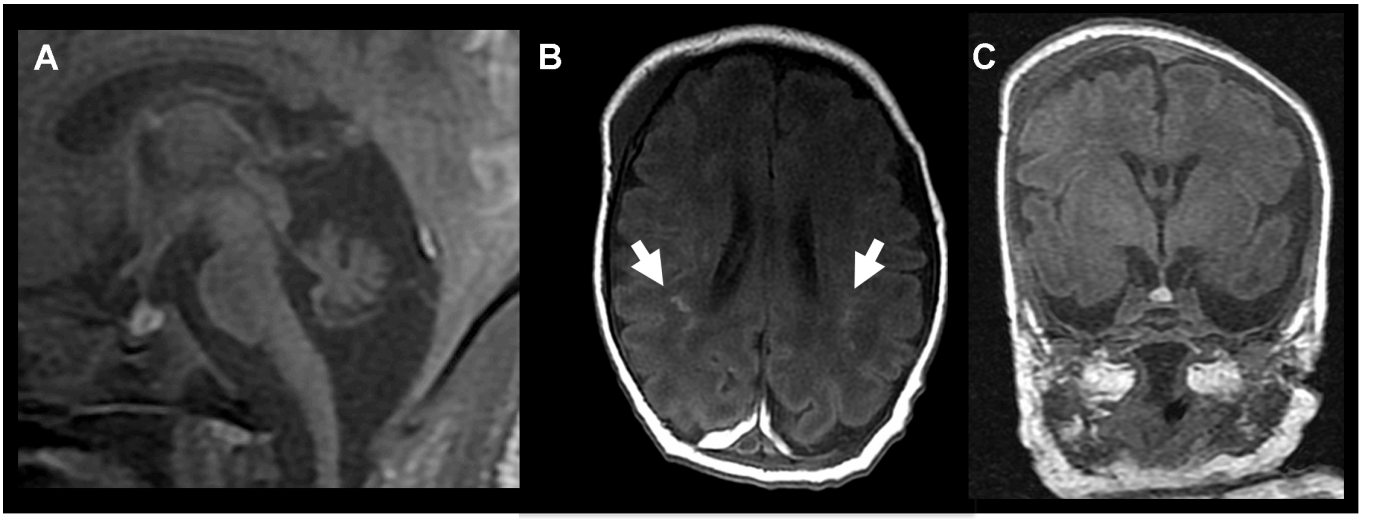
Patient I-2

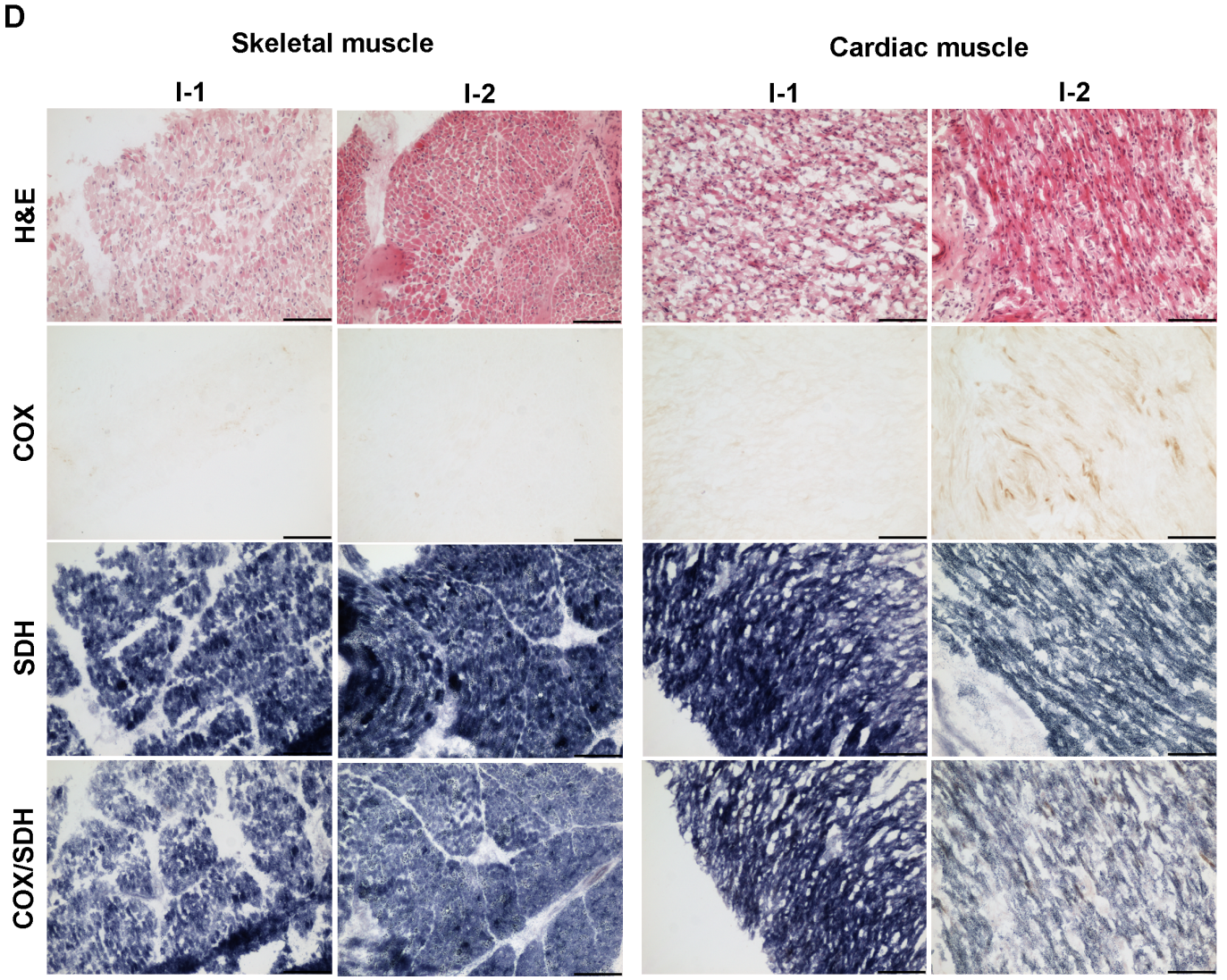
This pregnancy was closely monitored because of the previous neonatal death. Nuchal translucency was increased on ultrasound at 12 weeks (3.8 mm; 99th centile, 3.5 mm [12]). Because of concerns about fetal bradycardia, serial fetal echocardiograms were performed, which, at 20 weeks gestation, revealed mild biventricular hypertrophy in the absence of left or right outflow obstruction. From 29 weeks on the ventricular hypertrophy was more marked on the right than the left and was nonprogressive. Ultrasonography revealed transverse cerebellar diameter at 18.9 mm at 20^{+2} weeks and 19.5mm at 21^{+2} weeks on the 3rd percentile. Biparietal diameter was 43.8 and 49.6 mm at corresponding dates. Repeat ultrasound scan at 36 weeks showed polyhydramnios, right-sided pleural effusion, hyperechogenic bowel loops with bowel dilation, skin edema, and absence of fetal movements.

Labor was induced at 36^{+2} weeks, with vaginal delivery of a cyanotic, hypotonic, and bradycardic female weighing 2.340 kg (25th centile). APGAR scores were 3 at 1 minute and 4 at 5 minutes. Assisted ventilation rapidly improved both heart rate and color. She was intubated by 6 minutes of age because of absence of echocardiogram movements and absent respiratory effort. She remained ventilator dependent. Postnatal echocardiogram confirmed biventricular hypertrophy with good ventricular function and neither left nor right ventricular outflow obstruction. Neurologic examination revealed marked central and peripheral hypotonia, decreased reflexes and minimal spontaneous limb movements. No dysmorphic features were noted. Ocular and audiologic examinations were normal. On day 2, EEG suggested burst-suppression pattern. Blood and CSF lactate levels were elevated (blood persistently ~7 mmol/L with maximum 19.8 mmol/L on day 11 [reference range, 0.7–2.2 mmol/L], CSF 11.3 mmol/L on day 5 [reference range, 1.1–2.2 mmol/L]). She had low serum calcium on day 1 treated with intravenous calcium infusion and hyponatremia during the first week managed by fluid restriction. Urinary analyses showed moderate lactic aciduria, moderately increased 4-hydroxyphenylacetate, and mildly increased 4-hydroxyphenylpyruvate, consistent with liver dysfunction/immaturity. Cranial magnetic resonance imaging (MRI) was performed on day 8. After family discussion, intensive care was withdrawn on day 14, and death rapidly occurred. A full postmortem examination was conducted 1 day later with full consent.

Muscle Histology, Histochemistry, and Respiratory Chain Activity

Standard histologic and histochemical analyses of postmortem skeletal muscle and cardiac tissue were performed on





fresh-frozen 10-μm-thick cryosections. Standard methods included hematoxylin and eosin (H&E) stain, modified Gomori trichrome stain, and sequential cytochrome *c* oxidase/succinate dehydrogenase (COX/SDH) histochemistry to assess both complex IV (COX) and complex II (SDH) activities (13). Activities of individual respiratory chain complexes (I–IV) and citrate synthase were measured spectrophotometrically, as previously described (14).

Molecular Genetic Investigations

Genomic DNA was extracted from patient muscle biopsy according to standard protocols. Primer 3 was used to design primers specific to each coding exon of *RARS2* (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A746). Patient genomic DNA was polymerase chain reaction (PCR) amplified and Sanger sequenced using BigDye v3.1 chemistry (Applied Biosystems, Cheshire, UK) and capillary electrophoresis performed on an ABI3130xl platform (Applied Biosystems) (15). Patient sequencing chromatograms were compared with the Genbank reference sequence (NM_020320.3) and all variants were annotated using dbSNP build 138. Allele frequency of all gene variants was determined using ESP6500 and 10k genome project data. Parental DNA samples were referred to confirm a recessive inheritance of detected variants.

Complimentary DNA (cDNA) was derived from whole RNA extracted from patient fibroblasts cultured in standard Dulbecco modified eagle medium. Preservation of abnormal mRNA transcripts was accomplished by culturing confluent patient fibroblasts overnight in emetine-containing media at a final concentration of 100 μg/mL before harvesting (16). Reverse transcription of 2 μg RNA was performed using GoScript reverse transcription system and a poly dT primer (Promega, Hampshire, UK). PCR amplification of 2 overlapping cDNA fragments and electrophoresis of PCR amplified products through a 2% agarose gel facilitated identification of normal and abnormal length cDNA fragments.

Gel excision of cDNA fragments was performed using the Agarose Gel DNA Extraction Kit (Roche, Welwyn, Garden City, UK), according to the manufacturer's protocol and recovered amplicons were subject to DNA sequencing. In silico splicing prediction tools NNSPLICE, Human Splicing Finder, and MaxEnt were used to investigate the likely impact of a putative splicing variant in intron 8 identified by cDNA studies.

Neuropathology

Postmortem intervals for Patients I-1 and I-2 were 5 days and 1 day, respectively. After removal of the CNS, frontal and occipital brain samples from Patient I-2 were snap-frozen and kept at −80°C for long-term storage. Peripheral sciatic nerve tissues were also sampled and processed for electron microscopy. For both Patient I-1 and I-2, the remaining CNS was fixed in formalin for 7 and 11 days, respectively, and paraffin embedded before undergoing routine neuropathologic examination (14). To document the severity of the pathologic changes in patient tissues, CNS tissues from 2 disease controls were obtained from the Newcastle Brain Tissue Resource (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A747). Histology and immunohistochemistry were performed as previously described (14).

Mitochondrial Respiratory Chain Enzyme Histochemistry and Immunohistochemistry

Sequential COX/SDH histochemistry was undertaken on 10-μm-thick frozen sections of frontal and occipital cortex from Patient I-2, as previously described (17). Distribution and expression of key subunits of the mitochondrial respiratory chain complexes (complexes I–IV) and porin (a marker of mitochondrial number) were analyzed using immunohistochemistry performed on 5-μm formalin-fixed, paraffin-embedded frontal, parietal, temporal, and occipital cortices, basal ganglia, pons, and cerebellum, as previously described (14). To allow a direct comparison, patient and control tissues were included in all experiments.

RESULTS

Cranial MRI

Cranial MRI on Day 8 of Patient I-2 showed markedly decreased cerebellar diameter at 33 mm, reduced vermian height at 10.9 mm (Fig. 1A), and slightly decreased anteroposterior pontine diameter at 10 mm but with normal shape. No cerebellar cysts were identified. Cerebrum showed punctate foci in lobar white matter (Fig. 1B); corpus callosum and splenium were within normal limits; there were simple gyri with a possible area of polymicrogyria within 1 Sylvian fissure. Lateral ventricles were prominent. There was no midline fusion of either deep grey matter or cortical ribbon (Fig. 1C).

General Autopsy

Patient I-1

The head circumference was 32.5 cm (50th–75th centile). External gross examination showed no facial or other dysmorphism. Internal gross findings consisted of bilateral pleural effusions, hypoplastic lungs; the heart was normal. Microscopically, liver and kidney were normal.

Patient I-2

Head circumference was 32.0 cm (50th–75th centile). External gross examination revealed generalized subcutaneous edema, small midface, wide lower face, and depressed nasal bridge; limbs, hands, and feet were normal. There were pericardial, pleural, peritoneal effusions. Lungs were hypoplastic. The heart showed prominent right ventricular trabeculation with

FIGURE 1. Cranial MRI performed on Patient I-2 at age 8 days and effect of *RARS2* mutation on COX activity in postmortem skeletal muscle and heart sections. **(A)** Sagittal T1-weighted image demonstrating hypoplastic cerebellar vermis. **(B)** Transverse T1-weighted images illustrating punctate white matter signal changes (white arrows). **(C)** Coronal T1-weighted image illustrates no midline fusion of the deep grey matter or cortical ribbon. **(D)** COX/SDH histochemistry of patient tissues reveals a generalized loss of COX activity in both skeletal muscle and cardiac tissues. Scale bar = 100 μm.

TABLE. Assessment of Respiratory Chain Complex Activities in Postmortem Skeletal and Cardiac Muscle Homogenates From Patient I-2

	Complex I/CS	Complex II/CS	Complex III/CS	Complex IV/CS
Patient I-2 (muscle)	0.019	0.105	0.155	0.051
Controls (n = 25)	0.104 ± 0.036	0.145 ± 0.047	0.554 ± 0.345	1.124 ± 0.511
Patient I-2 (cardiac)	0.008	0.072	0.167	0.015
Controls (n = 25)	0.125 ± 0.048	0.152 ± 0.050	1.112 ± 0.386	1.258 ± 0.367

Enzyme activities are expressed as nmol NADH oxidized min^{-1}.unit citrate synthase $(CS)^{-1}$ for complex I, nmol DCPIP reduced min^{-1}.unit citrate $synthase^{-1}$ for complex II (succinate:ubiquinone-1 reductase) and the apparent first-order rate constant.sec^{-1}.unit citrate $synthase^{-1}$ for complexes III and IV ($\times 10^3$). Control values are shown as mean ± SD. DCPIP, 2,6-dichlorophenol-indophenol.

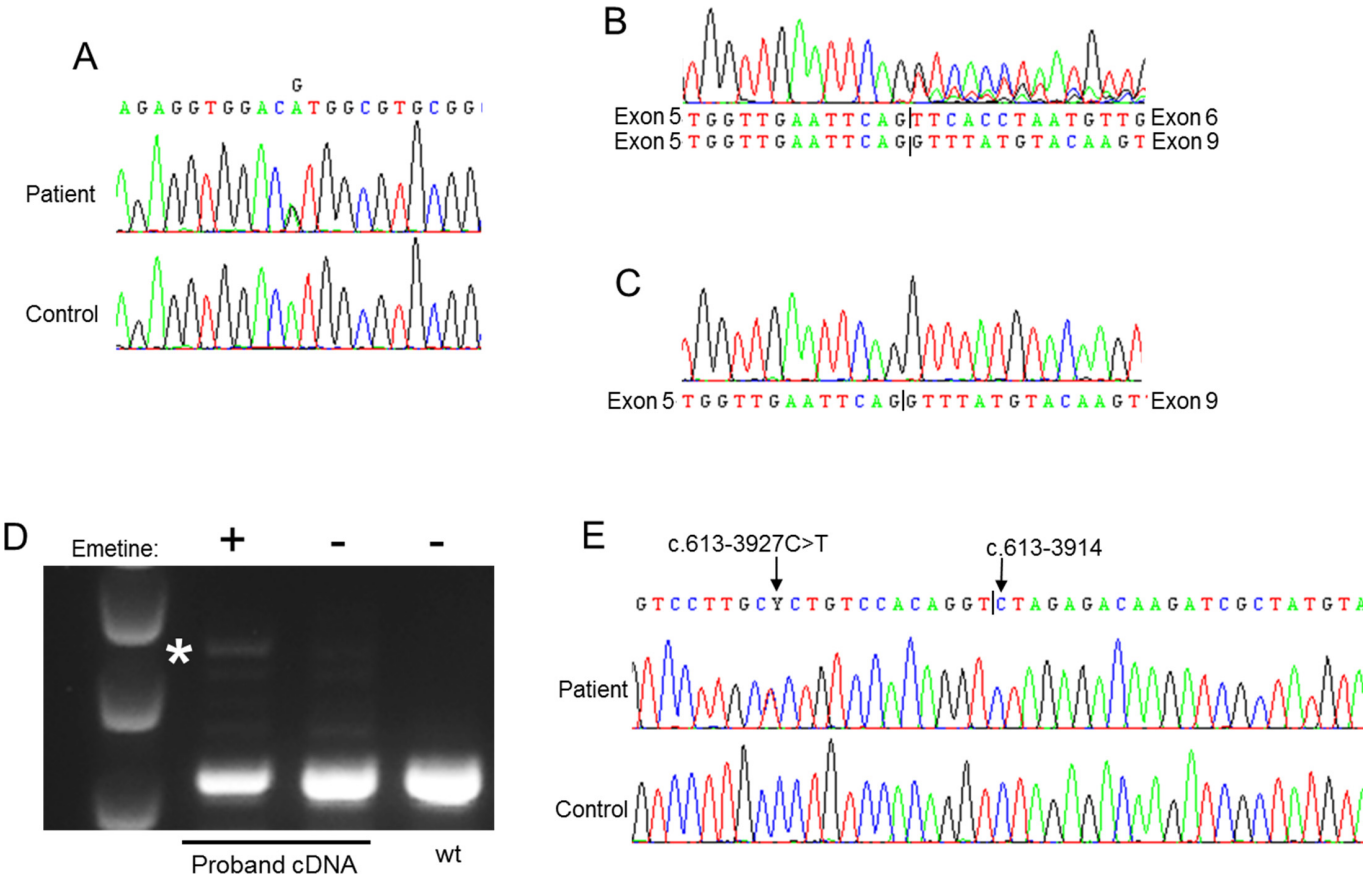


FIGURE 2. Molecular characterization of novel *RARS2* mutations. **(A)** Sequencing of the *RARS2* coding region identified a novel heterozygous c.1A>G *RARS2* variant predicted to abolish the initiating methionine (p.Met1?). **(B)** PCR and electrophoretic analysis of *RARS2* cDNA derived from the proband's fibroblasts revealed 2 populations, a wild-type amplicon plus an amplicon demonstrating skipping of exons 6–9. **(C)** Sequencing of a gel-purified cDNA amplicon clearly shows skipping of exons 6–9 in *RARS2* patient cDNA. **(D)** cDNA analysis of fibroblasts cultured in emetine-containing media reveal a novel transcript (asterisk). Sanger sequencing of this band following agarose gel excision revealed retention of 112 bp of intronic sequence (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A746). **(E)** Sequencing of surrounding genomic DNA revealed a novel c.613-3927C>T substitution upstream of the retained intronic sequence, which create a cryptic splicing acceptor at c.613-3916_613-3915; together with a cryptic splicing donor site at c.613-3793_613-3792, this causes the retention of 112 bp of intron 8 (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A746).

a thickened (0.9 cm) free ventricular wall. The left ventricle showed less trabeculation, but there was slight thickening of the free wall (1.2 cm); the myocardium was pale. The liver showed centrilobular congestion with focal cholestasis, microsteatosis, and lipid vacuolation of some hepatocytes. Renal tubules featured occasional Oil Red O–positive lipid vacuoles.

Muscle and Cardiac Morphology, Histochemistry, and Biochemistry

Patient I-1

Cardiac muscle featured instances of myocyte central clearing and abundant eosinophilic cytoplasmic puncta interpreted as mitochondria, some abnormally large, overall considered consistent with a mitochondrial cardiomyopathy. Oil Red O stain did not show accumulation of fine-droplet lipid in any myofiber.

Patient I-2

Microscopically, the heart showed myocyte hypertrophy without disarray. Some myocytes were swollen with perinuclear clearing and loss of cross-striation; contained numerous eosinophilic dots interpreted as mitochondria, some being abnormally large; and had an excess of fine droplets of Oil Red O–positive lipid. There was no inflammation, necrosis, or endocardial fibroelastosis.

COX/SDH histochemistry revealed a near global loss of COX activity despite sparing of SDH activity in both skeletal and cardiac muscle tissues from both patients (Fig. 1D). Although the postmortem interval was longer in Patient I-1 (5 days), it is our experience that samples retain COX and SDH activity after considerable postmortem delay and it is usually SDH activity that is most labile. Spectrophotometric assay of mitochondrial respiratory chain complexes showed a marked reduction in complex I, III, and IV activities in Patient I-2's skeletal and cardiac muscle compared with controls with a general sparing of complex II activity, indicating a generalized defect of mitochondrial translation (Table).

Molecular Genetics

The combination of radiologic cerebellar atrophy and multiple mitochondrial respiratory chain defects strongly suggest the diagnosis of PCH6 (OMIM 611523) due to recessive mutations in the *RARS2* gene (5). In confirmation, sequencing the entire coding region and intron-exon boundaries of the *RARS2* gene was performed for Patient I-2, revealing a single heterozygous c.1A>G variant that is predicted to abolish the initiator methionine, thereby resulting in a null allele (Fig. 2A). No further known or potentially pathogenic *RARS2* variants were identified in the coding region. cDNA investigations using fibroblast-derived RNA from Patient I-2 provided evidence of aberrant mRNA splicing with the presence of 2 differentially sized *RARS2* cDNA molecules, the wild-type transcript harboring the c.1A>G variant and a second transcript apparently lacking exons 6, 7, and 8 (Figs. 2B, C). Repeating the cDNA investigations using fibroblasts cultured in emetine, an inhibitor of mitochondrial translation, revealed a number of additional transcripts. One retained 123 bp of intronic sequence from intron 8, c.613-3916_613-3793 (Fig. 2D). Analysis of the surrounding genomic sequence revealed a novel c.613-3927C>T variant upstream of the retained sequence (Fig. 2E; Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A746), supporting creation of a cryptic splice acceptor. *In silico* investigations predict a very high score for the c.613-3916 cryptic splice acceptor site. The aberrantly spliced mRNA transcript that lacks exons 6–8 results in a frame-shift mutation.

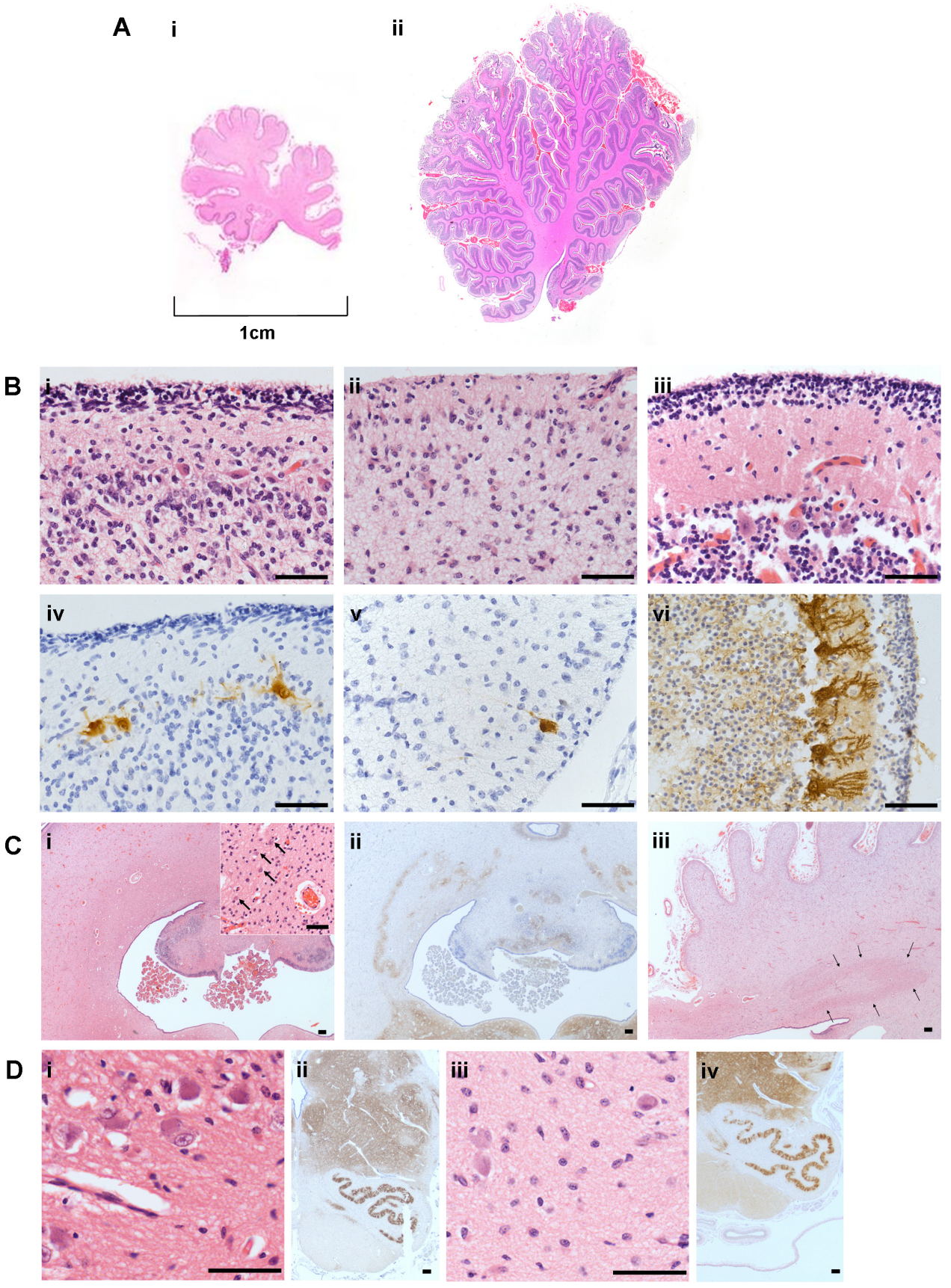
Analysis of familial DNA samples confirmed that both sisters harbored the paternal c.1A>G, p.? and maternal c.613-3927C>T *RARS2* variants, thereby confirming recessive inheritance. Prenatal testing of a chorionic villus sample for these variants in a third pregnancy revealed neither *RARS2* variant and a healthy child was born.

Neuropathology

Patient I-1

The fixed brain was micrencephalic weighing 257 g (reference range, 366 ± 50 g, n = 11 for 36–37 weeks gestation [18]). The combined cerebellum and brainstem weight of 11.5 g was similarly low for the age (reference range, 21.4 g ± 3.36 g). Externally, the gross appearance of forebrain and hindbrain was normal, and the cerebellar vermis and hemispheres were normally formed without any evidence of atrophy. Coronal sectioning of the hemispheres showed cortical pallor, consistent with ischemia-hypoxia, with lobar white matter discoloration without cystic degeneration or mineralization. The corpus callosum, fornix, septum pellucidum, and internal capsule were normal. The deep grey matter was unremarkable and without mineralization. The hippocampi were normal with mild compression in the lateral ventricles. The third ventricle was normal and there was no fusion across the midline. The midbrain aqueduct was compressed in the vertical plane, suggestive of mild cerebral swelling. Tectum and peduncles were normal. Cerebellum was sectioned horizontally and lacked distinct dentate nuclei. Medulla and pons were grossly normal.

Microscopically, the cerebellar cortex revealed focal depletion of neurons from the external granular cell layer, an abnormally thin molecular layer, and sparsely populated internal granular cell layer. Purkinje cell numbers were markedly reduced, with the remainder exhibiting a shrunken perikarya and eosinophilia (Fig. 3Bi). Calbindin-staining demonstrated the perikaryal change and added severe stunting of apical dendrites, with an absence of ''cactus bodies'' (dystrophic dendritic structures) (Fig. 3Biv). The dentate nucleus ribbon, best identified by synaptophysin immunoreactivity (Fig. 3Cii), had a normal undulating configuration but was abnormally thin and was very sparsely populated by neurons in H&E stain (Fig. 3Ci). In the medulla, synaptophysin immunostaining likewise demonstrated a normally formed neuropil ribbon for the inferior olivary nuclei, whereas H&E stain demonstrated its constituent neurons (Figs. 3Di, ii). Pyramidal and other long tracts appeared normal. The basal pontine nuclei contained normal neuron complement, although rare karyorrhectic bodies were identified (Fig. 4Bi). Basal pontine transverse fibers could not be demonstrated using antiphosphorylated neurofilament immunohistochemistry (Fig. 4Ci) contrasting with the positive immunoreactivity in the medial longitudinal fasciculus (Fig. 4Cii) and medial lemniscus (Fig. 4Ciii). The midbrain had a distinct red nucleus, but decussation of superior cerebellar peduncle could not be demonstrated. The cerebral aqueduct, cerebral peduncles,



and colliculi were normal. Although the hippocampi were normally formed, there was minor subicular neuronal karyorrhexis, microgliosis, and astrocytosis. Putamen, caudate, globus pallidus, and thalamus had normal neuronal complement, cytology, and neuropil consistency and did not show any mineralization. By immunohistochemistry, only the globus pallidus and lateral nuclei of the thalamus showed diffuse microgliosis; there was no astrocytosis. The optic tract and posterior limb of internal capsule appeared normal. The cerebral white matter was congested but lacked periventricular leukomalacia or other acquired abnormalities and the corpus callosum was normal. The neocortex demonstrated normal neuronal complement and cytology and no mineralization was observed.

Patient I-2

The fixed brain weighed 230 g, whereas the combined brainstem and cerebellum weight was disproportionately low at 10.4 g (4.5% of total fixed brain weight). Externally, cerebral leptomeninges loosely covered a normal arrangement of gyri where there was no sulcal widening. The medulla and cerebellum were much smaller relative to the remainder of the brain. Coronal sections of cerebrum showed neither sulcal widening nor malformation; however, macroscopically, there was dark discoloration of the central white matter with superimposed pale streaks within the occipital region (Fig. 4Di). The corpus callosum was of normal thickness, the anterior commissure and fornix were normal, and the septum pellucidum was caved anteriorly but otherwise normal. The deep grey matter showed no midline fusion. The hippocampi appeared normally formed and largely filled the temporal horns; the lateral and third ventricles were not dilated. The midbrain showed a normal aqueduct, peduncles, and colliculi. Pons had grossly normal tegmentum and basis pontis, whereas the medulla was tiny. Cerebellar vermis and hemisphere showed fewer folial subdivisions than normal (Figs. 3Ai, ii). A 5-cm sample of lower spinal cord displayed no convincing abnormalities.

Microscopic findings were generally similar to or supplemented those in the sibling. Sagittal sections through the cerebellar vermis clearly demonstrated a normal arrangement of principal lobules but much reduced folial branching (Fig. 3Ai). The marked reduction in thickness of individual cortical laminae, lack of Purkinje cells (Fig. 3Biii) and their cytologic changes (Fig. 3Bv), and reduced internal granule-cell packing-density were as in Patient I-1. The dentate nucleus and the internal olivary nuclei were both discernible and normally formed (Figs. 3Ciii and Diii, iv). The basis pontis of Patient I-2, however, contrasted strikingly with her sister's by displaying marked neuron loss (Fig. 4Bii) associated with astrogliosis (Fig. 4Biii), and microgliosis (Fig. 4Biv). Deep cerebral white matter showed mineralizing macrophage-rich foci of periventricular leukomalacia (Fig. 4Dii), corresponding to the macroscopic streaky pallor. The putamen, caudate, and globus pallidus exhibited sparse eosinophilic neurons consistent with ischemia-hypoxia, capillary prominence, and endothelial cell hypertrophy without excessive micromineralization. Immunohistochemistry for glial fibrillary acidic protein showed astrocytosis in the globus pallidus alone, whereas anti-CD68 showed moderate and minor microgliosis in globus pallidus and caudate-putamen, respectively. The hypothalamus showed normal nuclear groups and mammillary bodies, dorsolateral regions showed moderate diffuse microgliosis, and there was no astrocytosis. In contrast to Patient I-1, the internal capsule and optic tract showed moderate microgliosis.

Sciatic nerve (available for Patient I-2 only) showed rare small CD68-positive macrophages and similarly immunoreactive sparse spindly cells within the endoneurium by light microscopy. Phosphorylated neurofilament antibody showed axons of all sizes (Figs. 5A, B). There were no cellular infiltrates. Electron microscopy, although hampered by postmortem artefact, demonstrated considerably fewer myelinated fibers than expected for the age (Figs. 5C, D). Some myelinated fibers had an inappropriately thin sheath for axon diameter. In some others, the axon was absent, leaving only a collapsed myelin sheath. A few unmyelinated axons were large enough to be the result of demyelination, whereas the perineurium appeared mature, which does not suggest delayed maturation.

Mitochondrial Pathology in CNS Tissues

Mitochondrial densities, as judged by porin and nuclear-encoded SDHA immunoreactivity, were high in neurons throughout all CNS tissues analyzed. Immunohistochemical analysis of mitochondrial respiratory chain protein expression showed evidence of a specific and total loss of subunits comprising complex I, as judged by a lack of immunoreactivity for subunits NDUFB8 and NDUFS3, throughout all CNS tissues investigated in both Patients I-1 and I-2. This was prominent in the cerebellum where cell loss was severe (Fig. 6), the pontine nucleus (Fig. 7), and in other regions where neuronal population density was intact, including the occipital cortex (Fig. 8) and other brain regions, including frontal cortex and basal ganglia (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A748). Analysis of the other mitochondrial respiratory chain complexes revealed comparable immunoreactivity of complexes II and III to that observed in control tissues; however, the mitochondrially encoded COX1 was absent despite high immunoreactivity for COX4 of this complex. This loss of protein expression is selective for complexes I and COX1 throughout the brain and suggestive of respiratory chain deficiency due to decrease or absence

FIGURE 3. Histopathologic findings in the cerebellar tissues from Patient I-1 and I-2. **(A)** Macroscopically, the delayed development of the cerebellum is prominent in Patient I-2 (i) compared with an age-matched control (ii, H&E). **(B)** At a higher magnification, there is a marked reduction in Purkinje cells by H&E in both patients (I-1 = i, I-2 = ii) relative to an age-matched control (iii). Where Purkinje cells were present in Patient I-1 (iv) and Patient I-2 (v), they exhibited stunted apical dendrites compared with those in an age-matched control (vi). Calbindin immunohistochemistry. **(C)** The dentate nucleus was detected in both cases although the neuronal population density was reduced in Patient I-1 (i) and I-2 (iii), and the neuropil ribbon was thin (I-1, ii, synaptophysin). **(D)** The inferior olivary nuclei were present in both cases with intact neuronal population density shown with H&E in Patient I-1 (i) and Patient I-2 (iii). Neuropil ribbons are demonstrated in Patient I-1 (ii, synaptophysin) and I-2 (iv, synaptophysin). Scale bar = 100 μm.

