Cardiac manifestations of mitochondrial disease

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Abstract

Due to critical dependence of the heart on oxidative metabolism, cardiac involvement in mitochondrial disease is common and may occur as the principal clinical manifestation or part of multisystem disease. The basic features of cardiac mitochondrial disease expression remain uncertain and no effective treatment exists.

Previous research has suggested that cardiac involvement in mitochondrial disease is an important cause of morbidity and early mortality in paediatric populations. In this thesis, a retrospective study confirms the frequent occurrence of cardiac involvement in adults, and demonstrates a significant impact on survival; the importance of specific mt-tRNA mutations and age of symptom onset as predictors of cardiac involvement is also highlighted. Conversely, in children with end-stage cardiomyopathy of unknown aetiology, a prospective study identifies respiratory chain disease as an important cause of disease, altering patient management in a high-risk population in whom mitochondrial disease was not suspected.

Using histochemical and immunohistochemical analysis of cardiac tissue, profound complex I deficiency is demonstrated in all cardiomyocytes displaying any evidence of COX-deficiency but also in cells *without* COX deficiency, supporting the primacy of this factor in patients with well-characterised mt-tRNA mutations. Differences in cardiac complex I expression between patients harbouring m.3243A>G and m.8344A>G mutations may impact on cardiac phenotype; chamber-specific respiratory chain abnormalities are noted and, while tissue segregation may play a role in frequency and severity of cardiac involvement, skeletal muscle mitochondrial DNA mutation load is not a consistent marker of risk.

Advanced imaging techniques are used to demonstrate early concentric hypertrophic remodelling, and specific changes in intramyocardial strains and torsion, in patients harbouring the m.3243A>G or m.8344A>G mutations *without* clinical evidence of cardiac involvement. However, an endurance exercise interventional study shows that patients experience comparable cardiac hypertrophic and haemodynamic adaptations to sedentary controls and confirms the safety and efficacy of 16 weeks' training.

"As big as a cloud"

For William and Emma, who didn't exist when this all started, and are probably still a few years off reading it…

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Author's Declaration

This thesis is submitted for the degree of Doctor of Philosophy to Newcastle University. The research was performed within the Wellcome Trust Centre for Mitochondrial Research, Institute for Ageing and Health; the Clinical Research Facility, Royal Victoria Infirmary; and the Magnetic Resonance Centre, Newcastle University, and is my own work unless otherwise stated. All work was carried out under the supervision of Professors Douglass Turnbull, Robert Taylor and Bernard Keavney between August 2009 and September 2012. Throughout this time, I was funded by a Wellcome Trust Clinical Research Training Fellowship (BH092142).

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

Matthew Bates

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Publications

Data from studies in this thesis have been published and presented as listed below.

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Galna B, Newman JH, Jakovljevic DG, Bates MGD, Schaefer AM, McFarland R, Turnbull DM, Trenell MI, Gorman GS, Rochester L. Discrete gait characteristics are associated with m.3243A>G and m.8344A>G variants of mitochondrial disease and its pathological consequences. Journal of Neurology 2014;261:73-82.

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Online publications:

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Abbreviations

1D	One-dimensional
2D	Two-dimensional
A-VO_2 diff	Arterio-venous oxygen difference
AAR	Adaptive auto-regressive
ACE	Angiotensin-converting enzyme
AF	Atrial fibrillation
ANT	Adenine nucleotide translocator
ARB	Angiotensin receptor blocker
AT	Anaerobic threshold
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AV	Atrio-ventricular
AVB	Atrio-ventricular block
BER	Base excision repair
BH	BCL-2 homology
BMI	Body mass index
BPV	Blood pressure variability
BSA	Body surface area
BSE	British Society of Echocardiography
Ca^{2+}	Calcium ion
CM	Cristae membrane
CoA	Coenzyme A
COMPASS	Composite Autonomic Symptom Score
COX	Cytochrome *c* oxidase
CPEO	Chronic progressive external ophthalmoplegia
CRT-D	Cardiac resynchronization therapy with a defibrillator
CSB	Conserved sequence blocks
CSI	Chemical shift imaging
D-loop	Displacement loop
DAB	3,3’-diaminobenzidine

DBP	Diastolic blood pressure
DCM	Dilated cardiomyopathy
DNA	Deoxyribonucleic acid
DPG	Diphosphoglycerate
Drp	Dynamin-related protein
E/A ratio	Ratio of early to late ventricular filling velocity
ECG	Electrocardiograph
ECMO	Extra-corporeal membrane oxygenation
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
EMB	Endomyocardial biopsy
EPS	Electrophysiological study
ETC	Electron transport chain
FA	Flip angle
FAD	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
Fe-S	Iron-sulphur
FIS	Fatigue Impact Scale
FMN	Flavin mononucleotide
FOV	Field of view
GIT	Gastro-intestinal tract
GTP	Guanosine triphosphate
H & E	Haematoxylin and eosin
H strand	Heavy strand
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
HCM	Hypertrophic cardiomyopathy
HF	High Frequency
HRV	Heart rate variability
HSP	Heavy strand promoter
IBM	Inner boundary membrane

ICD	Implantable cardioverter defibrillators
IMS	Intermembrane space
KSS	Kearns-Sayre syndrome
L strand	Light strand
LF	Low Frequency
LGE	Late gadolinium enhancement
LHON	Leber's hereditary optic neuropathy
LSP	Light strand promoter
LV	Left ventricular
LVH	Left ventricular hypertrophy
LVMI	Left ventricular mass index
LVNC	Left ventricular non-compaction
LVOT	Left ventricular outflow tract
M/V ratio	LV mass to end-diastolic volume ratio
mCU	Mitochondrial calcium uniporter
MDRD	Modified diet in renal disease
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged red fibres
MHC	Mental health component
MIDD	Maternally inherited diabetes and deafness
MMR	Mismatch repair
MNGIE	Mitochondrial neuro-gastrointestinal encephalopathy
mnSOD	manganese superoxide dismutase
MPT	Mitochondrial permeability transition
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MRP	Mitochondrial RNA processing
MRS	Magnetic resonance spectroscopy
mt-RPOL	Mitochondrial RNA polymerase
mt-rRNAs	Mitochondrial rRNA

mt-tRNA	Mitochondrial tRNA
mtDNA	Mitochondrial DNA
mTERF	Mitochondrial termination factor
mtSSB	Mitochondrial single-stranded DNA binding
NAD	Nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia, pigmentary retinopathy
NBT	Nitro Blue Tetrazolium
NMDAS	Newcastle Mitochondrial Disease Adult Scale
NYHA	New York Heart Association
O_2^-	superoxide anion
OH^-	hydroyl radical
OM	Outer membrane
ORF	Open reading frame
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PDC	Pyruvate dehydrogenase complex
PHC	Physical health component
P_i	Inorganic phosphate
POLG	Mitochondrial polymerase γ
POLRMT	Mitochondrial RNA polymerase
POTS	Postural orthostatic tachycardia syndrome
PPM	Permanent pacemaker
PSD	Power Spectral Density
RCM	Restrictive cardiomyopathy
RER	Respiratory exchange ratio
RFA	Radio-frequency ablation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Rating of Perceived Exertion
RRI	RR interval

rRNA	Ribosomal RNA
SBP	Systolic blood pressure
SD	Standard deviation
SDH	Succinate dehydrogenase
SF-12	Short Form 12
SNARE	Soluble *N*-ethylaleimide-sensitive factor attachment protein receptor
SNHL	Sensori-neural hearing loss
SNP	Single nucleotide polymorphism
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TE	Echo time
TFAM	Mitochondrial transcription factor A
TFM	Task Force Monitor
TIA	Transient ischaemic attack
TR	Repetition time
tRNA	Transfer RNA
TSR	Torsion to endocardial circumferential strain ratio
U	Uracil
UDG	Uracil DNA glycosylase
VAD	Ventricular assist device
VDAC	Voltage-dependent anion channel
VLF	Very Low Frequency
VO_2	Peak oxygen uptake
VSD	Ventricular septal defect

Chapter 1.

Introduction

Mitochondria

1.1.1 Mitochondrial origins

Mitochondria are believed to have originated from free-living eubacteria that became entrapped in primitive eukaryotes around 1.5 billion years ago (Gray, 1992). The endosymbiotic theory states that bacterium and host form a symbiotic relationship: the bacterium gaining access to metabolisable substrate in the host cell while the eukaryote obtains access to adenosine triphosphate (ATP), derived through bacterial respiration (Margulis, 1971). The alternative hydrogen hypothesis suggests that eukaryotes arose from the symbiotic association of an 'anaerobic, strictly hydrogen-dependent, strictly autotrophic archaebacterium' and an eubacterium, capable of anaerobic, heterotrophic metabolism, that produced molecular hydrogen through respiration (Martin and Muller, 1998).

While the selective principles underlying the symbiotic relationship differ, both theories agree that the primitive eukaryote that engulfed the eubacterium was of archaebacterial origin, and that, over a lengthy period of reductive evolution, the endosymbiont became a highly dependent organelle with transfer of the majority of genes to the host nucleus. The original proposal that eukaryotes and archaebacteria possessed distinct ancestral lineage developed from the observation that some eukaryotes appeared not to contain mitochondria (Cavalier-Smith and Chao, 1996). However Müller's subsequent discovery of hydrogenosomes in some amitochondrial protozoa, the observation that underpinned the novel hydrogen hypothesis, led to the opposing belief that the first eukaryote ancestor already contained an endosymbiont. Recent research has demonstrated mitochondrial remnant organelles (mitosomes) in *Giardia intestinalis* (Tovar *et al*., 2003) and orthologous genes in microsporidia (Hirt *et al.*, 1997), finally establishing the endosymbiotic engulfment as an event preceding the divergence of the diplomonads and proving that the amitochondrial state of some eukaryotes is the result of drastic reductive evolution and not a primitive condition, as originally suggested.

1.1.2 Mitochondrial structure

Mitochondria are relatively large organelles approximately 1-2µm in length and 0.5-1.0µm in diameter. The critical relationship between mitochondrial structure and function is highlighted by the double plasma membrane that supports the eubacterial origin of mitochondria and compartmentalises the organelle (Palade, 1953). The outer membrane (OM) has a similar biochemical structure to the cell membrane and forms the boundary that separates the cytosol from the intermembrane space. An abundance of the voltage-dependent anion channel (VDAC) protein, porin, renders this membrane relatively permeable, allowing passive diffusion of low molecular weight molecules (<10kDa). The inner membrane is intrinsically impermeable to polar molecules and ions due to the absence of porins and the presence within the membrane of protein complexes that unfavourably alter the protein-phospholipid ratio. This membrane surrounds the central mitochondrial matrix that contains the mitochondrial genome; critical components of mitochondrial DNA (mtDNA) replication, transcription and translation; and enzymes involved in iron-sulphur (Fe-S) cluster production, the tricarboxylic acid cycle and the ß-oxidation of fatty acids (Alberts *et al.*, 2008).

Early electron microscopy studies noted that mitochondria possessed a 'system of internal ridges', termed *cristae mitochondriales* (Figure 1.1), that were suggested to be deep folds of the inner membrane (Palade, 1953). Different structural models were proposed including the 'baffle' model, commonly depicted in textbook illustrations of mitochondria (Palade, 1953), and the 'septa' model that postulated complete subdivision of the mitochondrial matrix into multiple compartments (Sjostrand, 1953). However later electron microscopic tomography studies established the pleomorphic and extensively-branching tubular nature of cristae (Figure 1.2), and revealed that the inner membrane represents two contiguous but distinct membranes connected at cristae junctions (Mannella *et al.*, 1997). The importance of this division of the inner membrane into inner boundary membrane (IBM) and cristae membrane (CM) was emphasised further in subsequent electron micrographic studies using quantitative immunohistochemistry that demonstrated differential protein content between IBM and CM (Vogel *et al.*, 2006).

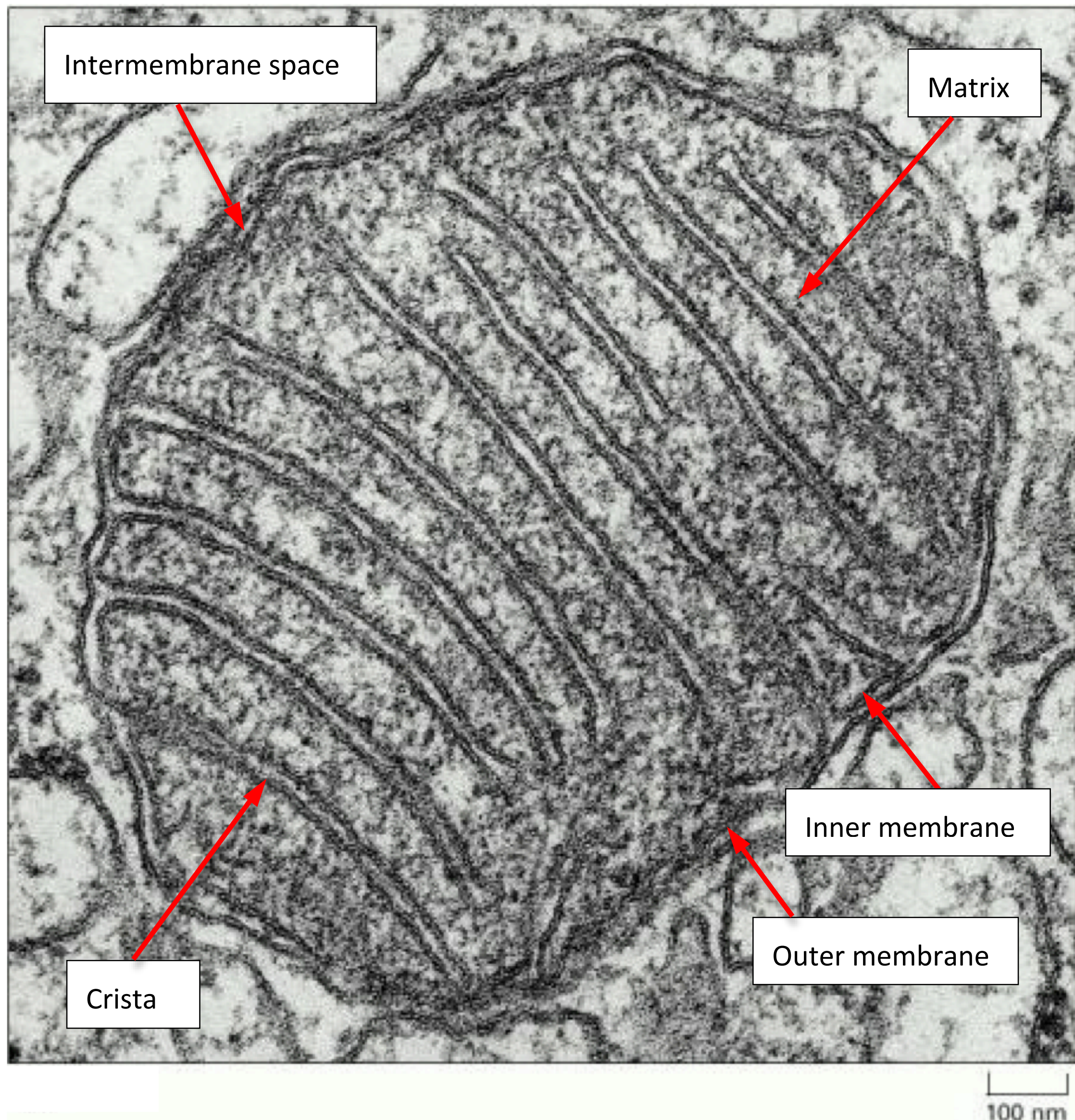


Figure 1.1 Electron micrograph of a mitochondrion. Early cross-sectional images revealed the inner and outer membranes of the mitochondrion and helped to establish the popular but over-simplistic 'baffle' structural model. Image modified from Molecular Biology of the Cell 5th Edition (Alberts *et al.*, 2008).

Consistent with the increased surface area provided by the presence of cristae, the CM is enriched in proteins involved in oxidative phosphorylation, Fe-S cluster biogenesis and the synthesis and transport of mtDNA-encoded proteins, while the IBM contains more proteins involved in mitochondrial fusion and transport of nuclear-encoded proteins. Although one interpretation of this finding would be that cristae junctions between IBM and CM represent absolute barriers to diffusion of membrane

components, the study authors actually concluded that the differential protein content arose through an alternative mechanism of ‘dynamic subcompartmentalization’. Already appreciating the importance of complex V of the respiratory chain in maintaining cristae morphology (Giraud *et al.*, 2002), they proposed that this complex may in fact localise other complexes (and indeed supercomplexes) in the CM, while interactions between the OM and the IBM may explain the specific protein content in this region (Vogel *et al.*, 2006). Central to this theory was the proposal that subcompartments of the inner membrane are dynamic and *not* separated by a rigid barrier at the cristae junction, thus permitting alterations in the distribution of proteins in response to stimuli (Vogel *et al.*, 2006; Zick *et al.*, 2009).

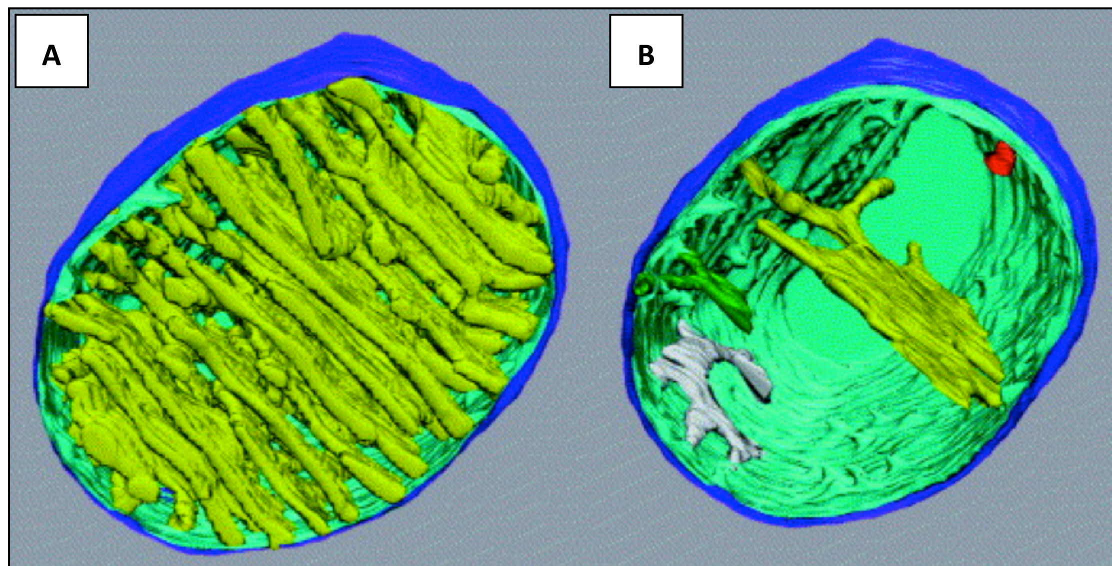


Figure 1.2 Computer model of mitochondrial membranes. Three-dimensional electron microscopic tomograms have been utilised to generate accurate models of mitochondrial membrane topography. (A) Complete model showing cristae (yellow), inner boundary membrane (green) and outer membrane (blue). (B) Partial model again showing inner boundary membrane (green) and outer membrane (blue) with four individual cristae in different colours, highlighting the pleomorphic nature of cristae. Image modified with permission from the original image (Frey and Mannella, 2000).

1.1.3 Mitochondrial dynamics

Although frequently depicted, and conveniently conceived, as discrete intra-cellular organelles, mitochondria are now known to exist as a dynamic network that is constantly remodelled through the processes of mitochondrial fission and fusion (Alberts *et al.*, 2008). Together these processes act to regulate the morphology, distribution and activity of mitochondria and therefore play a critical role in function.

Binary fission of mitochondria, further evidence of the eubacterial origin of the organelle, is dependent on the dynamin-related protein Drp1 and mutations in this gene cause highly interconnected mitochondria (Smirnova *et al.*, 2001). This GTPase localises to sites of fission and has been proposed as a mechanochemical enzyme that uses GTP hydrolysis to enable membrane division through constriction similar to the yeast orthologous protein Dnm1 (Ingerman *et al.*, 2005). Although the mechanisms underlying Drp1 recruitment from the cytosol to the OM are currently unclear, the small protein Fis1 likely plays a significant part in this process (James *et al.*, 2003), analogous to its role in Dnm1 recruitment in yeast (Mozdy *et al.*, 2000). Surprisingly, knockdown of Fis1 in mammalian cells does not affect Drp1 mitochondrial localization (Lee *et al.*, 2004).

Mitochondrial fusion involves the coordinated action of further GTPases localized to the OM. Mammalian cells posses two such mitofusins, Mfn1 and Mfn2, identified as homologues to the Drosophila protein, Fzo (Santel and Fuller, 2001). Mfn1 and Mfn2 knockout mice display fragmented mitochondria and mid-gestational lethality, but Mfn-null cells can be rescued by over-expression of either mitofusin indicating some functional degeneracy (Chen *et al.*, 2003). Both mitofusins contain two heptad repeats (HR1 and HR2), similar to the SNARE (soluble *N*-ethylaleimide-sensitive factor attachment protein receptor) proteins involved in vesicle docking, that act as physical tethers before fusion (Koshiba *et al.*, 2004). In addition to mitofusins, the dynamin family GTPase OPA1 is essential for mitochondrial fusion (Cipolat *et al.*, 2004). This intermembrane space protein associates with the inner membrane and knockout mice can undergo outer but not inner membrane fusion, demonstrating potential uncoupling of these two processes in mammals (Song *et al.*, 2009).

1.2 Mitochondrial Functions

While the principal function of mitochondria remains ATP synthesis via oxidative phosphorylation (OXPHOS), these organelles are also critically involved in other essential cellular processes including calcium homeostasis, the generation of reactive oxygen species, apoptosis and Fe-S cluster biogenesis.