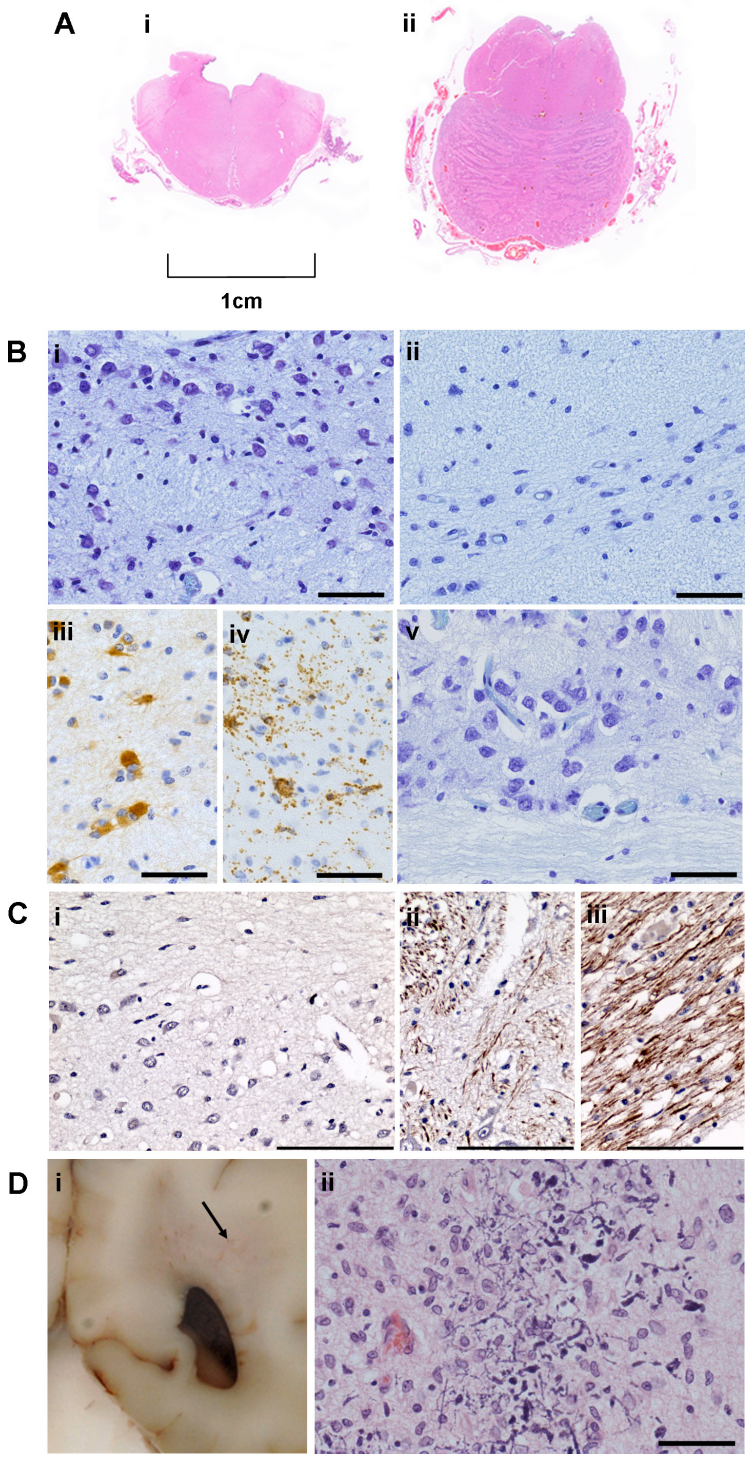


FIGURE 4. Histopathologic findings in the pons and occipital lobe tissues from Patient I-1 and I-2. **(A)** Macroscopic assessment of H&E-stained pons from Patient I-2 (i) revealed severe atrophy compared to an age-matched control (ii). **(B)** In a pontine nucleus in Patient I-1, there is relatively intact neuronal cell density (Luxol Fast Blue with Cresyl Fast Violet [LFB/CFV]) (i), whereas there was marked neuronal cell loss in Patient I-2 (ii, LFB/CFV) accompanied by astrogliosis (iii, glial fibrillary acidic protein immunohistochemistry [IHC]) and microgliosis (iv, CD-68 IHC), which was in contrast to normal neuronal density in an age-matched control (v, LFB/CFV). **(C)** In addition, there is a profound loss of phosphorylated neurofilaments in axons corresponding to the pontocerebellar fibers in Patient I-1 (i, SMI-31R IHC) in contrast to intact expression in medial longitudinal fasciculus (ii) and medial lemniscus (iii). **(D)** Deep white matter abnormalities were observed in the occipital lobe (i), consistent with the hyperintensity seen on neuroimaging; microscopically, this corresponded to the presence of mineralized axons (ii, H&E). Scale bar = 100 μm.

of expression of these components despite abundant mitochondria in these regions.

DISCUSSION

We present only the second autopsy series of neonatal pontocerebellar hypoplasia type 6 (PCH6) in 2 siblings born to nonconsanguineous parents. In the neonatal period, the siblings were hypotonic, lacked spontaneous respiratory effort, and deteriorated rapidly, leading to death on Day 1 and Day 14, respectively. Biochemical analysis of skeletal and cardiac muscle confirmed extensive respiratory chain deficiency involving complexes I, III, and IV, and enzyme histochemistry showed a complete loss of COX activity. Mitochondrial respiratory chain complex-specific immunohistochemistry confirmed a lack of immunoreactivity for complexes I and IV despite a high density of mitochondria throughout the CNS. This supports a generalized loss of respiratory chain activity due to a translation defect and is in agreement with previous findings with variable respiratory chain defects in muscle from patients with *RARS2* mutations (5, 10). Molecular genetic investigations identified a paternally inherited single heterozygous c.1A>G mutation that is predicted to abolish translation initiation. Despite the identification of just 1 heterozygous *RARS2* variant, the certain pathogenicity meant that it was highly likely that PCH6 was the clinical diagnosis in tandem with a second, latent *RARS2* variant. This initiated cDNA studies that led to the identification of a cDNA transcript lacking exons 6–8 due to a maternally

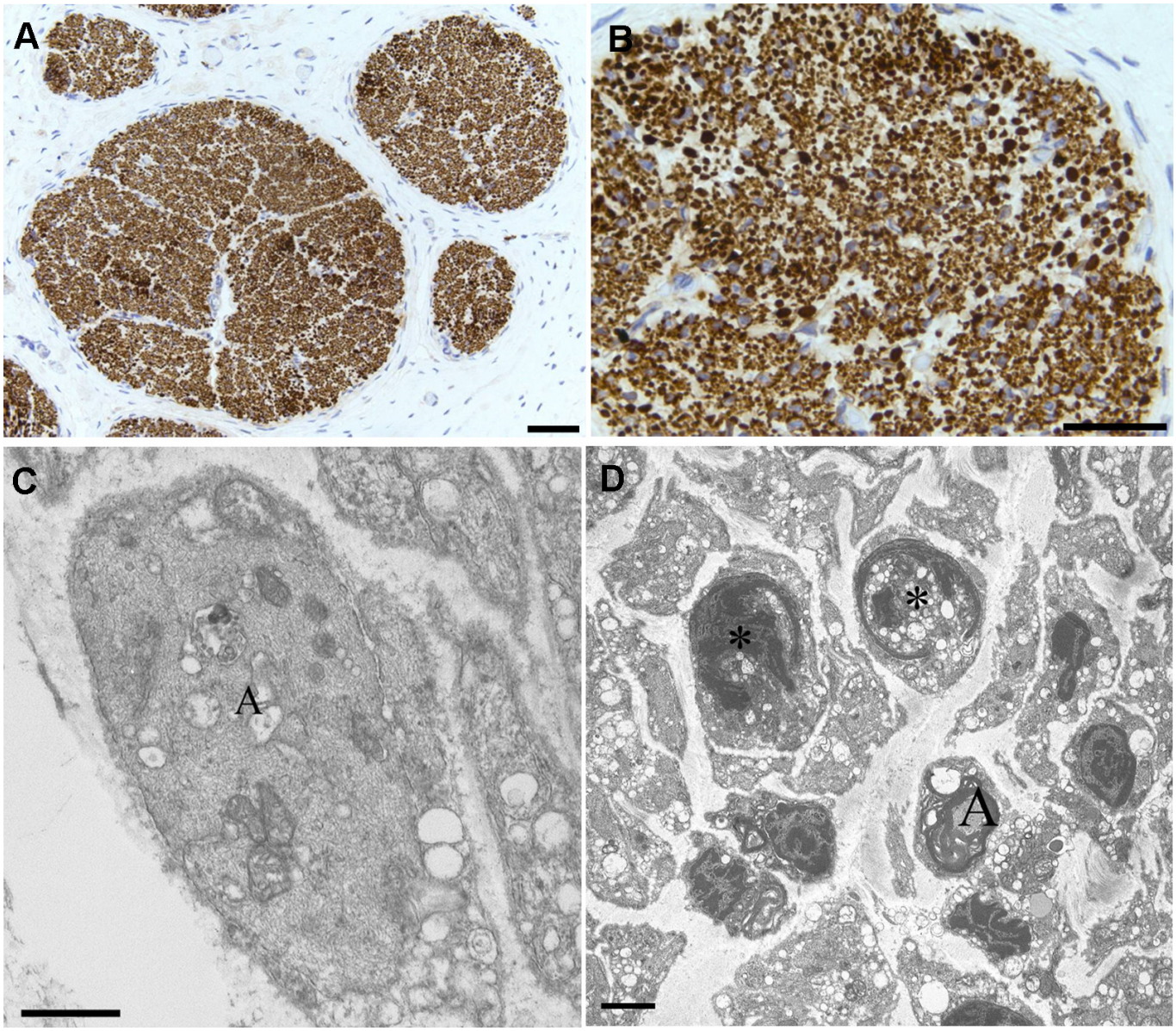
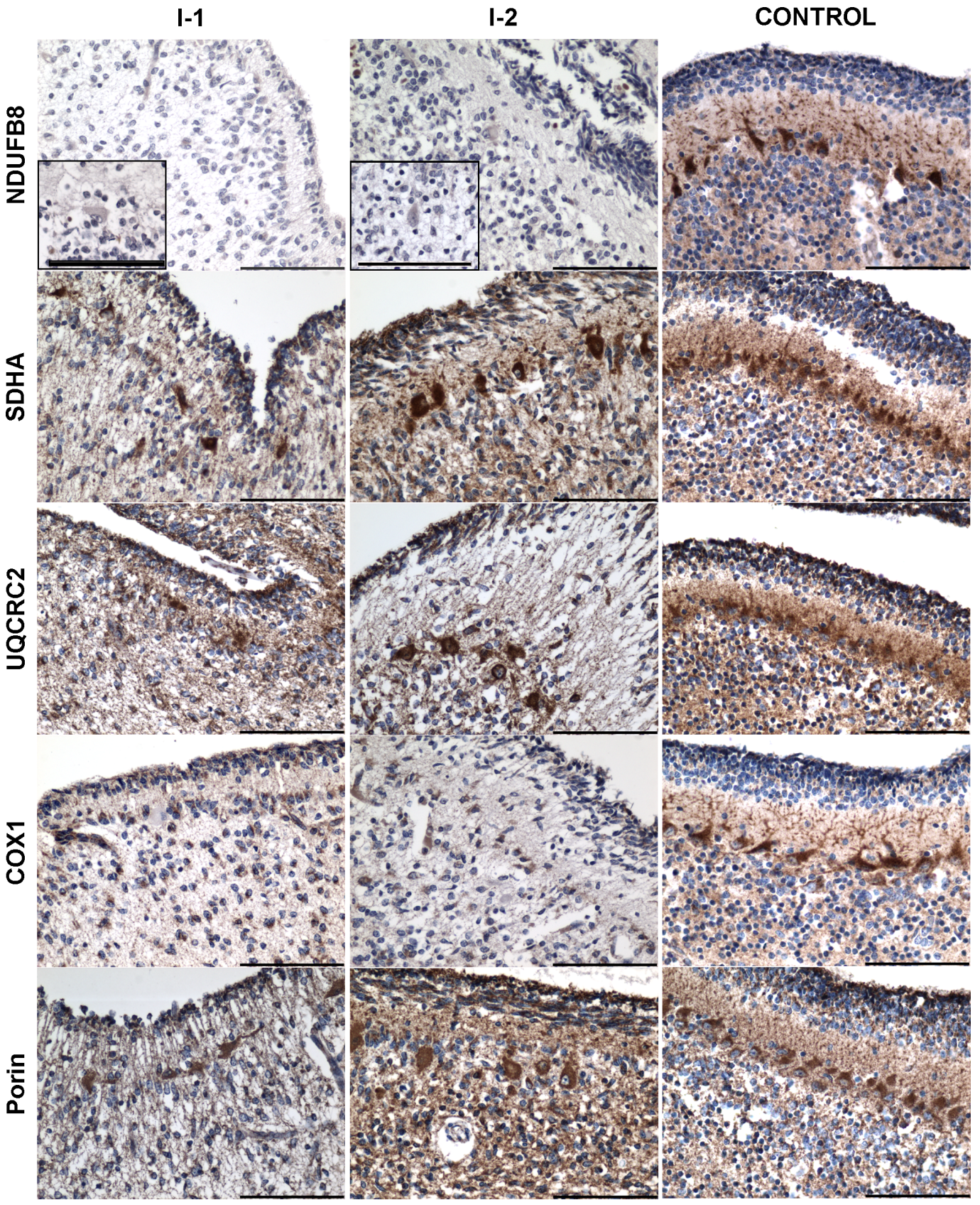


FIGURE 5. Sciatic nerve pathology in Patient I-2. **(A, B)** IHC revealed an irregular distribution and reduced number of large axons in the sciatic nerve (neurofilament IHC). **(C, D)** Electron microscopy revealed loss of myelin surrounding axons [A] and the presence of degenerating fibers (*), which are observed alongside intact axons. Scale bars: **A, B**, 100 μm; **C**, 1 μm; **D**, 2 μm.



inherited novel, deep-intronic variant, c.613-3927C>T, which activates a cryptic splice site. In addition to reporting 2 novel *RARS2* mutations, this study expands upon the clinical phenotypes and neuropathology associated with *RARS2* mutations in this rare autosomal recessive mitochondrial disease.

The canonical features of PCH6 detected in Patient I-2 included early-onset encephalopathy, raised lactate levels in blood and CSF, pontocerebellar hypoplasia (observed on MRI and confirmed by neuropathologic examination), and combined defects in oxidative phosphorylation (5, 7, 9, 10). In addition, we report features not previously associated with PCH6 including fetal echocardiograph findings consistent with a hypertrophic cardiomyopathy phenotype and hydrops fetalis. Cardiomyopathy was detected at 20 weeks gestation following concerns regarding fetal bradycardia and was ascribed to biventricular hypertrophy. Despite being a new finding in association with *RARS2* mutations, cardiomyopathy is a common clinical feature in other mitochondrial disease presentations in which neonatal presentation of the hypertrophic type (HCM) is associated with a high mortality (19). The high rate of mortality might reflect the metabolic shift from prenatal anaerobic glycolysis to postnatal metabolism that is driven by fatty acid oxidation, ketone catabolism, and oxidative phosphorylation (20). Primary mutations affecting mitochondrial DNA and mutations in the nuclear-encoded alanyl-tRNA synthetase (*AARS2*), *MTO1*, and *GTBP3* genes have also been implicated in severe infantile, prenatal-onset cardiomyopathy presentations with combined respiratory chain deficiencies (21–23). In addition to HCM, pulmonary hypoplasia was also reported in the patients described by Gotz et al and was thought to be secondary to the cardiac hypertrophy (23).

In view of the neuroradiologic imaging supporting the clinical diagnosis of PCH6, sequence analysis of the *RARS2* gene was undertaken. Sixteen PCH6 patients with recessive *RARS2* mutations have been reported to date, with missense variants being the major class (24). There is a paucity of truncating mutations, highlighting the importance of the full-length, functional transcript although splicing variants have accounted for 1 allele in 10 patients. No patient reported to date has presented with 2 null *RARS2* alleles, and this is reflected also in this family. The c.1A>G variant is predicted to entirely abolish the translation of the corresponding allele, whereas we hypothesize that the allele harboring the deep intronic splicing variant is predicted to confer some (albeit minimal) residual activity due to some normal transcript remaining. This would explain the limited compatibility with life that was observed in the clinically affected children of this family.

As a mutational mechanism, deep intronic variants are likely to be under-represented in the literature, possibly as a consequence of strategies employed in current genetic testing. First, genomic DNA is often the source for diagnostic sequencing investigations; therefore, splicing variants may remain undiagnosed unless they affect the consensus splicing donor or acceptor sites at +/−2 bp of the exon boundary; variants situated beyond +/− 10 bp of the exon/intron boundaries are often regarded as of ''uncertain significance.'' Exome-based, next-generation sequencing strategies share the same bias, although cDNA-based next-generation sequencing strategies and whole genome sequencing will hopefully elucidate the prevalence of both splicing variants and large-scale gene rearrangements. The deep intronic *RARS2* variant was only apparent following emetine-induced nonsense mediated decay arrest; the abnormal transcript was subject to nonsense mediated decay in normal culture conditions, leaving only a transcript lacking exons 6–8 in the mRNA corresponding to the maternal allele. This variant, although not subject to nonsense mediated decay, is likely to give rise to a nonfunctional, truncated protein. To our knowledge, only 1 deep intronic mutation has been reported previously in association with any mitochondrial disease presentation—a dominant *OPA1* mutation that causes progressive optic atrophy in adulthood (25). In addition to describing the first deep intronic recessive mutation in a fatal pediatric mitochondrial disease presentation, this study highlights the utility of cDNA investigations, particularly in patients with only 1 candidate mutation in a recessively inherited disease.

There is only 1 illustrated report of PCH6 neuropathology describing 2 cases (11), and 1 very brief description as part of a broader survey of pontocerebellar hypoplasia (7). Therefore, our findings double the number of neuropathologic descriptions of this condition and provide the first record of peripheral nerve pathology.

Our findings are similar to those of Joseph et al (11) in a number of respects but also reveal some significant differences. We found that the brains of our patients were also micrencephalic and the cerebellar vermis displayed much-simplified foliation, corresponding, at a rough estimate (26), to that of a 114-mm crown-rump (~18 week gestation) fetus and indicating an element of developmental delay, or arrest, that began in utero, in agreement with previous findings (11). The pons was abnormally small and basal pontine nucleus neurons were severely decreased in number in Patient I-2 but were within normal limits in Patient I-1. The small size of the pons might be attributed to delayed development, whereas neuronal cell loss might reflect regressive degeneration because of the presence of astrogliosis and microgliosis. Evidence in support of regressive degeneration is provided by the sibling who died only 1 hour after birth and who demonstrated intact neuronal density in the pontine nucleus. This was also verified by Joseph et al, where very few neurons were noted in this structure in both twins. This suggests that *RARS2* mutations adversely affect the pons and cerebellum and that the neurons within these structures are particularly vulnerable to degeneration. In fact, vulnerability of the developed cerebellum to perturbed mitochondrial function is particularly common (27–29). In these cases, however, it is likely to be a direct consequence of a

FIGURE 6. Mitochondrial respiratory chain deficiency in the cerebellum of Patients I-1 and I-2. Immunohistochemical staining of components of the mitochondrial respiratory chain revealed severe, combined respiratory chain deficiencies in remaining cells involving complex I (NDUFB8) and the mitochondrially encoded complex IV subunit I (COX1), despite preservation of complex II (SDHA) and complex III core 2 (UQCRC2) expression and the mitochondrial mass marker porin in serial sections. Scale bar = 100 μm.

mismatch between mitochondrial protein synthesis and the accelerated growth phase of the cerebellum during late gestation, rendering the cerebellum particularly susceptible (30).

We also provide further evidence corroborating deep white matter pathology in the occipital poles both radiologically and histopathologically that is comparable with the findings of Joseph

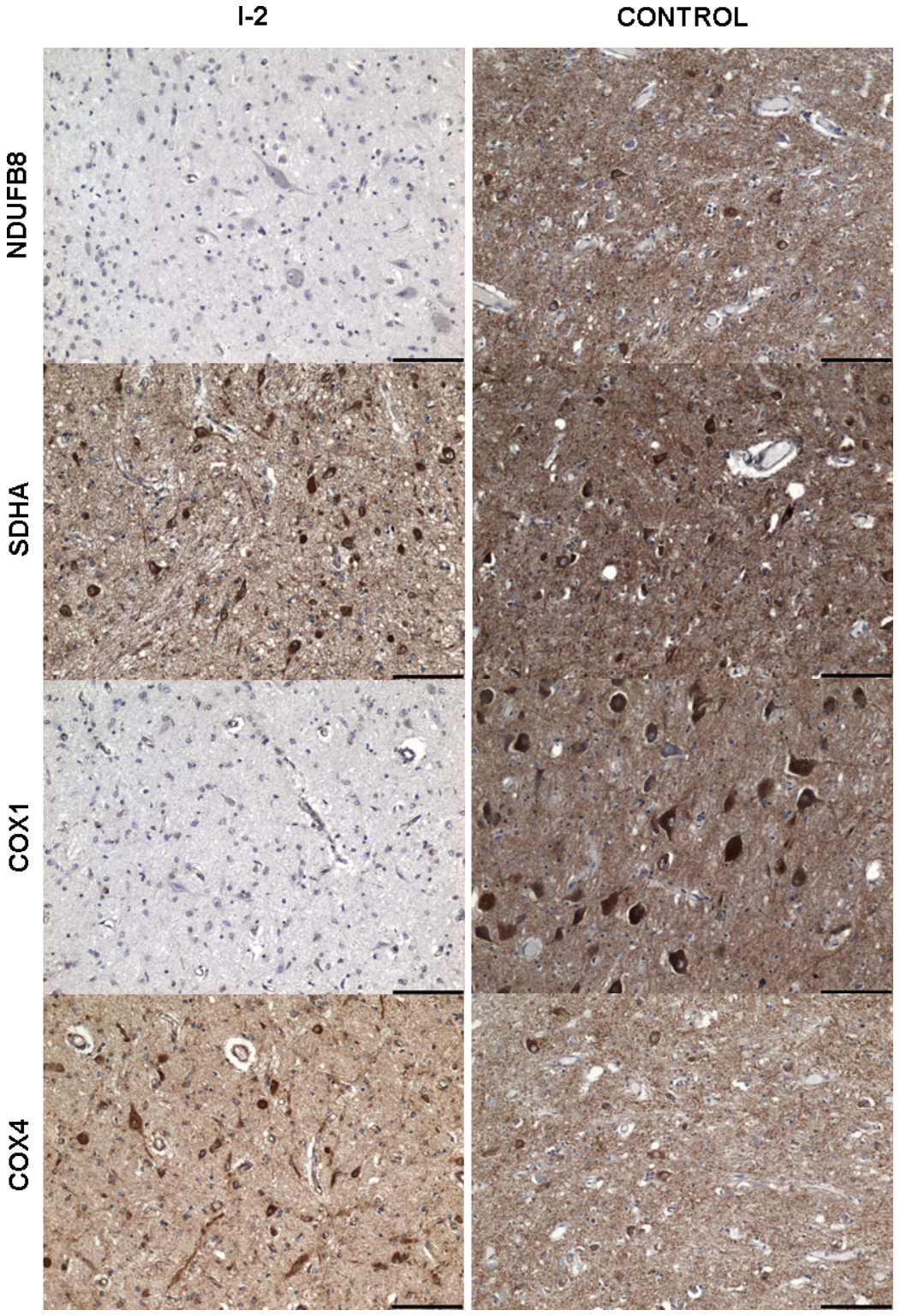


FIGURE 7. Mitochondrial respiratory chain deficiencies in the pontine nucleus. The mitochondrial respiratory chain proteins investigated showed high immunoreactivity in an age-matched control (right panels). Patient I-2 shows a profound absence of immunoreactivity for complexes I (NDUFB8) and IV (COX1), indicating respiratory chain deficiency. Scale bar = 100 μm.

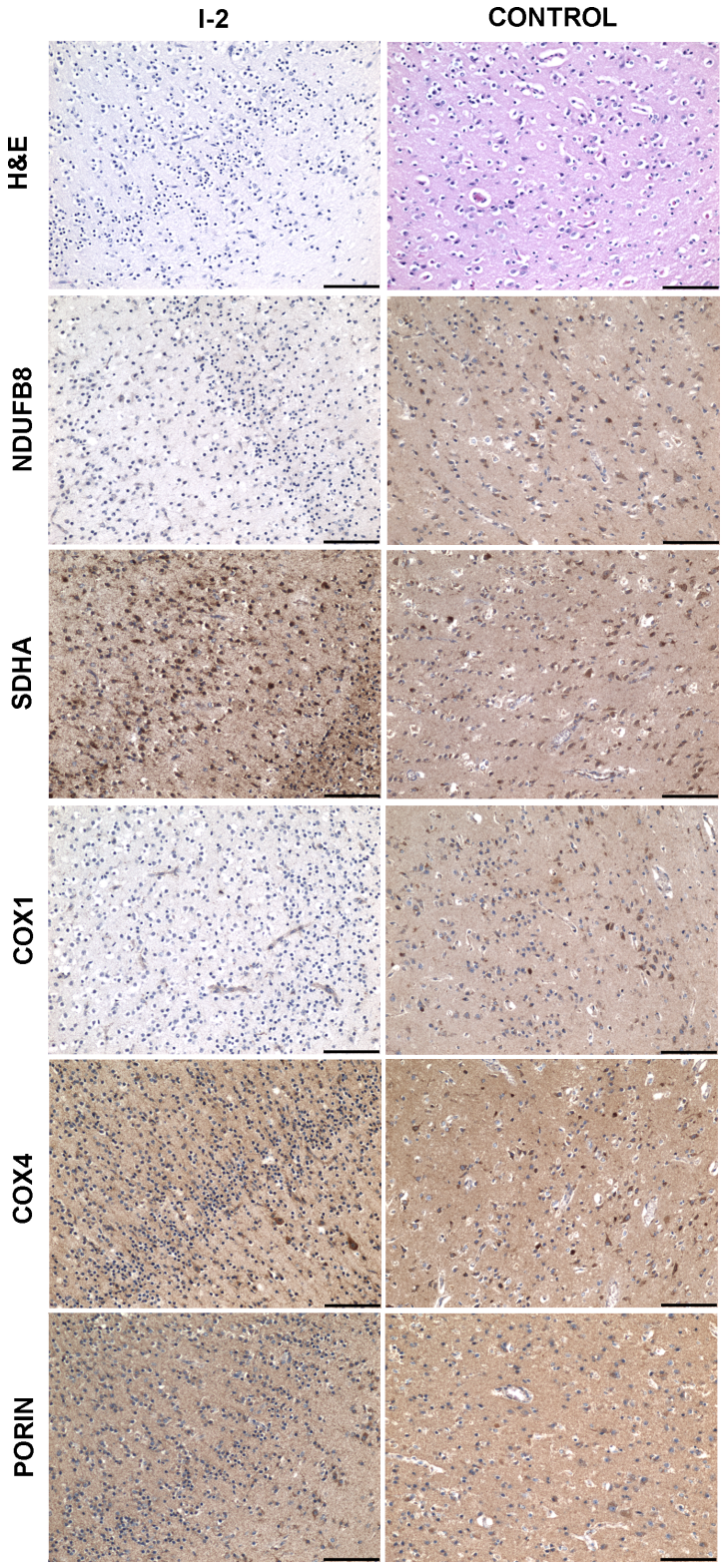


FIGURE 8. Mitochondrial respiratory chain deficiency in the occipital cortex. Neuron population densities were intact in Patient I-2 and control tissues (H&E). Immunohistochemical staining for components of the mitochondrial respiratory chain revealed a marked loss of complex I in Patient I-2 (NDUFB8) vs control (NDUFB8), and mitochondrially encoded subunit of complex IV (COX1) vs control (COX1), indicating complex I and IV deficiencies. Complex II (SDHA) and a nuclear-encoded subunit of complex IV (COX4) expression levels were preserved in conjunction with high mitochondrial density (porin) in both patient and control neurons. Scale bar = 100 μm.

et al, with discoloration, extensive gliosis, and few neurofilaments. Since both of our patients exhibited clinical complications associated with poor ventilation due to respiratory insufficiency, we cannot exclude the effects of hypoxic-ischemic injury contributing to the presence of karyorrhectic neurons in the pons and subiculum, eosinophilic neurons in the basal ganglia, and also periventricular leukomalacia. This was not a striking feature of the patients described by Joseph et al, but it remains an important consideration when evaluating the neuropathology since these are often features that emerge after severe hypoxic-ischemia (31).

We observed additional novel features not previously documented in PCH6 that expand on the neuropathologic features of the disorder. In both siblings, we identified the cerebellar dentate nucleus and inferior olivary nucleus, although we recognize the former demonstrated reduced neuronal cell density and gliosis. We were also able to identify a red nucleus, which was gliotic in Patient I-1. MRI in our cases did not reveal midline fusion of cerebral deep white matter and confirmed a full corpus callosum with well-formed splenium, in contrast to the findings of Joseph et al. Another important novel finding was the evidence of reduced axonal packing density and abnormally thin myelin sheaths in the peripheral nerve, which has not previously been described in PCH6.

Mitochondrial respiratory chain complex-specific immunohistochemistry was investigated in CNS tissues for the first time in both patients. This analysis revealed profound and rather selective complex I and IV deficiency, involving complex I subunits NDUFB8 and NDUFS3 and mitochondrially encoded COX1. This pattern of respiratory chain deficiency was widespread and not just restricted to the brainstem and cerebellum where the greatest pathology was evident.

The present study expands on the disease-causing variants of *RARS2*, provides further evidence of the clinical heterogeneity associated with *RARS2* mutations, and augments the neuropathologic features associated with PCH6. Our data expand on the pathogenesis of PCH6 disease caused by *RARS2* mutations and provide further characterization of the specific temporal and regional degeneration in patients with this rare disorder. Neuronal tissue is exquisitely sensitive to deregulation of protein synthesis, and even more so during the postmigratory phase of neuronal development when the demand for protein synthesis is high. Although we understand that RARS2 encodes for an enzyme that aminoacylates mt-tRNA^{Arg} in mitochondria, we do not fully understand why mutations in this gene cause a specific pattern of neurodegeneration leading to PCH6. We can speculate on the mechanisms based on *TSEN54* and *RARS2* knockdown studies performed in zebrafish in which overlapping phenotypes are observed implying a shared mechanism of degeneration (32). Based on this, we speculate that the demand for mitochondrial arginyl-tRNA synthetase is particularly high during the development of certain tissues, including the pontocerebellar and cardiac tissues. Indeed, the cerebellum undergoes a rapid phase of growth during late gestation, that is, at 28 weeks postconception, which could explain the specific vulnerability of this brain region to impaired *RARS2* function in patients harboring *RARS2* mutations (30).

Establishing a genetic diagnosis for families with mitochondrial respiratory chain dysfunction is crucial in the process of genetic counseling enabling a correct recurrence risk to be provided and reproductive options including prenatal diagnosis or preimplantation genetic diagnosis. Once 2 deleterious *RARS2* gene variants were identified, the parents of the patients in this report opted for analysis of a chorionic villus biopsy in a subsequent pregnancy. Our analysis showed that the fetus harbored neither of the familial *RARS2* mutations and a healthy boy has since been born.

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ORIGINAL INVESTIGATION

A recessive homozygous p.Asp92Gly *SDHD* mutation causes prenatal cardiomyopathy and a severe mitochondrial complex II deficiency

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Abstract Succinate dehydrogenase (SDH) is a crucial metabolic enzyme complex that is involved in ATP production, playing roles in both the tricarboxylic cycle and the mitochondrial respiratory chain (complex II). Isolated complex II deficiency is one of the rarest oxidative phosphorylation disorders with mutations described in three structural subunits and one of the assembly factors; just one case is attributed to recessively inherited *SDHD* mutations. We report the pathological, biochemical, histochemical and molecular genetic investigations of a male neonate who had left ventricular hypertrophy detected on antenatal scan and died on day one of life. Subsequent postmortem examination confirmed hypertrophic cardiomyopathy with left ventricular non-compaction. Biochemical analysis of his skeletal muscle biopsy revealed evidence of a severe isolated complex II deficiency and candidate gene sequencing revealed a novel homozygous c.275A>G, p.(Asp92Gly) *SDHD* mutation which was shown to be recessively inherited through segregation studies. The affected amino acid has been reported as a Dutch founder mutation p.(Asp92Tyr) in families with hereditary head and neck paraganglioma. By introducing both mutations into *Saccharomyces cerevisiae,* we were able to confirm that the p.(Asp92Gly) mutation causes a more severe oxidative growth phenotype than the p.(Asp92Tyr) mutant, and provides functional evidence to support the pathogenicity of the patient's *SDHD* mutation. This is only the second case of mitochondrial complex II deficiency due to inherited *SDHD* mutations and highlights the importance of sequencing all *SDH* genes in patients with biochemical and histochemical evidence of isolated mitochondrial complex II deficiency.

Introduction

Mitochondrial respiratory chain disease arises from defective oxidative phosphorylation (OXPHOS) and represents a common cause of metabolic disease with an estimated prevalence of 1:4300 (Gorman et al. 2015; Skladal et al. 2003). Under aerobic conditions, metabolised glucose, fatty acids and ketones are the OXPHOS substrates, shuttling electrons along the respiratory chain whilst concomitantly creating a proton gradient by actively transporting protons across the mitochondrial membrane. The resultant proton gradient is exploited by ATP synthase to drive ATP production. Under anaerobic conditions, for example where atmospheric oxygen is scarce or during periods of exertion, ATP synthesis is produced primarily during glycolysis (Horscroft and Murray 2014).