1.2.1 Oxidative phosphorylation

Pyruvate, generated together with the reduced co-factor nicotinamide adenine dinucleotide (NADH) and net gain of two ATP molecules through cytosolic glycolysis, is transported across the double membrane to the mitochondrial matrix (Berg *et al.*, 2007). There the pyruvate dehydrogenase complex (PDC) catalyses the decarboxylation of pyruvate to produce further NADH and acetyl Coenzyme A (CoA). Fatty acids are similarly activated in the cytosol and transported into mitochondria in the form of fatty acyl CoA. Through the 4 enzymatic steps of ß-oxidation, two carbons are removed from fatty acyl CoA to produce further acetyl CoA. In the tricarboxylic acid cycle, the acetyl group of acetyl CoA is transferred to oxaloacetate and, through a series of redox reactions, oxaloacetate is regenerated and CO_2 is produced along with further NADH, the alternative reduced co-factor flavin adenine dinucleotide ($FADH_2$) and one molecule of guanosine triphosphate (GTP).

OXPHOS is the final common pathway of aerobic respiration. The reduced cofactors NADH and $FADH_2$ are utilised in a process that releases ATP and water as a result of electron transport to O_2. Four enzyme complexes (I-IV), embedded in the mitochondrial inner membrane, form the electron transport chain (ETC) (Figure 1.3). Electrons pass through this chain from lower to higher redox potential compounds, releasing free energy that is then utilised to pump hydrogen ions (H^+) across the mitochondrial inner membrane and into the intermembrane space via complexes I, III and IV. An electro-chemical proton gradient is thereby generated, due to the impermeable nature of the inner membrane, and this provides the proton motive force that is utilised by complex V. Proton transport back into the mitochondrial matrix is linked to generation of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i), in a process described as ‘chemi-osmotic coupling’ (Mitchell, 1961).

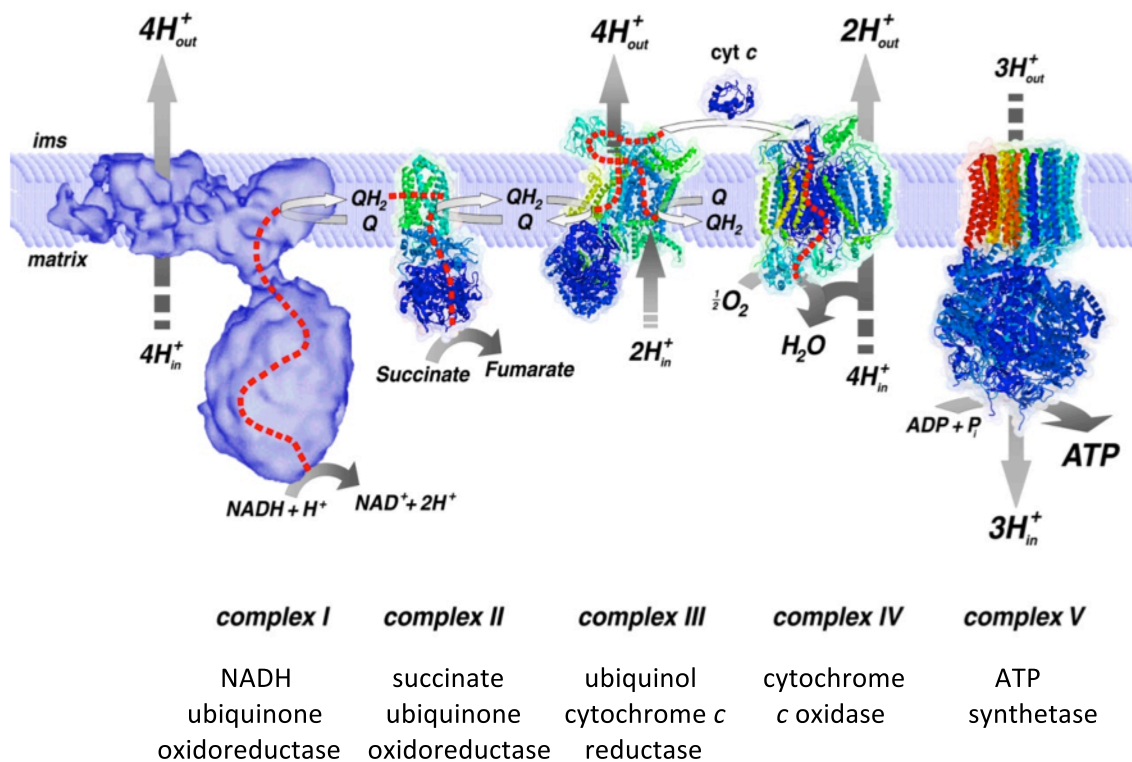


Figure 1.3 Schematic diagram of the mitochondrial electron transport chain. Complex I catalyses oxidation of NADH (produced via the TCA cycle and ß-oxidation of fatty acids) and reduction of ubiquinone (Q) to ubiquinol (QH_2), exporting 4 protons in the process. Complex II catalyses oxidation of succinate (via $FADH_2$) and reduction of further Q to QH_2, which is then re-oxidized by complex III, coupled to reduction of cytochrome *c* and export into the intermembrane space (IMS) of a further 4 protons (the Q cycle). Complex IV catalyses re-oxidation of cytochrome *c* and reduction of molecular oxygen to water, exporting 2 protons (Scheffler, 2008). Proton transfer back across the mitochondrial inner membrane into the matrix down an electro-chemical gradient via complex V generates ATP. Image modified with permission from original image (Nijtmans *et al.*, 2004).

The multi-subunit complexes of the ETC are all dually encoded by the mitochondrial and nuclear genomes with the exception of complex II (see Section 1.2.1.2), which is entirely nuclear encoded (Table 1.1). In addition to this bigenomic interaction, structural coordination between complexes I-V in the inner membrane is vital to the maintenance of the ETC. The binding of complexes I and III to form a structure known as a 'respirasome' is critical to the activity of complex I (Schagger *et al.*, 2004), and complex IV is often additionally bound to the complex I/III respirasome, forming a super-complex (Wittig and Schagger, 2009).

	Component gene location		mtDNA-encoded subunits
	Mitochondria	**Nucleus**	
OXPHOS enzyme complexes	13	76	
Complex I	7	38	ND1, ND2, ND3, ND4, ND4L, ND5, ND6
Complex II	0	4	-
Complex III	1	10	Cytochrome b
Complex IV	3	10	COX I, COX II, COX III
Complex V	2	14	ATPase 6, ATPase 8
Protein synthetic apparatus	24	>80	

Table 1.1 Gene location of mitochondrial components. The mitochondrial genome encodes 22 tRNAs, 2 rRNAs and 13 polypeptides that are all subunits of enzyme complexes I, III, IV and V of the ETC. Only complex II is entirely encoded by the nuclear genome. Table adapted with permission from original data (Taylor and Turnbull, 2005).

1.2.1.1 Complex I – NADH:ubiquinone oxidoreductase

Complex I is the largest enzyme complex of the ETC with a molecular mass ~1000kDa (Yamaguchi *et al.*, 2000) yet, despite recent advances in structural and functional knowledge, remains the least understood. Bovine complex I is composed of 45 subunits, seven of which are encoded by the mitochondrial genome (Hirst *et al.*, 2003). These subunits, encoded by the genes *MTND1-6* and *MTN4L*, together with seven other subunits encoded by nuclear genes, are all homologues to the 14 complex I subunits of prokaryotes and, being critical to the structure of complex I, are termed 'core subunits'. There is significant sequence conservation between eukaryotes and prokaryotes in these core subunit genes and overall functions, cofactors and inhibitors are identical. Thirty-one 'accessory subunits' complete complex I but their functions have been the subject of debate (Yamaguchi *et al.*, 2000; Yadava *et al.*, 2008).

The structure of complex I is L-shaped such that one membrane arm, containing relatively more hydrophobic subunits, is located within the lipid bilayer while the other peripheral arm extends into the mitochondrial matrix (Figure 1.4). All 7 mtDNA-encoded subunits are hydrophobic with numerous transmembrane helices (Hofhaus *et al.*, 1991; Zickermann *et al.*, 2009). The membrane arm of complex I contains the mtDNA-encoded subunits ND2, ND4 and ND5 that are homologous proteins and share distinct sequence similarity to the sodium-proton/potassium antiporter family. Recent studies reporting the X-ray structure of the membrane arm of complex I have confirmed the role of these proteins in the proton-pumping machinery that transports 4 protons across the inner membrane for every 2 electrons donated by NADH (Figure 1.4). A long, horizontal α-helical protein acts as a connecting rod coordinating the opening and closing of the antiporter-like channels in these 3 subunits (Efremov *et al.*, 2010; Ohnishi, 2010). The peripheral arm of complex 1 (Figure 1.4) contains all redox-active cofactors and can be further subdivided into N and Q modules. In the N module, a flavin mononucleotide (FMN) acts as the primary electron acceptor in the oxidation of NADH, with electrons subsequently transferred via 7 Fe-S clusters to the junction with the Q module, where Fe-S cluster N2 donates electrons directly to ubiquinone. Two further Fe-S clusters do not participate in this 'electron wire' and may serve alternative functions: N1a appears to play a role in the control of reactive oxygen species (ROS) generation (Esterhazy *et al.*, 2008), while N7 is essential for complex I assembly (Pohl *et al.*, 2007).

Complex I assembly is incompletely understood but most suggested models agree that the process is modular in nature with semi-sequential combination of large sub-assemblies forming the complete complex (Ugalde *et al.*, 2004; Lazarou *et al.*, 2009). Recently the use of complex I mutant cell lines derived from patients with mutations in different subunits has provided further detail about complex I assembly (Perales-Clemente *et al.*, 2010; Mimaki *et al.*, 2012), involving different entry points for critical mtDNA-encoded subunits (Figure 1.5).

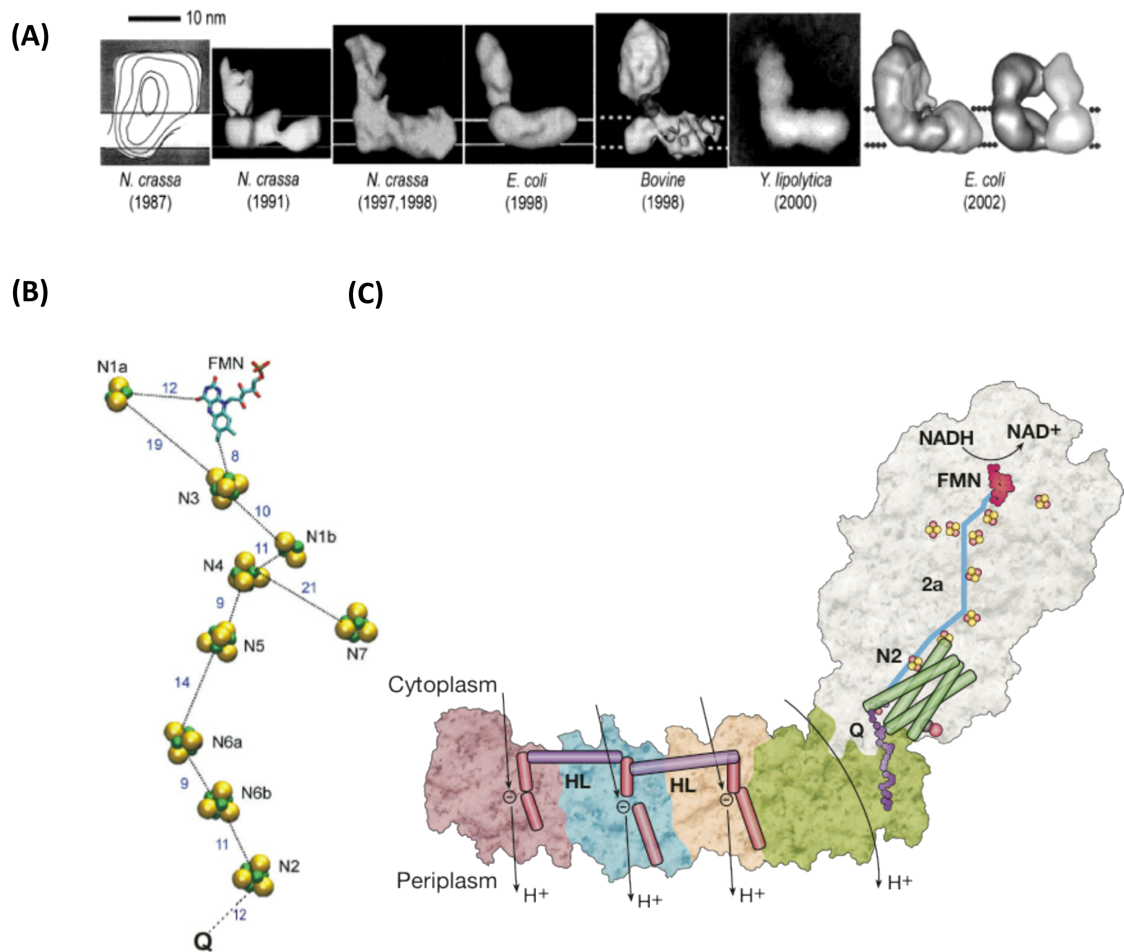


Figure 1.4 Structure and function of complex I. (A) Electron microscopic studies, initially in *Neurospora crassa*, revealed the characteristic and evolutionary conserved L-shape of complex I. Image modified with permission from original image (Hofhaus *et al.*, 1991). (B) The peripheral arm of complex I contains the 'electron wire' of 7 consecutive Fe-S clusters that transport electrons from the FMN (the primary electron acceptor from NADH oxidation) to the Q module, where N2 donates electrons to ubiquinone. Image modified with permission from original image (Verkhovskaya *et al.*, 2008). (C) Recent X-ray studies have demonstrated 3 repeated domains of fourteen transmembrane helices within the membrane arm of *E. coli* complex I. These mtDNA-encoded subunits, ND2, ND4 and ND5 (broken red bars) appear to function with a 'piston-like' mechanism achieved via a long, unusual amphipathic α-helix (purple bar) that acts as a connecting rod coordinating the simultaneous opening and closing of the antiporter-like channels. Schematic based on original image (Efremov *et al.*, 2010).

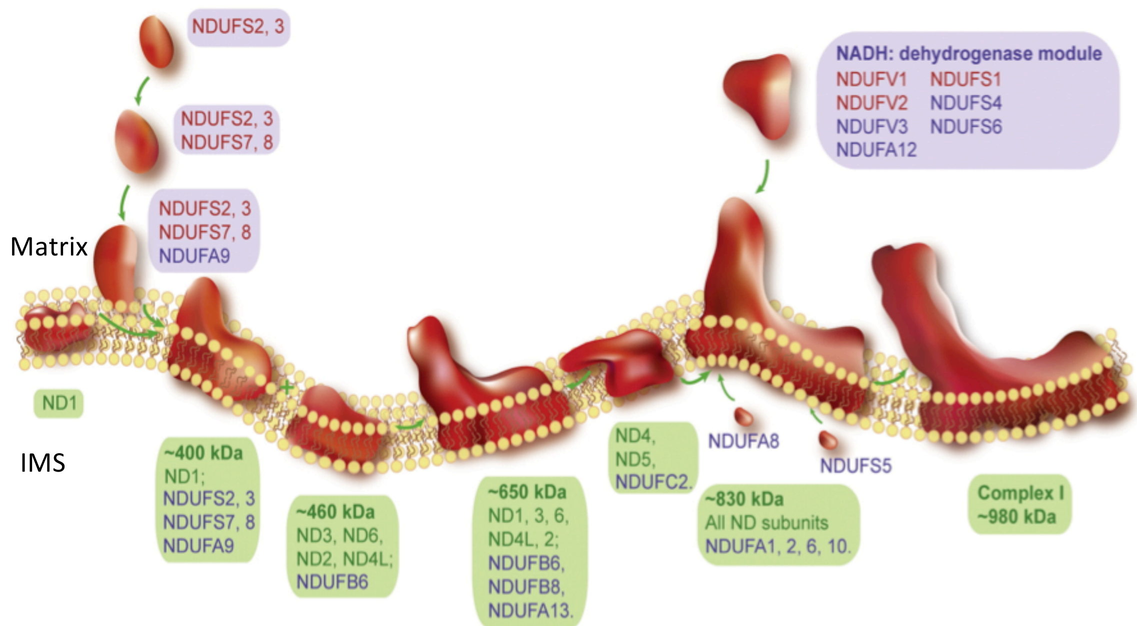


Figure 1.5 Schematic diagram of a proposed model of complex I assembly. Core subunits NDUFS2 and NDUFS3 form an early hydrophilic assembly complex, which expands with sequential incorporation of further hydrophilic subunits including NDUFS7, NDUFS8 and NDUFS9. This structure combines with a small membrane complex containing the mtDNA-encoded subunit ND1 to form ~400kDa assembly intermediate. Further integration with ~460kDa membrane complex containing ND3, ND6, ND2, ND4L and NDUFB6 forms the larger ~650kDa intermediate, and subsequent association with another membrane complex containing ND4, ND5 and NDUFC2 produces ~830kDa subunit. Another hydrophilic complex, the NADH dehydrogenase module (N module) is assembled using nuclear DNA-encoded subunits directly or indirectly involved in NADH oxidization. With addition of the N module and remaining subunits, including NDUFA8 and NDUFS5, mature complex I is assembled. Core subunits are displayed (red), together with remaining nuclear DNA-encoded subunits (blue) and mtDNA-encoded subunits (green), highlighting 'entry points' for mtDNA subunits in complex I assembly (Perales-Clemente *et al.*, 2010). Image modified with permission from original image (Mimaki *et al.*, 2012).

1.2.1.2 Complex II – Succinate:ubiquinone oxidoreductase

Succinate:ubiquinone oxidoreductase is the smallest complex of the ETC, the only complex to be entirely encoded by the nuclear genome and the only enzyme to participate in both the Tricarboxylic acid (TCA) cycle and the ETC. Complex II is made up of only 4 structural subunits. There are two subunits in the catalytic domain – subunit A is covalently bonded to the redox cofactor FAD while subunit B features 3 Fe-S clusters. Subunits C and D constitute the hydrophobic domain that anchors the complex in the inner mitochondrial membrane (Ikeuchi *et al.*, 2005).

Complex II catalyses the oxidation of succinate to fumarate linked to the reduction of FAD to $FADH_2$. The re-oxidation of $FADH_2$ is then coupled to the reduction of ubiquinone via two separate single electrode reactions at two ubiquinone binding sites (Ikeuchi *et al.*, 2005). Unlike at complexes I, III and IV, the passage of electrons through the redox centres of complex II does not release enough free energy to translocate protons across the mitochondrial inner membrane.

1.2.1.3 Ubiquinone (Coenzyme Q)

Ubiquinone is a small, mobile, hydrophobic molecule located in the inner mitochondrial membrane. It acts as a shuttle between enzyme complexes in the ETC, participating in a two-step sequential oxidation. Reduction of ubiquinone by a single electron forms the intermediate ubisemiquinone, while complete reduction by a second electron results in formation of ubiquinol.

1.2.1.4 Complex III – Ubiquinol:cytochrome c oxidoreductase

Complex III, with a molecular mass of ~248kDa, contains only one mtDNA-encoded subunit, cytochrome *b*, and 10 other subunits encoded by the nuclear genome (Table 1.1). The essential redox components of complex III are two *b*-type hemes, b_L and b_H, a *c*-type heme (c_1), and Fe-S cluster (the Rieske centre) and ubiquinone. Complex III catalyses the transfer of two electrons from ubiquinol to cytochrome *c*, through a complex series of redox reactions. This process is linked to the translocation of two further protons across the mitochondrial inner membrane into the intermembrane space by the proton-motive Q cycle (Mitchell, 1976). Mature, assembled complex III

also plays a significant role in complex I assembly and function. The co-dependence of the assembly of complexes I and III has been reported in mouse and human cultured cell models harbouring cytochrome *b* mutations, following appreciation that defects in genes encoding single complex III polypeptides can result in multiple complex deficiencies, affecting both complex I and III (Acin-Perez *et al.*, 2004).

According to the Q cycle model, a single electron is transferred from ubiquinol to the Rieske Fe-S centre, forming ubisemiquinone. Subsequently, this electron is then transferred to cytochrome c_1 and ultimately to cytochrome *c*. The newly-created ubisemiquinone then reduces the *b*-type heme b_L, which rapidly transfers an electron to b_H, and ubiquinone is then itself reduced by b_H to form a ubisemiquinone anion. Following commencement of a second Q cycle, a further ubiquinol molecule enters this pathway and the above process is repeated with reduction of the newly-created ubisemiquinone anion by b_H to form ubiquinol (Trumpower, 1990). Through this process, complex III is responsible for the production of superoxide anions (see Section 1.2.4).

1.2.1.5 Cytochrome c

Cytochrome *c* is a small, highly water-soluble, haem-containing protein encoded by a nuclear gene. Similar to ubiquinone, it acts as a shuttle between enzyme complexes in the ETC, specifically between complexes III and IV carrying a single electron. It has an additional role in the initiation of apoptosis (see Section 1.2.3)

1.2.1.6 Complex IV – Cytochrome c oxidase

Cytochrome *c* oxidase (COX) catalyses the oxidation of cytochrome *c* and the reduction of molecular oxygen to form water, coupled to the translocation of protons across the mitochondrial inner membrane. It is composed of 13 subunits, including three mtDNA-encoded subunits (COX I – III), and contains multiple metal ion sites for iron (haem$_a$ and haem$_{a3}$) copper (Cu_A and Cu_B), zinc and magnesium (Tsukihara *et al.*, 1996). The catalytic centre of the enzyme is formed by mtDNA-encoded subunits, COX I and COX II, whereas COX III, which associates in the transmembrane domain with COX I and II, may play a role in proton pumping (Wilson and Prochaska, 1990). Nuclear-encoded

subunits modulate enzyme activity (Arnold *et al.*, 1997) and confer stability to the fully assembled complex (Galati *et al*., 2007).

Assembly of complex IV is incompletely understood but follows a stepwise process via several intermediate subcomplexes (Tsukihara *et al.*, 1996). The assembly of COX I from apo-MTCO1 is an initial step in this sequence, and COX I and COX II are generally accepted to be accurate indicators of completed complex IV assembly, due to the quaternary structure of the enzyme complex and the central transmembrane location of these subunits (Tsukihara *et al.*, 1996; Nijtmans *et al.*, 1998). Assembly of complex IV is discussed in further detail in Section 3.5.3.3.

The electron shuttle cytochrome *c* binds to cytochrome *c* oxidase on the intermembrane space side of the enzyme complex and donates a single electron to the primary electron donor, Cu_A (Figure 1.6). This electron is transferred to $haem_a$ and subsequently to the active catalytic site, which contains $haem_{a3}$ and Cu_B. In total four electrons are required for the formation of water from molecular oxygen, with each electron donation to complex IV associated with a change in physical state of the active site and linked to the transfer of a single proton across the inner membrane (Faxen *et al.*, 2005). Denoted by the number of electrons transferred, the state transitions of the catalytic site of cytochrome *c* oxidase are: O^0 at baseline; E^1 with reduction of Cu_B; P^2 or 'peroxy' state with reduction of $haem_{a3}$, named due to the ability to bind oxygen that this change confers to the active site; F^3 or 'ferryl' state with further reduction; and finally O^4 or 'oxidised' state. This process completes the cycle, as the oxidised state of O^4 is the same as the initial state of O^0 (Faxen *et al.*, 2005)

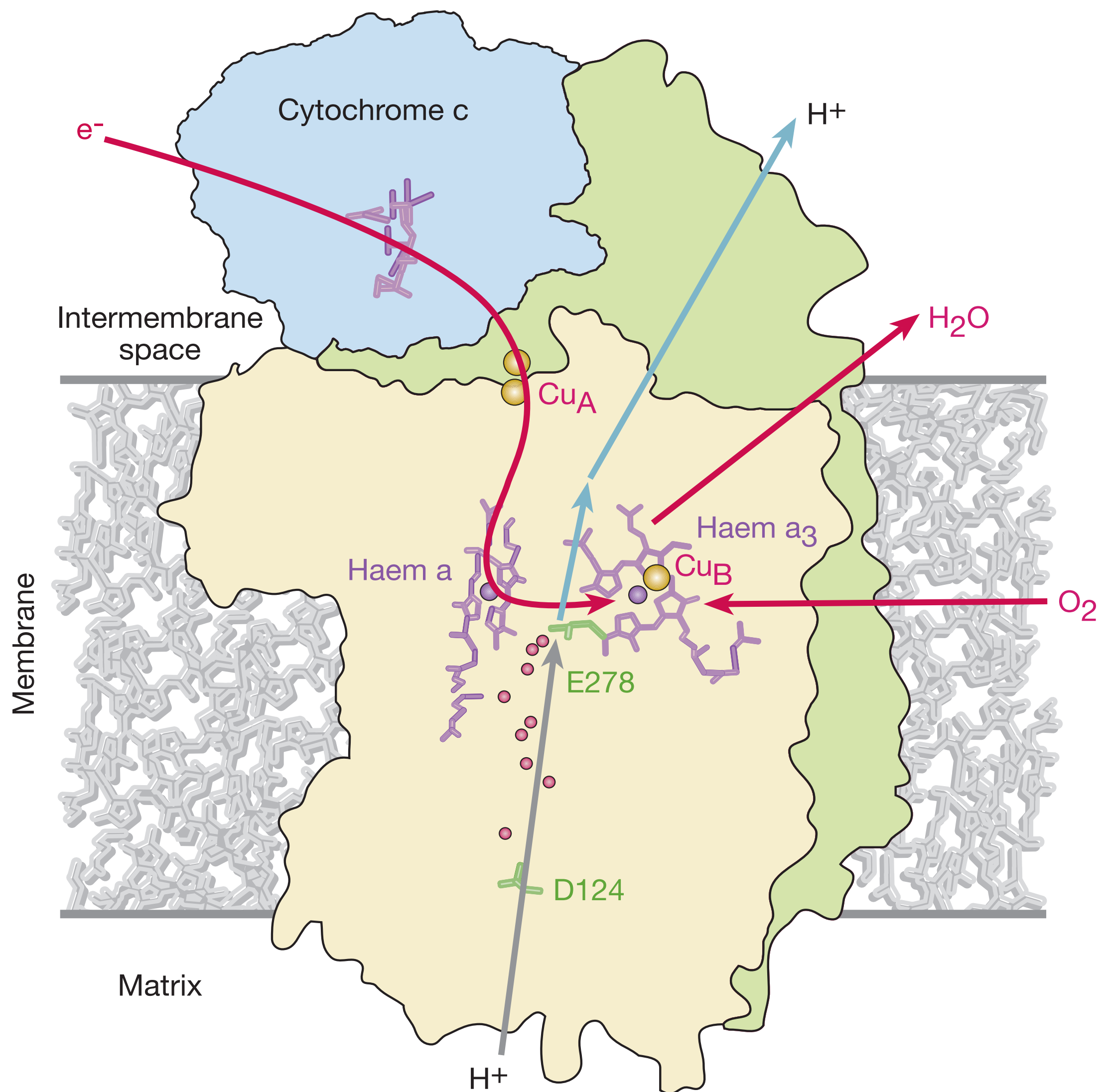


Figure 1.6 Schematic diagram of complex IV core. Cytochrome *c* (blue) donates single electrons to the primary electron donor (Cu_A) in the COX I subunit (yellow) that associates with the COX II subunit (green) in the inner mitochondrial membrane. Each electron is transferred to the active site that contains further metal binding sites ($Haem_{a3}$ and Cu_B). Molecular oxygen is the terminal electron acceptor and these redox reactions are linked to the translocation of protons from the matrix to the intermembrane space. This transfer occurs via the D pathway (grey) in which D124 and E278 signify important COX I amino acids (in *Paracoccus denitrificans*). Schematic based on the original image (Belevich *et al.*, 2006).

1.2.1.7 Complex V – ATP synthase

Complex V catalyses the generation of ATP from ADP and inorganic phosphate (Pi), linked to proton flow down the electro-chemical gradient created by the actions of complexes I – IV. ATP synthase is a large complex of ~500kDa and consists of 16 subunits, two of which are encoded by the mitochondrial genome (ATP 6 and 8).

Structurally complex V contains two distinct fractions (F_0 and F_1): F_0 is located in the mitochondrial inner membrane and consists of an *ab* complex and 8 helical, hydrophobic *c* subunits that form a proton channel; F_1 is a globular, water-soluble domain situated on the matrix side of the membrane that contains the active sites for ADP and Pi, and is composed of five different types of subunit (Figure 1.7).

Both F_0 and F_1 are rotary motors that can act in either direction to synthesise or hydrolyse ATP (Elston *et al.*, 1998). Utilising the electro-chemical gradient generated by complexes I – IV, protons flow from the intermembrane space to the matrix via the channel in F_0. Sequential protonation and deprotonation of glutamate residues within the channel formed by the c subunits of F_0 results in mechanical rotation of the whole domain (Elston *et al.*, 1998; Watt *et al.*, 2010). This action is transmitted via γ and ε subunits to cause rotation of F_1; and this movement then causes sequential conformational changes in the domain that enable binding of ADP and P_i at the active site, synthesis of ATP, and release of the catalytic product. Translocation of 8 protons across the mitochondrial inner membrane is required for complete 360 rotation of F1, which produces 3 molecules of the ATP yielding a bioenergetic cost of 2.7 H^+ / ATP molecule (Watt *et al.*, 2010).

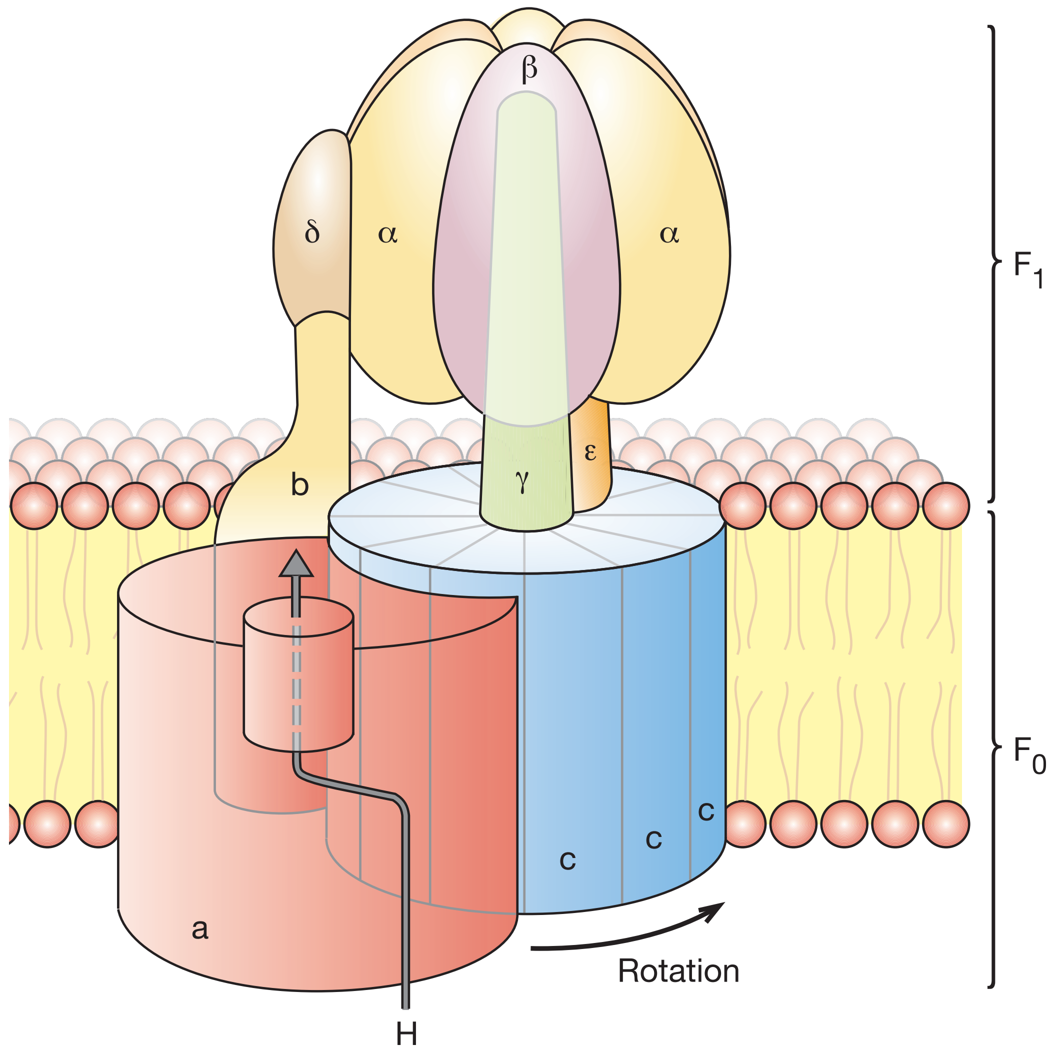


Figure 1.7 Schematic diagram of complex V. The F_0 portion of complex V is embedded in the mitochondrial inner membrane, while the F_1 portion is linked to the matrix side via a central stalk consisting of γ and ε subunits. Protons flowing down their electrochemical gradient through the central core of F_0 enable rotational conformational change in F_0 and subsequently F_1 via the γ and ε subunits. Sequential rotation of F_1 induces changes at the active catalytic site that enable ADP and P_i binding, ATP generation and ATP release. Schematic based on the original image (Alberts *et al.*, 2008).

1.2.1.8 Mitochondrial supercomplexes

Analysis of isolated bovine heart mitochondria using blue-native polyacrylamide gel electrophoresis (BN-PAGE) has demonstrated that, contrary to original beliefs, almost all complex I is associated with either complex III or complex III and IV (Schagger and Pfeiffer, 2000). Indeed the supercomplex formed of complexes I, III and IV represents the basic unit of mitochondrial respiration and is frequently termed a 'respirasome'. In this supercomplex, the binding sites for the mobile electron shuttles, ubiquinone and cytochrome *c*, are directly opposed on sequential complexes (Schafer *et al.*, 2007). The respirasome therefore improves the efficiency of electron transfer compared to the previous model reliant on random collision.

Supercomplex formation is also important in the assembly and stability of individual complexes of the ETC. The structure of complex I is highly dependent on the presence of the complex III dimer in both the common supercomplexes of I_1III_2 and $I_1III_2IV_1$ (Schagger *et al.*, 2004). This interaction between different complexes via the formation of supercomplexes modulates the effects of mtDNA mutations that encode individual ETC complex subunits. Recent studies in patient cell lines derived have suggested a central role for the COX I subunit in supercomplex stability – truncated COX I subunits are integrated into subcomplexes, the holocomplex and even into supercomplexes, but all these structures are unstable. Moreover the mechanism underlying pathogenicity in these patients appears to be rapid clearance of unstable respiratory complexes, as depletion of specific responsible proteases rescues the severe biochemical phenotype (Hornig-Do *et al.*, 2012).

1.2.1.9 Adenine Nucleotide Translocator

The adenine nucleotide translocator (ANT), a member of the skeletal muscle- and heart-specific solute carrier family 25, SLC25A4, and encoded by the nuclear gene *ANT1*, is the most abundant protein within the mitochondrial inner membrane and catalyzes the exchange of ATP and ADP across this membrane, maintaining the ADP supply for complex V synthesis of ATP.

1.2.2 Calcium homeostasis

Calcium ions (Ca^{2+}) are important second messengers in numerous cellular pathways. Mitochondria play a fundamental role in calcium homeostasis controlling both Ca^{2+} uptake and release.

At high cellular Ca^{2+} concentrations, Ca^{2+} bind to the specific mitochondrial Calcium Uniporter (mCU) on the cytoplasmic side of the mitochondrial OM. The mCU, under regulatory control of the mitochondrial uptake 1 protein (MICU1), catalyzes the passive uptake of Ca^{2+} into the mitochondrial matrix, driven by the favourable negative membrane potential generated by the ETC (Perocchi *et al.*, 2010). Further to their role in calcium sequestration and therefore control of cytoplasmic Ca^{2+} signalling, the calcium handling machinery of mitochondria can directly modulate ATP synthesis in response to cellular signals. Within the matrix, Ca^{2+} act to increase the rates of proton extrusion, oxygen utilisation and ATP generation with direct activation of pyruvate, isocitrate and oxoglutarate dehydrogenases (McCormack *et al.*, 1990).

Calcium plays an important role in both necrotic and apoptotic cell death via the process of mitochondrial permeability transition (MPT). The opening of MPT pores, in response to mitochondrial Ca^{2+} overloading, causes mitochondria swelling via increased permeability of the mitochondrial inner membrane to solutes, with subsequent cytochrome *c* release and cell death. Various factors, including cellular toxins and ROS generation, can modulate this process by alteration of the Ca^{2+} threshold or synergistic participation in MPT pore opening (Lemasters *et al.*, 2009).

1.2.3 Apoptosis

Apoptosis is a programmed form of cellular demise, distinct from the uncontrolled process of necrosis that is defined by premature cell death in response to injury. Two pathways of apoptosis have been described, which both converge on the cascading activation of caspases (cysteinyl aspartate-specific proteinases), which catalyze protein cleavage after aspartate residues (Nicholson and Thornberry, 1997). The extrinsic pathways is dependent on the activation of cell surface receptors by external agents (Figure 1.8), with recruitment and activation of procaspase-8 initiating a cascade of reactions culminating in activation of caspase-3 and DNA fragmentation. The intrinsic

pathway can be initiated by a variety of cellular factors but mitochondria coordinate the activation and execution of this important process (Wang and Youle, 2009).

The intrinsic pathway is regulated by the BCL-2 protein family (Figure 1.8), located primarily in the mitochondrial outer membrane (de Jong *et al.*, 1994), and the most potent signalling molecule of apoptosis is cytochrome *c* (Liu *et al.*, 1996). The BCL-2 proteins can be divided into anti-apoptotic and pro-apoptotic groups, that interact in a complex cascade in the intrinsic pathway (Figure 1.8). The anti-apoptotic proteins usually contain four BCL-2 homology (BH) domains, while pro-apoptotic proteins contain three such domains (e.g. BAK, BAX), or only one (e.g. BH3-only proteins BIM, PUMA). Ultimately BAK and BAX proteins enable cytochrome *c* release and, through recruitment of Drp-1, near simultaneous mitochondrial fission (Martinou and Youle, 2011). Following release, cytochrome *c* binds to APAF1 (Zou *et al.*, 1997), forming a heptameric protein called an apoptosome, with subsequent activation of caspases 3,the linking point of the intrinsic and extrinsic pathways, via caspase-9 (Youle and Strasser, 2008).

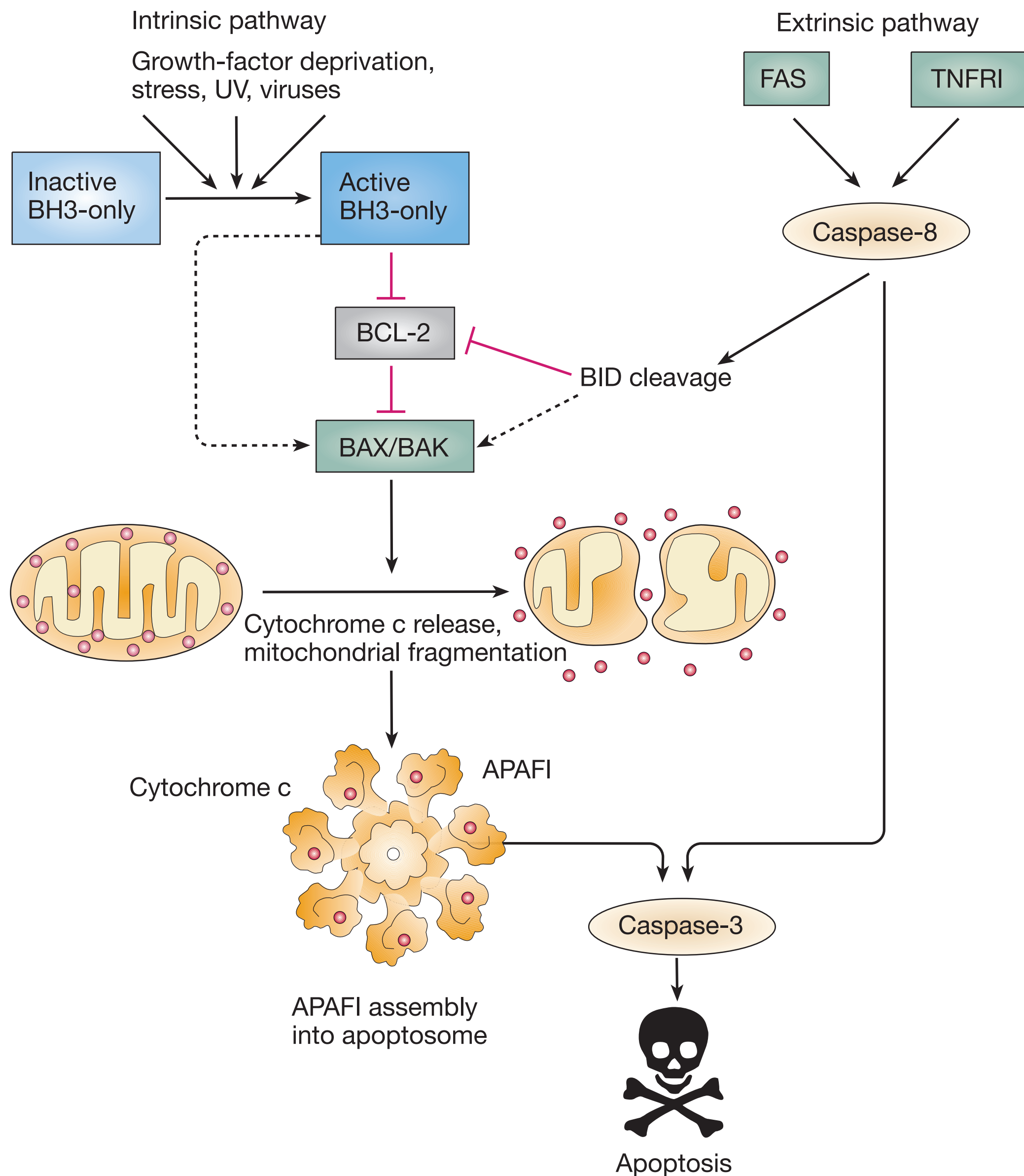


Figure 1.8 Intrinsic and extrinsic pathways of apoptosis. Apoptosis can be induced by intrinsic or extrinsic pathways. Cell surface receptors, such as FAS and tumour necrosis factor receptor-1 (TNFR1) initiate the extrinsic pathway with activation of the caspase cascade via caspase-8. Various factors including metabolic insults and genotoxic agents, can initiate the intrinsic pathway. BH3-only protein induction or post-translational activation results in inactivation of BCL-2 proteins, relieving inhibition of BAX and BAK activation, which promotes apoptosis. Some BH3-only proteins, such as BIM and PUMA, may be able to activate BAX and/or BAK (dotted line). Cytochrome *c*, release through mitochondrial fission, forms a heptameric protein ring with APAF1, called an apoptosome, which activates caspase-3. Whether resulting from activation of intrinsic or extrinsic pathway, the caspase cascade results in cleavage of substrates, activation of DNAses and coordination of cellular destruction. Cleavage of BID by caspase-8 links the two pathways, promoting apoptotic factor release. Image modified with permission from original image (Youle and Strasser, 2008).

1.2.4 Reactive oxygen species

Mitochondria are an important source of reactive oxygen species (ROS), such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroyl radical (OH^-), and contain a manganese superoxide dismutase (mnSOD). Mitochondria are therefore critically important in the control of ROS and their pathophysiological effects on diverse biological membranes and enzymes, including roles in cellular signalling pathways and the ageing process.