The mitoproteome consists of an estimated 1400 proteins (Pagliarini et al. 2008), including the 13 polypeptides and 24 non-coding tRNA and rRNA genes encoded by the

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mitochondria's own genetic material (mtDNA) that are exclusively maternally transmitted. The remaining genes of the mitoproteome are located on either the autosomes or sex chromosomes and as such are transmitted from parent to child in a Mendelian fashion. Defects in a number of mtDNA and nuclear-encoded genes have been linked to human disease, often associated with a vast genetic and clinical heterogeneity and further compounded by few genotype–phenotype correlations which help guide molecular genetic investigations.

Succinate dehydrogenase is a crucial metabolic enzyme complex that is involved in both the Krebs cycle and the mitochondrial respiratory chain. It is composed of two catalytic subunits (the flavoprotein SDHA, and Fe–S-containing SDHB) anchored to the inner mitochondrial membrane by the SDHC and SDHD subunits. All four subunits and the two known assembly factors are encoded by autosomal genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF1* and *SDHAF2*, hereafter referred to as *SDHx*). Congenital recessive defects involving *SDHx* genes are associated with diverse clinical presentations, including leukodystrophy and cardiomyopathy (Alston et al. 2012).

A recent review describes *SDHA* mutations as the most common cause of isolated complex II deficiency, with 16 unique mutations reported in 30 patients (Ma et al. 2014; Renkema et al. 2014); the next most common cause are mutations in *SDHAF1*, 4 mutations have been reported in 13 patients (Ghezzi et al. 2009; Ohlenbusch et al. 2012). Just one mitochondrial disease patient is reported to harbour either *SDHB* (Alston et al. 2012) or *SDHD* (Jackson et al. 2014) mutations and metabolic presentations have yet to be reported in association with *SDHC* or *SDHAF2*.

In addition to their role in primary respiratory chain disease, SDHx mutations can act as drivers of neoplastic transformation following loss of heterozygosity (LOH). One of the most common causes of head and neck paraganglioma (HNPGL) is LOH at the SDHD locus. These mutations are inherited in a dominant manner with a parent of origin effect; typically only paternally inherited SDHD mutations are associated with HNPGL development (Hensen et al. 2011).

Here, we report a neonate who presented prenatally with cardiomyopathy due to a novel homozygous *SDHD* mutation. This is the second report of recessive *SDHD* mutations resulting in a primary mitochondrial disease presentation and serves to characterise the biochemical, histochemical and functional consequences of our patient's molecular genetic defect. Moreover, the affected amino acid, p.Asp92, has been reported as a Dutch founder mutation in families with hereditary PGL, albeit the substituted residue differs. We have used the yeast, *Saccharomyces cerevisiae,* which has proven to be a useful model system to study the effects of SDHx gene mutations (Goffrini et al. 2009; Panizza et al. 2013), to provide functional evidence supporting the pathogenicity of the *SDHD* mutation identified in our patient and, to a lesser extent, that of the PGL-associated p.Asp92Tyr mutation.

Patient and methods

The patient is the third child born to unrelated Irish parents. Foetal heart abnormalities were identified on an anomaly scan at 31-weeks gestation, which prompted foetal echocardiography. A normally situated heart with normal systemic and pulmonary venous drainage was reported. Right to left shunting was noted at the patent foramen ovale and ductus arteriosus, consistent with gestational age. The left ventricle and left atrium were severely dilated with moderate–severe mitral regurgitation. There was severe left ventricular systolic dysfunction, but no evidence of pericardial effusion or ascites. Rhythm was normal sinus with a foetal heart rate between 100 and 120 beats per minute and subsequent weekly foetal echocardiogram showed no further progression of cardiac dysfunction or development of hydrops. Cardiac MRI at 32-weeks gestation showed marked left ventricular hypertrophy and dilation. A clinical diagnosis of dilated cardiomyopathy was considered and the parents were counselled that the prognosis for postpartum survival was poor. The proband was born by elective caesarean section at 37 + 6 weeks gestation with a birth weight of 2620 g (9th–25th centile) and occipital circumference of 34.5 cm (50th–75th centile). He had no dysmorphic features. He was transferred to neonatal intensive care on 100 % oxygen to maintain his saturations in the low 90 s. An additional heart sound and loud murmur were noted, along with hepatomegaly (4 cm below costal margin) but without splenomegaly. By 12 h of age, his condition had deteriorated significantly; echocardiogram showed dilation of the inferior vena cava, hepatic veins, right atrium and interatrial septal bowing. He had moderate tricuspid regurgitation and very poor biventricular function with non-compaction hypertrophy. The proband died the following evening following withdrawal of life support with parental consent. At postmortem examination, the heart weighed 43 g (normal = 13.9 ± 5.8 g). The right atrium was particularly enlarged. The endocardium of the right atrium but particularly also the right ventricle showed fibroelastosis. The right ventricle was remarkably diminutive and underdeveloped. The right atrium had a 7-mm-diameter patent foramen ovale. There was obvious non-compaction of the hypertrophic left ventricular myocardium (Fig. 1). Cardiac muscle, skeletal muscle and a skin biopsy were referred for laboratory investigations. Informed consent was obtained from the parents for the clinical and laboratory investigations and publication of the results.

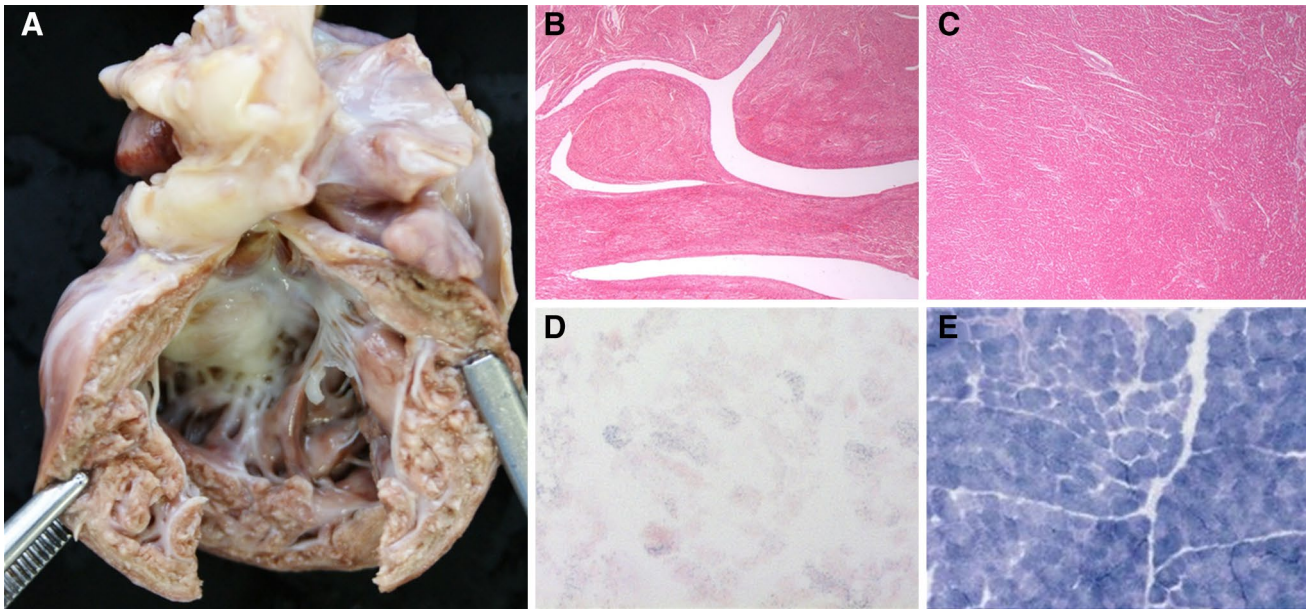

Fig. 1 Macroscopic and microscopic analysis of patient cardiac and skeletal muscle. **a** Macroscopic examination of patient heart revealed obvious non-compaction of the hypertrophic left ventricle. **b** Microscopic assessment of left ventricle confirms non-compaction compared to neonatal control tissue (**c**). Histochemical assessment of patient muscle biopsy shows a marked decrease in the activity of succinate dehydrogenase activity (**d**) compared with a control muscle (**e**)

Histochemical and biochemical assessment of metabolic function

Histological and histochemical assay of 10 µm serial sections of patient muscle biopsy was performed according to standard protocols. The measurement of respiratory chain enzyme activities was determined spectrophotometrically as described previously (Kirby et al. 2007). Fibroblast culture and measurement of β-oxidation flux in cultured fibroblasts using [9, 10-^{3}H] myristate, [9, 10-^{3}H]palmitate and [9, 10-^{3}H]oleate was performed as described elsewhere (Manning et al. 1990; Olpin et al. 1992, 1997).

Cytogenetic and molecular genetic investigations

Karyotyping of cultured fibroblasts and DNA extraction from patient muscle were performed according to standard protocols. Primers were designed to amplify each coding exon, plus intron–exon boundaries, of the *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF1* and *SDHAF2* genes. PCR amplicons were Sanger sequenced using BigDye3.1 chemistry (Applied Biosystems) and capillary electrophoresed on an ABI3130xl bioanalyser (Applied Biosystems) using standard methodologies. Resultant sequencing chromatograms were compared to the Genbank reference sequences: *SDHA* (NM_004168.2), *SDHB* (NM_003000.2), *SDHC* (NM_003001.3), *SDHD* (NM_003002.3), *SDHAF1* (NM_001042631.2) and *SDHAF2* (NM_017841.2). All gene variants were annotated using dbSNP build 138 whilst ESP6500 and 10 k genome project data allowed determination of allele frequencies. Parental DNA samples were screened to investigate allele transmission.

In silico pathogenicity prediction tools and structural modelling

The effect of the p.(Asp92Gly) substitution on SDHD function was predicted using the in silico tools SIFT (Ng and Henikoff 2003), Align GVGD (Tavtigian et al. 2006) and Polyphen (Adzhubei et al. 2010), all running recommended parameters. To determine whether the tertiary structure of the protein was affected by the mutation, the wild-type (NP_002993) and mutant SDHD protein sequences were input to PSIPRED (Jones 1999) and I-TASSER (Yang et al. 2014); I-TASSER output was visualised using UCSF Chimera (Pettersen et al. 2004).

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

Mitochondria-enriched pellets were obtained from 15 mg skeletal muscle as previously described (Nijtmans et al. 2002) and solubilised in 1× sample buffer (Invitrogen,

BN20032), 2 % dodecyl-β-d-maltoside (Sigma) and 5 % glycerol. Mitochondria were pelleted for 15 min at 100,000 g. Protein concentrations were determined by Pierce™ BCA Protein Assay Kit according to manufacturer's protocol (ThermoScientific). Protein samples (25 µg) were separated on NativePAGE™ Novex® 4–16 % Bis–Tris Protein Gels (Sigma) then transferred onto a PVDF membrane. Immunodetection of assembled respiratory chain complexes was performed using primary monoclonal antibodies (mitosciences) raised against complex-specific proteins: Complex I (NDUFB8, Abcam, ab110242), Complex II (SDHA, MitoSciences, MS204), Complex III (CORE2 Abcam, ab14745), Complex IV (COX1 Abcam, ab14705) and Complex V (ATP5A Abcam, ab14748). Following HRP-conjugated secondary antibody application (Dako), detection was undertaken using the ECL® plus chemiluminescence reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) and a ChemiDoc MP imager (Bio-Rad Laboratories).

Western blot

Mitochondria-enriched pellets prepared as above were lysed on ice in 50 mM Tris pH 7.5, 130 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF and 1 % NP-40. Protein concentration was calculated using the Bradford method (Bradford 1976). 13 µg of enriched mitochondrial proteins was loaded on a 12 % sodium dodecyl sulphate polyacrylamide gel with 1× dissociation buffer, electrophoretically separated and subsequently transferred onto a PVDF membrane. Immunodetection was performed using primary antibodies raised against complex II SDHA (MitoSciences, MS204) and SDHD (Merck Millipore, ABT110) and a mitochondrial marker protein, Porin (Abcam, ab14734). Following secondary antibody application (Dako), detection was undertaken using the ECL® plus chemiluminescence reagent (GE Healthcare Life Sciences, Buckinghamshire, UK) and ChemiDoc MP imager (Bio-Rad Laboratories).

Yeast strains and media

Yeast strains used in this study were BY4741 (*MATa*; *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its isogenic sdh4:kanMX4. Cells were cultured in yeast nitrogen base (YNB) medium: 0.67 % yeast nitrogen base without amino acids (ForMediumTM), supplemented with 1 g/l of drop-out powder (Kaiser et al. 1994) containing all amino acids and bases, except those required for plasmid maintenance. Various carbon sources (Carlo Erba Reagents) were added at the indicated concentration. For the respiration and mitochondria extraction, cells were grown to late-log phase in the YNB medium supplemented with 0.6 % glucose. Media were solidified with 20 g/l agar (ForMediumTM) and strains were incubated at 28 or 37 °C.

Construction of yeast mutant alleles

The *sdh4*Asp98Gly and *sdh4*Asp98Tyr mutant alleles were obtained by site-directed mutagenesis using the overlap extension technique (Ho et al. 1989). In the first set of PCR reactions, the *SDH4* region was obtained using the forward primer ESDH4F and the following reverse mutagenic primer sdh4R98G 5′-CATGACAG AAAAGAAAGA**ACC**AGCTGCAGTGGATAACGG AC-3′ and sdh4R98Y 5′-CATGACAGAAAAGAAAG A**GTA**AGCTGCAGTGGATAACGGAC-3′ where base changes are indicated in bold. The second *SDH4* region was obtained using the forward mutagenic primer sdh4F98G and sdh4F98Y, complementary to sdh4R98G and sdh4R98Y, and the reverse primer XSDH4R. The final mutagenized products were obtained using the overlapping PCR fragments as template with ESDH4F and XSDH4R as external primers. The products were then digested with *Eco*RI and *Xba*I and cloned in *Eco*RI–*Xba*I-digested pFL38 centromeric plasmid (Bonneaud et al. 1991). The mutagenized inserts were verified by sequencing and the pFL38 plasmid-borne *SDH4* and *SDH4* mutant alleles were transformed in the BY4741 using the lithium-acetate method (Gietz and Schiestl 2007).

Isolation of mitochondria, enzyme assay and respiration

Oxygen uptake was measured at 28 °C using a Clark-type oxygen electrode in a 1-ml stirred chamber containing 1 ml of air-saturated respiration buffer (0.1 M phthalate–KOH, pH 5.0) and 10 mM glucose (Oxygraph System, Hansatech Instruments, England). The reaction was initiated with the addition of 20 mg of wet weight of cells, as previously described (Goffrini et al. 2009). Preparation of mitochondria and succinate dehydrogenase DCPIP assay was conducted as described (Goffrini et al. 2009). The succinate:decylubiquinone DCPIP reductase assay was conducted as previously described (Jarreta et al. 2000; Oyedotun and Lemire 2001). Protein concentration was determined by the Bradford method using the Bio-Rad protein assay following the manufacturer's instructions (Bradford 1976).

Results

Pathological, histochemical and biochemical analysis

Histopathological examination of the patient's heart revealed non-compaction of the left ventricular myocardium (Fig. 1a, b). Histological investigations reported normal skeletal muscle morphology whilst histochemical analysis of fresh-frozen muscle biopsy sections revealed a global reduction of succinate dehydrogenase (complex II) activity compared to aged-matched control samples (Fig. 1d, e). Spectrophotometric analysis of respiratory chain function in patient muscle homogenate revealed a marked defect in complex II activity (patient 0.042 nmols/min/unit citrate synthase activity; controls 0.145 ± 0.047 nmols/min/unit citrate synthase activity ($n = 25$) representing ~30 % residual enzyme activity; the activities of complex I, complex III and complex IV were all normal (not shown). Fatty acid oxidation flux studies on cultured fibroblasts gave normal results which excluded virtually all primary defects of long- and medium-chain fatty acid oxidation as the cause of the underlying cardiac pathology. There was no evidence of an underlying aminoacidopathy and serum urea and electrolytes were within normal limits. Investigations of glucose and lactate levels were not performed. The monolysocardiolipin/cardiolipin (ML/CL) ratio on a postmortem sample was 0.03 and the neutrophil count was within normal limits.

Cytogenetic and molecular genetic investigations

Karyotyping reported a normal 46 XY profile, consistent with no large genomic rearrangements. Following identification of an isolated complex II deficiency, Sanger sequencing of all six *SDHx* genes was undertaken and a novel homozygous c.275A>G, p.(Asp92Gly) variant was identified in *SDHD* (ClinVar Reference ID: SCV000196921). Results from parental carrier testing were consistent with an autosomal recessive inheritance pattern, with each parent harbouring a heterozygous c.275A>G, p.(Asp92Gly) *SDHD* variant (Fig. 2a). The p.Asp92 *SDHD* residue is highly conserved (Fig. 2d) and the c.275A>G variant is not reported on either the ESP6500 or 1KGP suggesting that it is rare in the general population. Whilst the c.275A>G, p.(Asp92Gly) *SDHD* variant has not been previously reported, another mutation affecting the same residue—c.274G>T, p.(Asp92Tyr)—has been reported in association with familial PGL and PCC (Hensen et al. 2011). In silico predictions were strongly supportive of a deleterious effect; 100 % sensitivity and 100 % specificity were reported by SIFT and polyphen for both the p.(Asp92Gly) and p.(Asp92Tyr) variants. Both variants were assigned an aGVGD class of C65 (highly likely to be detrimental to protein function)—the p.(Asp92Gly) variant was reported to have a grantham difference (GD) value of 93.77, whilst the GD for the p.(Asp92Tyr) variant was 159.94 (GD > 70 is associated with C55/C65 variant classes). SDHD tertiary structure was not predicted to be markedly impacted by the patient's p.(Asp92Gly) substitution; no gross conformational change was reported by I-TASSER (Fig. 2b), whilst PSIPRED predicted only a mild alteration to the helix structure (Fig. 2c).

Functional effect of the c.275A>G, p.(Asp92Gly) SDHD mutation on protein expression and complex assembly

Having identified an excellent candidate mutation, assessment of respiratory chain complex assembly by one-dimensional BN-PAGE revealed a marked decrease in fully assembled Complex II, whilst levels of fully assembled complexes I, III, IV and V were comparable to controls (Fig. 3a). Western blot of mitochondrial proteins in patient muscle was performed which confirmed a significant reduction of the SDHD and SDHA proteins compared to both equally loaded control muscle samples and Porin, a mitochondrial marker protein (Fig. 3b).

Functional studies in a yeast model

To further assess the pathogenicity of the patient's novel p.(Asp92Gly) *SDHD* variant, we performed complementation studies using a strain of *S. cerevisiae* lacking the *SDH4* gene hereafter referred to as Δ*sdh4*. The *SDH4* gene is the yeast orthologue of human *SDHD* and although the human and yeast protein have a low degree of conservation (16 % identity and 36 % similarity) the p.Asp92 residue is conserved between the two species, corresponding to p.Asp98 in yeast (Fig. 2d). We then introduced the change equivalent to the human p.(Asp92Gly) variant into the yeast *SDH4* wild-type gene cloned in a centromeric vector thus obtaining the $sdh4^{D98G}$ mutant allele. Since another mutation involving the same residue, p.(Asp92Tyr), has been reported as a cause of paraganglioma, a second mutant allele, $sdh4^{D98Y}$, was also constructed to compare the phenotype between the two different amino acid substitutions. The *SDH4*, $sdh4^{D98G}$ and $sdh4^{D98Y}$ constructs and the empty plasmid pFL38 were then transformed into the Δ*sdh4* strain. To test the possible effects on mitochondrial function, we first evaluated the oxidative growth by spot assay analysis on mineral medium supplemented with either glucose or ethanol, at 28 and 37 °C.

A clear growth defect was observed for the Δ*sdh4/*$sdh4^{D98G}$ strain in ethanol-containing plates incubated both at 28 and 37 °C (Fig. 4a), with growth similar to that of the *sdh4* null mutant. Contrariwise, the Δ*sdh4/*$sdh4^{D98Y}$

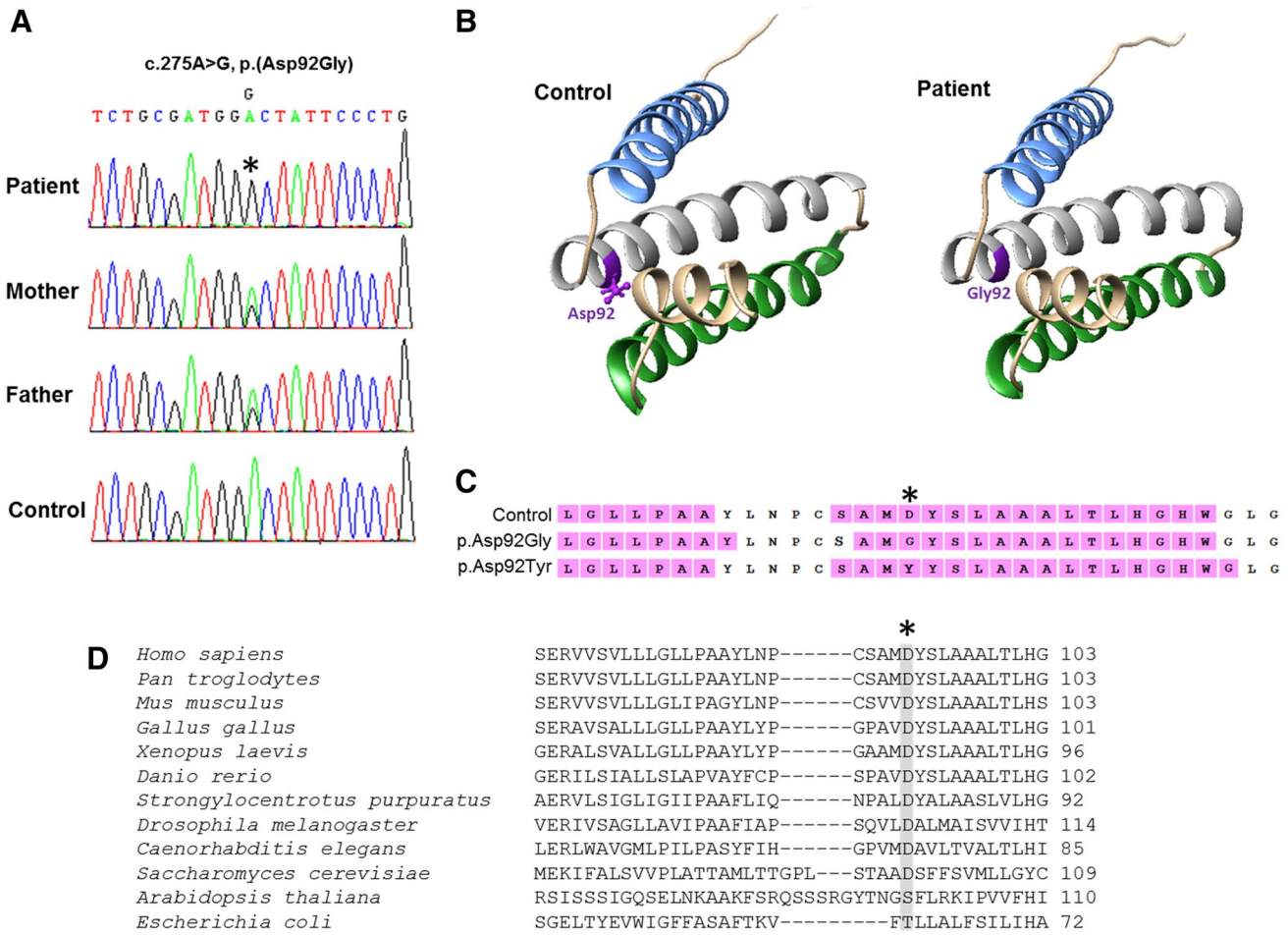

Fig. 2 Molecular genetic and in silico investigations. **a** Identification of a pathogenic *SDHD* mutation. A homozygous c.275A>G, p.(Asp92Gly) *SDHD* mutation was identified in the proband, with parental DNA screening supporting recessive inheritance. The mutation affects a highly conserved p.Asp92 residue in the *SDHD*-encoded subunit of succinate dehydrogenase (SDH). **b** Structural modelling. I-TASSER prediction of control and patient SDHD tertiary structure shows the p.Asp92 residue located within a transmembrane helix domain and the p.Asp92Gly substitution is predicted to have little impact on SDHD tertiary structure. **c** PSIPRED output predicts minor alterations to two of the SDHD helices from the patient p.(Asp92Gly) and HNPGL p.(Asp92Tyr) substitutions compared to control sequence. Predicted helix residues shown in *pink*; unshaded residues are located in coil domains. **d** Multiple sequence alignment of this region of the SDHD subunit was performed using ClustalW and confirms that the p.(Asp92Gly) mutation affects an evolutionary conserved residue (*shaded*). Alignments were manually corrected on the basis of the pairwise alignment obtained with PSI-BLAST

strain did not exhibit an OXPHOS-deficient phenotype at either temperature tested (Fig. 4b) or in either oxidative carbon source analysed (not shown). To further investigate the OXPHOS defect, oxygen consumption and SDH activity were measured. The oxygen consumption rate of the Δ*sdh4*/*sdh4*^{D98G} mutant was 55 % less than that of the parental strain Δsdh4/SDH4 (Fig. 5a), likewise, succinate dehydrogenase enzyme activities (PMS/DCPIP reductase and decylubiquinone reductase) were both severely reduced, with levels similar to those of the null mutant (Fig. 5b). Consistent with the results obtained from growth experiments the oxygen consumption rate of the Δ*sdh4*/*sdh4*^{D98Y} mutant was not impaired (Fig. 5a) but both SDH activities (PMS/DCPIP reductase and decylubiquinone reductase) were partially reduced (80 and 75 % residual activity) in Δ*sdh4*/*sdh4*^{D98Y} mitochondria (Fig. 5b). Together, these data support the pathogenicity of our patient's novel p.(Asp92Gly) *SDHD* variant.

Discussion

Mitochondrial complex II deficiency is one of the rarest disorders of the OXPHOS system, accounting for between 2 and 8 % of mitochondrial disease cases (Ghezzi et al. 2009; Parfait et al. 2000) with only ~45 cases reported in

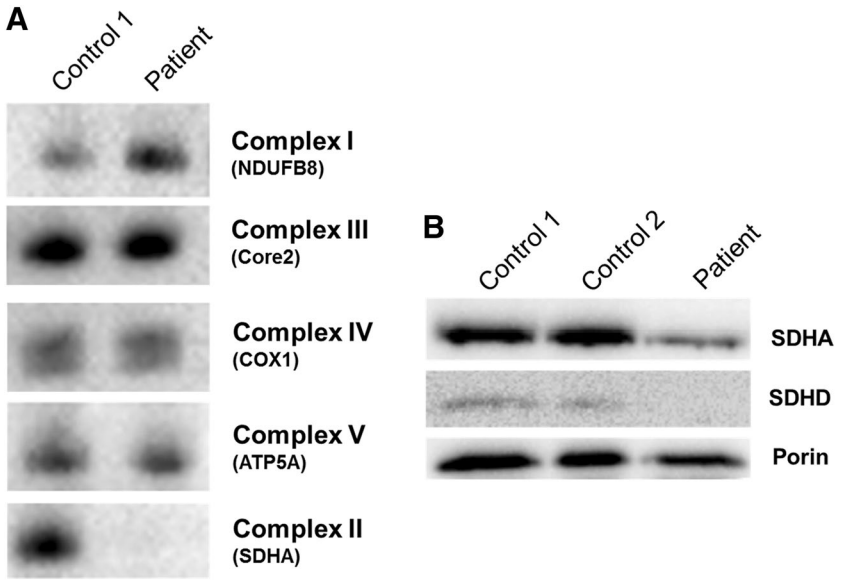

Fig. 3 Investigation of OXPHOS complex activities and protein expression in patient and controls. **a** BN-PAGE analysis of mitochondria isolated from patient and control muscle homogenates revealed a reduction of assembled complex II in patient muscle with normal assembly of all other OXPHOS complexes. **b** SDS-PAGE analysis of patient and control proteins probed with antibodies against Porin (a loading control) and the SDHA and SDHD subunits of succinate dehydrogenase revealed a stark reduction in SDH steady-state protein levels in patient muscle, consistent with subunit degradation thereby supporting the pathogenicity of the p.(Asp92Gly) variant

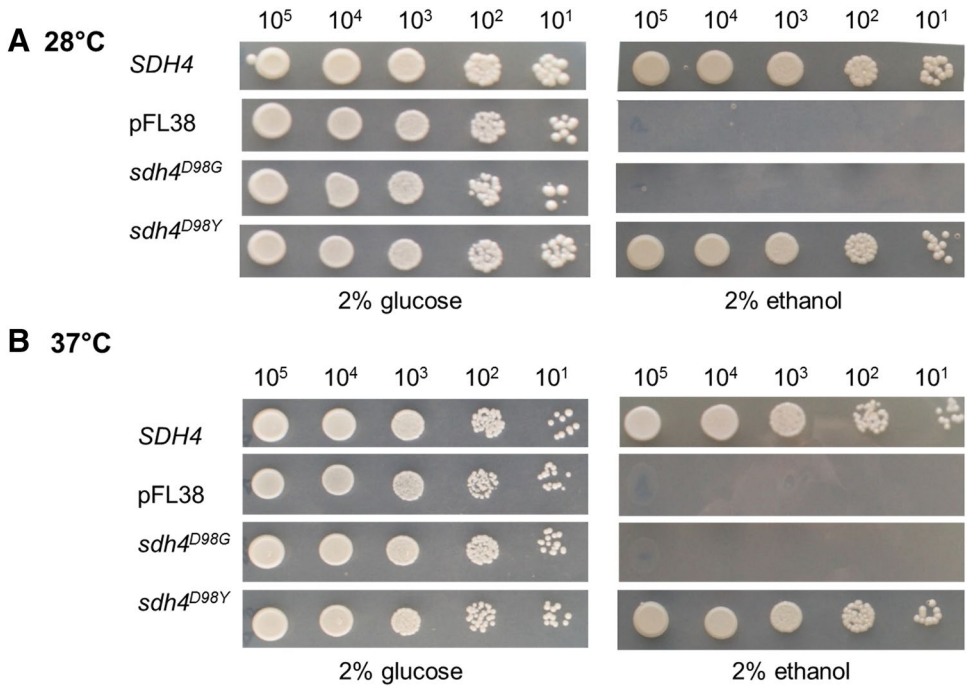

Fig. 4 Oxidative growth phenotype in yeast. The strain BY4741 Δ*sdh4* was transformed with a pFL38 plasmid carrying either the wild-type *SDH4*, the empty vector or the mutant alleles $sdh4^{D98G}$ and $sdh4^{D98Y}$. Equal amounts of serially diluted cells from exponentially grown cultures (10^5, 10^4, 10^3, 10^2, 10^1) were spotted onto yeast nitrogen base (YNB) plates supplemented with either 2 % glucose or 2 % ethanol. The growth was scored after 3-day incubation at 28 °C (**a**) and 37 °C (**b**)

the literature. We report a newborn boy presenting with left ventricular hypertrophy on foetal ultrasound at 32-weeks gestation who rapidly deteriorated after delivery due to cardiopulmonary insufficiency, dying on day one of life. Postmortem examination confirmed a non-compacted hypertrophic left ventricle but assessment of monolyso-cardiolipin and cardiolipin levels excluded a diagnosis of Barth syndrome. Biochemical analysis of his muscle biopsy revealed evidence of a marked isolated complex II deficiency. Sequencing the genes involved in succinate dehydrogenase structure and assembly was undertaken and revealed a novel homozygous c.275A>G, p.(Asp92Gly) *SDHD* mutation which was shown to be recessively inherited through segregation studies.

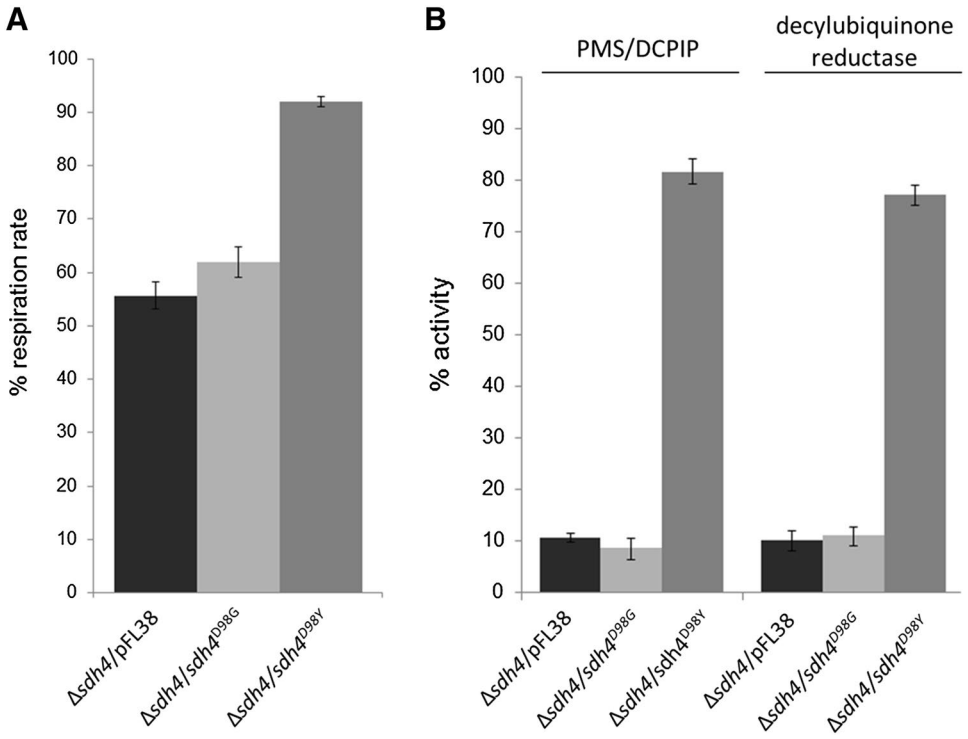

Fig. 5 **a** Oxygen consumption rates. Respiration was measured in cells grown in YNB supplemented with 0.6 % glucose at 28 °C. The values observed for the *sdh4* mutant cells are reported as a percentage of the wild-type *SDH4* cell respiratory rate, 40.46 ± 1.54 nmol min^{-1} mg^{-1}. **b** Complex II activity. PMS/DCPIP reductase and decylubiquinone reductase activities were measured in mitochondria extracted from cells grown exponentially at 28 °C in YNB supplemented with 0.6 % glucose. The values of the *sdh4* mutants are expressed as percentage of the activities obtained in the wild-type strain

Patients with an isolated complex II deficiency harbour either compound heterozygous or homozygous mutations in an SDH structural or assembly factor gene. The resultant loss of OXPHOS-driven ATP synthesis is associated with clinical presentations including Leigh syndrome, cardiomyopathy and leukodystrophy, that often present during infancy, though adult cases are reported (Taylor et al. 1996; Birch-Machin et al. 2000). Complex II deficiency is very rare, perhaps reflecting an incompatibility with life for many cases and our patient's clinical history with prenatal cardiomyopathy and rapid deterioration postpartum supports this hypothesis. Although the published cohort of patients with complex II deficiency is small, mutations which affect the ability of complex II to bind to the mitochondrial membrane are evolving to be the most deleterious.