Generation of O_2^- occurs in complexes I, II and III of the ETC. Although principally formed in the matrix at complex I and II, O_2^- at complex III can escape from the intermembrane space to the cytosol via VDAC (Han *et al.*, 2003), acting as a signalling molecule related to hypoxia-induced transcription via HIF-1α (Chandel *et al.*, 2000), and autophagy, the process by which eukaryotic cells degrade and recycle macromolecules and organelles (Scherz-Shouval *et al.*, 2007).

The free radical theory of ageing, first proposed in 1954, states that the sum effect of deleterious free radical reactions occurring throughout life constitutes, or is a major contributor to, the ageing process (Harman, 2009). Due to the close proximity of the mitochondrial genome to a site of ROS generation in the ETC, it was later postulated that damage to mtDNA by ROS could cause an age-associated accumulation of inactive mitochondria with deficient ATP and protein synthesis and cellular deterioration (Fleming *et al.*, 1982). Although subsequent studies have demonstrated an age-associated accumulation of mtDNA mutations in tissues such as colon (Greaves *et al.*, 2006), skeletal muscle (Bua *et al.*, 2006), the heart (Nekhaeva *et al.*, 2002), and the brain (Bender *et al.*, 2006), the clinical importance of these acquired mtDNA mutations in the general population remains debated and the precise mechanisms linking ROS, mtDNA mutations and the ageing phenotype are unclear.

1.2.5 Iron-sulphur cluster biogenesis

Iron-sulphur (Fe-S) clusters play a critical role in numerous cellular processes, from OXPHOS to DNA repair (Lukianova and David, 2005), and their biosynthesis is an essential evolutionary conserved function of mitochondria (Tovar *et al.*, 2003). Despite

this critical importance, the precise mechanisms of Fe-S cluster biogenesis in humans are currently only partly understood.

Iron enters mitochondria through the mitochondrial solute carrier family (SLC25) protein, mitoferrin (Shaw *et al.*, 2006), facilitated by frataxin, a nuclear-encoded protein localised to mitochondria. Defects in the *FXN* gene that encodes frataxin are responsible for Friedrich's ataxia, a rare autosomal recessive neurodegenerative disease with prominent cardiac involvement (Durr *et al.*, 1996). Sulphur is provided in mitochondria by IscS, a cysteine desulphurase that has been shown to associate with scaffold proteins involved in Fe-S cluster biosynthesis (Tong and Rouault, 2000).

1.3 Mitochondrial Genetics

Mitochondrial genetics are complex and display a number of unique characteristics that are critically important to our understanding of mitochondrial diseases (Taylor and Turnbull, 2005).

1.3.1 Mitochondrial Genome

The mitochondrial genome is the only source of extra-nuclear DNA in eukaryotes. Human mtDNA, located in the mitochondrial matrix in protein-DNA complexes termed nucleoids, is a closed circular, double-stranded molecule consisting of 16,569 base pairs. Consisting of complimentary heavy (H) outer and light (L) inner strands, the mitochondrial genome is compact and highly organised with no introns and only 2 non-coding regions, the origin of L strand replication and the more extensive displacement (D) loop, containing the origin of H strand replication and transcription major control elements (Figure 1.9).

The complete genome contains 37 genes that encode 22 transfer RNAs (mt-tRNAs), 2 ribosomal RNAs (mt-rRNAs), and 13 polypeptides that are all critical components of the OXPHOS enzyme complexes. The original sequence of the mitochondrial genome was published in 1981 and revised in 1999, with the majority of genes located on the H strand (Anderson *et al.*, 1981; Andrews *et al.*, 1999). All other proteins involved in all mitochondrial functions are encoded by the nuclear genome. This bi-genomic control of the mitochondrial proteome is a unique feature of mitochondrial biology.

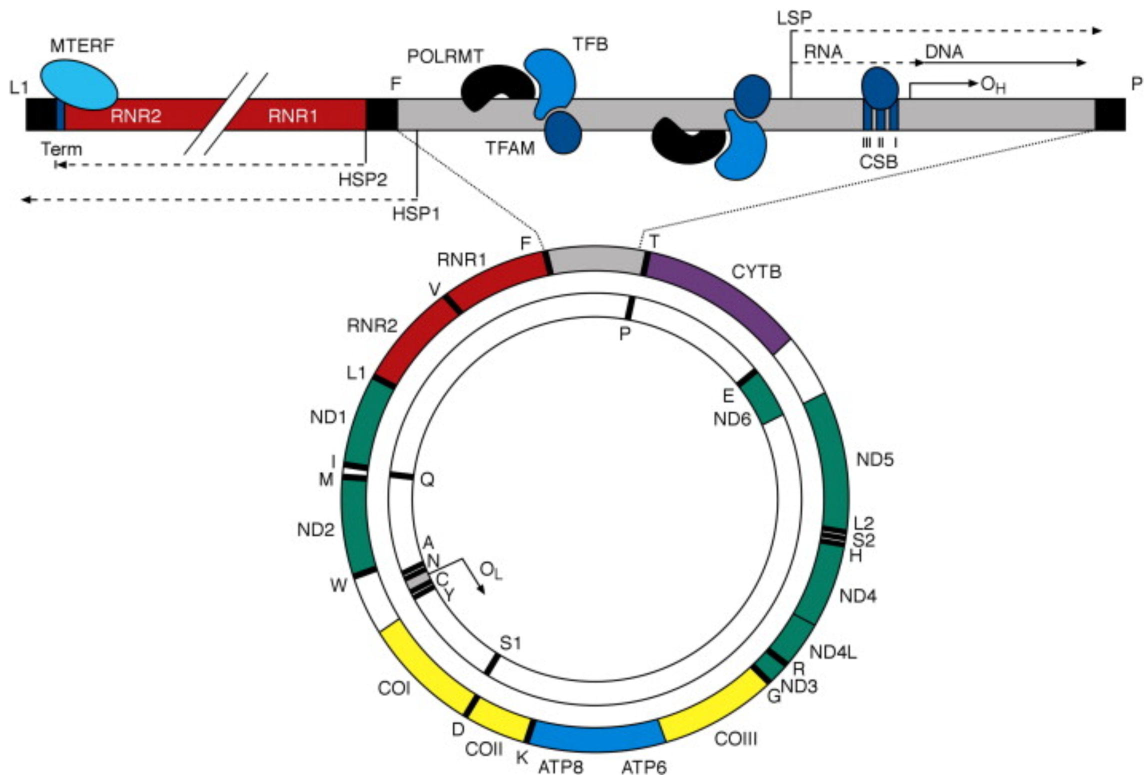


Figure 1.9 Human mitochondrial genome. Schematic diagram, with enhanced view of the mammalian D-loop and transcription termination regions. Thirteen essential components of the ETC are encoded by mtDNA: seven complex I genes (green), one complex II gene (purple), three complex IV genes (yellow), and two overlapping complex V genes (blue). Twenty-two mitochondrial genes, distributed between these protein-encoding genes, encode mt-tRNAs (thick black bars with single letter abbreviations for amino acids), and two further genes encode mt-rRNAs (red). Major non-coding regions (grey) include the 1.1kb D-loop, with the origin of H-strand replication (O_H), and origin of L-strand replication (O_L). H-strand transcription is initiated either from HSP1, generating a short transcript that terminates at the RNR2/MTTL1 boundary (Term) under the guidance of mTERF, or from HSP2, generating polycistronic transcripts of the entire H-strand. LSP denotes the L-strand initiation point that produces polycistronic transcripts for the L-strand and generates RNA precursors for H-strand replication initiation. Conserved sequence blocks (CSBs I-III) are conserved regions in human, mouse and rat that participate in formation of RNA primers for replication. Transcription from all promoters requires the upstream binding of transcriptional activator TFAM, together with a single subunit RNA polymerase (POLRMT), which forms a heterodimeric complex with the transcription factor TFB. The major arc of the mitochondrial genome spans the section from O_H to O_L and is the region most commonly associated with single, large-scale deletions. Image modified with permission from original (Tuppen *et al.*, 2010).

As discussed in section 1.1.1, the majority of the mitochondrial genome has been transferred to the nuclear genome through evolution, yet the reasons underlying the failure of completion of this process, and retention of this extra-nuclear genome, are unclear. Proposed theories include the suggestion that the mtDNA-encoded OXPHOS enzyme complex subunits are too hydrophobic to be translated in the cytosol, and the concept of retention of certain complex subunits in the highly polymorphic mitochondrial genome to permit rapid adaption of the OXPHOS system to future environmental change (Wallace, 2007).

1.3.1.1 Transcription

Transcription of the mitochondrial genome is a complex process initiated at separate regions in the two strands – the heavy strand and light strand promoters (HSP, LSP). Within these regions, transcription is initiated at one site in the LSP (L1) and two sites within the HSP (H1, H2). Transcription from L1 and H2 encompass almost the entire light and heavy strand respectively, resulting in the formation of large, polycistronic RNA molecules, while transcription from H1 includes only 2 mt-rRNAs and the mt-tRNAs for phenylalanine and valine (Figure 1.9) (Taanman, 1999). Following initial transcription, RNA processing in mitochondria is critically dependent on the position and structure of the mt-tRNA sequences (Ojala *et al.*, 1981).

The nuclear-encoded single subunit mitochondrial RNA polymerase (mt-RPOL) contains a promoter recognition loop that binds HSP/LSP and initiates transcription together with the mitochondrial transcription factor A (TFAM) and one of two mitochondrial transcription factor B homologous proteins (TFB1M, TFB2M) (Taanman, 1999; Falkenberg *et al.*, 2002). Although additional transcription factors exist, only these components (mt-RPOL, TFAM and TFB1M/TFB2M) are essential for transcription for the mitochondrial genome (Litonin *et al.*, 2010).

Four nuclear-encoded multi-functional proteins located within the mitochondrial matrix regulate the termination of transcription. These mitochondrial termination factors (mTERFs) are homologous proteins that display variable binding to specific sites within the mitochondrial genome and terminate transcription through interference with RNA elongation machinery (Yakubovskaya *et al.*, 2010). While the binding site (at

a specific site in mt-tRNA$^{Leu(UUR)}$) and action of mTERF1 have been determined, such details for mTERF2-4 have not yet been fully characterized. Knockout mice for mTERF2 display significant global OXPHOS impairment (Wenz *et al.*, 2009), while similar mTERF3 inactivation shows embryonic lethality (Park *et al.*, 2007). Cardiac tissue specific inactivation of mTERF3 however causes cardiomyopathy in mice with dysfunctional mtDNA transcription and OXPHOS impairment (Park *et al.*, 2007).

1.3.1.2 Translation

Mitochondrial translation displays distinct characteristics that differentiate the process from translation in both prokaryotes and the cytoplasm of eukaryotes. For example, due to the fact that uracil (U) in the wobble position of the mt-tRNA anticodon can recognise any base in the third position of the codon (Barrell *et al.*, 1980), the 22 tRNAs encoded by the mitochondrial genome are sufficient for the translation of all 13 mtDNA-encoded polypeptides (open reading frames, ORFs). In contrast, a minimum of 31 tRNAs are required for translation of the nuclear genome. Furthermore, mitochondrial ribosomes are distinct from both prokaryotic and eukaryotic ribosomes: both the 12S rRNA (in the 28S small subunit) and the 16S rRNA (in the 39S large subunit) are mtDNA-encoded and combine to form the unique 55S mitochondrial ribosome (Attardi and Ojala, 1971), a similar mass to the 70S bacterial ribosome but with atypically low RNA content and relatively high protein content (O'Brien, 2003).

The three descriptive phases of translation (namely initiation, elongation and termination) themselves involve several unique factors in mitochondria, distinct from their homologues in both prokaryotic and eukaryotic translation. The initiator tRNA (tRNA^{Met}) is responsible for both initiation and elongation of mitochondrial protein synthesis. In the presence of a formyl group (tRNA^{fMet}) and mitochondrial initiation factors 2 and 3 ($IF2_{mt}$, $IF3_{mt}$), this tRNA binds the small ribosomal subunit forming the initiator complex (Montoya *et al.*, 1981). Mitochondrial elongation factor Tu (mtEFTu) forms a ternary complex with GTP and an aminoacylated tRNA and carries the tRNA to the acceptor site of the mitochondrial ribosome (Janiak *et al.*, 1990). Following GTP hydrolysis, a further nuclear-encoded mitochondrial elongation factor, mtEFG1, allows the ribosome to progress by one codon so that the acceptor site is vacated (Jeppesen

et al., 2005). Termination of mitochondrial protein translation utilises the universal genetic code stop codons, UAA and UAG; however the mitochondrial genome also contains AGA and AGG, which were originally assigned as stop codons (Anderson *et al.*, 1981). While the mitochondrial release factor, mtRF1a, and the UAA and UAG codons accounted for 11 of the 13 ORFs in the mitochondrial genome, they did not permit release of the COX I and ND6 polypeptides. Recent research has shown that AGA and UAA in the corresponding transcripts result in a -1 reading frame shift, repositioning the mitochondrial ribosome at a universal UAG stop codon, due to the inability of tRNAs to recognize them directly (Temperley *et al.*, 2010).

Importantly pathogenic mutations have recently been identified in many of the nuclear-encoded factors involved in mitochondrial translation. For example, mutations in *TUFN* and *EFG1*, encoding mtEFTu and EFG1 respectively, have been linked to human mitochondrial disease (Coenen *et al.*, 2004; Valente *et al.*, 2007).

1.3.1.3 Mitochondrial DNA replication

Mitochondrial DNA is replicated independently of nuclear DNA, yet is entirely reliant on the nuclear genome for the processes of replication and repair. Replication of the mitochondrial genome occurs throughout the cell cycle and in all cells, including post-mitotic cells such as cardiomyocytes and neurones (Bogenhagen and Clayton, 1977). Two models have been proposed to describe the mechanisms of mtDNA replication.

1.3.1.3.1 Initiation of replication

Transcription of the L strand from the LSP is necessary for the expression of genes encoded by the L strand but also provides short mitochondrial transcripts that act as promoters for the initiation of H strand replication (Chang *et al.*, 1985). There are no differences in the initiation of these different processes, and the mechanisms that determine the outcome of this initial event as elongation of L strand transcription or H strand synthesis are currently unclear (Clayton, 1991). Critical to this transition from RNA synthesis to DNA synthesis are three conserved sequence blocks (CSBs) located downstream of O_H (Walberg and Clayton, 1981). In particular, at CBS II, the RNA/DNA hybrid may be cleaved by a ribonucleoprotein, named RNAse MRP (mitochondrial RNA processing), and the resultant product may be used by a DNA polymerase as a primer to synthesise a nascent H strand (Bennett and Clayton, 1990). An alternative model suggests an RNAse MRP-independent process achieving site-specific termination through the action of CBSII itself as a transcription termination element (Pham *et al.*, 2006).

1.3.1.3.2 Asynchronous strand displacement model

The strand displacement model proposes that double-stranded mtDNA is replicated in an asynchronous process, utilising two different sites of origin: beginning at O_H, mtDNA replication proceeds in a unidirectional manner until, approximately two-thirds of the way around the genome, O_L becomes exposed as a single-stranded template and replication of the L strand can be initiated. Following exposure, OL forms a loop-like structure that acts as a recognition site for mtDNA primase, and RNA again primes daughter L strand synthesis (Wong and Clayton, 1985). The two daughter H and L strands continue to lengthen in opposite directions, eventually forming a catenated

pair of rings. A type II topoisomerase reaction releases the two daughter monomeric mtDNA molecules (Clayton, 1982).

1.3.1.3.3 Synchronous leading and lagging strand model

More recently, a second mechanism has been proposed for mtDNA replication involving standard strand-synchronous replication of the kind found in the nucleus. Through the use of two-dimensional (2D) agarose gel electrophoresis, a distinct class of replication intermediates were identified that were resistant to single-strand nuclease digestion and displayed mobility properties consistent with coupled leading and lagging strand replication products (Holt *et al.*, 2000). Concluding that mtDNA replication occurs mainly, or exclusively, via this strand coupled mechanism, replication intermediates with partially single-stranded mtDNA (and hence supportive of synchronous replication) were suggested to result from RNA degradation during extraction of DNA from mitochondria (Yang *et al.*, 2002). Modification to the original model have involved demonstration of the initiation of mtDNA replication at multiple sites distant to OH (Reyes *et al.*, 2005), and bidirectional replication (Bowmaker *et al.*, 2003).

1.3.1.3.4 Protein components

The minimal replication machinery (replisome) of mitochondria contains distinct protein complexes – mitochondrial polymerase γ (POLG), the replicative mitochondrial helicase, TWINKLE, mitochondrial single-stranded DNA binding (mtSSB) protein, and mitochondrial RNA polymerase (POLRMT) (Figure 1.10). POLG is a 245kDa heterotrimer consisting of one catalytic and two accessory subunits (POLγA and POLγB, respectively) and possesses both polymerase and exonuclease activities for the replication and proof-reading of mtDNA (Gray and Wong, 1992). Recent research has shown that these functions are displayed by the catalytic subunit alone, and that the accessory subunit possesses non-specific double-stranded DNA binding capabilities, implicating a role for POLG in the maintenance of mtDNA copy number (Di Re *et al.*, 2009).

TWINKLE is a hexameric protein with 5’ to 3’ directionality that acts to unwind duplex mtDNA in an ATP-dependent process, stimulated by mtSSB protein (Korhonen *et al.*,

2003). The homotetrameric mtSSB proteins maintain the integrity of unwound mtDNA throughout the processes of replication and repair, by binding with high affinity to single stranded DNA (ssDNA) and therefore preventing ssDNA from forming stable intermediates that would hamper the actions of POLG (Yang *et al.*, 1997).

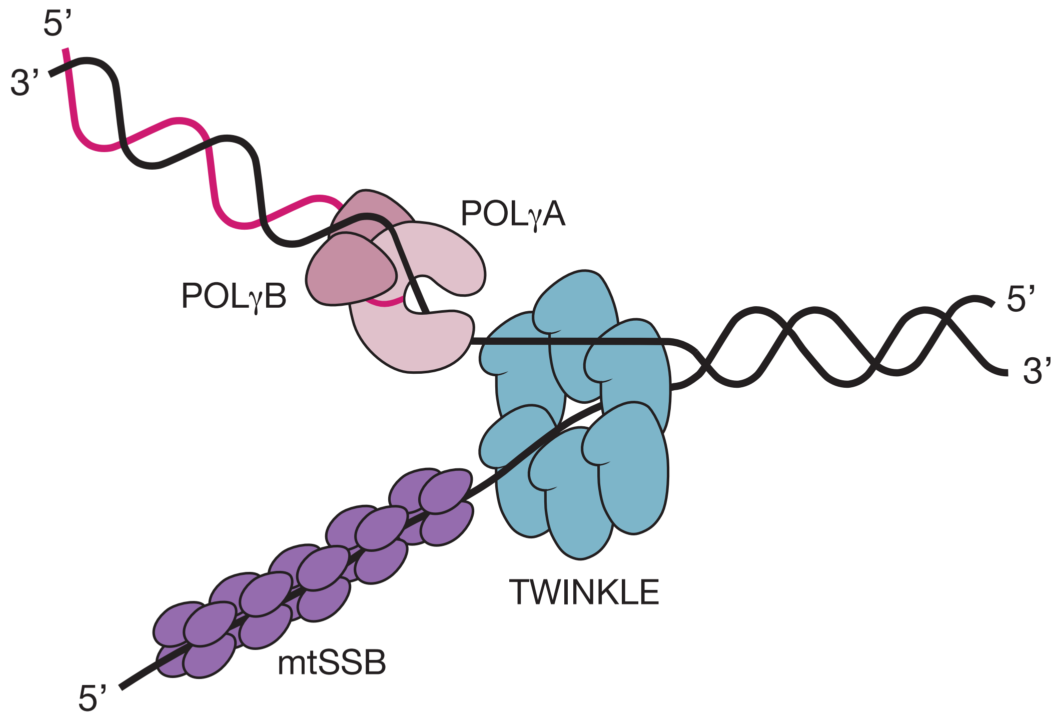


Figure 1.10 The minimal mitochondrial replisome. The TWINKLE helicase (blue) has 5'to 3' directionality, unwinding double-stranded mtDNA and enabling leading strand synthesis (red). The mtSSB protein (purple) stabilizes the unwound DNA and stimulates DNA synthesis by mitochondrial polymerase γ (POLγA and B, pink). POLRMT (not shown) can synthesize the RNA primer required for lagging strand mtDNA synthesis. Image modified with permission from the original image (Wanrooij and Falkenberg, 2010).

1.3.1.4 Mitochondrial DNA repair

Based on the original observation that ultra-violet light-induced pyrimidine dimers were not removed from mtDNA, it was previously believed that no mechanisms for mtDNA repair existed in mitochondria (Clayton *et al.*, 1974). In fact, more recent research has demonstrated that mitochondria do repair certain classes of damage such as abasic sites, single strand breaks, and oxidised bases (e.g. 8-oxodeoxyguanosine, 8-oxodG) (Croteau *et al.*, 1999).

The primary mechanism of mtDNA repair is the base excision repair (BER) pathway. Oxidative lesions and alkylation damage are initially excised by DNA glycosylases that recognise specific modified bases (e.g. 8-oxodG) and cleave the cleave the N-glysosylic bond between the modified base and the sugar, to produce an apurinic/apyrimidine (AP) site that is then processed by an AP endonuclease to cleave the DNA backbone. Uracil DNA glycosylase (UDG) was the first mitochondrial glycosylase to be identified, and is the produced from the same gene as nuclear UDG, with alternative splicing and use of a different start codon permitting fusion with a mitochondrial localisation sequence (Nilsen *et al.*, 1997). Following removal of the affected base, a one-nucleotide gap is formed, providing the 3'-hydroxyl (3'-OH) and 5'-deoxyribose-5-phosphate (5'-dRP) residues required for DNA synthesis. POLG then catalyzes the insertion of a nucleotide and DNA ligase seals the break (Croteau *et al.*, 1999). *In vitro* studies have demonstrated that when POLG is able to bypass an AP site, adenine is incorporated in >90% molecules resulting in a G>A change if the original base was a purine, and C>A or T>A for an original pyrimidine (Sagher and Strauss, 1983). Long patch BER (involving the replacement of ≥2 nucleotides) requires further proteins, FEN1 and DNA2, that bind and cleave the redundant original 5' strand created as DNA is synthesised by POLG to fill the gap (Liu and Demple, 2010).