The only other *SDHD*-deficient patient reported in the literature harboured compound heterozygous variants, one missense and one that extended the protein by three amino acids (Jackson et al. 2014). The clinical presentation of this individual differed from that of our patient who presented in utero with a cardiomyopathy that was incompatible with life. The previously described case was delivered at term after a normal pregnancy and presented at age 3 months with developmental regression following a viral infection with progressive neurological deterioration (epileptic seizures, ataxia, dystonia and continuous intractable myoclonic movements) and died at the age of 10 years. The patient described by Jackson et al. also had comparably low levels of SDHD protein on Western blot with greatly reduced levels of fully assembled complex II; a residual level of complex activity is therefore unlikely to account for the difference in presentation. We hypothesised that the p.(Asp92Gly) variant might have caused a conformational change given the location of the conserved acidic p.Asp92 residue at the N-terminus of one of the protein's helical domains. With this in mind, we modelled the predicted impact of the patient's p.(Asp92Gly) *SDHD* mutation on tertiary structure using in silico methodologies. Contrary to our expectations, neither I-TASSER nor PSIPRED predicted gross tertiary structural anomalies due to the substitution, despite being situated between two conserved cysteine residues; the pathogenicity is therefore assumed to lie in the nature of the amino acid properties as opposed to consequential protein misfolding. The location of the p.Asp92 residue at the helical N-termini may explain the discrepancy between the predicted Grantham scores and the functional data obtained following yeast modelling; leucine–tyrosine interactions are reported to act as stabilisers within alpha helices (Padmanabhan and Baldwin 1994) meaning the p.Asp92Tyr substitution (with a higher GD score) may therefore be less deleterious than the p.(Asp92Gly) substitution harboured by our patient. Moreover, the location of the substitution may also be important in capping the positive helical dipole, and replacement with a non-polar residue such as glycine would fail to provide the same charge stabilization. There was slight discordance between the helix predictions from

I-TASSER and PSIPRED (Fig. 2b, c) but on closer inspection of the discordant residues, there was low confidence in the predictions.

Mutations in *SDHD* and other *SDHx* genes have been implicated not only in primary metabolic dysfunction, but also as drivers of neoplastic transformation in various tumour types. There is a wealth of information in the literature describing the involvement of *SDHx* gene mutations in cases of hereditary and sporadic cancers including head and neck paraganglioma, pheochromocytoma and gastrointestinal stromal tumours (Miettinen and Lasota 2014). In the context of hereditary cancer, each somatic cell harbours one heterozygous germline mutation either inherited from a parent or occurring *de novo*. This single loss-of-function allele, alone, is insufficient to cause neoplastic transformation but if a "second hit" affects the wild-type allele, the loss of SDH activity disrupts ATP production. The inability of SDH to metabolise succinate causes a build-up of substrate, with elevated succinate levels stabilizing HIF1α. This in turn creates a pseudo-hypoxic state, prompting a switch to glycolytic respiration consistent with neoplasia (Hanahan and Weinberg 2011; Pollard et al. 2005). The metabolic stalling due to SDH dysfunction also acts to inhibit multiple 2-oxoglutarate-dependent histone and DNA demethylase enzymes resulting in widespread histone and DNA methylation, further adding to the tumorigenic burden of these already respiratory-deficient cells (Xiao et al. 2012).

To date, mutations reported in the SDHx genes are loss of function, either as tumour suppressors or in metabolic enzymes. The mutation harboured by our patient transcends these fields in that, although manifesting as a primary metabolic condition in our case, the p.Asp92 residue is recognised as a Dutch founder HNPGL mutation, p.(Asp92Tyr). Given the established link between tumorigenesis and this residue, further functional investigations were undertaken to determine whether the p.(Asp92Gly) variant—associated with primary metabolic dysfunction—was as deleterious, or indeed more so, than the founder HNPGL mutation. Functional investigations were supportive of a deleterious effect, Western blotting of patient muscle homogenates revealed a reduction in the steady-state levels evident for not only SDHD, but also for SDHA. This was supported by one-directional BN-PAGE, which confirmed a decrease in fully assembled complex II, consistent with the hypothesis that an inability to anchor the unstable complex within the mitochondrial membrane triggers the recycling of intermediates to prevent aggregation. This turnover is seen in other cases of mitochondrial complex dysfunction and prevents accumulation and aggregation of assembly intermediates and surplus complex subunits (Alston et al. 2012).

To assess the pathogenic role of the novel p.(Asp92Gly) SDHD substitution, we carried out a series of experiments in yeast devoid of *SDH4*, the yeast *SDHD* orthologue. The use of ethanol or glucose as a carbon source tested the strains' ability to rely upon either OXPHOS or fermentation for ATP synthesis. The SDHD residue p.Asp92 shows high evolutionary conservation and corresponds to p.Asp98 in yeast. Given that a germline mutation involving the same amino acid has been reported as a cause of paraganglioma [p.(Asp92Tyr)], two mutant alleles—$sdh4^{D98G}$ and $sdh4^{D98Y}$—were constructed to compare the phenotypes associated with the different substitutions. Consistent with the reduction of SDHD steady-state levels and fully assembled complex II found in our patient, the p.(Asp92Gly) mutation was detrimental to both oxidative growth and succinate dehydrogenase activity in yeast. Contrariwise, the p.(Asp98Tyr) HNPGL-associated substitution did not affect oxidative growth and showed a mild, albeit significant, reduction of SDH activity. Altogether the results obtained in the yeast model provide compelling functional evidence supporting the pathogenic role of the p.(Asp92Gly) mutation and show that this substitution conveys a more severe phenotype than the founder HNPGL *SDHD* mutation, this finding is not unique as other PGL-associated *SDHD* mutations were found to cause a milder phenotype when modelled in yeast (Panizza et al. 2013). Whilst our modelling suggests that the well-characterised p.(Asp92Tyr) *PGL* mutation is associated with what might be considered a mild phenotype in yeast, the phenotype in question is not a primary metabolic one and indeed it is only associated with oncogenesis in tandem with a second mutation, which is often a large-scale deletion or other null allele.

There were no reports of potential *SDHD*-associated cancers in the immediate family although further information from extended family members was unavailable. We previously reported inherited recessive *SDHB* mutations in association with a paediatric primary mitochondrial phenotype and this case also lacked a history of hereditary cancer (Alston et al. 2012). It is unclear whether germline carriers of the p.(Asp92Gly) *SDHD* mutation are at elevated risk of HNPGL and despite no tumours having been reported in the family, it is the opinion of their clinicians that surveillance was advisable and is ongoing.

Left ventricular non-compaction is a rare form of cardiomyopathy characterised by abnormal trabeculations in the left ventricle and associated with either ventricular hypertrophy or dilation. In some patients, LVNC arises from a failure to complete the final stage of myocardial morphogenesis, but this is not a satisfactory explanation for all cases, particularly those associated with congenital heart defects or arrhythmias. LVNC is genetically heterogeneous with many cases remaining genetically undiagnosed, but metabolic derangements are common and this form of cardiomyopathy is typical of Barth Syndrome, a disorder

of mitochondrial cardiolipin typically accompanied by neutropenia (Chen et al. 2002) and has also been observed in other mitochondrial disorders including those due to mutations in mtDNA (Pignatelli et al. 2003).

In conclusion, our case further expands the clinical and genetic heterogeneity associated with isolated complex II deficiency and demonstrates that sequencing analysis of all SDH subunits and assembly factors should be undertaken for patients in whom an isolated succinate dehydrogenase defect has been identified.

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Biallelic Mutations in *TMEM126B* Cause Severe Complex I Deficiency with a Variable Clinical Phenotype

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Complex I deficiency is the most common biochemical phenotype observed in individuals with mitochondrial disease. With 44 structural subunits and over 10 assembly factors, it is unsurprising that complex I deficiency is associated with clinical and genetic heterogeneity. Massively parallel sequencing (MPS) technologies including custom, targeted gene panels or unbiased whole-exome sequencing (WES) are hugely powerful in identifying the underlying genetic defect in a clinical diagnostic setting, yet many individuals remain without a genetic diagnosis. These individuals might harbor mutations in poorly understood or uncharacterized genes, and their diagnosis relies upon characterization of these orphan genes. Complexome profiling recently identified TMEM126B as a component of the mitochondrial complex I assembly complex alongside proteins ACAD9, ECSIT, NDUFAF1, and TIMMDC1. Here, we describe the clinical, biochemical, and molecular findings in six cases of mitochondrial disease from four unrelated families affected by biallelic (c.635G>T [p.Gly212Val] and/or c.401delA [p.Asn134Ilefs*2]) *TMEM126B* variants. We provide functional evidence to support the pathogenicity of these *TMEM126B* variants, including evidence of founder effects for both variants, and establish defects within this gene as a cause of complex I deficiency in association with either pure myopathy in adulthood or, in one individual, a severe multisystem presentation (chronic renal failure and cardiomyopathy) in infancy. Functional experimentation including viral rescue and complexome profiling of subject cell lines has confirmed TMEM126B as the tenth complex I assembly factor associated with human disease and validates the importance of both genome-wide sequencing and proteomic approaches in characterizing disease-associated genes whose physiological roles have been previously undetermined.

Complex I deficiency is the most common biochemical phenotype observed in subjects with mitochondrial disease.[1] It can occur as an isolated complex deficiency, where biochemical assessment of enzyme activities of other respiratory-chain components (complexes II, III, and IV) is normal, or as part of a multiple-respiratory-chain-complex deficiency with the involvement of other parts of the oxidative phosphorylation (OXPHOS) system. The latter is suggestive of a global mitochondrial defect involving, for example, mitochondrial maintenance, protein translation, or mitochondrial import. Mitochondrial complex I deficiency is phenotypically diverse, such that clinical presentations range from subacute necrotizing encephalomyelopathy (Leigh syndrome [MIM: 256000]) to pure myopathy and exercise intolerance.[1,2] In cases of isolated complex I deficiency, the genetic basis can be attributed to defects in the mitochondrial DNA (mtDNA) genes encoding seven structural subunits, in the nuclear genes encoding any of 37 other structural subunits, or in the increasing number of ancillary proteins that are responsible for faithful biogenesis and assembly of complex I. Such heterogeneity results in complicated diagnostic pipelines for clinical subjects. Massively parallel sequencing (MPS) strategies, whether in the form of whole-exome sequencing (WES)[3] or targeted capture

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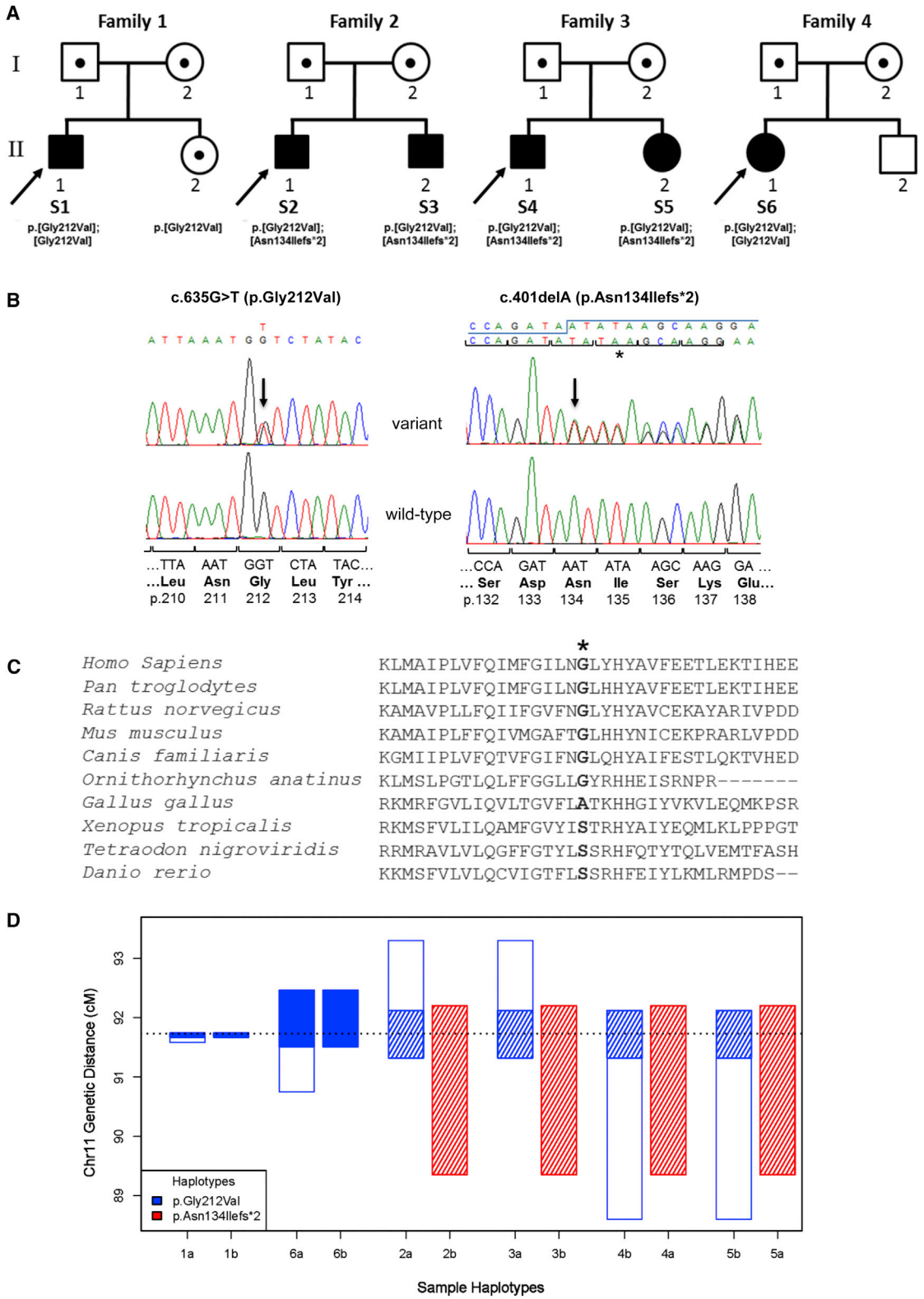

Figure 1. Autosomal-Recessive *TMEM126B* Variants Are Identified in Six Unrelated Subjects from Four Families Affected by an Isolated Complex I Deficiency

(A) Pedigrees and genotype of affected individuals harboring *TMEM126B* variants. Subject 1 harbors a homozygous c.635G>T (p.Gly212Val) *TMEM126B* variant; his parents and unaffected sister are heterozygous carriers of this variant. Subjects 2 and 3 harbor compound-heterozygous *TMEM126B* variants—a paternal c.401delA (p.Asn134Ilefs*2) variant and a maternal c.635G>T (p.Gly212Val) variant. Subjects 4 and 5 also harbor compound-heterozygous c.401delA (p.Asn134Ilefs*2) and c.635G>T (p.Gly212Val) *TMEM126B* variants; carrier testing confirmed that the subjects' mother harbors a heterozygous c.401delA (p.Asn134Ilefs*2) variant, but paternal DNA

(legend continued on next page)

(e.g., Ampliseq),[4] are proving extremely effective at establishing genetic diagnoses, particularly when mutations occur within known or candidate disease-associated genes. To date, mutations have been identified in all seven mtDNA-encoded structural subunits of complex I and 20 nuclear-encoded structural genes;[5–7] similarly, subjects have been reported with defects in nine assembly factors.[5] However, even after WES analysis, a significant proportion of subjects lack a genetic diagnosis—a common explanation is that their mutations affect an uncharacterized protein.[8,9] Here, we describe a cohort of six subjects who all harbor recessive mutations within the gene encoding TMEM126B, a protein recently identified as a complex I assembly factor by a proteomic study of knockdown cell lines.[10] Complexome profiling revealed TMEM126B to be a component of the mitochondrial complex I assembly (MCIA) complex alongside proteins ACAD9, ECSIT, NDUFAF1, and TIMMDC1, thus establishing *TMEM126B* (MIM: 615533) as a candidate gene for complex I deficiency.[10,11] With access to subjects harboring putative *TMEM126B* defects, we provide functional evidence to support the pathogenicity of these *TMEM126B* variants, unequivocally establishing this gene as a cause of complex I deficiency in association with either a severe multisystem presentation in infancy or pure myopathy in later child- or adulthood. This report describes the clinical, biochemical, and molecular findings in six cases of *TMEM126B*-related mitochondrial disease and validates the importance of proteomic approaches in identifying disease-associated genes whose physiological roles have been previously undetermined.

Subject 1 (family 1 subject II-1 in Figure 1A) was born to American parents without known consanguinity. He presented in childhood with pure exercise intolerance without muscle weakness. Exercise (running and swimming) caused leg fatigue, shortness of breath, and a rapid heart rate, often provoking vomiting and severe headache. Cardiology review in early adulthood showed normal electrocardiography (ECG) and echocardiography. Treadmill exercise testing caused fatigue after 2 min with a heart rate of 180 and elevated blood lactate (16 mmol/L; normal range $<$ 2.0 mmol/L), characteristic of mitochondrial dysfunction. He had normal creatine kinase (CK) levels, and there was no pigmenturia. Physical examination remains normal at 23 years of age.

Subjects 2 and 3 (family 2 subjects II-1 and II-2, respectively, in Figure 1A) are brothers who were born to non-consanguineous parents in Belgium. They presented in their early teens with exercise-induced dyspnea (subject 2), exercise intolerance (subjects 2 and 3), and post-exertional myalgia (subjects 2 and 3). Exertion was often followed by nausea and vomiting. Now in adulthood, currently aged 40 and 37 years, respectively, subjects 2 and 3 are wheelchair bound and have significantly impaired muscle strength affecting the lower limbs, particularly hip flexion and extension. Strength in the upper limbs is normal. Forced vital capacity, cardiac ultrasound, and cognitive development are normal, and neither subject has epilepsy, neuropathy, diabetes, or hearing impairment. Subject 2 has mild visual impairment (macular and peripheral retinal pigment migration) and had mild left ventricular hypertrophy in his twenties. CK was normal, but blood lactate (2.3–3.0 mmol/L in subject 2 and 3.2–3.8 mmol/L in subject 3) and cerebrospinal fluid lactate (5.8 mmol/L in subject 3) were elevated.

Subjects 4 and 5 (family 3 subjects II-1 and II-2, respectively, in Figure 1A) are affected siblings who were born in Belgium to unrelated parents with no other children. Their father died at the age of 47 years and complained of mild exercise intolerance; their mother is alive and complains of fatigue. Subjects 4 and 5 (currently aged 33 and 30 years, respectively) presented in adolescence with fatigue, exercise intolerance, and exercise-induced nausea. No other organs are affected, although subject 5 reports gastrointestinal problems. Cardiac, ophthalmic, and nephrologic examination, intellectual capacity, and CK were normal for both subjects. Cycloergometry (for both siblings) showed very low submaximal and maximal capacity. Both subjects are able to walk but cannot ride a bike or run, and they have reported improvements following coenzyme Q supplementation (200 mg/d).

Subject 6 (family 4 subject II-1 in Figure 1A) is female and the second child of healthy, unrelated parents living in Poland. She was born at 37 weeks of gestation with a weight of 2,150 g (third percentile [−1.88 SD]) and an Apgar score of 10. Patent ductus arteriosus and an atrial septal defect without ventricular hypertrophy were observed, and transient assisted respiration was required in the early neonatal period. At the age of 2 months, she was admitted to the hospital with very poor weight gain and vomiting, and during this period she went into cardiac arrest, attributed to gastroesophageal reflux and protracted

was unavailable for confirmatory testing. Subject 6 harbors a homozygous c.635G>T (p.Gly212Val) *TMEM126B* variant; both her parents are carriers, and her unaffected brother does not harbor the mutation.
(B) Sequencing chromatograms depict the recurrent c.635G>T (p.Gly212Val) and c.401delA (p.Asn134Ilefs*2) *TMEM126B* variants, which represent the disease alleles identified in our cohort of six affected subjects.
(C) Clustal Omega sequence alignment shows the evolutionary conservation of the p.Gly212 residue (marked with an asterisk).
(D) Shared maternal and paternal haplotypes in the region of interest for subjects 1–6, as inferred by SHAPEIT2. Subject 1 has a ~0.5 Mb homozygous region from 91.67 to 91.74 cM, whereas subject 6 has a ~2 Mb homozygous region from 91.51 to 92.46 cM (blue boxes). The two Belgian sibling pairs (subjects 2 and 3 and subjects 4 and 5) share the p.Gly212Val haplotype over a ~1.75 Mb region (91.31–92.12 cM: blue diagonal shade) and the p.Asn134Ilefs*2 haplotype over a ~4.6 Mb region (89.35–92.2 cM: red diagonal shade). Both haplotypes are shared over the 91.31–92.12 cM region. Boxed white areas represent regions shared with at least one other allele from a different family. The Polish subject 6 shares a ~2.2 Mb (91.31–92.46 cM) p.Gly212Val haplotype region with siblings 2 and 3 and a ~2.5 Mb (90.75–92.12 cM) region with siblings 4 and 5.

Table 1. Biochemical and Clinical Findings in Individuals with *TMEM126B* Variants

Subject Details		*TMEM126B* Variants	OXPHOS Activities in Skeletal Muscle				Clinical Features		
ID	Sex	cDNA (GenBank: NM_018480.4), Protein (GenBank: NP_060950.3)	RCC	Mean Enzyme Activity	Absolute Values	Control Mean (Reference Range)	Age at Onset	Clinical Course	Other Clinical Features and Relevant Family History
Subject 1[a]	male	c.[635G>T];[635G>T], p.[Gly212Val];[Gly212Val]	I	36% (↓)[b]	1.8	5.0 ± 0.8 (n = 28)	8 years	alive at 21 years	exercise intolerance, unable to perform sustained aerobic exercise, normal strength, normal ECG and echocardiography, normal resting lactate, normal CK
			II	210% (↑↑)	4.2	2.0 ± 0.6 (n = 44)			
			III	219% (↑↑)	23.6	10.8 ± 2.3 (n = 29)			
			IV	218% (↑↑)	8.5	3.9 ± 1.5 (n = 44)			
			CS	196% (↑↑)	24.1	12.3 ± 2.7 (n = 44)			
Subject 2[c]	male	c.[401delA];[635G>T], p.[Asn134Ilefs*2];[Gly212Val]	I	48% (↓)[b]	14	29 ± 13 (n = 30)	12 years	alive at 39 years, wheelchair bound	exercise intolerance, muscle weakness in lower limbs and pelvis, normal echocardiography, mild basal increases of lactate, normal CK, normal intelligence, retinitis pigmentosa
			II	138%	47	34 ± 14 (n = 30)			
			III	ND	ND	96 ± 31 (n = 30)			
			IV	82%	137	167 ± 58 (n = 30)			
			CS	237% (↑↑)	412	174 ± 70 (n = 30)			
Subject 3[c]	male	c.[401delA];[635G>T], p.[Asn134Ilefs*2];[Gly212Val]	I	14% (↓↓)[b]	4	29 ± 13 (n = 30)	10 years	alive at 36 years, wheelchair bound	clinically affected sibling of subject 2, exercise intolerance, muscle weakness in lower limbs and pelvis, normal echocardiography, mild basal increases in lactate, normal CK, normal intelligence, no retinitis pigmentosa
			II	179% (↑)	61	34 ± 14 (n = 30)			
			III	ND	ND	96 ± 31 (n = 30)			
			IV	103%	172	167 ± 58 (n = 30)			
			CS	281% (↑↑)	489	174 ± 70 (n = 30)			
Subject 4[a]	male	c.[401delA];[635G>T], p.[Asn134Ilefs*2];[Gly212Val]	I	10% (↓↓)[b]	3	29 ± 13 (n = 30)	8 years	alive at 32 years	exercise intolerance and fatigue
			II	253% (↑↑)	86	34 ± 14 (n = 30)			
			III	172% (↑)	165	96 ± 31 (n = 30)			
			IV	126%	210	167 ± 58 (n = 30)			
			CS	201% (↑↑)	350	174 ± 70 (n = 30)			
Subject 5[a]	female	c.[401delA];[635G>T], p.[Asn134Ilefs*2];[Gly212Val]	I	10% (↓↓)[b]	3	29 ± 13 (n = 30)	15 years	alive at 29 years	clinically affected sibling of subject 4, exercise intolerance and fatigue
			II	288% (↑↑)	98	34 ± 14 (n = 30)			
			III	129%	124	96 ± 31 (n = 30)			
			IV	238% (↑↑)	398	167 ± 58 (n = 30)			
			CS	259% (↑↑)	451	174 ± 70 (n = 30)			

(*Continued on next page*)

Table 1. *Continued*

Subject Details		*TMEM126B* Variants	OXPHOS Activities in Skeletal Muscle				Clinical Features		
ID	Sex	cDNA (GenBank: NM_018480.4), Protein (GenBank: NP_060950.3)	RCC	Mean Enzyme Activity	Absolute Values	Control Mean (Reference Range)	Age at Onset	Clinical Course	Other Clinical Features and Relevant Family History
Subject 6[a]	female	c.[635G>T];[635G>T], p.[Gly212Val];[Gly212Val]	I	17% (↓↓)[b]	3	17 ± 8 (n = 15)	2 months	alive at 5.5 years	multiorgan involvement manifesting in infancy (respiratory failure, cardiomyopathy, and renal acidosis), severe growth failure, chronic renal insufficiency, elevated serum lactate
			II	135%	13	10 ± 3 (n = 15)			
			III	64%	58	90 ± 52 (n = 15)			
			IV	82%	10	12 ± 9 (n = 15)			
			CS	228% (↑↑)	458	200.9 ± 48.5 (n = 15)			

For subject 1, respiratory-chain enzyme activities are expressed as U/min/g wet weight.[12] For subjects 2–6, enzyme activities are expressed as nanomoles of substrate/min/mg protein.[13] The following abbreviations are used: ↓, decreased; ↓↓, markedly decreased; ↑, increased; ↑↑, markedly increased; ECG, electrocardiography; and ND, not determined.
[a]Investigated by WES.
[b]Below the normal range.
[c]Investigated by targeted gene analysis (AmpliSeq capture or carrier testing).

renal failure with severe tubular acidosis (pH 7.21 [normal range = 7.35–7.43], 13.5 mmol/L $NaHCO_3$ [normal range = 22.0–26.0 mmol/L], 6.0 mmol/L potassium [normal range = 3.6–5.8 mmol/L], and 124 mmol/L sodium [normal range = 136–145 mmol/L]). Progressive hypertrophic cardiomyopathy, failure to thrive, and elevated blood lactate (8.1 mmol/L) prompted suspicion of mitochondrial disease. Currently aged 6 years, she is in good general condition and has age-appropriate motor and mental development but shows chronic renal failure (stage IV) and a marked growth deficit (−5.1 SD). She requires continuous administration of erythropoietin because of anemia and is supplemented with citrate and sodium because of tubular acidosis.

Muscle and/or skin biopsy was performed for each subject, and biochemical, histochemical, and molecular investigations were undertaken (Table 1). Informed consent for diagnostic and research studies was obtained for all subjects in accordance with the Declaration of Helsinki protocols and approved by local institutional review boards.

Histochemical analysis of all subjects' muscle biopsy revealed subsarcolemmal accumulation of mitochondria, suggestive of mitochondrial proliferation and evolving pathology of ragged-red fibers (Figure S1). Biochemical analysis of muscle respiratory-chain activities revealed a marked isolated complex I deficiency in all subjects, suggestive of a defect involving mtDNA or a nuclear-encoded protein implicit in complex I structure or assembly. The genetic basis was identified by previously described MPS strategies involving either a custom, targeted AmpliSeq panel (subjects 2 and 3) or WES (subjects 1 and 4–6) as described elsewhere.[14,15] For all cases, biallelic variants in *TMEM126B* (GenBank: NM_018480.4 and NP_060950.3) were identified—just two *TMEM126B* genotypes, either a homozygous c.635G>T (p.Gly212Val) missense variant (subjects 1 and 6) or a compound-heterozygous c.401delA (p.Asn134Ilefs*2) and c.635G>T (p.Gly212Val) genotype (subjects 2–5), account for the clinical phenotype of each subject in our cohort (Table 1 and Figures 1A and 1B). Where familial samples were available from parents and unaffected siblings, these variants were found to segregate with a clinically affected status. The c.401delA (p.Asn134Ilefs*2) variant is absent from dbSNP, the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (ESP6500), and the Exome Aggregation Consortium (ExAC) Browser (as of February 10, 2016). The c.635G>A (p.Gly212Val) variant is referenced in dbSNP (rs141542003) and recorded in ESP6500 (Europeans: 16/8,598 alleles [0.2%]) and the ExAC Browser (Europeans: 146/72,144 alleles [0.2%]; non-Europeans: 10/38,138 [0.02%]). No homozygous cases have been recorded (according to ExAC, ESP6500, and dbSNP data as of February 10, 2016), and subject 1 was the only individual to have rare potentially pathogenic biallelic *TMEM126B* variants in over 7,500 samples sequenced at the Institute of Human Genetics in Munich (where the c.635G>A variant was present in 15/15,134

alleles [0.1%]). Both *TMEM126B* variants have been submitted to ClinVar (see Accession Numbers).

Because the structure of TMEM126B has not been solved, in silico modeling of TMEM126B tertiary structure was performed with I-TASSER,[16] Phyre2,[17] and RaptorX,[18] and although confidence was low for overall structure predictions, each tool predicted the Gly212 residue to be located within a helical domain. Glycine, the smallest amino acid and the only one without a carbon-containing side chain, is often critical within helices because it is permissive in structure and allows the helix to twist. Its substitution for a branched-chain amino acid, such as valine, is likely to affect the tertiary structure and thus compromise protein function.[19,20] This is corroborated by in silico prediction tools including SIFT,[21] MutationTaster,[22] and PolyPhen-2,[23] which support a detrimental effect due to the p.Gly212Val substitution. Moderate evolutionary conservation of the Gly212 TMEM126B residue was suggested by Clustal Omega alignment of Ensembl-derived orthologs (Figure 1C).

Given that just two *TMEM126B* variants were identified in our ethnically diverse cohort of subjects (from Belgium, the United States, and Poland), we performed SNP genotyping to investigate a possible founder effect (Figure 1D and Tables S1 and S2). The most likely haplotype structure for the subjects was inferred with the SHAPEIT2 algorithm.[24] As anticipated, there was evidence of two alleles shared by state (0.81 cM region from 91.31 to 92.12 cM) for the Belgian sibling pairs from two apparently unrelated families (subjects 2 and 3 and subjects 4 and 5). Similarly, there was a shared haplotype (1.15 cM region from 91.31 to 92.46 cM) between subjects 2 (Belgian) and 6 (Polish), and this was echoed by a 1.37 cM shared haplotype from 90.75 to 92.12 cM in an analysis involving subjects 4 (Belgian) and 6 (Polish). Together, these data support common ancestors and the c.401delA (p.Asn134Ilefs*2) and c.635G>T (p.Gly212Val) variants as founder mutations. Subject 1, of European-American ancestry, was found to have a very small homozygosity-by-state (HBS) tract (0.07 cM, ~500 kb genomic distance), but on a background suggestive of first-cousin parentage. The homozygous c.635G>T (p.Gly212Val) variant occurs within the HBS tract but is intriguingly outside the large identity-by-descent tracts shared as a result of consanguinity. This suggests that a much more distant inbreeding loop leads to this HBS tract and that the first-cousin inbreeding loop is coincidental. The sharing of haplotypes in the cohort of subjects, and that some individuals share several megabases, suggests founder events for both haplotypes; with evidence of shorter shared haplotypes, HBS, and a slightly higher frequency than that of the p.Gly212Val variant, p.Asn134Ilefs*2 is likely to be the older founder event.

Extensive functional characterization of the identified *TMEM126B* variants was undertaken in muscle and fibroblast cell lines obtained from subjects 1–3. Blue native PAGE (BN-PAGE) analysis of fibroblasts from affected subjects revealed a marked reduction of fully assembled complex I in supercomplex form (Figure 2A) or holoenzyme form (Figure 2B) in subjects 2 and 3, who harbored a truncating mutation in *trans* with a p.Gly212Val missense variant. Conversely, complex I assembly was normal in fibroblasts from subject 1, suggesting an ability to function despite the biallelic p.Gly212Val variants (Figure S1). The accumulation of subcomplexes containing NDUFS3 in subjects 2 and 3 indicates that the matrix module containing NDUFS3 is made but is unable to be added to the membrane arm. SDS-PAGE and immunoblot analysis of select complex I subunits revealed strongly reduced levels in fibroblasts from subject 2 and 3, but not subject 1 (Figure 2C). Subsequent BN-PAGE analysis of muscle from subject 1 revealed severely diminished levels of fully assembled complex I (Figure 2D).[26] These results support a deleterious effect and recapitulate the biochemical enzyme assays in which markedly decreased complex I levels were observed in fibroblasts from compound-heterozygous subjects, whereas the fibroblasts from subject 1 retained complex I activities within the normal range (Figure 2E). Functional analysis of fibroblasts and muscle biopsy from additional individuals, notably subjects 4–6, revealed similar patterns of pathology (Supplemental Data). Two-dimensional BN-PAGE of mitochondria-enriched pellets from muscle biopsy of subjects 4 and 5 revealed a marked reduction of complex I subunits, whereas other complexes remained intact (Figure S2). Double immunofluorescence staining of fibroblasts from subjects 4 and 5 (Figure S3) or subject 6 (Figure S4) revealed decreased signal of TMEM126B directly (subjects 4 and 5) or clear evidence of reduced signal of complex I subunits in the case of subject 6 (NDUFS4 was used as a surrogate marker of complex I signal) in comparison to age-matched control subjects. Most noteworthy is the observation of a complex I biochemical defect in the cells from subject 6, who like subject 1, was homozygous for the p.Gly212Val variant yet presented much earlier in life with a more severe clinical phenotype (Table 1).

To provide further evidence that *TMEM126B* mutations are causative, we performed cellular rescue with *TMEM126B* variant 1 (GenBank: NM_018480.4) essentially as described previously[4] (Figure 3). Retroviral-mediated expression of *TMEM126B* in subject 2 fibroblasts largely restored the levels of assembled complex I (Figure 3A). In addition, after lentiviral-mediated expression of *TMEM126B*, enzyme activities were significantly increased in fibroblasts re-expressing *TMEM126B* from subjects 2 and 3, whereas fibroblasts from a healthy control or subject with recessively inherited, pathogenic *FOXRED1* variants (described previously[28]) showed no increased activity (Figure 3B).

TMEM126B was identified as a component of the MCIA complex, which also comprises the previously characterized assembly factors ACAD9, ECSIT, and NDUFAF1.[10,29] To gain deeper insight into the molecular consequences of the subjects' *TMEM126B* variants, we analyzed complex I assembly by complexome profiling.

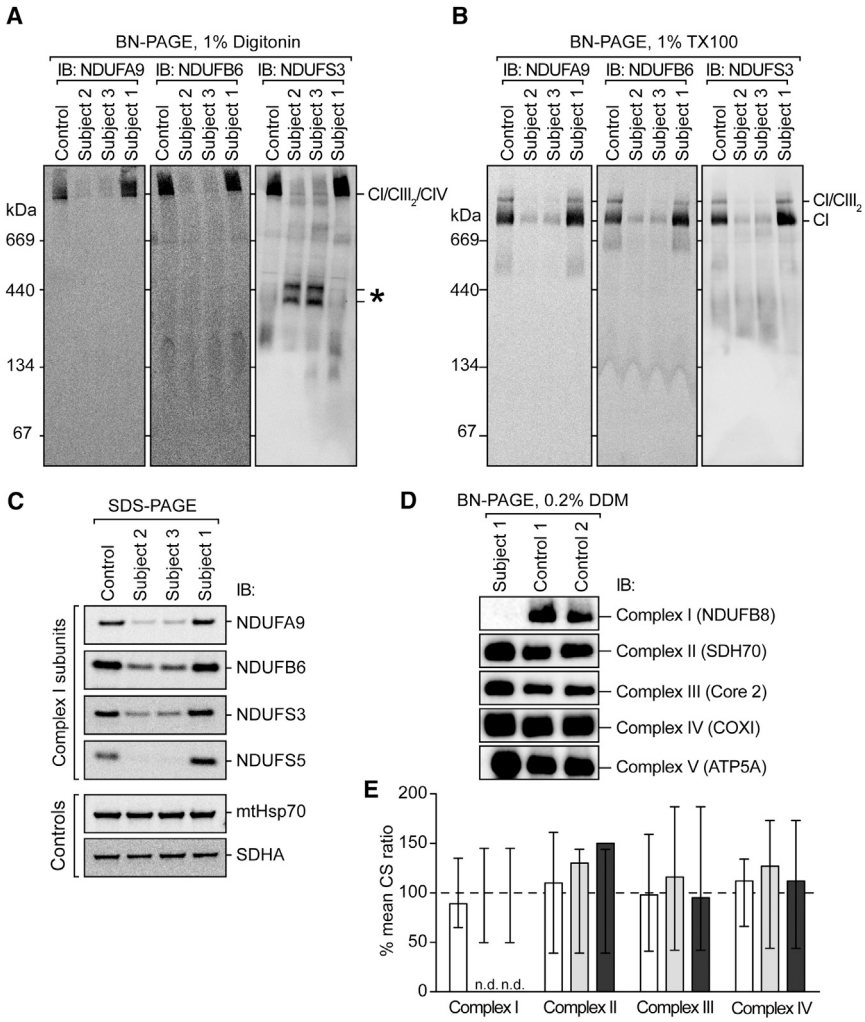

Figure 2. Biochemical Analysis of Subject Samples Demonstrates Tissue-Specific Complex I Deficiency

(A and B) Mitochondria isolated from cultured skin fibroblasts of control subjects and subjects 1–3 were analyzed by BN-PAGE after solubilization in (A) digitonin for maintaining supercomplex interactions or (B) Triton X-100 (TX100) for isolating holo-complexes according to published methodologies.[25] Immunoblotting was performed with antibodies as indicated. The blot probed with an antibody raised against NDUFS3 revealed the presence of additional, partially assembled complex I intermediates in the samples from subjects 2 and 3 (A, indicated by an asterisk).

(C) Mitochondria were isolated as described in (A) and (B) and analyzed by SDS-PAGE. Immunoblotting was performed with antibodies against complex I subunits or control proteins as indicated.

(D) Muscle samples derived from subject 1 and two control subjects were solubilized in n-dodecyl β-D-maltoside (DDM) and subjected to BN-PAGE and immunoblot analysis using antibodies directed to various OXPHOS complexes as indicated.

(E) Respiratory-chain enzyme activities in fibroblast mitochondria were assayed spectrophotometrically as described[12] and expressed as percentages of residual activity in relation to citrate synthase for subject 1 (white bars), subject 2 (light-gray bars), and subject 3 (dark-gray bars). Vertical lines represent the observed normal ranges for either 8 (subject 1) or 36 (subjects 2 and 3) normal control cell lines determined in Newcastle or Melbourne, respectively. The following abbreviation is used: ND, not detected.

As visualized in a heatmap (Figure 4), profiles of protein abundance confirmed a severe complex I assembly defect in fibroblasts from subjects 2 and 3 and a concomitant increase in the amount of free complex III. Prominent accumulation of a stalled assembly intermediate containing subunits of the Q module and assembly factors NDUFAF3, NDUFAF4, and TIMMDC1 was observed, reflecting the 315 kDa subcomplex reported by *TMEM126B* siRNA experiments in 143B cells.[29] Consistent with TMEM126B-knockdown cells,[10] subject mitochondria

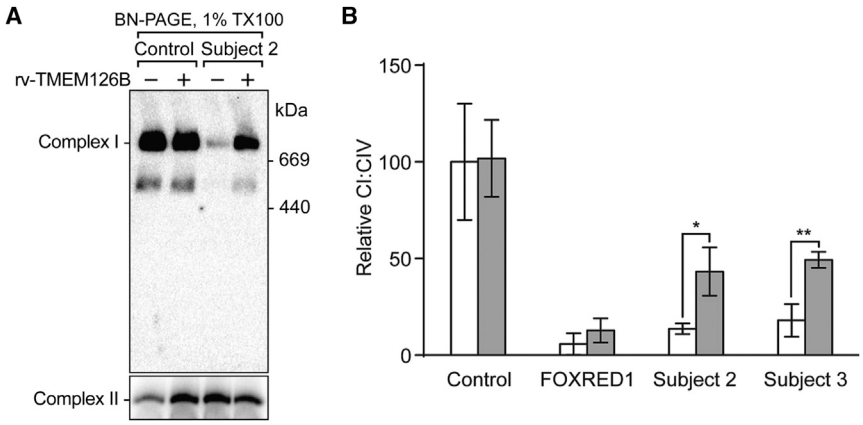

Figure 3. Re-expression of Wild-Type *TMEM126B* Can Lead to Increased Complex I Assembly and Activity in Subject Cells

(A) Wild-type *TMEM126B* mRNA was generated by retroviral expression in control and subject 2 fibroblasts as described previously.[27] After transduction and puromycin selection of cells, whole-cell lysates were solubilized in 1% Triton X-100 and analyzed by BN-PAGE and immunoblotting using antibodies against NDUFA9 (complex I) and SDHA (complex II) as a loading control.

(B) Wild-type *TMEM126B* mRNA was generated by lentiviral expression, and activities of complexes I and IV were assessed by enzyme dipstick analyses as described previously.[4] Barplots show complex I (CI) activity, normalized by complex IV (CIV) activity in control and subject fibroblasts,[28] after transduction with (gray bars) and without (white bars) wild-type *TMEM126B* mRNA. Data shown are means of three independent transfections ± SEM. *$p < 0.05$, **$p < 0.01$.

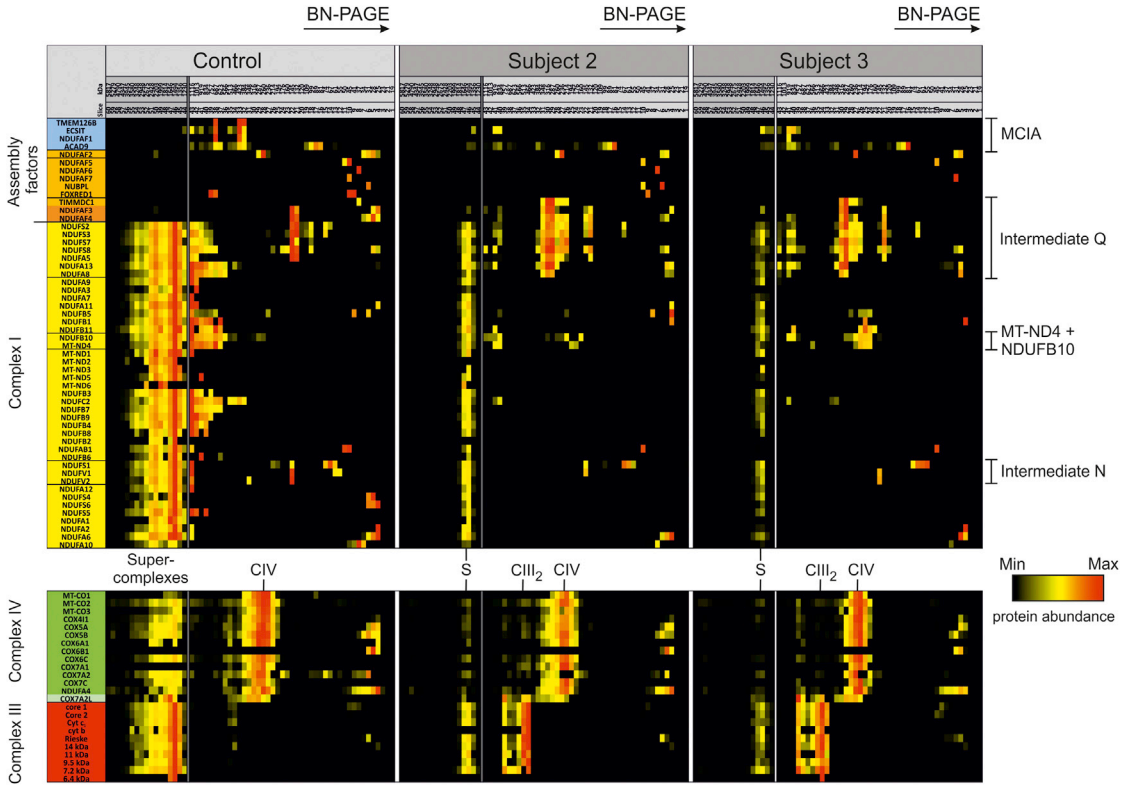

Figure 4. Complexome Profiling of Fibroblasts from Subjects 2 and 3 Identifies Stalled Complex I Assembly Intermediates
Prior to mitochondrial isolation, skin fibroblasts were cultured for 48 hr in medium supplemented with galactose as a carbon source. Mitochondrial protein complexes were solubilized with digitonin and separated by BN-PAGE.[30] Native gels were fixed and stained with Coomassie and cut into 60 equal fractions; proteins were digested with trypsin and analyzed by quantitative mass spectrometry. For direct comparison of protein-abundance profiles in control and affected subjects, intensity-based absolute quantification values[31] calculated by MaxQuant proteomics software[32] were normalized to the maximum over datasets (left part of each sample). Less abundant complex I assembly intermediates were normalized to the maximum within the mass region below 1,200 kDa (right part of each sample) for enabling better visualization within a heatmap. The native masses of gel slices were calibrated by exponential regression using positions of the human OXPHOS complex in the gel.[33] The left lane indicates assembly factors (orange), MCIA components (blue), and structural subunits of complex I (yellow), complex III (red), and complex IV (green). Abbreviations are as follows: MCIA, mitochondrial complex I assembly complex; $CIII_2$, complex III dimer; CIV, complex IV; and S, supercomplex containing complex I, a dimer of complex III, and one to four copies of complex IV.

formed a ~200 kDa subcomplex containing ND4 and NDUFB10, indicating that parts of the membrane arm can be assembled without a complete MCIA complex. Recent work on TIMMDC1-knockdown cell lines has suggested that the membrane protein TIMMDC1 connects the pre-assembled membrane subcomplex via interaction with MCIA components.[11,29] In subject mitochondria, only low levels of the membrane subcomplex were found in the large assembly intermediate of 830–1,000 kDa, reflecting an inefficient connection of the Q module and membrane modules. In contrast to TMEM126B-knockdown cells,[10] the subject cell lines showed markedly decreased levels of ECSIT, ACAD9, and NDUFAF1, the remaining MCIA components, together with complex I subunits in a mass region between ~800 and 950 kDa. The last part of the assembly sequence uses assembly factor NDUFAF2 and the preassembled NADH dehydrogenase module (N module) to complete complex I.[34–36] Fibroblasts from both affected subjects showed low amounts of preformed N module intermediates, indicating that assembly of the membrane part might control N module assembly such that it does not accumulate in the affected cells. NDUFAF2 was not detected in a complex with N module subunits but shifted from ~260 kDa in control cells to ~230 kDa in affected cells. It is known that once complex I assembly is completed, all assembly factors dissociate and leave a fully assembled complex I.[37] In cells from subjects 2 and 3, we identified minor amounts of ECSIT and ACAD9 still bound in the native mass region corresponding to supercomplex S1, containing complexes III and IV. It remains to be further determined whether these large assembly intermediates are already part of a supercomplex or co-migrate in this high-molecular-mass region. Another assembly factor, FOXRED1, recently identified to exhibit a function in late-stage assembly,[25] was identified as co-migrating with the large assembly intermediate of ~700–800 kDa in control fibroblasts. In subjects 2 and 3, FOXRED1 could be detected only at the electrophoretic front and not in a complex with complex I subunits, suggesting that the MCIA complex is a prerequisite for binding and function in the large intermediate.

Tissue specificity is a common phenomenon in OXPHOS disorders,[38] but this feature is particularly striking with the *TMEM126B* mutations we describe here. Five of the six subjects have relatively mild symptoms, confined mostly to myopathy, and all have normal cognitive development despite having a severe complex I defect in muscle and the fact that TMEM126B appears to be a ubiquitous complex I assembly factor. All tissues studied from subjects with *TMEM126B* mutations showed some residual complex I assembly, and the threshold level of complex I activity required by any tissue most likely depends on factors such as variation in the amounts of subunits and assembly factors, plus variation in protein turnover rates and basal and peak energetic demands. Inter-individual differences were also apparent—all subjects except subject 1 had a marked complex I defect in skin fibroblasts. We note that subject 6's congenital heart defects, low birth weight, and episode of acute dehydration most likely contributed to the severity of her symptoms. However, subjects 1 and 6 are both homozygous for the p.Gly212Val variant, but fibroblast complex I activity was normal in subject 1 and deficient in subject 6. Hence, the variation in outcomes also most likely relates to a combination of the severity of different mutations and as yet unknown genetic modifiers affecting the biochemical and clinical phenotypes. It is also possible that the alternative TMEM126B isoforms arising through alternative splicing of the *TMEM126B* mRNA transcripts might affect the clinical phenotype. The possibility of pathogenic variants within the *TMEM126B* paralog, *TMEM126A*, was excluded in subject 6 by analysis of the WES dataset; moreover, optic atrophy is a discriminatory feature in cases of *TMEM126A* pathology, and this individual has normal visual acuity.

Characterization of TMEM126B after proteomic screening and subsequent application of diagnostic MPS strategies has resulted in the diagnosis of six subjects from four families affected by *TMEM126B*-related mitochondrial disease. Our subjects suggest that a late-onset myopathic phenotype is the predominant clinical phenotype associated with *TMEM126B* defects. Functional experimentation including lentiviral rescue of subject fibroblasts establishes *TMEM126B* as the tenth complex I assembly factor associated with human disease, and this gene should be considered in the molecular genetic workup of subjects with biochemical evidence of an isolated complex I deficiency, particularly in European populations.

Accession Numbers

The accession numbers for variants c.401delA and c.635G>T are ClinVar: SCV000280578 and SCV000280579, respectively.

Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.05.021.

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Web Resources

Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Ensembl, http://www.ensembl.org/
ExAC Browser, http://exac.broadinstitute.org
I-TASSER, http://www.zhanglab.ccmb.med.umich.edu/I-TASSER
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org
Phyre2, http://www.sbg.bio.ic.ac.uk/phyre2/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RaptorX, http://raptorx.uchicago.edu
RefSeq, http://www.ncbi.nlm.nih.gov/refseq/
SHAPEIT2, https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html
SIFT, http://sift.jcvi.org/

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SHORT REPORT

A recurrent mitochondrial p.Trp22Arg *NDUFB3* variant causes a distinctive facial appearance, short stature and a mild biochemical and clinical phenotype

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ABSTRACT

Background Isolated Complex I deficiency is the most common paediatric mitochondrial disease presentation, associated with poor prognosis and high mortality. Complex I comprises 44 structural subunits with at least 10 ancillary proteins; mutations in 29 of these have so far been associated with mitochondrial disease but there are limited genotype-phenotype correlations to guide clinicians to the correct genetic diagnosis.

Methods Patients were analysed by whole-exome sequencing, targeted capture or candidate gene sequencing. Clinical phenotyping of affected individuals was performed.

Results We identified a cohort of 10 patients from 8 families (7 families are of unrelated Irish ancestry) all of whom have short stature (<9th centile) and similar facial features including a prominent forehead, smooth philtrum and deep-set eyes associated with a recurrent homozygous c.64T>C, p.Trp22Arg *NDUFB3* variant. Two sibs presented with primary short stature without obvious metabolic dysfunction. Analysis of skeletal muscle from three patients confirmed a defect in Complex I assembly.

Conclusions Our report highlights that the long-term prognosis related to the p.Trp22Arg *NDUFB3* mutation can be good, even for some patients presenting in acute metabolic crisis with evidence of an isolated Complex I deficiency in muscle. Recognition of the distinctive facial features—particularly when associated with markers of mitochondrial dysfunction and/or Irish ancestry—should suggest screening for the p.Trp22Arg *NDUFB3* mutation to establish a genetic diagnosis, circumventing the requirement of muscle biopsy to direct genetic investigations.

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INTRODUCTION

Mitochondrial respiratory chain disease is a significant cause of human disease with a population prevalence of approximately 1 in 5000 in adults and children.[1] Symptoms can manifest in the neonatal period but onset is often later in infancy, early childhood or even delayed to adulthood. Patients may present with disease affecting a single organ or have a multisystemic disorder typical of conditions such as Leigh syndrome. Approximately 70% of paediatric mitochondrial disease cases are caused by nuclear gene variants, while ~30% harbour defects involving mitochondrially encoded (mtDNA) genes.[2 3] Conversely, mtDNA mutations more often underlie adult mitochondrial disease presentations.[4] Beyond these prevalence statistics, the clinical and genetic heterogeneity results in a complex diagnostic pathway that usually relies on biochemical analysis of a muscle biopsy to direct genetic testing. Sanger sequencing of genes selected and prioritised according to clinical phenotype and biochemical results, as well as tissue biopsies, are being replaced by next-generation sequencing (NGS) strategies including candidate gene panels[5] and whole-exome sequencing.[6 7]

Investigation of isolated Complex I deficiency is particularly amenable to an NGS-based strategy given the number of genes implicated in its pathogenesis, with 44 structural subunits and at least 10 ancillary proteins required for enzyme assembly. It is the most common paediatric mitochondrial respiratory chain deficiency and mutations have been described in at least 29 genes to date,[8] almost all being associated with a poor clinical course and bleak prognosis.[8] Here we report the clinical and molecular genetic investigation of 10 patients from 8 unrelated families who all harbour an identical homozygous c.64T>C, p.Trp22Arg *NDUFB3* mutation, affecting a Complex I accessory subunit, previously reported in association with severe neurological presentations.[9 10] Most of our patients had considerably milder presentations despite harbouring the same variant. Recognition of mild dysmorphic facial features common to our initial patients prompted screening for the p.Trp22Arg *NDUFB3* variant in similar patients, leading to five further genetic diagnoses. This report demonstrates that the c.64T>C, p.Trp22Arg *NDUFB3* mutation can be associated with good long-term prognosis and that recognition of a cluster of physical

characteristics may enable rapid diagnosis of *NDUFB3*-related mitochondrial disease, circumventing invasive procedures or extensive genetic testing.

SUBJECTS AND METHODS

All patient samples were referred to the nationally commissioned 'Highly Specialised Mitochondrial Diagnostic Laboratory' in Newcastle upon Tyne for investigation of a putative mitochondrial defect. A clinical summary for each patient is given in table 1; detailed case reports are provided as online supplementary information. Informed parental consent was obtained.

Histochemical and biochemical analyses

Enzymatic activities of individual mitochondrial respiratory chain complexes were determined in patient muscle biopsies as previously described.[11]

Targeted next-generation sequencing

A custom 84.38 Kb Ampliseq panel was designed using the Ion Ampliseq Designer V.2.2.1 (http://www.ampliseq.com) to target 49 genes implicated in Complex I deficiency (see online supplementary table S1). To generate the barcoded Ampliseq target library using the Ion AmpliSeq Library Kit 2.0 and Ion Xpress Barcode Adapter 1–96 Kit, 40 ng patient DNA was used. Libraries were quantified using an Agilent 2100 Bioanalyser and pooled at 100 pM for emulsion PCR and enrichment using the Ion OneTouch2 and Enrichment system. Sequencing using the Ion PGM 200 Sequencing Kit was performed using 316 chips on an Ion PGM Sequencer, all according to the manufacturer's protocol. Torrent Suite V.4.2.1 was used to align reads against the human genome (hg19). The Variant Caller plugin was used to identify sequence variants that were annotated using wANNOVAR.[12]

Whole-exome sequencing

Targeted enrichment and sequencing was performed using 3 μg patient DNA. Enrichment was performed using the Illumina HiSeq Sure Select All Exon v5 Enrichment Kit, and sequencing was performed on an Illumina HiSeq 2500 sequencer, all as directed. Sequence data were mapped with BWA software to the human genome (hg19). Variants were called using GATK V.2.4.7 software and annotated using Ensembl V.72. Ensembl's 'defined consequence hierarchically' system retained the highest impacting gene variant. Filtering removed variants with ≤5× coverage, a minor allele frequency (MAF)>1%, those predicted to be non-functional, and those reported in dbSNP138 (unless seen in the Human Gene Mutation Database (HGMD)) or an in-house database (n=647 exomes).

Mutation screening, confirmation and carrier testing

The c.64T>C, p.Trp22Arg *NDUFB3* sequence variant was screened and confirmed using M13-tagged amplicons and Sanger sequencing with BigDye V.3.1 kit (Life Technologies). Capillary electrophoresis was performed using an ABI3130xl. Familial screening for the c.64T>C, p.Trp22Arg *NDUFB3* sequence variant was undertaken using parental and sibling DNA samples where available and appropriate.

Haplotype analysis

A putative founder effect was investigated by genotyping two proximal (D2S309 and D2S2214) and two distal (D2S116 and D2S2309), short tandem repeat (STR) markers flanking the *NDUFB3* gene. Corresponding PCR primers are listed on Ensembl. Mapping distance was calculated using MAP-O-MAT.[13]

Western blotting and blue native polyacrylamide gel electrophoresis

Mitochondrial fractions from control and patient muscle were prepared for western blotting and blue native polyacrylamide gel electrophoresis (BN-PAGE) as described previously.[14] Protein concentrations were determined with the Pierce bicinchoninic acid (BCA) Protein Assay Kit. Muscle protein extracts (100 μg) were loaded on Native PAGE 4–16% BisTris gels, electrophoretically separated in the first dimension before proteins were immobilised onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corporation) and subjected to standard immunoblotting analysis of oxidative phosphorylation (OXPHOS) complexes using primary and horseradish peroxidise conjugated secondary antibodies as described.[14] For western blotting, equal amounts of muscle protein (50 μg) were loaded on 12% gels and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by wet transfer to PVDF membrane and subsequent immunodetection.

RESULTS

Clinical findings

We describe five female and five male paediatric patients, each of whom are of short stature and share characteristic facial features. All weighed less than the 9th centile at birth, 8/10 were below the 2nd centile (80%). Clinical photography illustrates the prominent forehead, poorly defined philtrum and deep-set eyes (figure 1A, B). The majority of patients presented following a life-threatening metabolic crisis early in life followed by a period of sustained improvement. Subsequently, their clinical course has been largely benign but for occasional bouts of lactic acidosis associated with minor illnesses. A previous female sibling to patients 6 and 7 was born at term with growth restriction, became unwell and died on day 2 of life with profound lactic acidosis and multiorgan failure (no DNA was available for analysis). Patients 8 and 9 (siblings) presented to endocrinology for investigation of primary growth failure and were initially suspected to have 3M syndrome. Poor linear growth was seen in all patients, three patients have had growth hormone treatment with variable response.

Histochemical and biochemical analyses of mitochondrial respiratory chain enzymes

Where muscle biopsy had been performed, we identified an isolated Complex I deficiency (table 1). No muscle biopsy was available for patients 8 and 9 as a metabolic condition was not suspected.

Identification of a common underlying genetic defect

All patients in the cohort were found to harbour an identical homozygous c.64T>C, p.Trp22Arg *NDUFB3* sequence variant (table 1). Each of the three patients analysed by targeted NGS harboured between 54 and 57 genomic variants which were filtered to exclude those with a MAF >1% and variants outside the coding region ±10 bp of the intron/exon boundaries. For cases identified by whole exome sequencing (WES), from the 526 candidate variants compatible with autosomal recessive inheritance only a single, homozygous variant in *NDUFB3*, c.64T>C, p.Trp22Arg remained after filtering. All NGS-based strategies were confirmed by conventional Sanger sequencing.

The c.64T>C (chr2(hg38):g.201078946T>C) variant is referenced on dbSNP (rs142609245) and variant frequencies are recorded on ESP6500 (European: 14/8586 alleles (0.16%);

Table 1 Clinical and biochemical findings in the patient cohort

Patient (sex)	Ancestry	Clinical Presentation	Gestational age and birth weight (centile)	Age at latest review	Height at review (centile)	Lactate	Physical appearance			Residual CI activity*	Identified by
							Short stature	Prominent forehead	Long/thin philtrum		
1 (M)	English	RSV+ acute respiratory collapse and hypoglycaemia aged 8 weeks requiring intubation for 8 days. Pulmonary hypertension on echocardiogram. Maximum-recorded lactate 14 mmol/L. Discharged after 18 days. Normal cardiac function and morphology at 13 months.	Term <0.4th	9.5 years	<0.4th	+++	+	+	+	35%	Targeted NGS panel.
2 (F)	Irish	IUGR. Acute life-threatening event, age 20 days, required intubation. Hypertrophic cardiomyopathy.	30weeks 2nd	6 years	2nd	+	+	+	+	33%	Targeted NGS panel.
3 (F)	Irish	IUGR and oligohydramnios, FTT, mild hypertrophic cardiomyopathy.	34weeks 2nd–9th	3.5 years	0.4th–2nd	++	+	+	+	32%	Targeted NGS panel.
4 (F)	Irish	Growth restriction. Ketotic hypoglycaemia following vomiting illness. Short stature prompted endocrinology referral. Growth hormone therapy. MRI: high signal in periventricular white matter and dentate nuclei.	39weeks 0.4th–2nd	8 years	n.d.	++	+	+	+	24%	Mutation screen.
5 (M)	Irish	IUGR. Poor feeding. Congenital hypothyroidism (strong paternal family history). Developmental delay, growth failure, FTT, learning difficulties. Endocrinology review for short stature.	37weeks 0.4th–2nd	10 years	0.4th	+	+	+	+	35%	Mutation screen.
6 (F)	Irish	Oligohydramnios. IUGR. Poor feeding at birth. MRI brain and echocardiogram normal. Age-appropriate skills. Family history of previous neonatal death.	37weeks <0.4th	2.5 years	2nd–9th	+++	+	+	+	35%	Mutation screen.
7 (M)	Irish	Sib of P6. IUGR. Normal echocardiogram and cranial ultrasound. Normal development.	36weeks 2nd–9th	10 months	9th	++	+	+	+	n.d.	Mutation screen.
8 (M)	Irish	Initial poor feeding. Short stature prompted endocrinology review. Growth hormone therapy. MRI: high signal in globus pallidus. Echo: murmur. ECG: Wolff–Parkinson–White syndrome.	Term 0.4th–2nd	9.5 years	2nd	–	+	+	+	n.d.	Whole-exome sequencing; endocrinology.
9 (F)	Irish	Sib of P8. IUGR. Growth hormone therapy. Normal MRI brain, echocardiogram and ECG.	Term <0.4th	8 years	2nd	–	+	+	+	n.d.	Whole-exome sequencing; endocrinology.
10 (M)	Irish	IUGR, chronic lung disease, growth restriction and weight faltering. Dysmorphic with partial agenesis of corpus callosum. Acute collapse with rhinovirus bronchiolitis, severe pulmonary hypertension at 5.5 months. Elevated lactates with intercurrent illnesses.	31weeks <0.4th	11 months	<0.4th	+++	+	+	+	36%	Mutation screen.

*Residual Complex I activities, normalised to the activity of the matrix marker enzyme citrate synthase, are expressed as a percentage of mean control values.
FTT, failure to thrive; IUGR, intrauterine growth restriction; N.D., not determined; NGS, next-generation sequencing; RSV, respiratory syncytial virus.

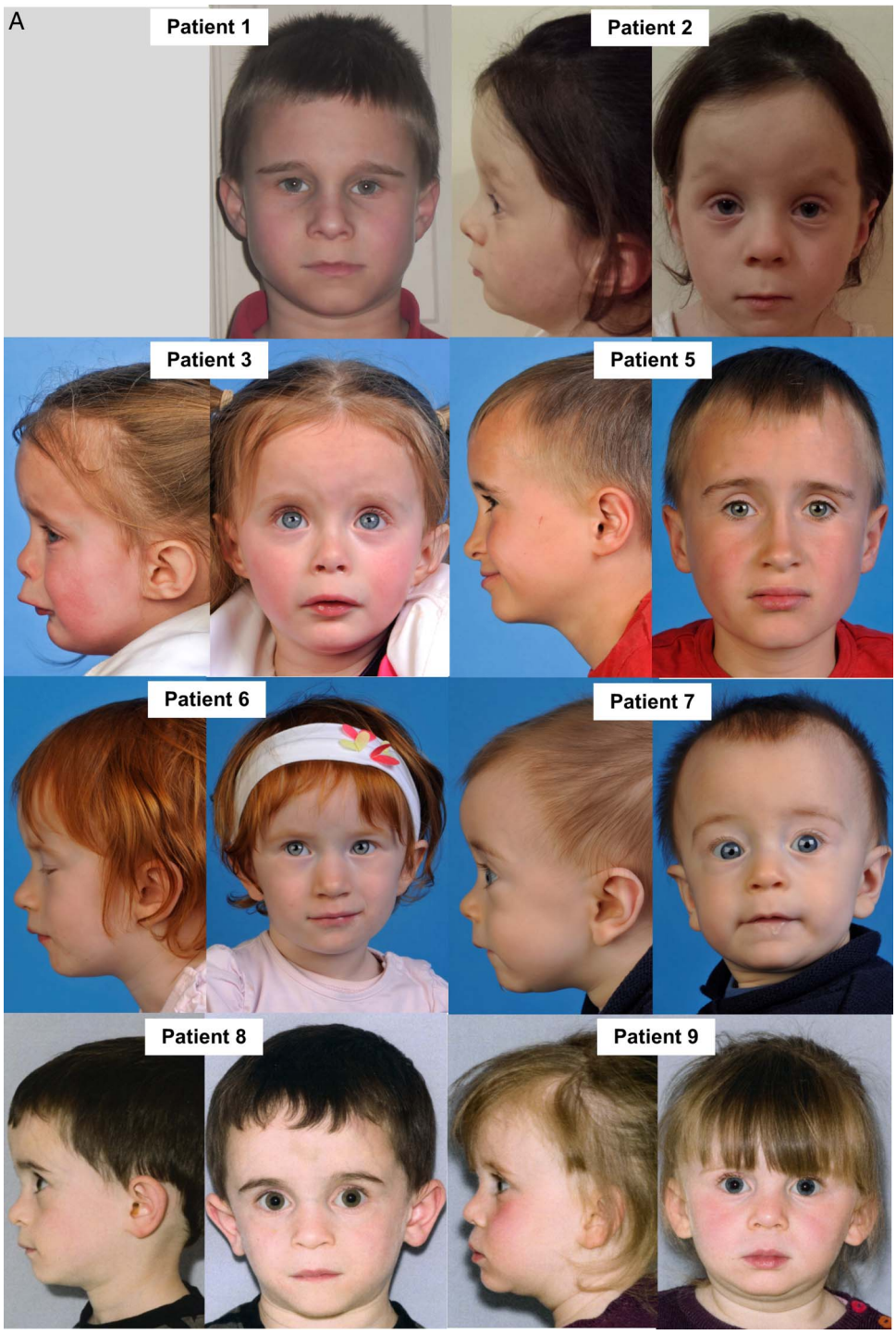

Figure 1 Clinical presentation associated with homozygous *NDUFB3* variant (A) Clinical photographs of eight patients harbouring a homozygous pathogenic c.64T>C, p.Trp22Arg *NDUFB3* variant. Patient 1 is of English descent, whereas the remaining cases are all of Irish heritage. Patients 6/7 and 8/9 are clinically affected sibling pairs. All have characteristic physical features including a prominent forehead, smooth philtrum, deep-set eyes and low-set ears. (B) Clinical photographs of patient 10, the youngest case within our cohort, illustrating the characteristic physical features associated with the p.Trp22Arg *NDUFB3* variant.

African-American: 2/4404 alleles (0.05%)) and ExAC (Non-Finnish Europeans: 69/66 604 alleles (0.1%); African: 2/10 390 alleles (0.02%); Latino: 1/11 568 alleles (0.01%); South Asian: 9/16 484 alleles (0.05%)). There are no homozygous cases recorded on either ESP6500[15] or ExAC[16] databases. Although the highest prevalence is recorded in European populations, the presence of the c.64T>C, p.Trp22Arg *NDUFB3* variant in non-European populations suggests other independent occurrences of this pathogenic mutation.

Carrier testing

With the exception of patients 4 and 5, where familial samples were unavailable, parental carrier testing confirmed recessive inheritance. Analysis of samples from the unaffected twin of

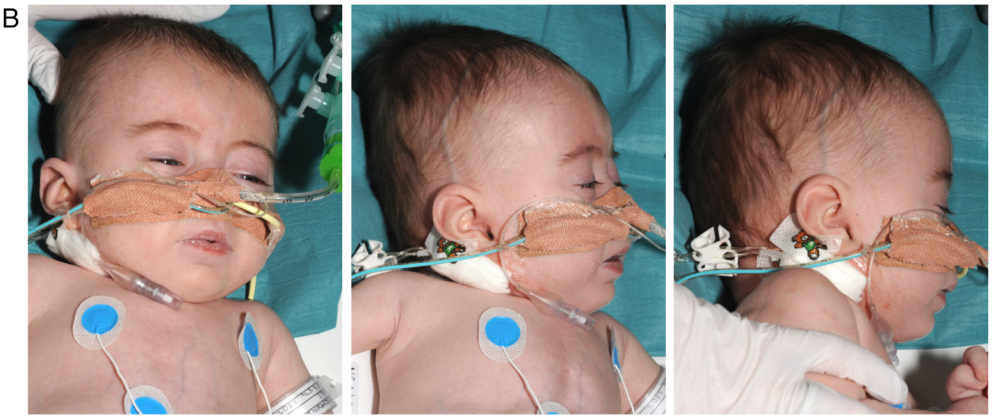

Figure 1 Continued

patient 1 and the three unaffected siblings of patients 8 and 9 confirmed the homozygous genotype segregates with a clinically affected status.