Mismatch repair (MMR) is the second mechanism of mtDNA repair that occurs in mitochondria. This ATP-dependent, selective and bidirectional process is responsible for the repair of mismatches and small loops generated through errors in replication (Mason *et al.*, 2003). Mitochondrial MMR is independent of the MSH2 enzyme, a protein that is essential in the nuclear MMR pathway, but involves the action of repair factor YB-1 (de Souza-Pinto *et al.*, 2009).

Despite the discovery of distinct mechanisms of mtDNA repair in mitochondria, the process is not comprehensive, and lesions that are not removed will likely block successful transcription by RNA polymerase and replication by POLG, and may explain the occurrence of deletions and rearrangements in mtDNA (Bogenhagen, 1999).

1.3.2 Susceptibility to mutation

The mitochondrial genome acquires mutations at a rate 10-17-fold higher than nuclear DNA (Wallace *et al.*, 1987), such that deleterious mutations are more likely to occur (Brown *et al.*, 1979). The higher mutation rate is thought to result from a combination of factors: (i) the relative proximity of mtDNA to the OXPHOS system and the associated exposure to ROS with the potential to cause mutation, (ii) a lack of histones for mtDNA, that in the nucleus provide protection for DNA, and (iii) fewer mechanisms for repair of mtDNA, compared to nuclear DNA (Tuppen *et al.*, 2010).

1.3.3 Homoplasmy and heteroplasmy

Most mammalian cells contain hundreds of mitochondria, each of which contains 2-10 copies of mtDNA, such that multiple copies of mtDNA exist within each cell. In the general population, although a small number of mtDNA molecules may contain mutations, their proportion is usually so small (<1%) that the tissue can be regarded as uniform for the normal mitochondrial genome (homoplasmy) (Monnat and Loeb, 1985). In contrast, in most pathogenic mtDNA mutations, two or more distinct mitochondrial genomes exist within the same tissue at high percentage (heteroplasmy) (Larsson and Clayton, 1995). Relaxed replication, in which mtDNA replicates independently of the cell cycle, permits the random replication and degradation of mtDNA molecules, and maintains a constant mtDNA copy number per cell. Changes in the balance of replication and degradation of different mtDNA molecules may account for *intracellular drift* over time in the level of heteroplasmy of a mutation (Chinnery and Samuels, 1999). Similarly, during cell division, mitochondria segregate at random to daughter cells, such the proportion of mutated mtDNA in different cell lineages of a heteroplasmic mutation may change, or even drift towards either homoplasmic state (Fernandez-Silva *et al.*, 2003).

1.3.4 Threshold

At a cellular level, most pathogenic mtDNA mutations behave recessively, only manifesting when the proportion of mutated mtDNA exceeds a certain threshold level. This threshold for mtDNA mutations is commonly given as the percentage of mutated molecules required to cause a biochemical defect in a cell (frequently loss of complex

IV activity) (Figure 1.11). However, the proportion of mutated molecules required for this cellular expression of the phenotype varies with the nature of the mutation and the energy demands of the cell (Attardi *et al.*, 1995). Typically the threshold for deleted mtDNA has been shown to be approximately 50-60%, while the threshold for point mutations in mtDNA may be considerably higher (Shoubridge, 1994).

Tissue mtDNA mutation load and tissue-specific threshold may affect the onset and clinical severity of mitochondrial disease (Chinnery *et al.*, 1997a). The recognition of pathogenic homoplasmic mtDNA mutations, which frequently result in isolated organ phenotypes including cardiomyopathy, emphasizes the fact that other genetic (e.g. expression of aminoacyl tRNA synthetases) or environmental factors can modulate the phenotype (Carelli *et al.*, 2003; Taylor *et al.*, 2003b; Perli *et al.*, 2012).

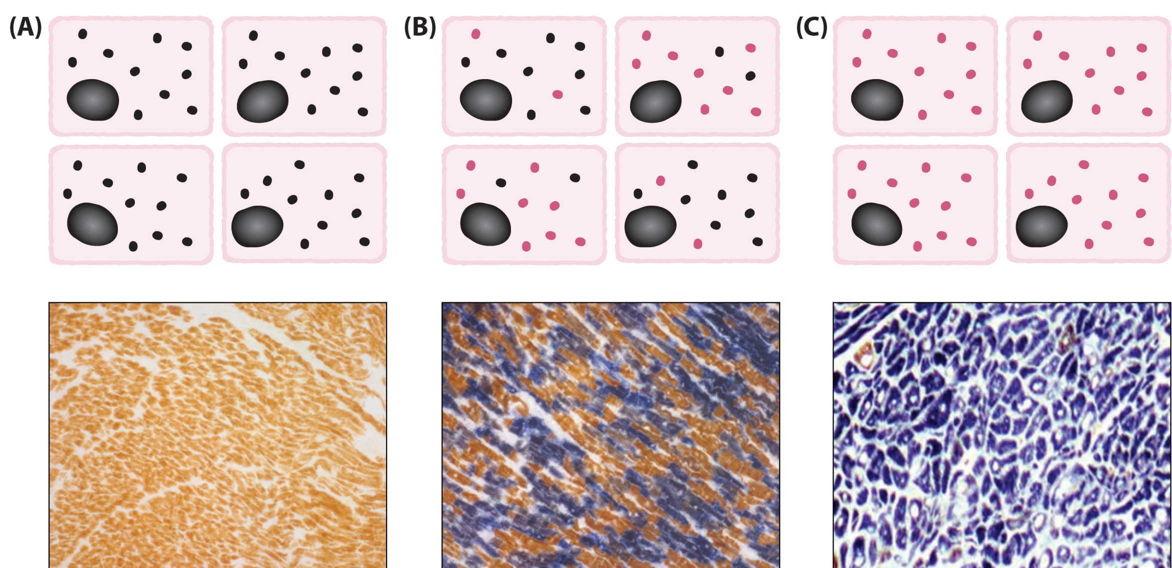


Figure 1.11 mtDNA mutations and patterns of cellular respiratory function. (A) In normal individuals, all cardiomyocytes contain multiple copies of wild type mtDNA (black circles, upper panel), with sequential cytochrome *c* oxidase (COX) / succinate dehydrogenase (SDH) histochemistry showing all cardiomyocytes as COX-positive (brown, lower panel). (B) In patients with heteroplasmic mtDNA mutations, different proportions of wild type (black) and mutated mtDNA (red) are present in individual cardiomyocytes (upper panel); COX/SDH histochemistry reveals mosaic pattern of COX-deficient and COX-positive cardiomyocytes, with cellular respiratory deficiency only apparent when a threshold proportion of mutated mtDNA is reached (lower panel). (C) In patients with homoplasmic mtDNA mutations, all cardiomyocytes contain multiple copies of mutated mtDNA (red, upper panel), with the majority of cells displaying COX-deficiency (blue, lower panel).

1.3.5 Clonal expansion

Clonal expansion describes the process through which mutations arising in a single mtDNA molecule accumulate to high levels, such that a cell or tissue can contain a majority of mtDNA molecules with this specific genotype. In the case of pathogenic mutations, this process can result in expression of a biochemical defect if the threshold level is exceeded. Although clonally-expanded mtDNA mutations have been demonstrated in a variety of tissues (Muller-Hocker *et al.*, 1993; Nekhaeva *et al.*, 2002), the mechanisms underlying this process remain unclear. Initial theories that were reliant on proposed replicative advantages of mutated over wild type mtDNA were rejected with demonstration that relaxed replication itself, in non-dividing cells such as skeletal muscle, was sufficient to result in clonal expansion of mutant mtDNA molecules through random genetic drift (Elson *et al.*, 2001). This mechanism of clonal expansion can result in an increase in the proportion of mutated mtDNA molecules even in post-mitotic cells, such as cardiomyocytes (Muller-Hocker, 1989).

Individual clonal expansions of mutated mtDNA correlate with age in a variety of human tissues, including the heart (Nekhaeva *et al.*, 2002), and it has been proposed that this process, rather than the continual accumulation of somatic mutations throughout life, is important in human ageing. The clonal expansion model suggests that mutations detected in later life may have first arisen much earlier in life, giving more time to accumulate to deleterious levels (Nicholas *et al.*, 2009). Evidence to support this theory arises from the mtDNA mutator mouse, which harbours a *POLG* knock-in mutation that prevents POLG proofreading during mtDNA replication, as, although these animals harbour multiple mtDNA mutations from mid-gestation onwards, the biochemical defect and ageing-related phenotype are not detectable until ~25 weeks of age (Trifunovic *et al.*, 2004). However this theory remains controversial and the clinical importance of these clonally expanded mtDNA mutations in the general population is debated. However in patients with mitochondrial disease possessing pre-existing high levels of a mtDNA mutation, this process can lead to profound changes in mtDNA mutation load and contribute to clinical disease progression.

1.3.6 Mitochondrial DNA inheritance

Human mtDNA exhibits strict maternal inheritance. As mtDNA already present in the oocyte prior to fertilisation gives rise to the entire population of mtDNA molecules in the offspring, with no contribution from the paternal mtDNA, heritable pathogenic mutations are transmitted via the maternal lineage and pedigrees exhibit maternal transmission of the clinical phenotype (Giles *et al.*, 1980; Case and Wallace, 1981). Disease exclusively in maternal relatives raises suspicion of mtDNA disease, and genetic counselling is manifestly different to that in nuclear genetic disorders. The nature of the defect also affects the likelihood of maternal transmission such that single, large-scale deletions are rarely transmitted from females to their offspring, while point mutations are frequently transmitted (Chinnery *et al.*, 2004).

The mechanisms that permit exclusive maternal inheritance are not clear, however selective degradation of paternal mtDNA is believed to occur through ubiquitination of sperm mitochondria outer membrane, in the fertilised oocyte, followed by proteolytic digestion (Sutovsky *et al.*, 2000; Thompson *et al.*, 2003).

1.3.7 The mitochondrial DNA bottleneck

Exclusive uniparental inheritance of mtDNA might be considered to result inevitably in slow random genetic drift, and exposure of the mitochondrial genome to the acquisition and accumulation of large numbers of deleterious mutations as predicted by Muller's Ratchet model of asexual reproduction in organisms with high mutation rates (Muller, 1964). However early research in Holstein cows recognised rapid shifts in mitochondrial genotype over single generations, suggesting that a mechanism existed for rapid mtDNA variation (Hauswirth and Laipis, 1982). During female germline development, the number of mtDNA molecules within each cell is dramatically reduced before being re-amplified to a final number >100,000. Assuming heteroplasmy in germline cells, differential amplification during this '*genetic bottleneck'* could then lead to rapid shifts in genotype between generations (Hauswirth and Laipis, 1982). Partitioning of mtDNA molecules into different cells, with segregation of replicating mtDNA between proliferating primordial germ cells, has been demonstrated to be a critical event during embryogenesis, that partly explains

the rapid segregation of genotypes (Cree *et al.,* 2008). Since human mtDNA disease is frequently associated with heteroplasmic mtDNA mutations, and the level of the mutation is often linked to the severity of the phenotype (Chinnery *et al.*, 1997a), the mechanisms underlying the mtDNA bottleneck have been the subject of intense study. Rapid shifts in the level of heteroplasmy between single generations can result in an asymptomatic mother giving birth to a child with severe disability, such that an understanding of this process may have profound impacts on the clinical management and counselling of patients with mtDNA disease (Carling *et al.,* 2011).

Although precise mechanisms of the mtDNA bottleneck remain unclear and the extent of the reduction in mtDNA copy number in primordial germ cells is disputed, studies in mouse embryos with benign heteroplasmic mitochondrial genomes have confirmed that the individual offspring of a single heteroplasmic mouse can have widely different levels of heteroplasmy but that the mean levels of mutation in the offspring are similar to the parent (Jenuth *et al.*, 1996). Observations in mouse models harbouring a mt-tRNA mutation have shown that differences in heteroplasmy levels are largely determined pre-natally during oocyte development. Such deleterious mt-tRNA mutations are not subject to purifying selection during this process, resulting in oocyte heteroplasmy levels that are determined by random genetic drift (Freyer *et al.,* 2012). Research in human patients has been used to conclude that the variation in offspring heteroplasmy levels is entirely due to the mtDNA bottleneck and random genetic drift (Chinnery *et al.*, 2000; Brown *et al.*, 2001). Nevertheless this area remains controversial. Diverse processes involving transcription, biogenesis, mitochondrial dynamics, nucleotide pool regulation and ROS production have been shown to influence mtDNA copy number and may therefore be important in regulation of the mtDNA bottleneck. Understanding how these processes contribute to mammalian mtDNA transmission is an important subject for future study with implications for counselling and care (Carling *et al.,* 2011).

1.4 Mitochondrial disease

Mitochondrial disease includes various clinical disorders that occur as a result of dysfunctional OXPHOS, due to a primary genetic defect. Clinical disease-based prevalence studies suggest that mitochondrial disease, due to mutations in mtDNA alone, affects 9.2/100,000 adults aged <65 years, with a further 16.5/100,000 children and adults aged <65 years at risk of development of disease (Schaefer *et al.*, 2008). These figures derive from regional referral patterns, excluding patients with nuclear DNA mutations, and are therefore likely to be an underestimation of the true prevalence of mitochondrial disease. The m.3243A>G mutation alone is present in ~1 in 300 of the general population and, while many individuals will possess low levels of mutation and remain asymptomatic, mitochondrial disease, due to mtDNA mutations, appears more common than previously thought, causing disease in ~1 in 5000 individuals (Elliott *et al.*, 2008), making mitochondrial disease one of the most common inherited neuromuscular disorders.

1.4.1 Genotype and phenotype

Although mtDNA mutations are the commonest cause of mitochondrial disease in adults, identified in ~70% patients, and presenting unique challenges in diagnosis and management, mitochondrial disease can be caused by defects in either mitochondrial or nuclear DNA, causing a diverse range of phenotypes. Three separate categories of mitochondrial disease exist, based on the location of the primary genetic defect.

Point mutations and single, large-scale deletions of mtDNA are regarded as primary mtDNA defects, while *multiple* DNA deletions and mtDNA depletion are secondary mtDNA defects, as they result from mutations in nuclear genes, predominantly involved in the replication or maintenance of the mitochondrial genome (e.g. polymerase γ, TWINKLE). A third group of mitochondrial disease exists in which mutations occur in nuclear genes encoding subunits or cofactors of the enzyme complexes of the ETC or mitochondrial translational factors. In these disorders, while OXPHOS is dysfunctional, there is no specific defect of the mitochondrial genome itself.

Significant advances have been made in our understanding of the molecular biological basis of mitochondrial disease and this has had an important impact on genetic counseling and disease management. However, regardless of the nature of the underlying genetic mutation, the relationship between genotype and phenotype in mitochondrial disease is generally quite weak, although notable exceptions to this statement do exist.

1.4.2 Clinical features

The clinical spectrum in mtDNA disease is wide with both isolated organ involvement and more frequent multisystem disease recognized (Figure 1.12). Presentation may be at any age and in almost any organ, but those with high energy requirements including brain, eye, skeletal muscle and heart are most frequently involved (McFarland *et al.*, 2010; Tuppen *et al.*, 2010). Indeed natural history studies have demonstrated that cardiac involvement in mtDNA disease is progressive and an independent predictor of morbidity and early mortality (Scaglia *et al.*, 2004; Limongelli *et al.*, 2010). Cardiac and neurological diseases are the commonest causes of early death in patients with mitochondrial disease due to the m.3243A>G mutation, while sudden death, often with a suspected cardiac aetiology, is frequently reported (Majamaa-Voltti *et al.*, 2008). Hence, cardiologists are likely to become increasingly involved in the multi-disciplinary care of patients with mitochondrial disease.

The manifestations of mtDNA disease vary from oligosymptomatic states (e.g. type 2 diabetes mellitus or migraine) to complex syndromes often involving neurological, ophthalmological, cardiological, gastroenterological or endocrine features (Tuppen *et al.*, 2010). Proximal skeletal myopathy may be slowly progressive while ophthalmological manifestations including ptosis, ophthalmoplegia, cataracts and optic atrophy are common presenting symptoms. Central nervous system involvement is often associated with more severe disease, incorporating deafness, migraine, epilepsy, ataxia, encephalopathy, stroke and dementia. Diabetes is common in patients with mtDNA disease while liver, renal and other endocrinological abnormalities are more rarely described.

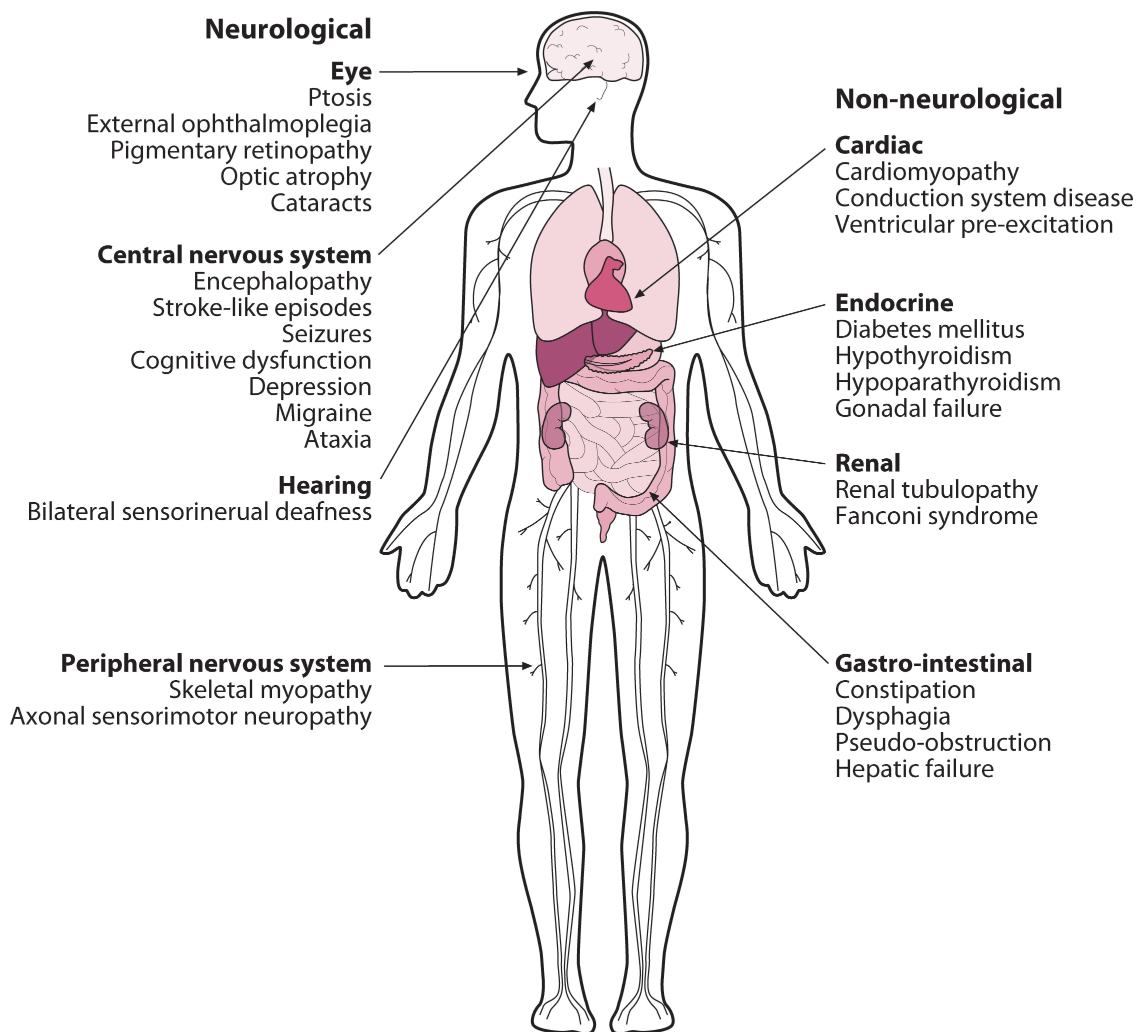


Figure 1.12 Clinical features of mtDNA disease. Diverse organ systems can be affected in mtDNA disease either within an individual or a family. Patterns of distant organ involvement (e.g. diabetes and deafness) or family history may prompt consideration of mitochondrial aetiology. Original image reproduced with permission (Bates *et al.*, 2012).

Clinical syndromes of mtDNA disease (Table 1.2), originally described in individual families, have permitted investigations of well-characterized groups of patients (Majamaa-Voltti *et al.*, 2006; Majamaa-Voltti *et al.*, 2008; Limongelli *et al.*, 2010). However it is recognized that many patients with mtDNA disease do not fit into clinical categories. Patients may present with features suggestive of mtDNA disease, such as involvement of distant organs (e.g. deafness and diabetes), or familial history of isolated organ involvement (e.g. hypertrophic cardiomyopathy) (Taylor *et al.*, 2003b).