Haplotype analysis

Analysis of the *NDUFB3*-flanking STR markers across 0.5cM support multiple, independent occurrences of the c.64T>C, p.Trp22Arg variant (see online supplementary figure). Analysis of the markers most proximal to the *NDUFB3* gene (D2S309 and D2S2309), those most likely to be in linkage disequilibrium, shows three discrete haplotypes (1-1, 2-1 and 1-2). When including the distal STR markers in the analysis, this increases to seven haplotypes (*a-f*, plus #). There is one particularly prevalent haplotype ('*a*') in the patient cohort that is present in the heterozygous state in 8/10 cases and homozygous for 1/10 cases, supporting a founder allele. Additionally, the '*b*' and '*c*' haplotypes are present in two unrelated families. Haplotype analysis of the two previously reported cases shows the variants are also on the background of either the '*a*' or '*b*' haplotypes, suggesting a shared founder. We infer that the '#' haplotype corresponds to the allele harbouring the truncating *NDUFB3* mutation reported by Haack *et al*, as patient RC1 harboured a p.Trp22Arg variant in compound heterozygosity with p.Gly70*.

Steady-state levels of respiratory chain components and complexes

The p.Trp22Arg variant affects an evolutionary conserved amino acid residue (figure 2A). We investigated the steady-state protein levels of OXPHOS subunits in muscle available from three patients harbouring a homozygous p.Trp22Arg *NDUFB3* variant by SDS-PAGE and immunoblotting. The steady-state levels of Complex I subunit proteins NDUFB8 and NDUFA9 were decreased in all three patients while levels of protein components of Complexes II, III, IV and V were normal (figure 2B). Analysis of the assembly of OXPHOS complex subunits into mitochondrial respiratory chain complexes was undertaken by BN-PAGE, showing a decrease of fully assembled Complex I in P6, P2 and P3 muscle—correlating with the recorded biochemical defect—while the assembly profile of Complexes II, III, IV and V were all normal (figure 2C). Immunoblotting with NDUFB8 appeared to show partially assembled Complex I intermediates of ~650 kDa in patient muscle, consistent with other defects involving subcomplex Iβ of the hydrophobic membrane arm, of which NDUFB3 and NDUFB8 are both integral components.[17–19]

DISCUSSION

Mitochondrial disease presentations are frequently heterogeneous, with a paucity of genotype-phenotype correlations to direct molecular genetic testing even with a known biochemical diagnosis. We present a cohort of 10 patients from 8 non-consanguineous families who harbour a homozygous c.64T>C, p.Trp22Arg *NDUFB3* variant; together these patients represent a distinct clinical presentation. The majority of patients presented with intrauterine growth restriction (IUGR) and share characteristic facial features including a prominent forehead, smooth philtrum, deep-set eyes and low-set ears. All patients are short (height <9th centile) and while short stature is not uncommon in mitochondrial disorders, dysmorphic features are rare with the exception of *PUS1*[20] and *FBXL4*[21] mutations. *NDUFB3* encodes a structural Complex I subunit, and contrary to reported Complex I-deficient cases there were surprisingly few persistent features of mitochondrial disease; blood lactate levels were typically normal, although transient acidotic events were reported following illness leading, in some cases, to hospital admission before recovery. There were no seizures, ataxia or other neurological deficit noted; patients 2 and 3 had hypertrophic cardiomyopathy on echocardiography, but this resolved with time. All patients are reported to be well, with good levels of energy, attaining developmental milestones and making good progress at school (where appropriate). Patient 10 (<1 year of age) is much younger than the rest of our patient cohort, but is making excellent developmental progress (see online supplementary case reports).

With the exception of one patient (patient 1), all are reported to be of Irish ancestry. Interestingly, analysis of the *NDUFB3*-flanking STR markers supports multiple, independent occurrences of the c.64T>C, p.Trp22Arg variant, despite its prevalence in the Irish population. Across the 0.5cM region analysed, there are six different p.Trp22Arg alleles; given that this region is not a recognised recombination hot spot, it is likely that the mutation has arisen independently and recurrently although our data suggest a common founder for some cases and cannot fully exclude recombination as a contributory factor.

The c.64T>C, p.Trp22Arg *NDUFB3* variant is represented on the ExAC server (0/81/121214 (homozygous/heterozygous/alleles); MAF=6.6×10^{-4}) and has been reported in the literature twice previously, once in compound with a nonsense mutation and once as a homozygote; functional complementation experiments confirmed *NDUFB3* as the causative gene defect in both cases.[9 10] The homozygous case reported by Calvo *et al*[9] had IUGR (weight <3rd centile) and presented with hypotonia

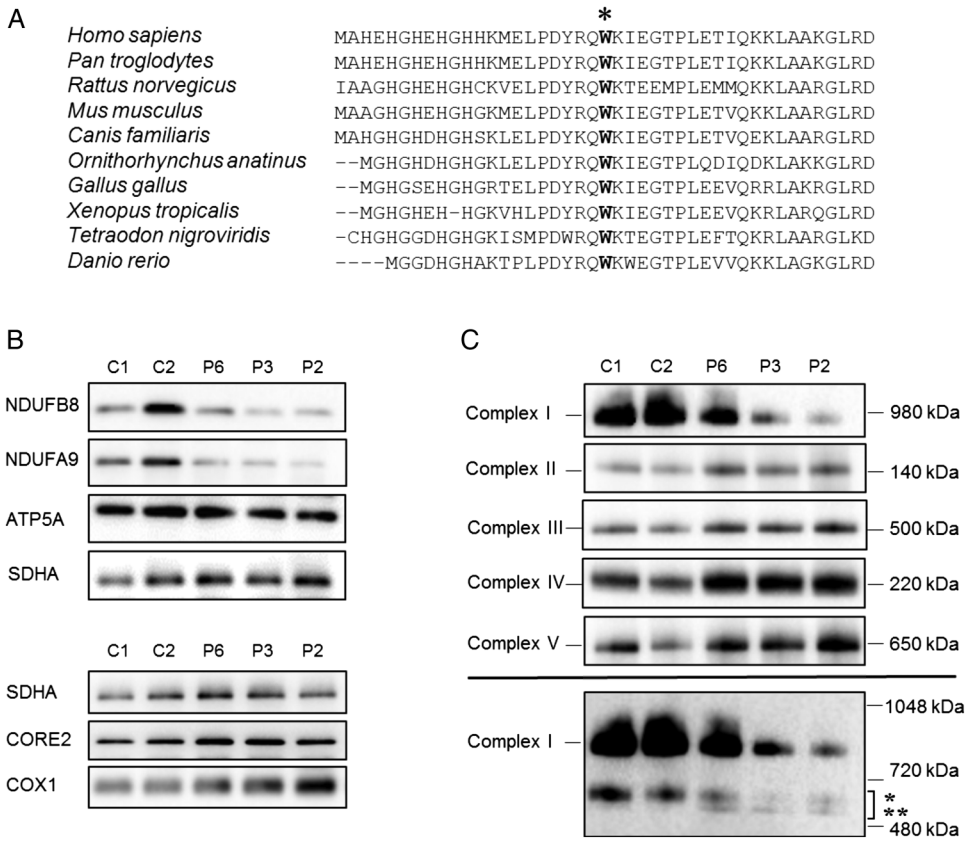

Figure 2 Analysis of OXPHOS complex assembly and protein expression levels (A) Clustal Omega sequence alignment shows the evolutionary conservation of the p.Trp22 residue (marked with asterix), based on the human sequence (amino acids 1–43). (B) Immunoblot analysis of steady state levels of OXPHOS subunits in mitochondrial lysates isolated from control (C1, C2) and patient skeletal muscle samples (P6, P3, P2). OXPHOS subunit-specific antibodies against the indicated proteins showed a marked decrease in Complex I subunits (NDUFB8 and NDUFA9) in patient samples compared with controls. (C) One-dimensional blue native polyacrylamide gel electrophoresis (PAGE) (4–16% gradient) analysis showing a defect in the assembly of Complex I in patients with the homozygous *NDUFB3* variant. Individual OXPHOS complexes were detected by immunoblotting using subunit-specific antibodies (Complex I (NDUFB8), Complex II (SDHA), Complex III (UQCRC2), Complex IV (COX1) and Complex V (ATP5A)). The assembly of Complexes II–V was normal in all three patient samples when compared with age-matched controls. The lower panel suggests a presence of additional, partially assembled Complex I intermediates in both control and patient samples; the upper band (indicated by *) is likely to represent the ~650 kDa Iβ subcomplex of the hydrophobic membrane arm while the lower band (indicated by **) represents partially assembled intermediates which are only visible in patient samples. These were detected by probing with an antibody raised against NDUFB8 and are in agreement with published studies.[17] In (B) and (C), SDHA (Complex II) was used as loading control.

and lactic acidosis, required ventilation and died at 4 months of age. The other reported case was born at 35 weeks gestation with low birth weight (3rd centile), with severe lactic acidosis and ketosis developing by day 2. Despite an initially severe presentation, her symptoms ameliorated and she is reported to remain of short stature but suffers illness-induced bouts of lactic acidosis. An older sibling of patients 6 and 7 in our series died on day 2 of life with profound lactic acidosis and multiorgan failure. No underlying cause was identified but a metabolic disorder was suspected, prompting early metabolic investigation of subsequent siblings.

Functional investigation of available patient muscle biopsy revealed a marked decrease in steady state levels of Complex I structural subunits, and although BN-PAGE analysis showed fully assembled Complex I to be diminished, some fully assembled Complex I remains. This is in stark contrast to many Complex I structural subunit defects, where levels of fully assembled Complex I are often entirely depleted,[22] and could provide an explanation for the milder clinical phenotypes observed in our patient cohort. Nuclear modifiers or mtDNA background could influence the prognosis but therapeutic intervention (see online supplementary case reports) and effective life support measures are most likely to contribute to the survival rate in our patients who presented in acute metabolic crisis. Evidence of mitochondrial proliferation was present in available muscle biopsy samples with elevated citrate synthase activity and ragged-red fibres indicating a cellular response to metabolic dysfunction. Our cohort and previously reported cases demonstrate this is a successful strategy for some but not all. Typically, paediatric mitochondrial disease patients progressively decline, with the exception of some patients with *TRMU* mutations or the m.14674T>C/G mt-tRNA^{Glu} 'reversible cytochrome *c* oxidase (COX) deficiency' variants. We show that the p.Trp22Arg *NDUFB3* mutation can also be associated with good long-term survival, even when some patients present in acute metabolic crisis with an isolated Complex I deficiency in muscle.

Another unique aspect of this case series involves patients 8 and 9; all other cases were referred by metabolic paediatricians but these children were diagnosed by paediatric endocrinologists investigating primary short stature. Blood lactates were normal for both children but patient 9 presented with Kussmaul-type respiration aged 2 years, which could be consistent with lactic acidosis. In light of the reported c.64T>C, p.Trp22Arg *NDUFB3* cases, cardiac

screening was performed, revealing Wolff–Parkinson–White (WPW) syndrome in patient 8, a rare cardiac conduction defect which is over-represented in patients with mitochondrial disease.[23] The initial manifestation of WPW syndrome can be sudden death and the diagnosis might facilitate interventions including non-invasive risk stratification and/or therapeutic ablation.[24]

Many cases of isolated Complex I deficiency associated with nuclear gene mutations are discrete entities and no common variant accounts for more than a few apparently unrelated cases.[25] We present 10 patients from 8 families who harbour the same homozygous *NDUFB3* variant and share a plethora of unifying physical features, an unprecedented finding in association with isolated Complex I deficiency. Recognition of the distinctive facial features in combination with short stature should suggest screening for the c.64T>C, p.Trp22Arg *NDUFB3* mutation, even in the absence of 'classic' metabolic symptoms, and particularly when Irish ancestry is involved.

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Contributors CLA, RM, MO and RWT contributed to the project design, analysis of the data and/or the drafting of the manuscript. CH, PGM, SO'S, GD, JPHS, AAM, IK, PM, AAMM, DRT, HP, PEC, JH and EC recruited patients and family members and phenotypically characterised the families. CLA, MO, SAH, LH and IPH performed the biochemical and molecular genetic studies. All authors critically revised the manuscript text. RWT supervised the study.

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REPORT

Sudden Cardiac Death Due to Deficiency of the Mitochondrial Inorganic Pyrophosphatase PPA2

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We have used whole-exome sequencing in ten individuals from four unrelated pedigrees to identify biallelic missense mutations in the nuclear-encoded mitochondrial inorganic pyrophosphatase (*PPA2*) that are associated with mitochondrial disease. These individuals show a range of severity, indicating that *PPA2* mutations may cause a spectrum of mitochondrial disease phenotypes. Severe symptoms include seizures, lactic acidosis, cardiac arrhythmia, and death within days of birth. In the index family, presentation was milder and manifested as cardiac fibrosis and an exquisite sensitivity to alcohol, leading to sudden arrhythmic cardiac death in the second decade of life. Comparison of normal and mutant PPA2-containing mitochondria from fibroblasts showed that the activity of inorganic pyrophosphatase was significantly reduced in affected individuals. Recombinant PPA2 enzymes modeling hypomorphic missense mutations had decreased activity that correlated with disease severity. These findings confirm the pathogenicity of *PPA2* mutations and suggest that PPA2 is a cardiomyopathy-associated protein, which has a greater physiological importance in mitochondrial function than previously recognized.

Inorganic pyrophosphate (PPi, also termed diphosphate) is formed by several important nucleotide triphosphate-dependent reactions necessary for DNA, RNA, protein, and lipid synthesis. Pyrophosphate has to be hydrolyzed to orthophosphate (Pi). An enzyme that catalyzes this reaction is termed inorganic pyrophosphatase (PPA [Enzyme Commission number EC 3.6.1.1]) and provides Pi for biomolecules via synthesis of ATP, the terminal product of cellular energy metabolism. PPAs are found in all kingdoms of life. Type I enzymes present in *Escherichia coli* and eukaryotes depend on divalent ions, preferably Mg^{2+} ions.[1] Humans, similar to the yeast *Saccharomyces cerevisiae*, have two PPAs that share sequence homology. These comprise a cytoplasmic soluble PPA1 and a mitochondrial-located PPA2. For the latter it has been proposed that the soluble catalytic part binds to a yet uncharacterized inner mitochondrial membrane protein.[2] Knockout of the cytoplasmic *PPA1* (MIM: 179030) ortholog *IPP1* leads to a loss of viability in yeast.[3,4] Knockout of the mitochondrial *PPA2* results in a growth defect on non-fermentable carbon sources and loss of mitochondrial DNA in *S. cerevisiae*.[3]

We have identified hypomorphic missense mutations in the human gene of the mitochondrial inorganic pyrophosphatase encoded by *PPA2* (MIM: 609988) in a multicenter study by exploring undiagnosed cases with presumed mitochondrial disease using whole-exome sequencing (WES). In agreement with the Declaration of Helsinki, informed consent for genetic and biochemical studies was obtained from all study participants or their guardians.

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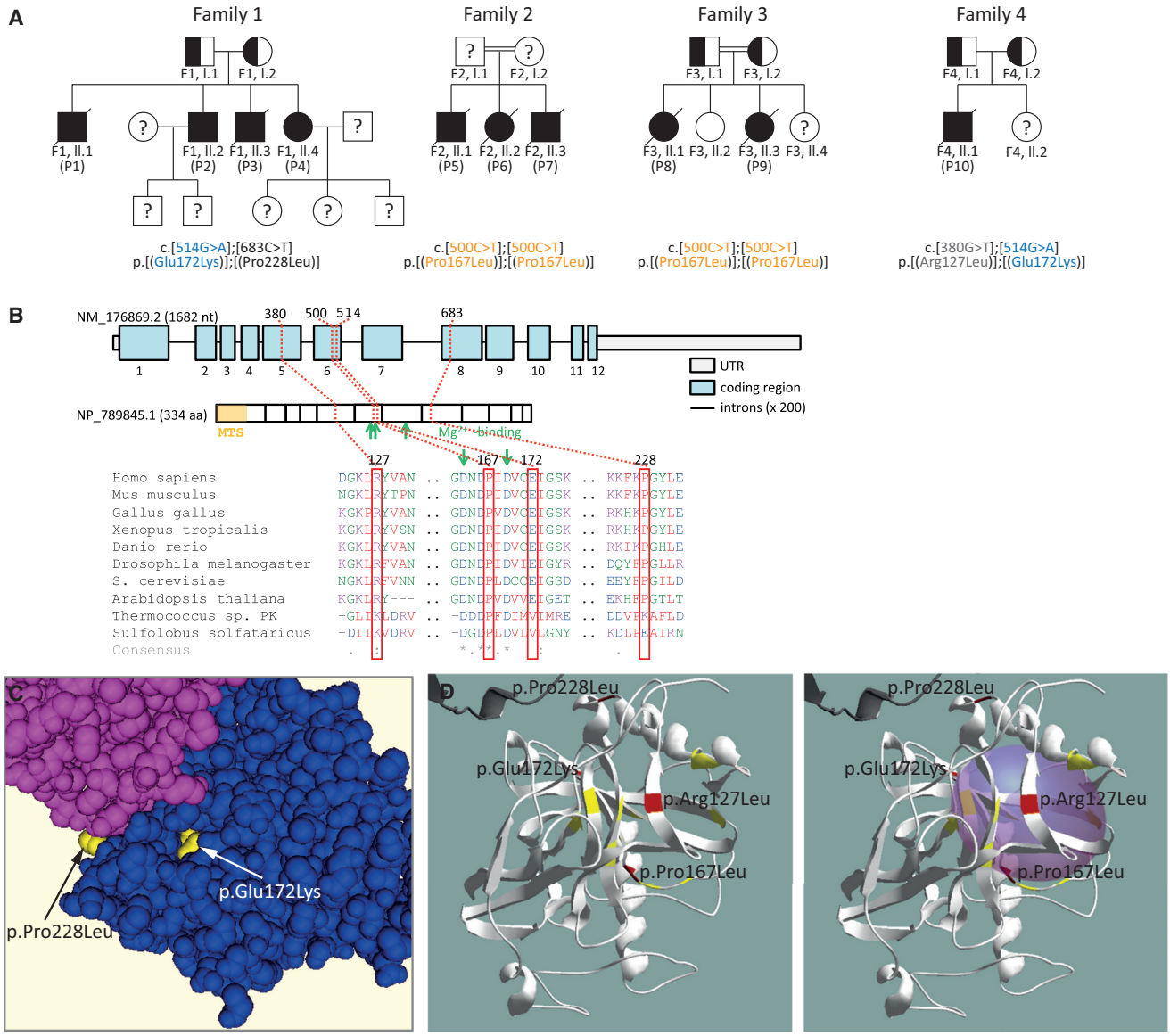

Figure 1. Pedigree Structure, *PPA2* Genomic Organization Conservation, and Family 1 Variant Modeling
(A) Pedigrees of four families identified with mutations in *PPA2* (GenBank: NM_176869.2) encoding the mitochondrial inorganic pyrophosphatase. Individuals with a question mark have not been tested. Mutations found in more than one family are colored.
(B) Location of mutations within the gene, and phylogenetic conservation of the predicted missense mutations.
(C) Space fill model showing position of p.Pro228 at boundary of dimers and p.Glu172 in the active site produced in CN3D with reference PDB: 1M38.
(D) Left: Structural model of one molecule of PPA2 showing the position of four mutations in folded structure (red). Residues that are known to be critical to PPA2 function in *S. cerevisiae* are highlighted in yellow.[19] Right: Space fill of the PPA2 active site showing three substitutions are located at the surface of the active site. Models produced using Swiss-PdbViewer[20] (with reference PDB: 1M38).

The study was approved by the Ethics Committee of the Technische Universität München and South Yorkshire Research Ethics Committee.

Family 1 consists of four affected individuals (P1–P4) born to healthy unrelated parents of European descent from New Zealand (Figure 1). This family was identified after the sudden death of two children. The first child, P1, was well until the age of 15 years when he collapsed and died after ingestion of a small volume of beer. He had no prior cardiac symptoms, but had exhibited exquisite sensitivity to alcohol in medicine and food, which was common to all four siblings in childhood. This was manifest by pallor and severe chest and arm pain after consumption of small amounts of alcohol (<0.1 g ethanol). The only abnormalities observed post mortem were in the heart, with both ventricles found to be slightly dilated. A diagnosis of myocarditis and sudden arrhythmic cardiac death was made. Individual P3 died suddenly at 20 years of age after ingestion of a small amount of alcohol (approx. 10 g ethanol). He was previously well and had no prior cardiac symptoms, but had also been exquisitely sensitive to alcohol. At post mortem

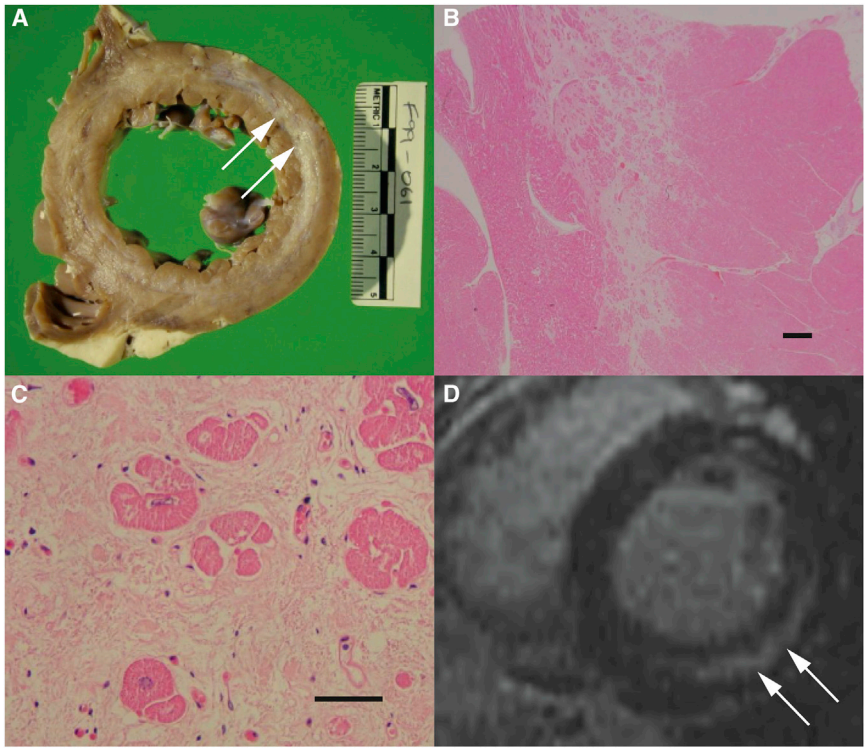

Figure 2. Cardiac Fibrosis in PPA2 Deficiency
(A) Affected individual P3, post mortem section through left ventricle showing a virtually circumferential lamina of scarring in midmyocardium with focal subendocardial involvement. Fibrosis is marked by arrows.
(B and C) Low-power (B) (bar equals 1 mm) and high-power (C) (bar equals 25 μm) microscopy of the posterior freewall of the left ventricle showing prominent midmyocardial loose fibrosis in P3 (hematoxylin and eosin staining).
(D) Cardiac MRI showing prominent midmyocardial fibrosis in affected individual P4 (at 25 years of age), marked by arrows.

examination the heart weighed 395 g (normal 300 g). The left ventricle was dilated with a virtually circumferential lamina of scarring in mid-myocardium. Microscopic examination revealed very widespread, mostly mature scarring of mid-myocardium in all sectors (Figure 2). Two living siblings, P2 and P4 (currently 38 and 34 years of age, respectively) were assessed based on their family history and their sensitivity to alcohol. Cardiac MRI showed marked mid-myocardial fibrosis in both siblings (P4 shown in Figure 2). They subsequently each received an implantable defibrillator for primary prophylaxis of sudden arrhythmic cardiac death, although no events have occurred to date. Despite extensive investigations into the cause of sudden death in this family over a period of >20 years, no definitive diagnosis was made (for more clinical details see Supplemental Data).

Whole-exome sequencing (see Table S1 for details) was performed on the two living siblings to elucidate the underlying molecular defect. Given that both parents appeared unaffected, we searched for rare non-synonymous variants common to the two affected children in a recessive disease model of inheritance. Four candidate genes were identified with compounding missense mutations and with an association to cardiomyopathy and/or mitochondrial function: *KCNJ12* (MIM: 602323), *TTN* (MIM: 188840), *AARS2* (MIM: 612035), and *PPA2*. Of these four genes, variants in all but *PPA2* were excluded based on non-segregation with disease (Table S2). Both affected children were compound heterozygous for *PPA2* mutations c.[514G>A];[683C>T] causing the predicted coding changes p.[Glu172Lys];[Pro228Leu], with each parent carrying one mutation. Sanger sequencing confirmed compound heterozygosity of *PPA2* mutations in the two deceased individuals, establishing the same genotype for all four affected individuals (Figure S1).

We considered that PPA2 dysfunction was the likely underlying cause of sudden cardiac death in our index family. We identified an additional three families with a further six affected individuals harboring compound heterozygous or homozygous *PPA2* mutations (Figure 1) in large exome datasets from individuals suspected of a disorder in mitochondrial energy metabolism. Family 2 (c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]) (GenBank: NM_176869.2) comprises three affected siblings born to consanguineous parents from Sri Lanka. Family 3 (c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]) consists of two affected and two healthy siblings born to consanguineous parents of Pakistani origin. Family 4 (c.[380G>T];[514G>A], p.[Arg127Leu];[Glu172Lys]) has one affected and one healthy sibling born to unrelated healthy parents from the United Kingdom.

Unlike the individuals from family 1, all the affected individuals in these three families presented with classical mitochondrial disease symptoms and died within the first 2 years of life of cardiac failure (Table 1). The identification of these individuals suggests that a spectrum of severity is conferred by different biallelic *PPA2* mutations. In affected individuals homozygous for c.500C>T (p.Pro167Leu), the clinical presentation involved lactic acidosis, seizures, hypotonia, and cardiac arrhythmia within the first months of life. Myocyte loss, disarray, or fibrosis was present in all individuals. Respiratory chain function varied from normal to moderate reduction in complex I and IV activities in cardiac tissue and was normal in fibroblasts and skeletal muscle tissue. The individual harboring compound heterozygous c.[380G>T];[514G>A], p.[Arg127Leu];[Glu172Lys] mutations first presented with short seizures at 10 months and developed dilated cardiomyopathy and multiorgan

Table 1. Genetic and Clinical Findings in Individuals with *PPA2* Variants

ID	Sex (M/F)	*PPA2* Variants: cDNA (NM_176869.2), Protein (NP_789845.1)	OXPHOS Activities (Normal Ranges in Brackets)	Clinical Features			
				AO	Age at Death	Cardiac Phenotype	Other Findings
F1, II:1, P1	M	c.[514G>A];[683C>T], p.[Glu172Lys];[Pro228Leu]	ND	4 years	15 years	autopsy: slight dilation of both ventricles; small pale area in the epicardium of the left ventricle, evidence of focal inflammation with neutrophils, lymphocytes, and eosinophils	sensitive to small amounts of alcohol
F1, II:2, P2	M	c.[514G>A];[683C>T], p.[Glu172Lys];[Pro228Leu]	ND	14 years	alive at 38 years	cardiac MRI: myocardial fibrosis; received implantable defibrillator	sensitive to small amounts of alcohol
F1, II:3, P3	M	c.[514G>A];[683C>T], p.[Glu172Lys];[Pro228Leu]	ND	10 years	20 years	autopsy: dilation of the left ventricle, circumferential lamina of scarring in midmyocardium with focal subendocardial involvement; very widespread mostly mature scarring of midmyocardium in all sectors	sensitive to small amounts of alcohol
F1, II:4, P4	F	c.[514G>A];[683C>T], p.[Glu172Lys];[Pro228Leu]	normal in skeletal muscle	9 years	alive at 33 years	cardiac MRI: myocardial fibrosis; received implantable defibrillator.	sensitive to small amounts of alcohol; immunohistochemical studies of skeletal muscle showed changes suggestive of a mild chronic myopathy
F2, II:, P5	M	c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]	ND	10 days	11 days	autopsy: herds of fresh necrosis mainly of the right heart and interstitial lymphocyte infiltration; electron microscopy: myocard showed mitochondria with degeneration of cristae	elevated plasma lactate levels; tachypnoea and tachycardia; tonic-clonic seizures; death after severe bradycardia
F2, II:2, P6	F	c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]	normal in skeletal muscle and fibroblasts	14 days	14 days	autopsy: acute and subacute necrosis more pronounced in the right heart more severe than in the left heart; electron microscopy: myocardium showed mitochondria with degeneration of cristae like in P5	metabolic acidosis with elevated plasma lactate levels; tachypneoa; vomiting, generalized seizure; cardio-respiratory decompensation; death 6.5 hr after onset of symptoms; multiple subacute necroses in the semioval center of both cerebral hemispheres
F2, II:3, P7	M	c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]	normal in skeletal muscle and fibroblasts; heart muscle: LV: CI 4.1 (5.5–51.5) and CIV 64 (73.2–516.6) decreased, RV: CI not detectable, CII 9.0 (25.8–40.7), CIV 42 (73.2-516.6)	3 days	32 days	cardiac tachyarrhythmia; ECG showed hypodynamic right ventricle; autopsy: myocardium without necrosis and inflammatory infiltrations; myocytes with reduced amount of myofibrils; region of fibrosis, partially fat tissue in the right heart	elevated plasma lactate, transaminases, lactate dehydrogenase, creatine kinase (CK), CK-MB, and troponin levels

(*Continued on next page*)

Table 1. *Continued*

ID	Sex (M/F)	*PPA2* Variants: cDNA (NM_176869.2), Protein (NP_789845.1)	OXPHOS Activities (Normal Ranges in Brackets)	Clinical Features		Cardiac Phenotype	Other Findings
				AO	Age at Death		
F3, II:1, P8	F	c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]	ND	5.5 months	5.5 months	autopsy: evidence for long-standing myocyte loss, increased interstitial myocyte loss, increased interstitial collagen, focal myocyte fiber disarray in the left ventricle and interventricular septum	24 hr history of vomiting and diarrhea, 1× seizures; multiple cardiac arrests; hypoxic injury of the brain; the liver showed mild fatty change
F3, II:3, P9	F	c.[500C>T];[500C>T], p.[Pro167Leu];[Pro167Leu]	normal in skeletal muscle	8 months	11 months	autopsy: extensive fibrosis of the heart muscle	plasma lactate elevated, diarrhea, vomiting; focal seizure then generalized seizure; cardiac arrest
F4, II:1, P10	M	c.[380G>T];[514G>A], p.[Arg127Leu];[Glu172Lys]	normal in skeletal muscle; CI decreased heart muscle 0.026 (0.125 ± 0.048)	10 months	2 years	echocardiography: ejection fraction of 74%, mild left ventricular hypertrophy; autopsy: extensive transmural fibrosis of the left ventricle, acute myocardial ischemia	seizures, urinary organic acids: increased 3-hydroxybutyrate, acetoacetate, and C14:1, C14, C16:1 acylcarnitine elevation in blood.

Abbreviations are as follows: AO, age at onset; F, female; M, male; ND, not determined.

failure at 1 year, necessitating intensive care for several weeks. All affected individuals died from cardiac failure after sudden deterioration. Interestingly, both individuals from family 3 and the affected individual from family 4 had viral infections at the time of hospital admission before their final heart failure (Figure S2). In the older siblings from family 2, vomiting (among other symptoms of metabolic decompensation prior to admission) was reported, one of them having loose stools once, but viral infection was not confirmed (Table 1). Clinical case histories of all affected individuals are provided in the Supplemental Data.

Western blotting showed normal amounts of PPA2 protein in fibroblast mitochondria from individuals P5, P6, and P7 but decreased amount in P9 (Figure S3). In autopsy muscle of P9, the amount of PPA2 protein was decreased, although it appeared to be normal in P6, who carried the same *PPA2* mutation (Figure S4). In heart autopsy material from P10, we noted decreased PPA2 levels as well as decreased levels of a complex I structural protein (subunit NDUFS4), correlating with the observed decrease in complex I activity in this tissue (Figure S5). In the cardiac autopsy sample of P7, PPA2 and complex I subunit levels were decreased as was the expression of the mitochondrial marker proteins porin and citrate synthase, suggestive of a more general reduction of mitochondrial number possibly due to changes in tissue composition (Figure S5).

All four missense variants involve residues of high evolutionary conservation (Figure 1) and are predicted to have a pathogenic effect on PPA2 function in silico (SIFT, PolyPhen-2, and MutationTaster) (Table S3). The high homology between the human and yeast (*S. cerevisiae*) PPA proteins facilitated predictive modeling of these human variants based on the known yeast structure of the cytosolic/nuclear pyrophosphatase IPP1 (MMDB ID: 21720; PDB: 1M38) (Figure 1). Glutamine to lysine substitution at residue 172 is predicted to disrupt at least three hydrogen bonds between interacting protein chains near the surface of the enzyme's active site. Any disruption of the active site may impair enzymatic function of PPA2. A substitution of proline to leucine at residue 228, located on the outside surface where dimer association occurs, is also predicted to disrupt the secondary structure of PPA2. Proline is a known peptide turning point amino acid and is likely to affect the orientation of the two helices between which it lies in this enzyme. We suggest that disruption of the conformation of the outer surface may impair correct dimerization of PPA2 molecules.

All four *PPA2* mutations identified in our cohort are present in the Exome Aggregation Consortium (ExAC) database (12/2015) at a frequency < 0.005, equating to 59/60,400 individuals heterozygous for p.Glu172Lys, 30/60,134 individuals heterozygous for p.Pro228Leu, 20/60,677 individuals heterozygous for p.Arg127Leu, and 3/60,457 individuals heterozygous for p.Pro167Leu (Table S1). None of these *PPA2* variants is reported in a homozygous state in ExAC, the NHLBI Exome Sequencing Project

(ESP6500) database, or 7,000 control exomes of an in-house database (Munich). Due to the complete growth defect of yeast PPA2 knockouts on non-fermentable carbon sources, it can be speculated that biallelic loss-of-function mutations of *PPA2* are incompatible with life in humans. In total, 13 LOF variants (found in 18 alleles) are published in the ExAC database and furthermore ExAC contains 71 different missense mutations (in 237 alleles) with a SIFT score ≤ 0.05 (cut-off for mutations to be considered pathogenically relevant). The cumulative heterozygous carrier frequency of these likely pathogenic *PPA2* mutations is 0.0024, which would result in a calculated prevalence for compound heterozygous or homozygous pathogenic *PPA2* mutations of 0.58 per 100,000 (1 in 170,000).