Syndrome	Principal clinical features	Mitochondrial DNA mutation
CPEO	External ophthalmoplegia, myopathy	single or multiple mtDNA deletions
Kearns-Sayre syndrome	Pigmentary retinopathy, ataxia, cardiac conduction defects	single, large-scale mtDNA deletion
Leigh syndrome	Subacute necrotizing encephalopathy, basal ganglia lesions	Complex I, IV and V gene mutations
LHON	Acute / sub-acute visual loss	Complex I gene mutations
MELAS	Myopathy, encephalopathy, lactic acidosis, stroke-like episodes	mt-tRNA gene mutations
MERRF	Myoclonus, epilepsy, ataxia	mt-tRNA gene mutations
NARP	Neuropathy, ataxia, pigmentary retinopathy	Complex V mutations
Pearson's marrow-pancreas syndrome	Sideroblastic anaemia, exocrine pancreatic insufficiency, hepatopathy, nephropathy	single, large-scale mtDNA deletion

Table 1.2 Clinical syndromes of mitochondrial disease. CPEO = chronic progressive external ophthalmoplegia; LHON = Leber's hereditary optic neuropathy; MELAS = myopathy, encephalopathy and lactic acidosis with stroke-like episodes; MERRF = mitochondrial encephalopathy with ragged red fibres; mtDNA = mitochondrial DNA; NARP = neurogenic ataxia and retinitis pigmentosa.

1.4.3 Single, large-scale mitochondrial DNA deletions

Rearrangements of mtDNA include both single, large-scale deletions and duplications, but deletions are more common, with a large proportion of these defects occurring in the region flanked by the 13 base-pair perfect tandem repeat sequences (Schon *et al.*, 1989). The intervening DNA is lost due to errors occurring during mtDNA replication or repair, with incorrect pairing of tandem repeats (Shoffner *et al.*, 1989). Although the size and precise location of single, large-scale deletions can vary, a 4.9kb deletion in

the major arc has been identified as the commonest specific deletion in patients with a variety of phenotypes of mitochondrial disease (Schon *et al.*, 1989).

Single, large-scale deletions are frequently sporadic mutations, and rarely, if ever, inherited; they are believed to occur during development and undergo subsequent clonal expansion. No deletions removing O_H, O_L or LSP have been described, supporting a critical role for these regions in mtDNA replication. Deletions are exclusively heteroplasmic mtDNA mutations so that clinical disease is only apparent when a threshold level of the mutated mtDNA is exceeded (MITOMAP: A Human Mitochondrial Genome Database, http://www.mitomap.org, 2013), and the expressed phenotype is at least partly dependent on the stage of development at which the deletion occurs and the pattern of tissue segregation. As shown in Table 1., the three clinical phenotypes of mitochondrial disease associated with single, large-scale deletions are Kearns-Sayre syndrome (KSS) (Zeviani *et al.*, 1988), Chronic Progressive External Ophthalmoplegia (CPEO) (Moraes *et al.*, 1989), and Pearson's marrow-pancreas syndrome (Rotig *et al.*, 1990).

KSS was originally identified in 1958 as a syndrome featuring retinal pigmentation and heart block (Kearns and Sayre, 1958), with later refinement of diagnostic criteria to include: retinitis pigmentosa, occurring before the age of 20 years, CPEO, and one of three additional criteria: (i) ataxia, (ii) atrio-ventricular (AV) conduction block, or (iii) elevated cerebo-spinal fluid protein (Bau and Zierz, 2005). Subsequent recognition of the multi-system nature of many conditions involving CPEO lead to development of the term "ophthalmoplegia-plus syndromes", with KSS representing one end of the spectrum in this broad group (Drachman, 1968). In common with other clinical phenotypes of mitochondrial disease, CPEO can be caused by a variety of different abnormalities of both the nuclear and mitochondrial genome. Nuclear mutations in genes encoding essential proteins in the maintenance and replication of mtDNA can cause multiple mtDNA deletions and CPEO, including defects in *ANT1* (Kaukonen *et al.*, 2000), *POLG1* (Agostino *et al.*, 2003b), *POLG2* (Longley *et al.*, 2006), *PEO1* (Spelbrink *et al.*, 2001), *OPA1* (Hudson *et al.*, 2008), and *RRM2B* (Tyynismaa *et al.*, 2009). Furthermore point mutations in mtDNA can also give rise to the CPEO phenotype including the m.3243A>G mutation (Goto *et al.*, 1990). Never-the-less, single, large-

scale deletions remain the most frequent cause of CPEO-related phenotypes, accounting for > 90% KSS and ~50% CPEO cases (Holt *et al.*, 1989; Laforet *et al.*, 1995), with the common 4.9kb deletion alone accounting for over one third of such CPEO patients (Schon *et al.*, 1989).

1.4.4 Point mutations of mitochondrial DNA

In contrast to sporadic single, large-scale deletions, point mutations of mtDNA are usually maternally inherited, giving rise to pedigrees of patients that can display a wide variety of clinical phenotypes. Pathogenic mtDNA mutations can be differentiated from benign single nucleotide polymorphisms (SNPs) through the application of canonical criteria involving segregation of mutations within tissues and families, evolutionary conservation of affected nucleotides or amino acids and occasionally biochemical studies in cultured cells. The two most common clinical phenotypes of mitochondrial disease associated with mtDNA point mutations are mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) (Goto *et al.*, 1990), and myoclonic epilepsy with ragged red fibres (MERRF) (Wallace *et al.*, 1988).

1.4.4.1 Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes

A clinical presentation of mitochondrial disease distinct from KSS and MERRF was first described in 1975 (Shapira *et al.*, 1975), and given the acronym MELAS, several years later (Pavlakis *et al.*, 1984). Clinical criteria for the diagnosis subsequently required the presence of (i) stroke-like episodes before the age of 40 years; (ii) encephalopathy, characterized by seizures, or dementia, or both; and (iii) lactic acidosis, or ragged-red fibres, or both (Hirano *et al.*, 1992). Even at that point however it was recognised, in this multisystem condition, that common additional features included migraine, gastro-intestinal involvement, ataxia, hearing loss and short stature. Although subsequent research has demonstrated that patients with MELAS are in fact a genetically heterogenous group, with causative mutations identified, for example, in the complex I protein-coding genes *ND5* and *ND6* (Santorelli *et al.*, 1997; Ravn *et al.*, 2001), the m.3243A>G mutation remains the most common cause of MELAS, found in > 80% cases (Goto *et al.*, 1991).

The m3243A>G mutation in the dihydrouridine loop (D-loop) of the mitochondrial $tRNA^{Leu(UUR)}$ was first identified as a genetic cause of MELAS in 1990 (Goto *et al.*, 1990). However the coincident finding in that original study of the m.3243A>G mutation in a patient with CPEO forced the conclusion that this mutation was not exclusive to the MELAS phenotype. Although persistently referred to as 'the MELAS mutation', the m.3243A>G mutation actually displays exceptional phenotypic diversity: for example, the mutation is known to occur in patients with CPEO without mtDNA deletions, but also represents an important cause of diabetes mellitus accounting for 1-3% patients with diabetes, giving mitochondrial diabetes a prevalence in the general population of Western Europe of ~1 in 1500 (Gerbitz *et al.*, 1995). Throughout all such epidemiological studies, bilateral sensori-neural deafness (SND) has been recognised in pedigrees of patients with diabetes due to the m.3243A>G mutation, occurring in > 60% patients. The term 'maternally inherited diabetes and deafness' (MIDD) was therefore introduced (van den Ouweland *et al.*, 1994), and subsequent reports have noted an association of this phenotype with retinopathy, neuropsychiatric symptoms, myopathy, cardiomyopathy and chronic kidney disease (Guillausseau *et al.*, 2001).

Widely regarded as the most common heteroplasmic mtDNA point mutation, the m.3243A>G mutation can cause extremely varied disease in individual patients (Nesbitt *et al.*, 2013). In fact, while descriptive clinical syndromes have enabled epidemiological and natural history studies to support patient counselling, their relevance to individual patients has been questioned and multisystem treatment should be patient-specific, taking into account the nature and extent of organ involvement.

1.4.4.2 Myoclonic epilepsy with ragged red fibres

Myoclonic epilepsy with ragged red fibres (MERRF) was first described as a clinical syndrome with recognition that a subgroup of patients were distinct from those with other forms of mitochondrial disease, including KSS and Leigh's disease (Fukuhara *et al.*, 1980). As suggested by the acronym, key clinical features of MERRF include myoclonus and epilepsy in association with marked numbers of ragged red fibres in skeletal muscle biopsies, but additional findings include ataxia, muscle weakness and

atrophy, truncal lipomatosis and cardiac manifestations. Maternal inheritance and combined deficiencies of the enzyme activities of complexes I and IV were recognized in original large pedigrees (Wallace *et al.*, 1988), while the pathogenic m.8344A>G mutation was identified a few years later (Shoffner *et al.*, 1990). The mutation was subsequently revealed to have profound effects in a variety of tissues, preventing post-transcriptional modification of uridine, in the anti-codon wobble position, and resulting in dysfunctional translation of lysine and deficiencies in ETC enzyme complexes that contain mtDNA-encoded subunits (Yasukawa *et al.*, 2000a).

Unlike the m.3243A>G mutation and the clinical syndrome of MELAS, the relationship between genotype and phenotype is stronger for the m.8344A>G mutation and MERRF (Hammans *et al.*, 1993). The m.8344A>G mutation is reported only rarely in association with phenotypes other than MERRF, including Leigh's disease and atypical single system presentations. The mutation accounts for > 90% of cases with MERRF but alternative pathogenic mutations have been described. Indeed a second mutation in the mt-tRNA^{Lys}, m.8356T>C, was reported in association with the MERRF phenotype (Silvestri *et al.*, 1992), however subsequent studies have demonstrated symptoms of migraine and stroke-like episodes in these patients, resulting in the diagnosis of a MERRF/MELAS overlap syndrome (Zeviani *et al.*, 1993). Interestingly, while the m.8344>G mutation shows a more uniform distribution in different tissues with a high threshold for clinical disease, the heteroplasmic m.8356T>C mutation demonstrates a lower mutation load in blood than skeletal muscle, where it can approach homoplasmic levels (Silvestri *et al.*, 1992). This finding of differential tissue segregation with low levels of heteroplasmy in blood is much more in keeping with the m.3243A>G mutation, and perhaps linked to the overlap with MELAS clinically.

1.4.5 Nuclear DNA mutations

As explained in previous sections, mitochondrial disease can result from pathogenic mutations in nuclear DNA as well as in mtDNA, due to the bigenomic control of the mitochondrial proteome. A classification of nuclear mutations that can cause mitochondrial disease has been proposed based on their genetic and clinical effects. Mutations may therefore occur in nuclear genes that: (i) alter the stability of mtDNA,

(ii) encode structural subunits or co-factors of the ETC enzyme complexes, (iii) encode proteins indirectly related to ETC function, or (iv) cause dysfunction of non-protein components of the ETC (Zeviani and Di Donato, 2004).

Firstly, recent years have seen the identification of pathogenic mutations in several nuclear genes, involved in the replication and repair of mtDNA, that result in multiple mtDNA deletions and clinical mitochondrial disease. For example, autosomal dominant progressive external ophthalmoplegia (PEO) is commonly caused by defects in one of three nuclear genes: *ANT1*, encoding the skeletal muscle-heart specific isoform of the mitochondrial adenine nucleotide translocator; *TWINKLE*, encoding the mtDNA helicase that unwinds duplex mtDNA prior to replication; or *POLG1*, encoding the catalytic subunit of the mtDNA-specific polymerase γ (Agostino *et al.*, 2003b; Zeviani and Di Donato, 2004). While the role of these genes in the maintenance of mtDNA explains the effect of the associated mutations, defects in other genes can impact on the overall stability of the mitochondrial genome. Mutations in *TP*, the gene encoding thymidine phosphorylase are associated with the clinical syndrome of mitochondrial neuro-gastrointestinal encephalopathy (MNGIE) (Hirano *et al.*, 1994). Such defects reduce the activity of the thymidine phosphorylase, resulting in an accumulation of dTTP and an imbalance in the nucleotide pools that control the rate and fidelity of mtDNA replication (Nishino *et al.*, 1999).

Secondly, mutations in nuclear genes encoding structural subunits of ETC enzyme complexes and their assembly cofactors have been associated with mitochondrial disease. Mitochondrial proteome analysis has been used to identify >1000 genes encoding mitochondrial proteins in humans, and mutations have been reported in >220 of these genes in association with clinical mitochondrial disease (Koopman *et al.,* 2012). For example, autosomal recessive mutations in *NDUFS1*, *NDUFS2*, *NDUFS4*, *NDUFS7* and *NDUFS8* have all been reported in patients with complex I deficiency and mitochondrial disease, primarily Leigh's disease, and frequently in association with cardiomyopathy (Morris *et al.*, 1996; Triepels *et al.*, 2001). In contrast to complex I, all four protein subunits of complex II are nuclear-encoded and *SDHA* gene mutations are a rare cause of Leigh's disease and complex II deficiency (Bourgeron *et al.*, 1995). An isolated defect of complex III activity has also been reported in association with a

mutation in the *UQCRB* gene, that encodes subunit VII of complex III, in a patient with mitochondrial disease and lactic acidosis (Haut *et al.*, 2003). Additional nuclear genes encoding assembly co-factors of ETC enzyme complexes III, IV and V have been reported in association with mitochondrial disease. For example, nuclear mutations in *SURF1*, *SCO1*, *SCO2*, *COX10* and *COX15* all cause complex IV deficiency with the frequent clinical presentation of cardio-encephalo-myopathy (Agostino *et al.*, 2003a).

Thirdly, nuclear gene mutations can result in abnormalities of the function of the ETC by effecting non-protein factors. Barth syndrome describes the clinical constellation of myopathy, cardiomyopathy, short stature, neutropaenia and 3-methyl glutaconic aciduria. It is associated with abnormalities of cardiolipin metabolism, which is an important constituent of the phospholipid milieu of the mitochondrial inner membrane. Nuclear mutations account for Barth syndrome and coenzyme Q10 deficiency, further extending the mechanistic spectrum of mitochondrial disease caused by defects in nuclear genes (Zeviani and Di Donato, 2004).

Finally, deficiencies of nuclear-encoded proteins that have important mitochondrial functions, indirectly related to the ETC, have recently been reported in association with mitochondrial disease. For example, mutations in *OPA1*, a gene encoding a dynamin-related protein embedded in the mitochondrial inner membrane, are a recognised cause of autosomal dominant optic atrophy, having important impacts on mitochondrial dynamics (Agier *et al.*, 2012). Similarly, the c.1250G>A mutation in *RMND1*, a gene encoding a mitochondrial membrane protein essential for meiotic nuclear division 1, has been reported in association with early infant fatality and a mitochondrial translational defect affecting all OXPHOS complexes with mtDNA-encoded subunits, suggesting a possible role in coordinating the assembly or maintenance of the mitochondrial ribosome (Janer *et al.,* 2012).

Historically, identification of causative nuclear genetic mutations in patients with mitochondrial disease was dependent on biochemical analysis, mitochondrial and nuclear DNA sequencing, and candidate gene studies. The evolving application of new technologies such as whole exome sequencing will revolutionise this process and likely lead to a large increase in the number of known causative nuclear gene defects.

1.5 Cardiac manifestations of mitochondrial disease

More than 250 different pathogenic mtDNA mutations have been reported in humans, many in association with cardiac disease, which ranges from cardiomyopathy to electropathy, including conduction disease and ventricular pre-excitation. This diversity combined with the absence of a cardiac phenotype that is unique to patients with mtDNA disease present significant challenges to the managing cardiologist.

1.5.1 Prevalence and natural history

The true prevalence of mtDNA-related cardiomyopathy is unknown, although based on the prevalence of mtDNA disease and the frequency of cardiac involvement at least ~1 in 10-15,000 of the general population will be affected. Public databases of mtDNA mutations associated with human disease exist and will be an important resource in determining prevalence (MITOMAP: A Human Mitochondrial Genome Database, http://www.mitomap.org, 2013; OMIM: Online Mendelian Inheritance in Man, An Online Catalog of Human Genes and Genetic Disorders, http://www.omim.org, 2013). However such databases are currently not completely accurate as, due to extensive variability of the mitochondrial genome and lack of adherence to strict canonical criteria for determining pathogenicity, some non-disease causing variants are listed. Challenges lie ahead with regard to the analysis of such bio-informatic data (Zaragoza *et al.*, 2010; Zaragoza *et al.*, 2011; Elson *et al.*, 2012).

Natural history studies have demonstrated both the high prevalence of cardiac disease in mtDNA disease and the deleterious effects on outcome of a cardiac presentation. A significant difference in survival to age 16 years was apparent in 113 children with mitochondrial disease (18% and 92% respectively, in those with and without cardiomyopathy) (Scaglia *et al.*, 2004). This result, in a cohort including mitochondrial and nuclear DNA mutations, has subsequently been confirmed in other large paediatric cohorts (Holmgren *et al.*, 2003; Debray *et al.*, 2007). Adult studies, in patients with mtDNA mutations exclusively, have established the progressive nature of cardiac involvement (Okajima *et al.*, 1998; Majamaa-Voltti *et al.*, 2006; Wahbi *et al.*, 2010), with important impacts on morbidity and early mortality (Anan *et al.*, 1995; Majamaa-Voltti *et al.*, 2008).

In common with many newly recognized disorders, early reports of cardiac involvement in mtDNA disease featured patients with severe phenotypes. Family genetic screening has undoubtedly broadened the spectrum of mtDNA disease to include more asymptomatic or oligosymptomatic adults, perhaps limiting the applicability of early studies. A recent study of 32 adult patients demonstrated that, although cardiac involvement was apparent in 78% patients, minor ECG abnormalities represented the most common manifestation, with cardiomyopathy present in 25% patients (Limongelli *et al.*, 2010). Progressive systolic dysfunction and high-grade atrio-ventricular (AV) block did occur in a minority but the incidence of severe cardiovascular complications was relatively low over median follow-up of 4 years. Large multi-centre prospective clinical cohort studies are already underway and will provide novel insights into the natural history and response to intervention of adult mtDNA disease (Nesbitt *et al.*, 2013).

1.5.2 Pathogenetic mechanisms

The molecular events linking mtDNA defects to cardiac dysfunction are poorly understood. While several factors including rarity of the disorder, limited access to human cardiac tissue and an absence of reliable animal models of mtDNA disease have played a role in limiting such investigations, the weak nature of genotype-phenotype correlations is undoubtedly a critical factor. The development of cardiomyocyte cell lines from patients with mtDNA disease using inducible pluripotent stem cell technology may therefore be an important step forward in this area.

Early mechanistic insights developed from observation of patterns of disease. Although patients with specific mtDNA mutations may present with different cardiac phenotypes (Wahbi *et al.*, 2010), and similar cardiac involvement occurs in patients with different mtDNA mutations (Majamaa-Voltti *et al.*, 2002; Wahbi *et al.*, 2010), cross-sectional studies suggest patterns of cardiac involvement do exist (Table 1.3). For example, cardiomyopathies, often with a hypertrophic phenotype, are more frequently reported in association with mt-tRNA gene mutations, while AV block is a feature of KSS, which is commonly caused by single, large-scale deletions in mtDNA (Limongelli *et al.*, 2010). The only cardiac phenotype reported in association with the

m.1555A>G mt-rRNA gene mutation, is a restrictive cardiomyopathy (Santorelli *et al.*, 1999). Although differential effects of mutations in mt-tRNA, mt-rRNA and polypeptide genes on mitochondrial transcription, translation and protein function may be expected, the mechanisms underlying this genotype-phenotype relationship are unclear. While tissue specificity of mutation load is widely recognized as a factor in the diverse clinical features of mtDNA disease generally, a similar phenomenon at a tissue level may be equally important. Higher mutation load of a single, large-scale mtDNA deletion has been reported in post-mortem AV nodal and His-Purkinje system tissue than in contractile myocardium from a patient with KSS, suggesting a reason for the apparent sensitivity of the conduction system (Muller-Hocker *et al.*, 1998).

Marked induction of mitochondrial biogenesis is a prominent feature of end-stage mtDNA-related cardiomyopathy (Heddi *et al.*, 1999; Hansson *et al.*, 2004; Sebastiani *et al.*, 2007), and has been demonstrated in diverse tissues from patients with mtDNA disease. Although in skeletal muscle this response can partially compensate for OXPHOS dysfunction, experimental and clinical evidence suggest that it may have a detrimental effect in cardiac muscle (Wredenberg *et al.*, 2002; Russell *et al.*, 2004).

Gene	mtDNA mutation	Electropathy		Cardiomyopathy				
		V Pre	Cond	HCM	DCM	RCM	LVNC	Hist
	Common							
MTTL1	m.3243A>G	++	+	++	+	+	+	-
MTTI	m.4300A>G	-	-	++	+	-	-	-
MTTK	m.8344A>G	++	+	++	++	-	-	+
MTND4	m.11778G>A	++	-	+	-	-	-	-
	single, large-scale mtDNA deletion	-	++	-	+	-	-	-
	Rare							
MTRNR1	m.1555A>G	-	-	-	-	+	-	-
MTTV	m.1624C>T	-	-	+	+	-	-	-
MTTL1	m.3252T>C	-	+	-	+	-	-	-
	m.3260A>G	+	-	+	+	-	-	-
	m.3303T>C	-	+	+	+	-	-	-
MTND1	m.3337G>A	-	-	+	+	-	-	-
	m.3460G>A	+	-	+	-	-	+	-
MTTI	m.4269A>G	-	-	-	+	-	-	-
	m.4277T>C	-	-	+	-	-	-	-
	m.4284G>A	-	+	+	+	-	-	-
	m.4317A>G	-	-	+	+	-	-	-
	m.4320C>T	-	-	+	-	-	-	-
MTTK	m.8363G>A	-	-	+	+	-	-	-
MTATP8/ MTATP6	m.8528T>C	-	-	+	-	-	-	-
	m.8529G>A	-	-	+	-	-	-	-
MTATP6	m.8993T>G	-	-	+	-	-	-	-
MTTG	m.9997T>C	-	-	+	-	-	-	-
MTND4	m.11778A>G	-	-	-	+	-	-	-
MTTL2	m.12297T>C	-	-	-	+	-	-	-
MTND5	m.13513G>A	+	+	-	-	-	-	-
MTND6	m.14484T>C	-	-	-	+	-	-	-
MTCYB	m.14849T>C	-	-	+	-	-	-	-
	m.15498G>A	-	-	-	-	-	-	+

Table 1.3 Cardiac phenotypes associated with pathogenic mtDNA mutations. Pathogenic mitochondrial DNA mutations were identified from online databases (18,19), excluding rare single nucleotide polymorphisms and haplogroup markers. mtDNA = mitochondrial DNA; V Pre = ventricular pre-excitation; Cond = Conduction disease; HCM = hypertrophic cardiomyopathy; DCM = dilated cardiomyopathy; RCM = restrictive cardiomyopathy; LVNC = left ventricular non-compaction; Hist = histiocytoid cardiomyopathy; ++ = reported in cross-sectional cohort study with >10% frequency; + = reported in single case report(s) / families series only; - = not reported.