In order to investigate effects of PPA2 deficiency on the cellular metabolism, we measured oxygen consumption rate (OCR) by micro scale respirometry (XF96 Seahorse Biosciences).[5] Basal respiration and oligomycin-inhibited OCR was similar in affected individuals (P5, P6, and P7) but after the addition of the mitochondrial uncoupler FCCP, a higher activity was observed in affected individuals compared to control subjects. The difference between basal and FCCP-stimulated OCR, termed reserve respiratory capacity (RRC), was twice as high in PPA2-deficient fibroblasts compared to controls (Figures S6A and S6B). High RRC observed in PPA2-deficient cells might be due to a limitation in ATP synthesis because of insufficient Pi supply within mitochondria (Figure S7). Actually, the investigation of cells with proven ATP synthase deficiency due to mutations in either *TMEM70*[6] (MIM: 612418) or *ATP5E*[7] (MIM: 606153) revealed a similar OCR-profile with high RRC. Because high RRC is not a specific finding, further investigations were required to demonstrate decreased ATP synthesis in PPA2 deficiency.

We next determined pyrophosphatase activity in isolated mitochondria from fibroblasts of control and affected individuals, which were prepared from 540 ccm of confluent primary fibroblasts. After harvesting by trypsinization and washing twice with phosphate-buffered saline cells, the weight of the cell pellet was determined. Cells were suspended in the 10-fold amount (e.g., 500 μL per 50 mg of cell pellet) of ice-cold, hypotonic homogenization buffer (10 mmol/L Tris [pH 7.4]) and homogenized by the use of a tight fitting Potter Elvehjem homogenizer. Immediately after homogenization, 1.5 mmol/L sucrose (20% of the homogenization volume) was added to preserve mitochondria. After centrifugation at 600 × *g*, the mitochondria containing supernatant was centrifuged at 10,000 × *g* and the mitochondria-containing pellet was washed twice with SEKT buffer (250 mmol/L sucrose, 2 mmol/L EGTA, 40 mmol/L KCl, 20 mmol/L KCl [pH 7.4]). The mitochondrial pellet was finally suspended in the equal amount of SEKT buffer (50 μL per 50 mg cell pellet) and stored at −80°C prior to further investigations.[7,8] The hydrolysis of PPi and quantification of orthophosphate (Pi) formed was determined according to previously published methods[9,10] with minor modifications. The incubation buffer contained 50 mmol/L Tris ([pH 8.0] 0.1 mmol/L EGTA) and the indicated concentrations of $MgCl_2$ and PPi. The reaction was started by the addition of enzyme in a final volume of 100 μL, incubated at 37°C, and stopped by the addition of 100 μL reagent A (0.70% (w/v) ammonium heptamolybdate tetrahydrate, 1.26 mol/L H_2SO_4), developed by the addition of 40 μL reagent B (0.35% (w/v) polyvinylalcohol, 0.035% (w/v) malachite green oxalate at room temperature for 20 min. The activity of PPA was significantly decreased in isolated fibroblast mitochondria from affected individuals P5, P7, and P9 at each PPi (0.001–0.1 μmol/L) and $MgCl_2$ (0.5 or 3.0 mmol/L) concentration investigated (Figures 3A and 3B). Inactivation by $CaCl_2$ was similar in affected individuals compared to control subjects (Figure 3C). Fibroblasts from affected individuals P6 and P10 did not grow sufficiently to collect enough cells for the isolation of mitochondria and from individuals P1–P4 and P8 no fibroblasts were available.

For the expression of recombinant human PPA2, wild-type *PPA2* cDNA was cloned into the expression vector pRSET B (Invitrogen) using the cloning sites BamHI and BglII.[11] The first 96 nucleotides corresponding to the cleavable N-terminal mitochondrial targeting sequence were omitted from the construct. The c.500C>T (p.Pro167Leu), c.514G>A (p.Glu172Lys), and c.683C>T (p.Pro228Leu) variants were introduced into the wild-type *PPA2* sequence by site-directed mutagenesis using Gibson assembly (New England Biolabs) with appropriate primers for PCR amplification (Phusion, New England Biolabs) and the correct coding regions of all four constructs was confirmed by Sanger sequencing. Recombinant protein was expressed in the *Escherichia coli* strain BL21(DE3)pLysS at 37°C starting at OD_{600} of 0.2 and using 1 mmol/L IPTG for 2 hr. The bacterial suspension was harvested and sonified in homogenization buffer and the supernatant was bound to HisPur cobalt spin columns (Thermo).[11] The amount of the recombinant proteins was determined by western blotting with a human PPA2 antibody (Abcam cat# ab177935). Equal amounts of either wild-type or mutant recombinant PPA2 proteins were used for the pyrophosphatase activity assay. Compared to wild-type, the p.Pro167Leu and p.Glu172Lys variants showed 5%–10% residual activity at PPi substrate concentrations 18–500 μmol/L. The p.Pro228Leu variant had a residual activity of 24%–28% in this concentration range compared to wild-type (Figure 3D). The activities of wild-type and mutants were similarly sensitive to inhibition by Ca^{2+} (data not shown).

As previously reported, *PPA2* knockout strain from *S. cereviseae* is unable to grow on aerobic media.[3] We also detected a growth defect of *PPA2* knockout yeast on diamide-containing media, which lowers antioxidant concentrations (Figure S8).[12] These antioxidants protect the cell against reactive oxygen species that are also natural by-products of mitochondrial respiratory chain function. The increased diamide sensitivity of PPA2-deficient yeast therefore suggests reduced levels of antioxidants.

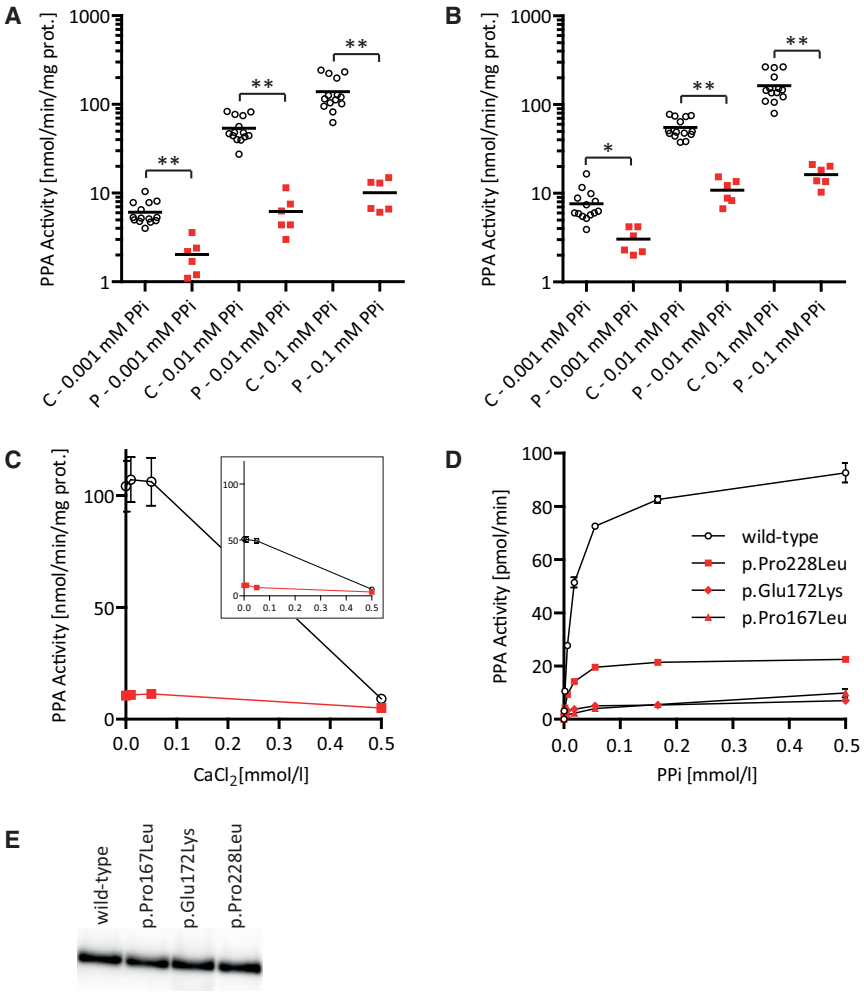

Figure 3. Inorganic Pyrophosphatase Activity in Fibroblast Mitochondria and Recombinant Enzymes

(A and B) Activity of inorganic pyrophosphatase in different fibroblast mitochondria isolations from affected individuals (P) P5, P7, and P9 compared to 14 control subjects (C) at different PPi concentrations and either (A) 0.5 mmol/L $MgCl_2$ or (B) 3.0 mmol/L $MgCl_2$.

(C) Inhibition of inorganic pyrophosphatase in fibroblast mitochondria from affected individual P5 (red squares) and three control subjects (black circles) incubated at 0.5 mmol/L $MgCl_2$ and different $CaCl_2$ concentrations and either 0.1 mmol/L PPi or 0.01 mmol/L PPi (small insert).

(D) Pyrophosphatase activity of equal amounts of recombinant proteins at different PPi concentrations.

(E) Protein amount of recombinant PPA2 protein was adjusted by western blot analysis and silver staining (Figure S9).

*$p < 0.01$, **$p < 0.0001$ in Student's unpaired t test. The error bars in this graph indicate the standard error of the mean.

In the case of family 1, our data suggest that p.Pro228Leu is a relatively mild variant, given that PPA2 function is only moderately reduced. This hypothesis is supported by investigation of the activity of recombinant PPA2 enzyme activity. The p.Pro228Leu substitution resulted in a reduction of PPA activity to approximately 25% of wild-type (Figure 3). These individuals show chronic accumulation of cardiac fibrosis, and death occurred after ingestion of alcohol to which they were already known to have acute sensitivity. We propose that alcohol acted as a trigger in these case subjects, whose PPA2 dysfunction created chronic mitochondrial sensitivity and whose hearts were consistently deprived of adequate ATP resulting in fibrosis. Ingestion of alcohol appears to have increased the stress on the already sensitive mitochondria/fibrotic heart, causing cardiac arrhythmia and death. There is a link between alcohol metabolism and inorganic pyrophosphatase function that might underlie the pathology of affected individuals. Ethanol is oxidized to acetaldehyde and further to acetic acid.[13] Resulting acetic acid has to be activated to acetyl-coenzyme A, which is accompanied by the formation of equimolar amounts of PPi (Figure S7). This esterification reaction is catalyzed by short-chain acyl-CoA synthetases encoded, for example, by *ACSS1* (MIM: 614355), an enzyme with high expression in heart mitochondria.[14] In cases of severe PPA2 dysfunction, ATP depletion has an acute effect and lactic acidosis and cardiomyopathy occurs prior to chronic damage developing, which could lead to acute symptoms in the presence of secondary triggers. It is interesting to note, however, that both affected individuals in family 3 had a history of vomiting, diarrhea, and seizures prior to admission to hospital, and viral infection (rotavirus [1st] and norovirus [2nd]) was confirmed in stool samples taken at time of admission. A norovirus infection was also found in P10 from the United Kingdom. This may indicate that a viral stressor was responsible for adversely affecting mitochondrial metabolism in families 3 and 4, in the same way that alcohol was a trigger for arrhythmia in the index family. In the oldest sibling of family 2, there was also some vomiting prior to hospital admission but viral illness was not confirmed. In the two younger siblings, vomiting occurred among other initial symptoms of metabolic compensation, in the youngest sibling who was hospitalized from birth already on the third day of life. Of note, symptoms like vomiting,[15] diarrhea,[16] and seizures[17] are also typical for other disorders of the mitochondrial energy metabolism.

All affected individuals died from cardiac failure. Sudden, unexpected cardiac death was especially observed in P1, P3, P5, P6, and P8. As clearly seen in cardiac MRIs from the two living individuals from family 1, midmyocardial fibrosis is a pre-existing condition (Figure 2) even though no cardiac symptoms were experienced by these

individuals. Using late gadolinium enhancement (LGE), myocardial fibrosis can be clearly determined and is also a common finding in other disorders of the mitochondrial energy metabolism such as MELAS (MIM: 540000) due to the common m.3243A>G mutation of the mitochondrial DNA.[18]

In conclusion, we have identified biallelic missense mutations in *PPA2* as cause of mitochondrial cardiomyopathy and sudden cardiac death. This finding highlights a critical role of PPA2 in mitochondrial function and warrants further functional investigation. Importantly, mild mutations in *PPA2* may not have an immediate life-threatening effect until triggered by a stressor such as viral illness or alcohol metabolism, predisposing otherwise healthy individuals to sudden cardiac death. Considering the relatively high frequency of *PPA2* mutations present in the ExAC database, it is important that clinically suspicious individuals are screened for *PPA2* mutations in addition to evidence of heart fibrosis by cardiac MRI. Moreover, application of an implantable cardioverter defibrillator may prevent sudden cardiac death in at-risk individuals who harbor biallelic *PPA2* mutations.

Supplemental Data

Supplemental Information includes supplemental case reports, nine figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.06.027.

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Web Resources

Cn3D, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml
Enzyme Nomenclature (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology [NC-IUBMB]), http://www.chem.qmul.ac.uk/iubmb/enzyme/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
MMDB, http://www.ncbi.nlm.nih.gov/structure
MutationTaster, http://www.mutationtaster.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do
SIFT, http://sift.bii.a-star.edu.sg/
Swiss PDB Viewer, http://www.expasy.org/spdbv/

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JAMA Neurology | Original Investigation

Clinical Features, Molecular Heterogeneity, and Prognostic Implications in *YARS2*-Related Mitochondrial Myopathy

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IMPORTANCE *YARS2* mutations have been associated with a clinical triad of myopathy, lactic acidosis, and sideroblastic anemia in predominantly Middle Eastern populations. However, the identification of new patients expands the clinical and molecular spectrum of mitochondrial disorders.

OBJECTIVE To review the clinical, molecular, and genetic features of *YARS2*-related mitochondrial disease and demonstrate a new Scottish founder variant.

DESIGN, SETTING, AND PARTICIPANTS An observational cohort study was conducted at a national diagnostic center for mitochondrial disease in Newcastle upon Tyne, England, and review of cases published in the literature. Six adults in a well-defined mitochondrial disease cohort and 11 additional cases described in the literature were identified with *YARS2* variants between January 1, 2000, and January 31, 2015.

MAIN OUTCOME AND MEASURES The spectrum of clinical features and disease progression in unreported and reported patients with pathogenic *YARS2* variants.

RESULTS Seventeen patients (median [interquartile range] age at onset, 1.5 [9.8] years) with *YARS2*-related mitochondrial myopathy were identified. Fifteen individuals (88%) exhibited an elevated blood lactate level accompanied by generalized myopathy; only 12 patients (71%) manifested with sideroblastic anemia. Hypertrophic cardiomyopathy (9 [53%]) and respiratory insufficiency (8 [47%]) were also prominent clinical features. Central nervous system involvement was rare. Muscle studies showed global cytochrome-*c* oxidase deficiency in all patients tested and severe, combined respiratory chain complex activity deficiencies. Microsatellite genotyping demonstrated a common founder effect shared between 3 Scottish patients with a p.Leu392Ser variant. Immunoblotting from fibroblasts and myoblasts of an affected Scottish patient showed normal YARS2 protein levels and mild respiratory chain complex defects. Yeast modeling of novel missense *YARS2* variants closely correlated with the severity of clinical phenotypes.

CONCLUSIONS AND RELEVANCE The p.Leu392Ser variant is likely a newly identified founder *YARS2* mutation. Testing for pathogenic *YARS2* variants should be considered in patients presenting with mitochondrial myopathy, characterized by exercise intolerance and muscle weakness even in the absence of sideroblastic anemia irrespective of ethnicity. Regular surveillance and early treatment for cardiomyopathy and respiratory muscle weakness is advocated because early treatment may mitigate the significant morbidity and mortality associated with this genetic disorder.

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Supplemental content

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Childhood-onset mitochondrial disease is among the most common inherited neurologic disorders, with an estimated minimum point prevalence of 5 (95% CI, 4.0-6.2) in 100 000 population.[1] Moreover, recent studies have estimated the prevalence of mitochondrial disease in affected adults at 9.6 per 100 000 caused by mutations in mitochondrial DNA (mtDNA) and 2.9 per 100 000, caused by nuclear mutations.[2] Hence, overall disease burden is potentially extensive. Patients exhibit marked phenotypic and genotypic heterogeneity, with mitochondrial respiratory chain complex deficiency a hallmark feature in tissues.

The amino-acyl transfer RNA (tRNA) synthetases are a group of enzymes critical for protein synthesis that are required for the recognition and conjugation of specific amino acids. Two sets of synthetases are encoded by separate genes in human cells, distinguished by cytoplasmic (referred to as *ARS*) or mitochondrial (referred to as *ARS2*) localization. *ARS2* mutations have emerged as an important cause of mitochondrial translation disorders that first appeared to manifest in tissue and cell-type specific phenotypes, including central nervous system involvement (*CARS2*, *DARS2*, *EARS2*, *FARS2*, *GARS*, *IARS2*, *MARS2*, *NARS2*, *PARS2*, *RARS2*, *TARS2*, and *VARS2*); myopathy, lactic acidosis, and sideroblastic anemia (MLASA) (*YARS2*); hypertrophic cardiomyopathy (*AARS2*); sensorineural hearing loss and ovarian dysgenesis (Perrault syndrome) (*LARS2* and *HARS2*); and hyperuricemia, pulmonary hypertension, renal failure, and alkalosis (*SARS2*).[3-18]

Mutations of *YARS2* encoding mitochondrial tyrosyl-tRNA synthetase have been described predominantly in patients with infantile- to childhood-onset autosomal recessive MLASA syndrome with mitochondrial respiratory chain complex deficiencies.[4,19-23] The MLASA syndrome was first described in patients with *PUS1* mutations,[24] with *YARS2* mutations later identified in patients with a clinically similar phenotype,[4] and most recently in 2 patients with a novel de novo heteroplasmic m.8969G>A (*MT-ATP6*) and recessive *LARS2* variants.[25,26] Although rare, sideroblastic anemia is a prominent feature in Pearson syndrome caused by single, large-scale mtDNA deletions.[27,28]

We present 6 adult patients with *YARS2* variants, including 4 previously unreported patients of Scottish descent, and demonstrate a new *YARS2* founder effect within this population, with immunoblotting and yeast modeling of novel missense variants to confirm pathogenicity. Together with previous cases from the literature, we review the spectrum of clinical features and progression of *YARS2*-associated mitochondrial disease to fully delineate the clinical phenotype and genotype correlates.

Key Points

Question What is the genotypic and clinical phenotypic spectrum of *YARS2*-related mitochondrial disease?

Findings In this retrospective, observational study, the triad of myopathy, lactic acidosis, and sideroblastic anemia was not identified in all cases. The p.Leu392Ser variant is likely a newly identified founder *YARS2* mutation in the Scottish ancestry.

Meaning Testing for pathogenic *YARS2* variants should be considered in patients presenting with mitochondrial myopathy and lactic acidosis even in the absence of sideroblastic anemia, irrespective of ethnicity.

Methods

Patients

Six patients (1.1, 1.2, 2, 3, 4.1, and 4.2) were referred to a national diagnostic and clinical center in Newcastle upon Tyne, England (clinical vignettes provided in the eAppendix in the Supplement). Patients 1.1 and 1.2 were initially reported in 1974.[29] Total genomic DNA was extracted and muscle biopsies were taken with written informed consent by standard procedures. We performed a literature review to identify all previously published cases with *YARS2* variants.[4,19-23] This study was approved and performed under the ethical guidelines issued by North East-Newcastle and North Tyneside institutional review board and complied with the Declaration of Helsinki.[30] Patients provided written informed consent.

Muscle Histochemistry and Respiratory Chain Enzyme Analysis

Muscle biopsy samples were subjected to cytochrome-*c* oxidase (COX), succinate dehydrogenase (SDH), and sequential COX-SDH histochemical reaction.[31] Measurement of mitochondrial respiratory chain complex activities in skeletal muscle homogenates was performed as previously described.[32]

Mitochondrial DNA Studies

Whole mitochondrial genome sequencing was performed using skeletal muscle homogenate to exclude pathogenic point mutations. Mitochondrial DNA rearrangements and mtDNA depletion were excluded using established, diagnostic, long-range polymerase chain reaction, and quantitative real-time polymerase chain reaction assays, respectively. Haplogroups were classified using HaploGrep.[33]

Identification of Pathogenic *YARS2* Variants

YARS2-targeted gene screening or variants identified by whole exome sequencing were confirmed by Sanger sequencing using custom-designed primers for all 5 exons and intronic regions of *YARS2* (GenBank NM_001040436.2). Patient 4.1 was the first to have the diagnosis identified by whole exome sequencing,[34] followed by patients 2 and 3 with a separate exome filtering pipeline.[35] Targeted *YARS2* gene screening was performed on patient 1.1.

Identification of Founder *YARS2* Mutation

Short tandem repeat tracts flanking the *YARS2* gene were identified using the Repeat Masker track of the University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu),[36] and primers were designed using Primer3 (http://primer3.ut.ee/).[37] Patient DNA was amplified across the various short tandem repeat loci by using polymerase chain reaction. Use of a fluorescently tagged forward primer facilitated electrophoresis on an ABI3130xl

genetic analyzer. Sizing and genotyping were performed (Peak Scanner, version 1.0; Applied Biosystems) with standard analysis parameters; ROX500 (Applied Biosystems) was used as a size standard.

Immunoblotting

Total protein aliquots from fibroblasts and myoblasts of patient 3 plus 2 controls for each cell line were separated through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were probed with antibodies specific to YARS2 (AP7838; Abgent), SDHA (ab14715; Abcam), ATP5B (ab14730; Abcam), UQCRC2 (ab14745; Abcam), MT-COI (ab14705; Abcam), MT-COII (ab110258; Abcam), and NDUFB8 (ab110242; Abcam). β-Actin (A5316; Sigma-Aldrich) and VDAC1/Porin (ab14734; Abcam) were used as loading controls.

Yeast Modeling

Yeast strains were generated and cultured as described in the eMethods in the Supplement. Human YARS2 is highly conserved in phylogenesis including yeast. The human residue Leu392 is conserved from human to yeast (Leu411) (eFigure, C in the Supplement), and the mutant allele corresponding to the novel missense variant identified in the patient of our cohort was generated (msy^{L411S}). The human residue Cys369 is, however, not conserved in yeast (Leu391) (eFigure, C in the Supplement). For this nonconserved residue, we mutagenized the yeast sequence to replace the amino acid leucine of the wild-type Msy1 with the amino acid cysteine of the wild-type human YARS2, thus creating the so-called "humanized" version (hL391C). If the humanized wild-type variant is able to complement the oxidative growth defect of the *msy1*Δ strain, it is possible to evaluate the effects of the novel missense variant identified in our cohort (Cys369Tyr) by creating the corresponding yeast mutant allele $msy1^{L391Y}$.

Oligonucleotides are provided in eTable 1 in the Supplement. Oxidative growth and mitochondrial respiration were measured as described previously.[19] The full methodology is provided in the eMethods in the Supplement.

Statistical Analysis

A paired, 2-tailed *t* test was performed to examine the yeast mitochondrial respiratory rate, and the level of statistical significance was set at $P \leq .05$. Microsoft Excel 2016 (Microsoft Corp) was used for statistical analysis.

Results

Clinical Features

We present 6 patients referred to our national diagnostic center with confirmed *YARS2* variants (**Table 1**), including 4 individuals of Scottish ancestry. A literature review identified an additional 11 patients with *YARS2* variants.[4,19-23] Hence, there is a total of 17 patients with *YARS2* variants (eTable 2 in the Supplement). A summary of the clinical features of all 17 patients (13 pedigrees) is given in **Table 2**. The median (interquartile range [IQR]) age at onset was 1.5 [9.8] years (range, 1 week to 31 years); 14 patients (82%) presented within the first decade of life. One patient presented in adolescence and 2 patients presented in adulthood. Six patients (35%) died (median [IQR] age at death, 25.5 [46.5] years; range, 3 months to 52 years). Two patients who died at age 3 months both presented with symptoms within 8 weeks of birth. A third patient presenting at age 10 weeks survived into adolescence, and the longest-surviving patients presented in childhood and survived into adulthood. Death in all 6 of these patients was preceded by progressive respiratory muscle weakness, which often required noninvasive ventilation, and cardiac failure (**Figure 1**).

Muscle Histochemistry and Muscle Respiratory Chain Enzyme Activity Analysis

Skeletal muscle biopsy specimens were available for 8 of the 17 patients (47%) (eTable 2 in the Supplement). Global COX deficiency was evident in 4 patients (**Figure 2**); ragged red fibers were observed in 2 patients. Patient 7 had a marked increase in SDH activity, but patient 13.1 exhibited only a minimal increase. Other abnormalities included lipid vacuoles (patient 9) and a few myopathic hypoatrophic fibers (patient 13.1).

Muscle respiratory chain enzyme activity was measured in 10 patients. Combined complex I, III, and IV deficiencies were found in 6 patients; complex I and IV deficiencies were found in the remaining 4 individuals.

Identification of Pathogenic *YARS2* Variants

In our cohort, we identified 3 novel pathogenic *YARS2* variants (c.1106G>A p.Cys369Tyr, c.1147_1164dup p.Val383_Glu388dup, and c.1175T>C p.Leu392Ser) and a previously reported variant (c.137G>A p.Gly46Asp).[22] The p.Cys369Tyr substitution is absent from external databases (ExAC, ESP6500, and 1000 Genomes Project) and affects a highly conserved residue; the impact of the missense substitution is predicted to be deleterious according to SIFT and MutationTaster; PolyPhen2 and AlignGVGD are more conservative with their predictions. Because of the proximity of the c.1106G>A nucleotide to the intron-exon boundary, the c.1106G>A substitution may cause aberrant messenger RNA splicing and, in addition, it is predicted to abolish a SC35 exonic splicing enhancer according to the in silico splicing prediction tool ESEfinder. The p.Val383_Glu388dup variant was found in 2 of 121 360 (1.648×10^{-5}) alleles in ExAC from 2 non-Finnish European individuals. The p.Leu392Ser missense change was found in 1 of 121 354 alleles in ExAC in a single non-Finnish European individual and is predicted to be deleterious according to SIFT and MutationTaster; however, PolyPhen2 and AlignGVGD predict a milder effect of the p.Leu392Ser substitution (ExAC, ESP6500, and 1000 Genomes Project frequency data, accessed June 22, 2016). To date, there is a total of 9 pathogenic variants described in the *YARS2* gene (**Figure 3**A). All variants lead to missense changes except for a 6-amino acid duplication within the S4-like domain and a nonsense variant that is likely to lead to degradation of messenger RNA by nonsense-mediated decay.[21] All patients harbored homozygous missense changes except for patients 3 and 12, who had compound heterozygous changes.

Table 1. Clinical, Molecular, and Genetic Details of Patients With *YARS2*

	Patient					
Characteristic	1.1	1.2	2	3	4.1	4.2
Sex	Male	Male	Female	Male	Male	Male
Age at onset, y	12	2	Childhood	4	<10	<10
Current age decade, y	50s[a]	40s[a]	70s	20s	30s[a]	30s
Ethnicity	Scottish	Scottish	Scottish	Irish/Scottish	Jordanian	Jordanian
Consanguinity	No	No	Yes	No	No	No
Family history	Sibling of 1.2	Sibling of 1.1	No	No	Sibling of 4.2	Sibling of 4.1
Clinical features						
Myopathy[b]	4/5	Yes	4−/5,Wheelchairat 59 y	4/5	4/5	3/5
Respiratory insufficiency	NA	NA	NIV at 55 y	Severe restrictive pattern	Yes	NIV at 33 y
Cardiomyopathy	High-output cardiac failure	High-output cardiac failure	No	Yes	End-stage cardiac failure	No
Sideroblastic anemia	Yes	Yes	No	No	Yes	No
Transfusion	No	No	No	No	Yes	No
Lactic acidosis	Yes	Yes	No	Yes	Yes	Yes
Other features	No	No	Facial weakness	No	No	Scapular winging
Muscle biopsy						
Histochemistry	Global COX deficiency	NA	Global COX deficiency	Global COX deficiency, RRFs	Global COX deficiency	RRFs
RC enzyme deficiency	CI, CIII, CIV	NA	CI, CIV	CI, CIII, CIV	CI, CIV	ND
YARS2 variant						
cDNA change	Homozygous c.1175T>C	ND	Homozygous c.1175T>C	c.1106G>A/ c.1147_1164dup	Homozygous c.137G>A	ND
Amino acid change	Homozygous p.Leu392Ser	ND	Homozygous p.Leu392Ser	p.Cys369Tyr/ p.Val383_Glu388dup	Homozygous p.Gly46Asp	ND
mtDNA haplogroup	H1m1	ND	G2a3a	T2e1a	HV13	ND

Abbreviations: cDNA, complementary DNA; CI, complex I; CIII, complex III; CIV, complex IV; COX, cytochrome-*c* oxidase; mtDNA, mitochondrial DNA; NA, not available; ND, not done; NIV, noninvasive ventilation; RC, mitochondrial respiratory chain; RRFs, ragged red fibers.

[a] Age at death.

[b] Scored according to the Medical Research Council scale for muscle strength (https://www.mrc.ac.uk/research/facilities-and-resources-for-researchers/mrc-scales/mrc-muscle-scale/). The muscle scale grades muscle power on a scale of 0 to 5 in relation to the maximum expected for that muscle: 0, no contraction; 1, flicker or trace of contraction; 2, active movement, with gravity eliminated; 3, active movement against gravity; 4, active movement against gravity and resistance; and 5, normal power. Grades 4−, 4, and 4+ may be used to indicate movement against slight, moderate, and strong resistance, respectively.

The Lebanese founder p.Phe52Leu variant was the most frequent pathogenic *YARS2* variant, occurring in 5 patients.

We identified 2 patients (1.1 and 2) from 2 apparently unrelated families of Scottish ancestry with a homozygous p.Leu392Ser variant in the S4-like domain. Patient 2 was born to consanguineous parents. Formal genetic testing of patient 1.2, a clinically affected sibling of patient 1.1, was not feasible; however, their clinically unaffected sister was tested and did not harbor the familial variant. We also identified a third patient of Scottish ancestry (patient 3) with unreported (p.Cys369Tyr and p.Val383_Glu388dup) compound heterozygous variants in which familial segregation studies confirmed recessive inheritance.

Identification of Founder *YARS2* Mutation

Microsatellite genotyping of dinucleotide repeat regions flanking the *YARS2* gene supported a common founder across a region of at least 183 kb shared between unrelated patients 1.1 and 2 with a T-1-2-3 haplotype consisting of the *YARS2* p.Leu392Ser mutation plus markers ms3, ms2, and ms1. Analysis of DNA from the unaffected sister of patient 1.1, whose genetic status was homozygous wild type at the p.Leu392Ser locus, was as anticipated: consistent with inheritance of the normal paternal and maternal alleles (Figure 3B).

Assessment of Mitochondrial DNA

Pathogenic or likely pathogenic mtDNA point mutations, rearrangements, and depletion were excluded. All of our patients belonged to different haplogroups (Table 1 and eTable 3 in the Supplement).

Immunoblotting Analysis

Immunoblotting of total protein aliquots from cultured skin fibroblasts and myoblasts of patient 3 showed no change in immunoreactive YARS2 levels (eFigure, A and B in the Supplement). A minimal reduction of MT-COI was observed in both fibroblasts and myoblasts, whereas a marked decrease in NDUFB8 was observed only in myoblasts.

Table 2. Summary of the Clinical Features of All 17 Patients

Clinical Features	No. (%)
Myopathy/exercise intolerance	15 (88)
Lactic acidosis	15 (88)
Sideroblastic anemia	13 (76)
Transfusion dependent	7 (41)
Spontaneous recovery	3 (18)
MLASA triad	12 (71)
Hypertrophic cardiomyopathy	9 (53)
Respiratory insufficiency	8 (47)
Ventilatory support	5 (29)
Facial muscle weakness	2 (12)
Ptosis	2 (12)
Ophthalmoparesis	1 (6)
Strabismus	1 (6)
Nystagmus	1 (6)
Delayed motor milestones	1 (6)
Scoliosis	1 (6)
Seizure	1 (6)
Gastrointestinal symptoms	1 (6)
Hepatomegaly	1 (6)
Proximal renal tubulopathy	1 (6)

Abbreviation: MLASA, myopathy, lactic acidosis, and sideroblastic anemia.

Yeast Modeling

Oxidative growth of the strains expressing msy^{L411S} and the humanized version $msy1^{hL391C}$ was similar to the wild type at 28°C and 36°C, whereas growth of the strain expressing $msy1^{L391Y}$ was reduced, particularly at 36°C (eFigure, D in the Supplement). Oxygen consumption of cells grown at 28°C showed a reduction for both of the mutants: mutant $msy1^{L391Y}$ showed a 27% reduction of the respiratory rate with respect to its humanized version msy^{hL391C} and mutant $msy1^{L411S}$ showed a 21% reduction with respect to the wild-type strain (eFigure, E in the Supplement).

Discussion

We describe the variable clinical phenotype of 17 patients with *YARS2* variants, including 6 new adult cases identified by targeted *YARS2* gene sequencing or whole exome sequencing. Our study suggests that the classic clinical triad of MLASA is not essential for identifying *YARS2*-related mitochondrial disease. Demonstrable proximal muscle weakness may be subtle during the early stage of *YARS2*-related mitochondrial disease onset. Episodic vomiting coupled with fatigue, headache, and cardiac palpitations following physical exertion in children and adults should raise a clinical suspicion of exercise intolerance with associated lactic acidemia. We show that almost all affected patients (n = 15) exhibited lactic acidemia together with marked exercise intolerance and progressive myopathy.

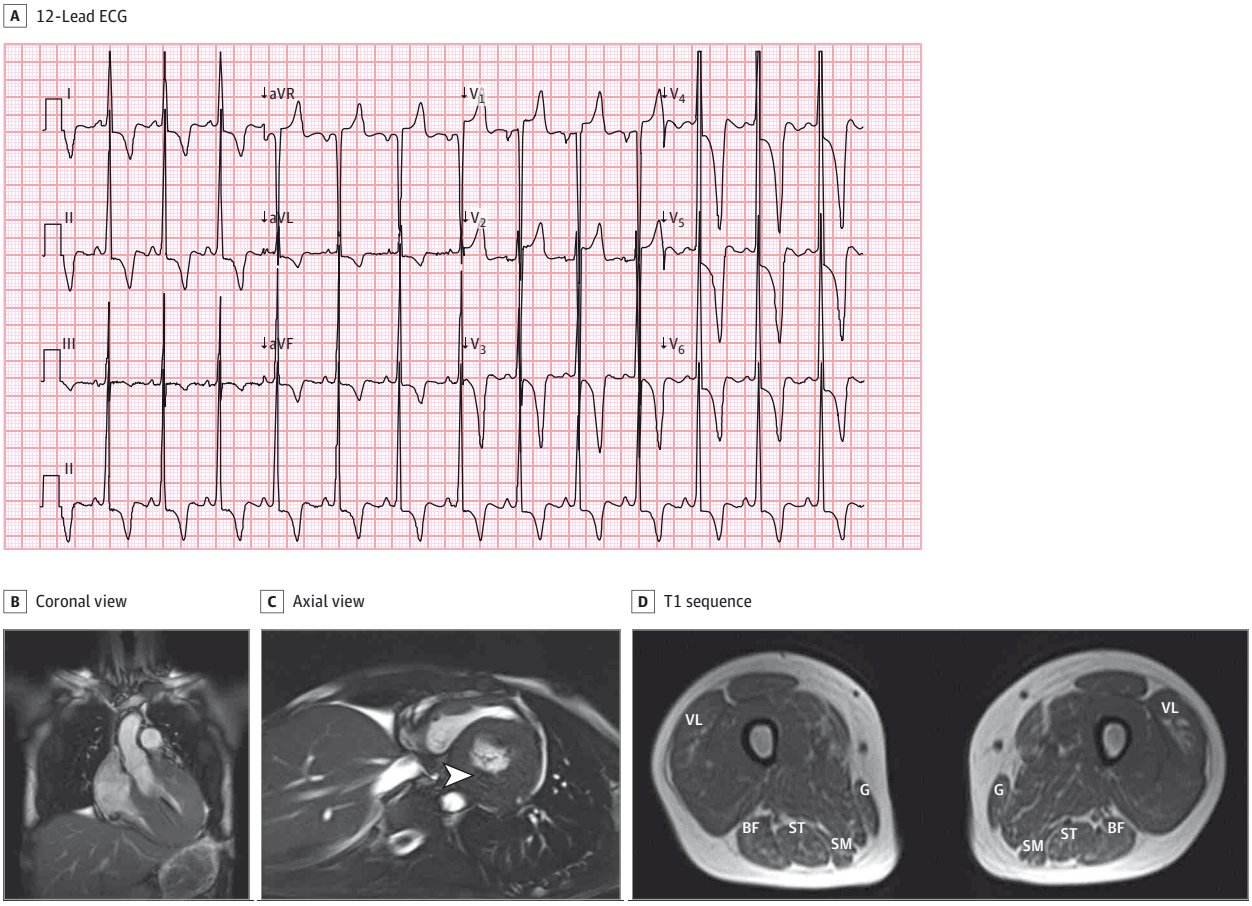
Sideroblastic anemia has been considered to be an important clinical feature of Pearson syndrome-,[26] *PUS1*-,[24] or *YARS2*-related[4] mitochondrial disorders. Pearson syndrome, characterized by infantile-onset exocrine pancreatic failure and sideroblastic anemia,[27] can be differentiated from *PUS1*- and *YARS2*-related mitochondrial disease by its clinical phenotype and presence of a single, large-scale mtDNA deletion in blood and other tissues. Both *YARS2* and *PUS1* mutations have been reported in MLASA, but there may be additional clinical pointers to help discern between them. Mutations in *PUS1* have been associated with microcephaly,[38-41] variable degrees of learning disability and cognitive impairment,[38-40,42] dysmorphic features,[38-40] and failure to thrive or to experience growth restriction[39,41,43]; these features are conspicuously absent in YARS2-related mitochondrial disease. In contrast, hypertrophic cardiomyopathy is a clinical feature that has been identified independently in 8 family pedigrees with *YARS2* mutations (eTable 2 in the Supplement) compared with only a single case of *PUS1* mutations.[41] Although there are emerging phenotypic differences observed between these 2 genetic mutations, caution should be applied on drawing firm conclusions given the relatively small number of patients in these 2 cohorts to date.

We have observed full resolution of sideroblastic anemia in 2 of our patients and other previously reported patients with *YARS*2 mutations. The exact mechanism of such a phenomenon in *YARS2* mutations is unknown given the other salient clinical features, including myopathy and cardiomyopathy, frequently progress over time. In another form of mitochondrial disease, the spontaneous resolution of sideroblastic anemia in Pearson syndrome has been speculated to be the result of negative selection of mutant mtDNA in the bone marrow.[44] This is a feature that warrants further work but is beyond the scope of this article.

Severe respiratory muscle weakness leading to ventilation failure, especially in adult patients, is a rare clinical feature in mitochondrial disease[45-47] compared with other genetic muscle diseases.[48] Analysis of all cases would suggest this is a key, terminal feature combined with hypertrophic cardiomyopathy and/or refractory transfusion-dependent sideroblastic anemia in the natural progression of *YARS2*-associated mitochondrial disease. These findings highlight the importance of regular surveillance of respiratory and cardiac function. Furthermore, early instigation of supportive treatment, such as noninvasive ventilation and pharmacologic cardiac remodeling agents,[49] may slow disease progression.

The muscle biopsy findings of severe, global COX deficiency in muscle fibers were consistent with a diagnosis of mitochondrial myopathy and prompted further investigation. Immunoblotting of fibroblasts and myoblasts from patient 3 demonstrated no change in YARS2 protein levels; immunoblotting of fibroblasts, myoblasts, or MyoD-forced myotubes from 2 patients (8.2 and 12) also showed no decrease.[4,21] In contrast, YARS2 levels were undetectable in myoblasts and myotubes from patient 7.[22] This may reflect the tissue-specific nature of *YARS2* and other aminoacyl tRNA synthetase mutations and variation in the threshold for expression between different individuals and tissues. One factor contributing to this variation may be the rate of protein turnover in an individual tissue. Our data illustrating only a minor decrease in MT-COI

Figure 1. Findings of Cardiac Investigations and Magnetic Resonance Imaging (MRI) of the Muscle in Patient 3

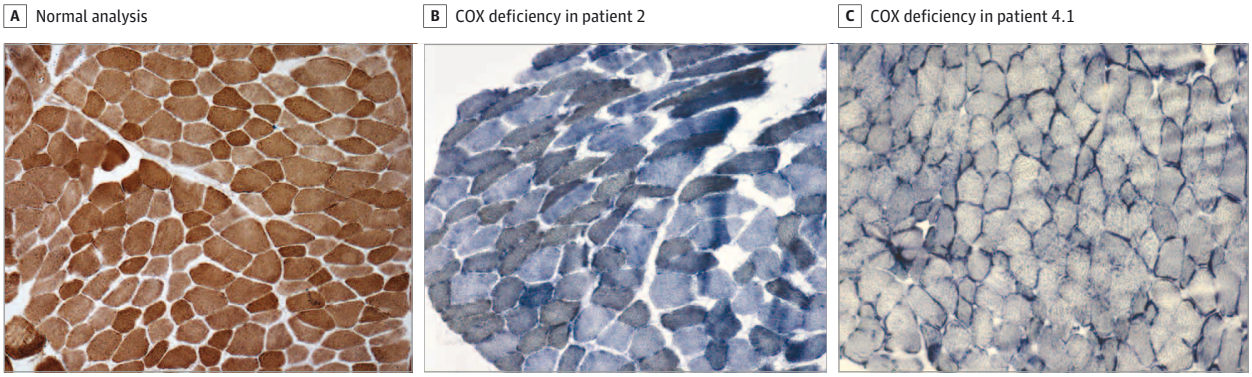


A, Twelve-lead electrocardiogram (ECG) showed voltage criteria consistent with left ventricular hypertrophy (S wave in V_1 + R wave in V_6 = 48 mm + 53 mm = 101 mm; >35 mm or 7 large squares are suggestive of left ventricular hypertrophy) with strain pattern. B and C, Coronal and axial views of cardiac MRI. Concentric hypertrophic cardiomyopathy was evident (arrowhead). D, T1-sequence MRI of the muscle showed mild atrophy affecting the vastus lateralis (VL), bicep femoris (BF), semitendinosus (ST), semibranosus (SM), and gracilles (G).

and MT-COII levels in patient fibroblasts and myoblasts despite marked COX deficiency in muscle lend support to this argument.

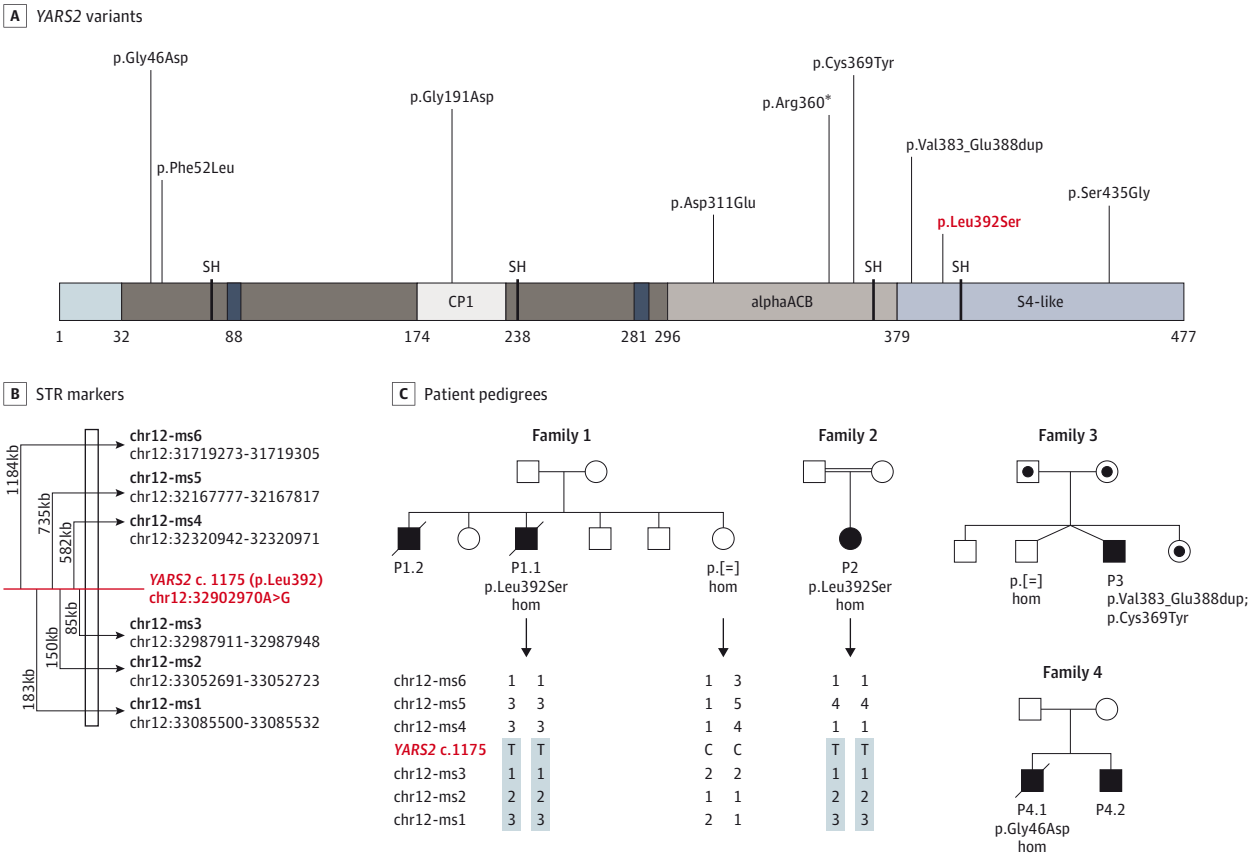
Yeast modeling to confirm pathogenicity of the reported p.Asp311Glu variant, corresponding to *msy*^{D333E}, had been performed previously demonstrating an OXPHOS defect by fail-

Figure 2. Skeletal Muscle Histochemistry Analyses



Normal cytochrome-*c* oxidase (COX)–succinate dehydrogenase histochemical analysis from a healthy control sample (A) compared with a global COX deficiency observed in the muscle fibers of patient 2 (B) and patient 4.1 (C) (scale bar, 100 µm).

Figure 3. Genetic Characterization of Patients



A, All reported pathogenic *YARS2* variants. Novel p.Leu392Ser founder mutation from this study is shown in red. B, Location of short tandem repeat (STR) markers used for analysis and their genomic distance relative to *YARS2*. C, Pedigrees of patients (P), including 1.1, 1.2, and 2 with STR haplotype results (phase assumed when no parental samples are available) and a shared haplotype (highlighted in blue) involving the *YARS2* locus (red type), ms1, ms2, and ms3 STR markers. Pedigrees 3 and 4 are also shown.

ure to grow on media containing ethanol.[19] We replicated this for our unreported missense changes, p.Leu392Ser and p.Cys369Tyr, corresponding to msy^{L411S} and msy^{L391Y}. Both mutated alleles showed mild OXPHOS defects, although msy^{L391Y} had a slightly more severe OXPHOS phenotype. However, both mutants were phenotypically milder than the previously characterized msy^{D333E} mutant. More important, when compared, all modeled missense changes reflected the human clinical phenotypes.

Predicting the severity and speed of disease progression is challenging given the genetic heterogeneity of this disorder. Homozygous mutations in p.Gly46Asp appear to manifest later in childhood compared with the p.Phe52Leu and p.Ser435Gly variants. We now describe 3 unrelated patients of Scottish ancestry with homozygous p.Leu392Ser mutations who presented with slower disease progression and survival into late adulthood. It has been hypothesized that mtDNA haplotype background may influence phenotypic expression.[21,50,51] Within our patient cohort, haplotype H was associated with slower disease progression and a less severe phenotype as previously observed.[21] Patient 2, who presented with the mildest phenotype of all *YARS2* patients, belonged to haplogroup G (Table 1 and eTable 3 in the Supplement), which, to our knowledge, has not been previously reported. However, as with previous phylogenetic analysis and characterization of mtDNA haplogroups in patients with *YARS2*,[21] there are likely additional, indeterminate genetic factors that modify phenotypic expression of disease.

The advent of next-generation sequencing has permitted the identification of patients with *YARS2* mutations who do not manifest with the full MLASA syndromic features, thus further expanding the phenotypic and genotypic heterogeneity of these mitochondrial disorders. This is exemplified by the identification of a 73-year-old patient with characteristic global COX deficiency in muscle who harbored recessive *YARS2* variants and manifests remarkably indolent, progressive, mitochondrial myopathy.

Conclusions

YARS2-related mitochondrial disease is phenotypically heterogeneous and has a variable prognosis ranging from infantile-onset—and often fatal—MLASA syndrome to later adolescent-

onset, slowly progressive myopathy. Progressive respiratory muscle weakness and cardiomyopathy are the major causes of death in these patients. We suggest that *YARS2*-associated mitochondrial disease may be an underdiagnosed clinical entity, particularly in populations outside the Middle East and in adult patients who do not exhibit the full clinical spectrum of MLASA, as we demonstrate a new Scottish *YARS2* founder effect within our patient population.

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ARTICLE

A national perspective on prenatal testing for mitochondrial disease

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Mitochondrial diseases affect >1 in 7500 live births and may be due to mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). Genetic counselling for families with mitochondrial diseases, especially those due to mtDNA mutations, provides unique and difficult challenges particularly in relation to disease transmission and prevention. We have experienced an increasing demand for prenatal diagnostic testing from families affected by mitochondrial disease since we first offered this service in 2007. We review the diagnostic records of the 62 prenatal samples (17 mtDNA and 45 nDNA) analysed since 2007, the reasons for testing, mutation investigated and the clinical outcome. Our findings indicate that prenatal testing for mitochondrial disease is reliable and informative for the nuclear and selected mtDNA mutations we have tested. Where available, the results of mtDNA heteroplasmy analyses from other family members are helpful in interpreting the prenatal mtDNA test result. This is particularly important when the mutation is rare or the mtDNA heteroplasmy is observed at intermediate levels. At least 11 cases of mitochondrial disease were prevented following prenatal testing, 3 of which were mtDNA disease. On the basis of our results, we believe that prenatal testing for mitochondrial disease is an important option for couples where appropriate genetic analyses and pre/post-test counselling can be provided.
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BACKGROUND

Mitochondrial diseases are clinically and genetically heterogeneous conditions affecting >1 in 7500 live births[1] and causing significant morbidity and mortality.[2,3] Genetic counselling for families with mitochondrial disease can provide some unique and difficult challenges, particularly in relation to disease transmission and prevention. A clear understanding of the inheritance patterns in families with mitochondrial disease, the reproductive and prenatal testing options available, their application to mitochondrial disease, and the risks involved is crucial if accurate and appropriate advice is to be imparted to prospective parents.

Mitochondria are small multifunctional intracellular organelles present in all eukaryotic cells and primarily responsible for generating adenosine triphosphate by oxidative phosphorylation. Their efficient functioning is determined by two genomes; the nuclear genome and also the maternally inherited 16.6-kb mitochondrial genome.[4,5] Disease may occur as a result of mutations in either of these genomes. Defects in nuclear DNA (nDNA) can cause problems with mitochondrial DNA (mtDNA) maintenance and repair, defects in translation or structural defects of respiratory chain complexes.[5,6] mtDNA is present in multiple copies within cells and in oocytes the copy number can exceed 1×10^5 per cell.[7] A consequence of this high intracellular copy number is the phenomenon of heteroplasmy, where two or more species of mtDNA co-exist. This situation arises when a mtDNA mutation occurs within only a proportion of the mtDNA present. Importantly though, disease will only occur when a tissue-specific threshold has been exceeded (usually >60%). This threshold is not the same for all mtDNA mutations and the level of heteroplasmy may change slowly with time.[4,5,8,9]

Patients may develop their first symptoms in adulthood and it is not unusual for the mtDNA mutation to be transmitted to children before their mother becomes symptomatic. In addition, asymptomatic mothers with low levels of mtDNA mutation may have symptomatic children with very high levels because of a genetic phenomenon known as the 'mtDNA genetic bottleneck'. A main component of this bottleneck occurs during formation and initial divisions of primordial germ cells when mtDNA copy numbers are reduced to <200 per cell. This decrease in copy number, together with the random segregation of mtDNA molecules to daughter cells, can result in a markedly skewed distribution of mutation when mature oocytes are formed.[8,10,11]

Approximately 30 000 women per year are offered invasive prenatal testing in the United Kingdom.[12] Included in this number is a small, but growing, group of women who are offered prenatal testing for mitochondrial disease. There is no cure for mitochondrial disease and effective treatments are lacking. Reproductive options for families affected by mitochondrial disease are important, particularly those who have already lost a child to the disease or for those women known to harbour mtDNA mutations. Prenatal testing for mitochondrial disease is only possible, however, with known causative mutations, where there is sufficient evidence from segregation and disease linkage studies, biochemical and functional assays to confirm the pathogenicity of these mutations. The primary aim of prenatal diagnosis for mitochondrial disease is to provide an

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accurate assessment of the risk of the foetus developing mitochondrial disease either *in utero* or in childhood.[13–16]

Where mitochondrial disease is inherited in an autosomal recessive manner, as is most often the case in childhood-onset mitochondrial disease, and the genetic changes identified are novel, then carrier status should be confirmed in each parent with additional evidence provided from functional studies supporting pathogenicity. When a mtDNA mutation is responsible, then heteroplasmy levels in blood and urine should be determined in the mother and, where possible, in maternal relatives, especially previously affected children. For a minority of mtDNA mutations, there is a clear correlation between the level of heteroplasmy and disease severity, but this does differ between families and assessment of foetal risk should be made in the context of how other family members have been affected.[16]

Since establishing the nationally commissioned mitochondrial diagnostic service in 2007, we have experienced an increasing demand for prenatal diagnostic testing from families affected by mitochondrial disease. We have retrospectively reviewed the analyses performed over the period April 2007 to January 2013 to inform the debate and plan future testing strategies.

MATERIALS AND METHODS

Data regarding prenatal diagnostic testing were collected from two (Newcastle and Oxford) of the three centres (Newcastle, Oxford and London) commissioned by the National Health Service to provide a clinical and diagnostic service for Rare Mitochondrial Diseases of Adults and Children (the service in London did not undertake any prenatal investigations during this period). We are not aware of any other genetic testing centres in the United Kingdom undertaking mtDNA heteroplasmy assessment and although it is possible that other centres have performed prenatal assessment for nuclear mutations, the availability of a free nationally commissioned service makes this somewhat unlikely. Three reviewers examined the diagnostic reports and medical notes over the 69-month period between April 2007 and January 2013. Discrepancies and omissions in the clinical information were clarified with the clinical team.

Assessing mutation status during diagnostic chorionic villus biopsy (CVB) testing

A variety of molecular techniques are used for prenatal testing depending upon the causative familial mutation. Presence or absence of nuclear gene mutations is ascertained by direct Sanger sequencing of PCR-amplified products. Prenatal assessment of heteroplasmy levels of familial mtDNA point mutations was achieved using mutation-specific pyrosequencing assays.[17] These assays have been established in the Newcastle laboratory for a variety of pathogenic mutations and have been shown to be sensitive in their detection of 1% mutation load.[18–21] Mutation screening for large-scale single mtDNA deletions is performed using a combined methodology involving quantitative fluorescent real-time PCR (QPCR) and long-range PCR.[22] The QPCR assay compares the copy number of the commonly deleted *MTND4* gene against the copy number of the *MTND1* gene, which is seldom deleted in patients with large-scale mtDNA rearrangements.[23]

RESULTS

Sixty-two prenatal diagnoses were made in the 69-month study period, of which 59 were CVB samples and 3 were amniocentesis. Gestation at time of testing ranged from 8 weeks 5 days to 15 weeks for CVB, with the amniocenteses being performed between 15 weeks 5 days and 17 weeks 3 days. Prenatal testing was requested in the majority of patients as a consequence of having had a previously affected child ($n=58$). Other requests were due to being a known mtDNA mutation carrier (although clinically asymptomatic; $n=3$), or having severely affected siblings ($n=1$).

mtDNA mutations

Of the prenatal samples assessed, 17 were for mutations in mtDNA (m.3243A>G ($n=4$), m.11777C>A ($n=2$), m.9176T>C ($n=2$), m.14453G>A ($n=1$), m.13513G>A ($n=1$), m.8344A>G ($n=1$), m.8993T>G ($n=1$), m.8993T>C ($n=1$), m.10191T>C ($n=1$), m.10158T>C ($n=1$), m.3688G>A ($n=1$) and a single, large-scale mtDNA deletion ($n=1$); Table 1). In addition to accurately determining the level of mtDNA mutation in the foetal DNA sample, all mtDNA mutations were further categorised as having low (<30%), intermediate (30–70%) or high (>70%) mtDNA loads. Of the 17 prenatal samples, high heteroplasmy levels were detected in CVB samples from patients 6 and 8 and in both cases termination of pregnancy was performed. Patient 8 herself harboured relatively high levels of the m.8993T>G mutation in blood DNA (73% heteroplasmy) although the threshold for a severe neurological phenotype, such as Leigh Syndrome, in association with the m.8993T>G mutation is typically very high.[24] In contrast, patient 6 did not have the mutation detectable in her blood or urine. Intermediate heteroplasmy levels were identified in six samples (patients 1, 5, 9, 12, 14 and 16), one of which resulted in termination of pregnancy (patient 1), and the outcome for the remainder is not known. Five of these mothers themselves had intermediate heteroplasmy levels of the respective mutation in DNA derived from their urinary epithelium; one had high heteroplasmy levels (patient 9), but (as is the case for the m.8993T>G and indeed the m.9176T>C mutation) the m.8993T>C mutation is associated with a very high threshold.[24–26] Low level mutation load was detected in the CVB samples from patients 7 and 15, both of whom continued their pregnancies. Patient 15, who harboured low heteroplasmy levels in blood and intermediate levels in buccal and urinary epithelium of m.3688G>A, went on to deliver a clinically unaffected child. The outcome for the child born to patient 7, in whom we could not detect the familial m.9176T>C mutation in blood or urine, is not known. The familial mutation was not detected in seven prenatal samples, from four mothers in whom we found no evidence of the familial mutation in blood or urine (patient 2 (m.3243A>G), patient 4 (m.3243A>G), patient 10 (m.14453G>A), patient 17 (single deletion)), two mothers harbouring low heteroplasmy levels (patient 3 (m.3243A>G) and patient 13 (m.10191T>C)) and one mother with intermediate levels in blood (patient 11 (m.13513G>A)). Postnatal screening for the familial mutation was performed for three cases using blood samples; the results identified no evidence of the corresponding mutation in each instance and they remain clinically well (patient 4 (m.3243A>G), patient 10 (m.14453G>A) and patient 11 (m.13513G>A)).

nDNA mutations

The remaining 45 prenatal samples were screened for recessive nDNA mutations (*POLG* ($n=19$), *SURF1* ($n=14$), *NDUFS2* ($n=2$), *MPV17* ($n=2$), *RARS2* ($n=2$), *DGUOK* ($n=2$), *NDUFV1* ($n=1$), *TK2* ($n=1$), *SUCLA2* ($n=1$), *TMEM70* ($n=1$)) (Supplementary Table 2). Of these, 12 inherited both parental mutations, 8 of which resulted in termination of the pregnancy, 2 pregnancies continued resulting in the birth of affected infants (mutations confirmed on infant blood DNA for one case) and the outcome of the remaining 2 cases is not known. There were 15 heterozygous carriers and the familial mutations were not detected in 18 cases. Pregnancy was terminated in one of these cases for an incidental finding of the XXY karyotype (patient 26).

Table 1 Prenatal testing for mtDNA mutations

Pt	*Reason for CVB*	*Screening mutation*	*Maternal mutation and heteroplasmy level*	*Result of CVB*	*Clinical outcome*
1	Mother known mutation carrier	m.3243A>G	m.3243A>G (55% urine, 12% blood)	68% m.3243A>G[a]	Termination of pregnancy
2	Previously affected child	m.3243A>G	m.3243A>G not detected in blood/urine/ buccal samples	No mutation detected	Pregnancy continued
3	Mother known mutation carrier	m.3243A>G	m.3243A>G (1% blood, 18% urine)	No mutation detected	Pregnancy continued
4	Maternal grandmother and brother affected	m.3243A>G	m.3243A>G not detected in blood/urine	No mutation detected	Baby clinically well; no mutation detected in blood DNA at 11 weeks
5	Previously affected child	m.8344A>G	m.8344A>G (35% blood, 35% urine)	46% m.8344A>G[a]	Data not available
6	Previously affected child	m.9176T>C	m.9176T>C not detected in blood/urine	98% m.9176T>C[a]	Termination of pregnancy
7	Previously affected child	m.9176T>C	m.9176T>C not detected in blood/urine	9% m.9176T>C[a]	Pregnancy continued
8	Previously affected child	m.8993T>G	m.8993T>G (73% blood)	98% m.8993T>G[a]	Termination of pregnancy; PM samples confirmed mutation levels 97%
9	Previously affected child	m.8993T>C	m.8993T>C (79% blood, 86% urine, 82% buccal)	58% m.8993T>C[a]	Pregnancy continued
10	Previously affected child	m.14453G>A	m.14453G>A not detected	No mutation detected	Clinically unaffected baby girl born
11	Previously affected child	m.13513A>G	m.13513G>A (45% blood)	No mutation detected	Baby clinically well; no mutation detected in blood DNA at 12 weeks
12	Maternal brother affected	m.11777C>A	m.11777C>A (30% blood, 36% urine, 43% buccal)	46% m.11777C>A[a]	Data not available
13	Mother known mutation carrier	m.10191T>C	m.10191T>C (2% blood, 16% urine)	No mutation detected	Pregnancy continued
14	Previously affected child	m.10158T>C	m.10158T>C (5% blood, 33% urine, 16% buccal)	52% m.10158T>C[a]	Data not available
15	Previously affected child	m.3688G>A	m.3688G>A (20% blood, 50% urine, 43% buccal)	3% m.3688G>A[a]	Clinically unaffected baby girl born
16	Previously affected child	m.11777C>A	m.11777C>A (18% blood, 32% urine, 20% buccal)	54% m.11777C>A[a]	Alive and well at 5 years
17	Previously affected child	Single large-scale mtDNA deletion	No deletion in mtDNA detected	mtDNA deletion not detected	Pregnancy continued

Seventeen women referred for prenatal testing because of a personal or maternal family history of mtDNA disease, results of the prenatal test and clinical outcome where known.
Reference sequence: Revised Cambridge Reference Sequence;[35] GenBank Reference NC_012920.1.
[a]Information submitted to the publicly available MITOMAP database (http://www.mitomap.org/MITOMAP).

DISCUSSION

The aim of prenatal diagnostic testing in mitochondrial disease is to identify foetuses harbouring mutations that will cause severe disease and offer termination at a relatively early stage of pregnancy. In our experience, prenatal testing is usually requested as a consequence of having had a previously affected child (57/62 requests).

CVB, usually performed at 11–14 weeks gestation, is the procedure most commonly offered to women at risk of having a child affected by mitochondrial disease, and particularly for those harbouring mtDNA mutations. Amniocentesis, a procedure that relies on aspirating cellular material (largely shed foetal epithelial cells) from the amniotic sac, may be less reliable in detecting and quantifying mtDNA mutations and tends to be offered to those women presenting later in pregnancy or where a nDNA mutation is responsible for the mitochondrial disease. Prenatal diagnostic testing procedures also bring additional risk of miscarriage; approximately 1% and 2% higher than spontaneous miscarriage for amniocentesis and CVB, respectively.[12] No spontaneous miscarriages occurred as a result of prenatal testing in our cohort. Percutaneous umbilical blood sampling is not routinely offered in mitochondrial disease because of the risk of maternal contamination of the sample and the increased risk of foetal loss ($<3\%$) associated with the procedure.[12] We do not offer a biochemical prenatal diagnosis, although this has been performed (rarely) at other international centres where a biochemical deficiency has been established in a previously affected child, but no genetic diagnosis achieved. This type of biochemical diagnosis has a number of specific requirements and as a result worldwide experience is very limited.[16]

For women known to harbour pathogenic mtDNA mutations, interpretation of prenatal testing is complex and it is important to consider factors such as heteroplasmy of the mtDNA mutation, threshold levels, phenotypic expression of the mutation in maternal relatives and the strength of association between genotype and phenotype. Only through evaluating all of these aspects can the clinician provide clear, accurate genetic advice regarding the likely outcome for the foetus affected by a mtDNA mutation.[13,15,16,20] Recent studies indicate that prenatal samples provide an accurate prediction of mutation load in the postnatal period.[27] In addition, studies analysing heteroplasmy levels in blastomeres from disaggregated eight cell stage embryos, have indicated that there is less variation between cells than was previously considered to be the case, implying that sampling bias in prenatal diagnosis is minimal.[28] Further research to ascertain the correlation between genotype, mutation load and phenotype is required with regards to some mtDNA mutations. In the seven cases in which the mutation was not detected, these families could be reassured that their child would not be clinically affected by mitochondrial disease. Counselling mothers with foetuses harbouring mtDNA mutations in the intermediate range ($n=11$) can prove more difficult particularly for mutations where the relationship between genotype and phenotype is far less clear (eg, m.3243A>G). In this context, information regarding levels of heteroplasmy in affected and unaffected close relatives is very important. This was particularly true for patient 1 (Table 1), who was the only intermediate result to lead to termination of pregnancy. Extensive pre- and post-result counselling included a discussion of m.3243A>G heteroplasmy levels present in patient 1's affected and

unaffected maternal relatives, who gave permission to disclose this information for the purposes of counselling. The two women with foetuses demonstrating high heteroplasmy levels on prenatal testing both opted for termination of pregnancy following appropriate counselling with both a specialist in mitochondrial disease and a genetic counsellor. Although all families affected by mitochondrial disease should be aware of available reproductive options, it is these women, harbouring high levels of pathogenic mtDNA mutations, who may wish to consider alternative options for future pregnancies. Such options include gamete (egg or sperm) donation, or adoption as an alternative to pregnancy. Techniques to reduce or prevent transmission of mtDNA and nDNA mutations, such as pre-implantation genetic diagnosis, are already available.[14,29,30]

One of the shortfalls of this retrospective collection of data on prenatal diagnoses in patients with mitochondrial disease is the paucity of information available regarding follow-up on children born after prenatal diagnosis. It appears that for mothers with low or intermediate levels of mtDNA mutation, routine outpatient follow-up is infrequent and therefore information regarding their children scant. However, as a national service, we are not aware of any children presenting with mitochondrial disease symptoms following prenatal mtDNA diagnosis during the period April 2007–January 2013.

Interpretation of prenatal testing is relatively straightforward in those families harbouring nDNA mutations where classic Mendelian rules of inheritance apply. Parents can be given much clearer results with regards as to whether or not their child will be affected. In keeping with current UK practice, at parental request we have also provided information on the carrier status of the foetus.[31]

For homoplasmic maternally transmitted mtDNA disease, where PGD is not an option, further strategies are being developed. Techniques such as pronuclear transfer, where male and female pronuclei from the patient's fertilised oocyte are transferred to an enucleated healthy fertilised donor oocyte, and metaphase II spindle transfer, which involves the transfer of the metaphase II spindle from a mature affected oocyte into an enucleated healthy donor oocyte before fertilisation, are at an advanced stage of development with successful metaphase II spindle transfer having been performed in Macaque monkeys.[32–34] In March 2013, the Human Fertilisation and Embryology Authority (HFEA) agreed its advice to UK Government on the ethics and science of IVF-based techniques designed to prevent the transmission of maternally inherited mitochondrial disease. Further work assessing the safety, efficacy and applicability of this technique is planned (www.hfea.gov.uk/6896.html).

CONCLUSION

We have experienced an increasing demand for prenatal diagnostic testing from families affected by mitochondrial disease, with 62 prenatal diagnoses made since the service was first offered in 2007 and at least 11 cases of mitochondrial disease prevented.

Information on reproductive options including prenatal diagnosis (CVB or amniocentesis), egg donation and sperm donation, techniques to reduce or prevent transmission (eg, PGD), or adoption as an alternative to pregnancy, should be presented to all families affected by mitochondrial disease to facilitate informed reproductive choices. Pronuclear transfer and metaphase II spindle transfer continue to be developed, but are not yet available. The present availability of PGD and the future possibility of nuclear transfer mark an exciting phase in preventing the transmission of mitochondrial disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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