Proliferation of intermyofibrillar mitochondria mechanically interferes with sarcomeric function, contributing to adverse cardiac remodelling (Russell *et al.*, 2004; Sebastiani *et al.*, 2007). Induction of genes involved in mitochondrial biogenesis and fatty acid oxidation (FAO) in mtDNA-related cardiomyopathy increases oxygen consumption and contrasts with other pathologies, including unexplained left ventricular hypertrophy (LVH), where cardiac energy metabolism shifts from FAO to glucose oxidation to *reduce* oxygen consumption (Lehman and Kelly, 2002). Moreover, in the absence of induction of antioxidants, an increased mass of mutated mitochondria causes increased ROS (Esposito *et al.*, 1999; Sebastiani *et al.*, 2007). The pathogenetic role of ROS has been confirmed in animal models of nuclear mitochondrial disease but data in mtDNA disease are lacking (Li *et al.*, 1995; Dai *et al.*, 2010).

1.5.3 Cardiomyopathy

1.5.3.1 Hypertrophic cardiomyopathy / Left ventricular hypertrophy

Hypertrophic remodelling is the dominant pattern of cardiomyopathy in all forms of mitochondrial disease (Majamaa-Voltti *et al.*, 2002; Sorajja *et al.*, 2003; Vydt *et al.*, 2007; Limongelli *et al.*, 2010), occurring in up to 40% patients (Scaglia *et al.*, 2004; Limongelli *et al.*, 2010) and can mimic hypertrophic cardiomyopathy (HCM). The prevalence of HCM within the general population is ~1 in 500 yet sarcomeric protein mutations are identified in only ~60% of HCM patients. mtDNA-related cardiomyopathy represents a potential phenocopy of HCM and may partly account for this discrepancy similar to single gene disorders that have already been identified in HCM cohorts such as Anderson-Fabry and glycogen storage diseases (Sachdev *et al.*, 2002; Arad *et al.*, 2005). Cardiologists need to be alert to the presence of extra-cardiac features, or possible maternal inheritance patterns, in this population to enable consideration of the diagnosis of mtDNA disease.

Point mutations in mtDNA can cause sporadic or maternally-inherited cardiomyopathy, which may be the only or presenting feature. Recent cohort studies using echocardiography have identified LVH in 38-56% patients harbouring the m.3243A>G mutation and have revealed a correlation between skeletal muscle mutant load and indexed left ventricular mass (Majamaa-Voltti *et al.*, 2002; Vydt *et al.*, 2007). Patients with high mutation load may therefore be at increased risk of development of

cardiomyopathy. LVH is recognized in patients with other mtDNA mutations including several mt-tRNA genes (e.g. m.8344A>G in *MTTK*, m.4269A>G and m.4317A>G in *MTTI*) and infrequent polypeptide genes (e.g. m.8993T>G in *MTATP6* and m.8528T>C in the *MTATP6/MTATP8* overlap region) (Tanaka *et al.*, 1990; Taniike *et al.*, 1992; Pastores *et al.*, 1994; Ware *et al.*, 2009; Wahbi *et al.*, 2010). Patients with high mutation load may therefore be at increased risk of development of cardiomyopathy. The homoplasmic m.4300A>G mutation, in the mt-tRNA^{Ile} (*MTTI*) gene has now been identified in several families with isolated mtDNA-related cardiomyopathy and may play a more important role in inherited cardiomyopathy than previously appreciated, although this is yet to be confirmed through systematic analysis of HCM cohorts (Taylor *et al.*, 2003b).

There are important differences in the cardiac phenotype and natural history of HCM and mtDNA-related cardiomyopathy. Left ventricular outflow tract (LVOT) obstruction is rarely observed in mtDNA-related cardiomyopathy yet it appears that there is an increased likelihood of progression to ventricular dilatation and heart failure than in HCM (Vydt *et al.*, 2007). A longitudinal study with 6.9 years mean follow-up duration demonstrated that the degree of LVH correlated positively with chamber dilatation and negatively with systolic function in patients harbouring m.3243A>G (Okajima *et al.*, 1998). Heart failure with ventricular dilatation and impaired systolic function has been reported in patients with LVH and m.3243A>G or m.8344A>G mutation (Okajima *et al.*, 1998; Wahbi *et al.*, 2010).

1.5.3.2 Dilated cardiomyopathy

While dilated cardiomyopathy (DCM) can be the initial pattern of cardiac involvement in mtDNA disease (Stalder *et al.*, 2012), it more commonly represents progression of pre-existing hypertrophy with chamber dilation and systolic dysfunction (Ito *et al.*, 1990; Seibel *et al.*, 1991; Wahbi *et al.*, 2010). One patient with DCM was identified among 17 patients with mitochondrial disease, while a recent study of 18 patients with m.8344A>G mutation confirmed DCM in 22% patients (Anan *et al.*, 1995; Wahbi *et al.*, 2010). DCM is rarer than the hypertrophic phenotype in association with other mt-tRNA point mutations including m.3243A>G, m.4269A>G and m.4317A>G (Tanaka *et al.*, 1990; Taniike *et al.*, 1992; Stalder *et al.*, 2012), and appears to be an infrequent

and late phenomenon in KSS, described in only 2% of published patients (Tveskov and Angelo-Nielsen, 1990; Hirano and DiMauro, 1996).

Due primarily to phenotypic rarity, data are lacking concerning natural history in patients with mtDNA disease and DCM phenotype. Mouse models of DCM and mitochondrial disease do exist (Ashrafian *et al.*, 2010; Dai *et al.*, 2010; Narula *et al.*, 2011), but do not feature mtDNA point mutations or single deletions and have little direct relevance to patients with these specific mutations. Cardiac symptoms may be limited in patients with multisystem mtDNA disease due to progressive skeletal myopathy restricting physical activity. However limited echocardiographic studies in adults suggest progression of DCM may be slow and, at least in some patients responsive to conventional heart failure therapies (Wahbi *et al.*, 2010; Stalder *et al.*, 2012).

1.5.3.3 Rarer cardiomyopathies

1.5.3.3.1 Restrictive cardiomyopathy

Restrictive cardiomyopathy is a rare presentation of cardiac involvement in mtDNA disease but has been reported in association with maternally-inherited deafness and diabetes due to the m.3243A>G mutation (Thebault *et al.*, 2008), and as the only clinical finding in a subject with m.1555A>G mutation (Santorelli *et al.*, 1999).

1.5.3.3.2 Left ventricular non-compaction

Left ventricular non-compaction (LVNC) is caused by abnormal compaction of myofibrils during cardiac development and results in progressive ventricular dilatation and systolic dysfunction. Differentiation from normal variants can be difficult, diagnosis remains controversial and the natural history is unclear (Oechslin *et al.*, 2000; Kohli *et al.*, 2008). Mutations in sarcomeric or ion channel genes account for only a small proportion of LVNC cases (Klaassen *et al.*, 2008). LVNC has recently been recognized as a cardiac manifestation of mtDNA disease, particularly in paediatric populations, and most commonly as part of multisystem disease (Scaglia *et al.*, 2004; Finsterer, 2009). A recent report of an association between a m.3398T>C *MTND1* variant and LVNC supports the assertion that mtDNA mutations may be important in pathogenesis (Tang *et al.*, 2010).

1.5.3.3.3 Histiocytoid cardiomyopathy

Histiocytoid cardiomyopathy is another rare cardiomyopathy characterized by pathognomonic histiocyte-like cells within subendocardium. Reported cases frequently document aggregates of structurally abnormal mitochondria (Shehata *et al.*, 1998), and have been linked to the m.8344A>G mutation and a mutation in the *MTCYB* gene that encodes an complex III enzyme subunit (Andreu *et al.*, 2000; Vallance *et al.*, 2004).

1.5.4 Electropathy

1.5.4.1 Conduction system disease and bradyarrhythmias

Conduction system disease occurs commonly in patients with mtDNA disease, and prevalence increases with age as in the general population. Heart block forms part of the diagnostic criteria of KSS such that a review of the published literature suggests a prevalence of conduction system disease of 84% (Hirano and DiMauro, 1996). Conduction system disease occurs, albeit less commonly, in ~5-10% of patients in other forms of mtDNA disease with AV or interventricular conduction disturbances reported in association with m.3243A>G and m.8344A>G mutations (Majamaa-Voltti *et al.*, 2002; Wahbi *et al.*, 2010). Although mechanisms are currently unknown, differences in mutation load or in sensitivity of different cardiac cell types to different mtDNA mutations (threshold), may account for this phenotypic discrepancy (Muller-Hocker *et al.*, 1998).

Importantly in patients with neuromuscular disease, including mtDNA disease, progression to high-grade AV block is often unpredictable necessitating prompt recognition of any conduction system disease and consideration of early intervention (Vardas *et al.*, 2007; Epstein *et al.*, 2008). Early deaths in patients with KSS may be directly attributable to infra-nodal heart block (Roberts *et al.*, 1979). Risks of progression and clinical outcomes associated with conduction system disease in other forms of mtDNA disease are unknown.

1.5.4.2 Ventricular pre-excitation and tachyarrhythmias

Ventricular pre-excitation and Wolff-Parkinson-White syndrome may be more common in patients with mtDNA disease than in the general population. First observed in association with Leber’s hereditary optic neuropathy, ventricular pre-excitation has

been reported in 10% patients and 8% maternal relatives compared to 1.6% of paternal relatives (Nikoskelainen *et al.*, 1994). While supported by several studies, the failure of some groups to replicate this finding has stimulated debate as to whether these results represent chance findings or evidence of a direct aetiological link (Sorajja *et al.*, 2003). Evidence in support of the latter is provided by reports of ventricular pre-excitation occurring in association with m.8344A>G and m.3243A>G mutations, where manifest pre-excitation was observed in 3-27% of patients (Anan *et al.*, 1995; Okajima *et al.*, 1998; Sproule *et al.*, 2007; Wahbi *et al.*, 2010). While ventricular pre-excitation has been reported in association with mtDNA-related cardiomyopathy (Finsterer *et al.*, 2001), this combination does not appear as common as in other forms of inherited disease such as that caused by *PRKAG2* gene mutations (Arad *et al.*, 2005). Symptomatic patients with mtDNA disease and manifest ventricular pre-excitation have undergone successful radio-frequency ablation (RFA) of accessory pathways but natural history remains unclear and invasive management of asymptomatic patients is controversial.

Supraventricular and ventricular tachyarrhythmias have both been reported in patients with mtDNA disease, particularly in children and in those with cardiomyopathy (Oginosawa *et al.*, 2003; Scaglia *et al.*, 2004). While prolongation of the QT interval has been identified in some patient groups (Karanikis *et al.*, 2005), determination of the true incidence of this finding and the risk of ventricular arrhythmia requires larger longitudinal studies.

1.5.4.3 Congenital heart disease

Mitochondria play a critical role in early cardiac development and mitochondrial dysfunction has been implicated in the aetiology of congenital heart disease. Congenital structural heart defects, such as ventricular septal defects (VSDs), have been reported in patients with mitochondrial disease (Farag *et al.*, 2002). However with such high prevalence of muscular VSDs in neonates, it is difficult to establish whether this association represents more than a chance event. Mouse models with severe cardiac structural defects exclusively feature nuclear mutations with limited application to mtDNA disease (Larsson *et al.*, 1998; Chen *et al.*, 2007). Biochemical evidence of mitochondrial dysfunction has been reported in congenital heart disease,

but these are likely to represent secondary abnormalities of unclear significance (Mital *et al.*, 2004; Shinde *et al.*, 2007).

1.5.5 Diagnosis

The diagnosis of mtDNA disease is complex and requires a multidisciplinary approach (Figure 1.13). A maternal inheritance pattern or the presence of extra-cardiac features of mtDNA disease may raise suspicion of the diagnosis. While these extra-cardiac manifestations include common or non-specific features (Figure 1.12), particular patterns of organ involvement (e.g. diabetes and deafness) should alert the cardiologist to the possibility of mtDNA disease.

1.5.5.1 Molecular genetic testing

Emerging evidence supports screening of peripheral lymphocytes or urine samples for mtDNA mutations (e.g. 3243A>G, m.4300A>G) in specific clinical scenarios. In patients with unexplained LVH not fulfilling standard criteria for HCM, symmetrical hypertrophy and the absence of LVOT obstruction may favour an alternative diagnosis, such as mtDNA-related cardiomyopathy (Gersh *et al.*, 2011). Sequencing of the mitochondrial genome may be an appropriate next step in investigation. However, with more pronounced variation than the nuclear genome, challenges exist in the determination of pathogenesis (Zaragoza *et al.*, 2011). Comparison to published databases is necessary but true determination of the pathogenicity of novel mtDNA mutations is complex and reliant on canonical criteria involving segregation of mutation within tissues and families, evolutionary conservation of affected nucleotides or amino acids and occasionally biochemical studies in cultured cells.

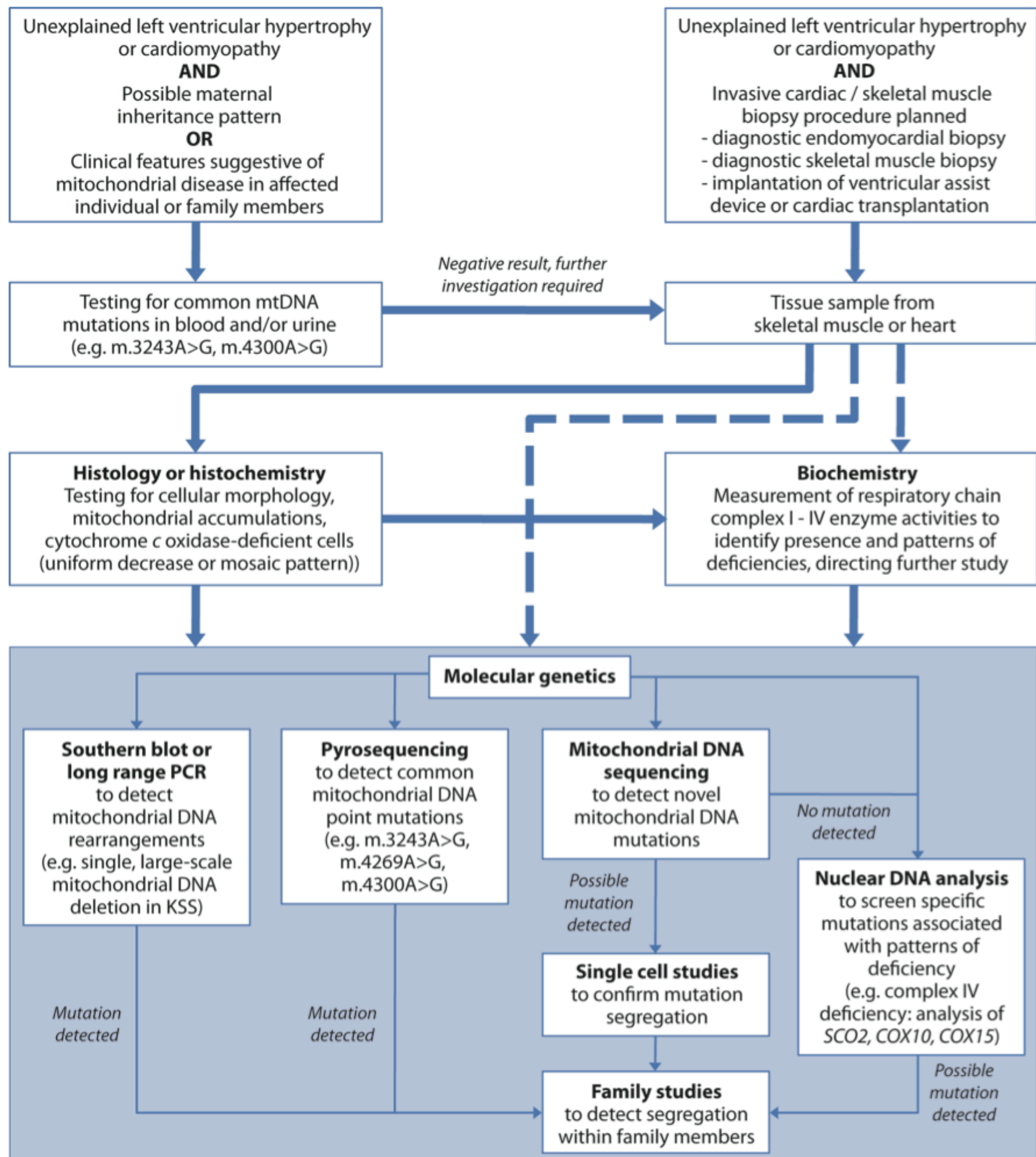


Figure 1.13 Algorithm for investigation of mitochondrial disease in cardiomyopathy. Ideally, mitochondrial disease should be investigated in the most affected tissue. However, this is often not possible and skeletal muscle biopsy can serve as an alternative, even in the absence of clinical myopathy. Histochemical analysis, although not always possible due to limited tissue availability, can direct genetic investigation but it may be necessary to perform biochemical or molecular genetic analysis directly (dashed arrows). mtDNA = mitochondrial DNA; PCR = polymerase chain reaction.

1.5.5.2 Invasive biopsy analysis

While molecular genetic testing may expedite a diagnosis of mitochondrial disease in some patients, in many, particularly those with novel mutations, analysis of invasive biopsy tissue remains important.

Pathological studies of the myocardium are available from a small number of patients with mtDNA-related cardiomyopathy (Taylor *et al.*, 2003b; Sebastiani *et al.*, 2007). Common but relatively non-specific histological findings are diffuse cellular hypertrophy with swollen, often vacuolated, cardiomyocytes FIG Interstitial fibrosis varies but myofibre disarray, typical of HCM, is absent and ultrastructural examination reveals proliferation of abnormal mitochondria with sarcomere displacement (Leone *et al.*, 2011). On cardiac frozen sections, the sequential assay of cytochrome *c* oxidase (COX) / succinate dehydrogenase (SDH) activities can demonstrate the typical mosaic appearance of COX deficiency (Figure 1.14).

Skeletal muscle biopsy is a low risk procedure that can provide similar evidence for mtDNA disease, even in patients without evidence of myopathy. However the tissue specificity of biochemical defects due to mtDNA mutations is such that in isolated or prominent cardiomyopathy, examination of endomyocardial biopsy (EMB) tissue may be relevant. This procedure is associated with a serious complication rate of ~1% and remains controversial (From *et al.*, 2011). International guidelines suggest pathological methodologies and clinical scenarios where EMB can reasonably be performed, including in the investigation of possible mtDNA-related cardiomyopathy (Cooper *et al.*, 2007; Leone *et al.*, 2011). Indeed, in such patients, opportunistic assessment of cardiac tissue obtained during other invasive cardiac procedures (e.g. ventricular assist device implantation) should be considered (Bates *et al.*, 2012). A recent consensus statement supports attempts to maximize the diagnostic utility of such specimens (Stone *et al.*, 2012).

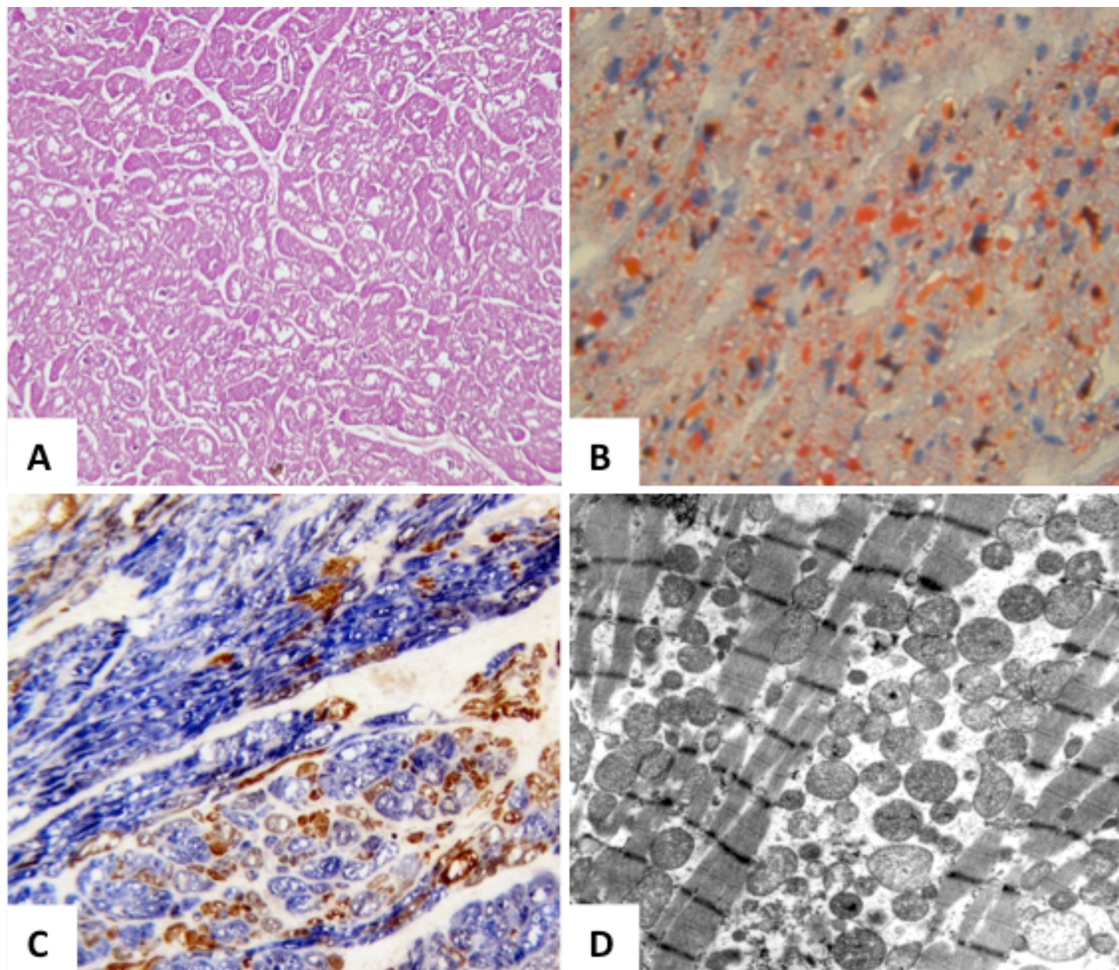


Figure 1.14 Histological, histochemical and ultrastructural features of mtDNA-related cardiomyopathy. (A) Histological examination of explanted left ventricular tissue from a patient with a homoplasmic mt-tRNA^{Ile} mutation reveals enlarged cardiomyocytes with prominent cytoplasmic vacuolization (H&E, 20x). (B) Vacuoles contain lipid droplets that stain with Oil Red O (40x). (C) Sequential COX/SDH histochemistry shows several COX-deficient cardiomyocytes (blue) with scattered COX-positive cells (brown, 40x). (D) Ultrastructural analysis reveals proliferation of polymorphic mitochondria and displacement of sarcomeres (uracyl acetate lead citrate, 3150x). Images reproduced with permission from original image (Bates *et al.,* 2012)

1.5.6 Cardiac investigations

Cardiac involvement in mtDNA disease can remain asymptomatic until an advanced stage, often due to limited mobility of patients. Although the utility of screening is debated in mtDNA disease given variability in clinical course, best practice supports a high index of suspicion and instigation of regular surveillance (Figure 1.15). Multidisciplinary care is essential given potential involvement of organs that can cause symptoms associated with cardiac disease. Exercise intolerance, for example, may

result from skeletal myopathy or respiratory muscle weakness, as well as cardiomyopathy or arrhythmia. A cardiologist with an understanding of mtDNA disease should be involved in the care of all patients with confirmed cardiac involvement (Bates *et al.,* 2012).

In common with a number of other rare neuromuscular or metabolic conditions, there are few clear recommendations for disease management. There is general agreement that all patients with mtDNA disease, unaffected carriers of a known mutation, and obligate carriers should have baseline cardiac assessment. This should include clinical history and examination, 12-lead electrocardiograph (ECG) and an assessment of cardiac structure and function, typically echocardiography, as a minimum standard in all forms of mtDNA disease as, although specific cardiac phenotypes are associated with different mtDNA mutations (e.g. single, large-scale mtDNA deletion and AV block), diverse cardiac phenotypes can occur.

Although the initiation, nature and frequency of cardiac screening has not been subject to specific study, many experienced centres use an initial 12-month interval for repeated ECG and functional assessments consistent with guidelines for HCM and different forms of neuromuscular disease, with extension of this interval to 3-5 years if normal findings are repeated (Bates *et al.,* 2012). Magnetic resonance imaging (MRI) may reveal cardiac involvement when standard evaluation is unremarkable (Nakanishi *et al.*, 2007), and permits imaging without reliance on acoustic windows, often absent in patients with skeletal or respiratory muscle disease. Cardiac MRI also permits accurate tissue characterization using late gadolinium enhancement (Stalder *et al.*, 2012), an area where ongoing studies may reveal important features of mtDNA-related cardiomyopathy.

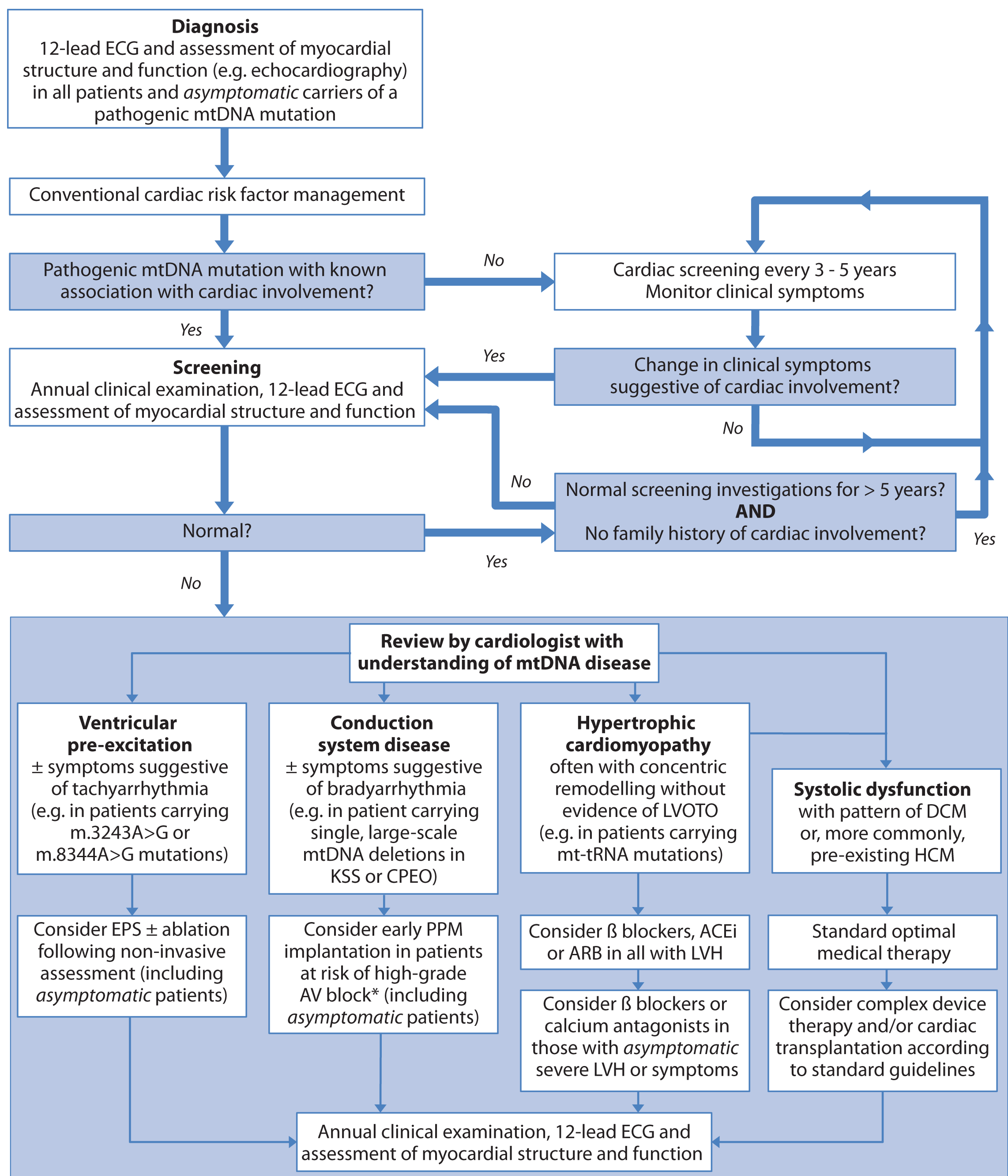


Figure 1.15 Clinical algorithm for cardiac screening and management in mtDNA disease. International guidelines support early intervention for conduction disease in patients with mtDNA disease (Vardas *et al.*, 2007; Epstein *et al.*, 2008). ACEi = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; AV = atrio-ventricular; CPEO = chronic progressive external ophthalmoplegia; DCM = dilated cardiomyopathy; EPS = electrophysiological study; HCM = hypertrophic cardiomyopathy; KSS = Kearns-Sayre Syndrome; LVH = left ventricular hypertrophy; LVOTO = left ventricular outflow tract obstruction; mtDNA = mitochondrial DNA; PPM = permanent pacemaker; * many experienced centres regard rapid progression of conduction disease, severe surface ECG abnormalities and/or HV interval >70ms as high-risk features for block (Lazarus *et al.*, 2002; Groh *et al.*, 2008).

Several lines of evidence suggesting a central role of disrupted energy metabolism in HCM and phenocopies, including mtDNA disease (Ashrafian *et al.*, 2003; Crilley *et al.*, 2003). Abnormal cardiac bioenergetics have been demonstrated in patients with m.3243A>G mutation and structurally normal hearts on echocardiography (Lodi *et al.*, 2004). Contemporaneous assessments of myocardial bioenergetics, fibrosis, and myocardial deformation, using cardiac tagging, may permit early identification of patients at risk of developing cardiomyopathy. The preferred approach may therefore involve both cardiac MRI and echocardiography at diagnosis to establish a baseline, with subsequent screening performed with echocardiography alone. However, larger longitudinal studies are necessary to clarify the role of such investigations in patients with mtDNA disease.

1.5.7 Management of mtDNA-related cardiac disease

Although clinical trials are underway, a recent Cochrane review suggests that there is no current drug treatment that has shown clear clinical benefit in the primary outcome in patients with mtDNA disease, or has been approved by regulatory bodies for the treatment of mitochondrial disease (Pfeffer *et al.*, 2012). For example, idebenone, a co-enzyme Q_{10} analogue that has been approved for the treatment of Friedrich's ataxia, has recently been studied in a large, randomized, placebo-controlled trial in patients with LHON and, although it failed to meet the primary endpoint, there was a suggestion of benefit in patients with discordant visual activities (Klopstock *et al*., 2011). Resistance and endurance exercise training programmes both improve symptoms in mtDNA disease but effects on cardiac structure and function are currently unknown, and benefits are lost on cessation of exercise with deconditioning (Taivassalo *et al.*, 2006; Murphy *et al.*, 2008). Patients with mtDNA disease remain at risk of common acquired cardiac disorders and current guidelines to address conventional risk factors should be followed.

1.5.7.1 Cardiomyopathy

Recommendations for the management of hypertrophic remodelling in mtDNA disease are reliant on clinical studies in HCM and LVH, with interventions based on reasonable clinical assumptions of similar treatment effects, together with reports of successful

outcomes (Tranchant *et al.*, 1993; Bonnet *et al.*, 2001; Stalder *et al.*, 2012). Non-dihydropyridine calcium channel antagonists and beta-blockers are recommended in symptomatic patients or those with asymptomatic severe LVH in HCM (Maron *et al.*, 2003; Gersh *et al.*, 2011). ß blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers have been demonstrated to reduce LVH in the general population. Given the progressive nature of hypertrophic remodelling in mtDNA disease, these drugs are often started with the first appreciation of LVH.

Standard optimal medical therapies for heart failure with systolic dysfunction are used in mtDNA disease, with reports of both clinical improvement and progression despite therapy (Nakanishi *et al.*, 2007; Stalder *et al.*, 2012). ACE inhibitors have been shown to slow onset and progression of cardiomyopathy associated with Duchenne muscular dystrophy, and reduce mortality (Duboc *et al.*, 2005). Complex device therapy including the use of implantable cardioverter defibrillators (ICDs) and cardiac resynchronization therapy should be considered in patients with mtDNA disease provided conventional guidelines are met, including life expectancy of >1 year. Cardiac transplantation, although controversial in metabolic disease with potential multisystem involvement, has been performed successfully in patients with mtDNA disease (Tranchant *et al.*, 1993; Morgan *et al.*, 1996; Bonnet *et al.*, 2001; Schmauss *et al.*, 2007). Clinical outcomes appear to be dependent on the extent of extra-cardiac involvement in addition to complications of transplantation itself, although data are lacking.

1.5.7.2 Electropathy

International guidelines recommend permanent pacemaker (PPM) implantation at an earlier stage of conduction system dysfunction in patients with neuromuscular disease, including mtDNA disease, than in the general population due to unpredictable progression (Vardas *et al.*, 2007; Epstein *et al.*, 2008). In these patients, any degree of AV block, including first degree block and/or any degree of fascicular block are Class IIb indications for PPM implantation, irrespective of symptoms. Such prophylactic PPM implantation however remains controversial. Severe surface ECG abnormalities (PR interval > 240ms, QRS duration > 120ms, rhythm other than sinus, or high-grade AV block) and an HV interval > 70ms are high-risk features for sudden death in myotonic dystrophy (Lazarus *et al.*, 2002; Groh *et al.*, 2008). Recent evidence suggests that an

invasive strategy to assess AV conduction in those with high-risk non-invasive features is associated with improved survival (Wahbi *et al.*, 2012). Many centres use similar criteria in patients with mtDNA disease, particularly KSS. Although sudden deaths have been reported in patients with functioning PPMs and a variety of neuromuscular diseases, there is no data to guide ICD implantation in patients with mtDNA disease out with standard primary and secondary indications.

Conventional medications for symptomatic supraventricular arrhythmias can be used in patients with mtDNA disease. Ventricular pre-excitation can lead to symptomatic re-entrant tachyarrhythmia in patients with mtDNA disease and is, in other patients, associated with a small risk of sudden cardiac death. Consistent with international guidelines, and following non-invasive assessment including an exercise ECG, consideration should therefore be given to invasive electrophysiological study (EPS) in all patients with mtDNA disease and non-intermittent pre-excitation (Blomstrom-Lundqvist *et al.*, 2003; Cohen *et al.*, 2012). Asymptomatic pre-excitation is a class IIa indication for EPS ± RFA in adults, class IIb in children >5 years of age but class III (i.e. not indicated) in those <5 years of age.

1.6 Research Aims

Cardiac involvement in mtDNA disease appears to be common and may be an important predictor of morbidity and early mortality in adults. Comprehensive clinical algorithms for cardiac disease are vitally needed, and considerable international collaborative efforts will be required to achieve this aim. Nevertheless, cardiologists are becoming increasingly involved in the care of patients with mtDNA disease as recognition of the clinical spectrum of these disorders increases. However specific disease-modifying therapies do not yet exist, and data are scarce concerning the detailed phenotype, natural history, appropriate screening strategies and clinical management of cardiac involvement in mtDNA disease. With particular emphasis on patients with mt-tRNA mutations, this thesis aims to describe the clinical cardiovascular phenotype, identifying predictors of cardiac involvement, and to establish the prevalence of mtDNA-related cardiac disease, while investigating the pathogenetic mechanisms underlying mitochondrial cardiomyopathy and the response to clinical intervention.

Chapter 2.

Materials and Methods

2.1 Materials

2.1.1 Equipment and Consumables

2.1.1.1 Equipment

Equipment	Supplier
Autoclave	Astell
Balance: Adventurer OHAUS	Jencons-PLS
Binder General Purpose Incubator	Philip Harris
BodPod air displacement plethysmograph	Cosmed
Cardiac Image Modelling software (Tagging analysis)	Auckland UniServices Ltd
Cardiac Echocardiography software (Horizon Cardiology)	McKesson
Cryostat (Cryo-star HM 560)	Microm International
Cycle ergometer (Corival)	Lode
Dry Heat Block	Techne
Echocardiography scanner (Vivid 7)	GE
Gilson Pipetteman (P10, P20, P200, P1000)	Anachem
Light Microscope	VWR International
Magnetic Resonance Scanner (3-Tesla Intera Achieva)	Philips
MetaLyzer expired gas analyzer	Cortex
NANOpure II Water Purification System	Barnstead
NICOM bioreactance cardiac output monitor	Cheetah Medical
3510 pH Meter	Jenway
Phosporus-31 magnetic resonance surface coil	Pulseteq
SenseWear Armband multi-array physical activity monitor	Bodymedia
Stereo Investigator Confocal SI System for Stereology	MBF Bioscience
CX9000 digital camera	
Olympus BX51 microscope	

TaskForce Monitor heart rate and blood pressure analyzer	CNSystems
Viewforum workstation	Philips
Vortex-genie 2	Scientific Industries
Zeiss Imaging System	Zeiss
Axioplan 2iE Light Microscope	
AxioCam HRc digital camera	
AxioVision imagecapture software	
KS 300 densitometric software	

2.1.1.2 Consumables

0.2ml Strip-tubes with flat caps	Starlab
1.5ml / 2.0ml Eppendorf tubes	Starlab
Autoclave tape	Fisher Scientific
Coverslips (22x22mm, 22x40mm, 22x50mm)	Merck
Falcon tubes (15ml and 50ml)	Costar
KIMCARE Medical Wipes	Kimberly-Clark Professional
Microscope Slides (76x26x1.0-1.2mm)	Merck
Parafilm	Scientific Laboratory Supplies
Pasteur pipettes (plastic)	Fisher Scientific
Pipette tips	Starlab
Scalpels	Fisher Scientific
Thermometers	Fisher Scientific
Whatman Grade IV filter paper	Merck

2.1.2 Solutions and Chemicals

2.1.2.1 Solutions

Unless otherwise specified in the text, all solutions listed were prepared using nanopure (18 megaOhms activity) water.

5% Bovine Serum Albumin	10g bovine serum albumin in 1l dH_2O
Formal Calcium	3.6% (v/v) Formaldehyde
	1.1% (w/v) Calcium Chloride
4% Paraformaldehyde	20g paraformaldehyde in 250ml nanopure water
	250ml 0.2M phosphate buffer
Phosphate Buffer (pH 7.0)	200ml 0.2M disodium hydrogen phosphate
	50ml 0.2M sodium dihydrogen phosphate
Phosphate Buffered Saline	Prepared from tablets: 1 Tablet per 100ml water
TBS (and TBST) pH 7.4	3g Trisma base in 5l dH_2O
	42.5g NaCl (+ 0.1% Tween 20)

2.1.2.2 Chemicals

2.1.2.2.1 Tissue preparation

Isopentane	Merck
Liquid Nitrogen	BOC
OCT cryo-embedding matrix	Raymond Lamb

2.1.2.2.2 Histology, histochemistry and immunohistochemistry

Calcium Chloride	Sigma
Catalase	Sigma

Cytochrome *c*	Sigma
3,3'Diaminobenzidine Tetrahydrochloride	Sigma
Disodium Hydrogen Phosphate	Sigma
DPX mountant	Merck
Eosin	Merck
Ethanol AnalaR	Merck
Formaldehyde	Merck
Haematoxylin	Merck
Histoclear	National Diagnostics
Hydrogen Peroxide	BDH
Nitro Blue Tetrazolium	Sigma
MenaPath Liquid Stable DAB	Menarini diagnostics
MenaPath X-Cell Plus HRP Polymer Detection Kit	Menarini diagnostics
Methanol AnalaR	Merck
Paraformaldehyde	Sigma
Phenazine Methosulphate (PMS)	Sigma
Phosphate Buffered Saline Tablets	Oxoid
Primary antibodies	Cambridge Biosciences
Sodium azide	Sigma
Sodium chloride	Sigma
Sodium succinate	Sigma
Triton X-100	Sigma

2.2 Methods

2.2.1 Patients

All samples and data in these studies were obtained from patients referred to the Newcastle NCG Mitochondrial Diagnostic Service (www.mitochondrialncg.nhs.uk) that is supported by the Newcastle upon Tyne Hospitals NHS Foundation Trust and NHS Specialized Services. The majority of patients were also participants in the Medical Research Council (MRC) Centre for Neuromuscular Diseases Mitochondrial Disease Patient Cohort (UK) study. Ethical approval was obtained from North East Research Ethics Committees (Newcastle and North Tyneside 2 or Sunderland). All participants provided informed consent, samples were subject to the relevant regulations of the Human Tissue Act (2007), and patient confidentiality was maintained.

2.2.1.1 Retrospective cohort study

Patient data for cross-sectional analysis of the prevalence of the cardiac manifestations of mitochondrial disease and the retrospective study of disease progression and outcome were obtained from two specific sources: participants in the MRC Centre for Neuromuscular Diseases Mitochondrial Disease Patient Cohort (UK) study and attendees of a specialist mitochondrial disease clinic in Newcastle upon Tyne over a 25-year period from 1985-2010.

2.2.1.2 Retrospective post-mortem cardiac tissue study

The database of the Newcastle NCG Mitochondrial Diagnostic Service was searched for adult patients carrying the m.3243A>G or m.8344A>G mutations with suitable post-mortem cardiac tissue, obtained and stored with informed consent for studies.

2.2.1.3 Prospective cardiac tissue study

Patient samples for the prospective cohort study of mitochondrial respiratory chain abnormalities in children <18 years of age with end-stage cardiomyopathy were obtained from consecutive patients with idiopathic or familial cardiomyopathy, undergoing orthotopic cardiac transplantation or ventricular assist device (VAD) implantation at the Newcastle upon Tyne Hospitals NHS Foundation Trust between January 2009 and December 2010.

2.2.1.4 Prospective cardiac imaging studies

Patients for the cross-sectional magnetic resonance imaging analysis of early cardiac involvement in mtDNA disease and the endurance exercise training interventional study were recruited from patients with mitochondrial disease due to either the m.3243A>G or m.8344A>G mutation, who were consecutive attendees at a specialist outpatient clinic between August 2010 and July 2011.

2.2.2 Human tissue preparation

Human tissue samples for these studies were obtained from either (i) open surgical biopsy at the time of cardiac transplantation or VAD implantation, or (ii) post-mortem tissue. All tissues samples were mounted on Whatman grade IV filter paper using OCT adhesive and snap-frozen by immersion in a bath of isopentane that had been pre-cooled to -160^{0}C in liquid nitrogen. A 24-hour service for processing explanted cardiac tissue from children with end-stage cardiomyopathy undergoing transplantation or VAD implantation was established to ensure a time delay of <60 minutes from surgical biopsy to freezing. Similarly a time delay to freezing of <8 hours post-mortem was required for inclusion of tissue samples in the post-mortem studies. Human tissue was transported in liquid nitrogen and stored at -80^{0}C. All tissue was sectioned at -19^{0}C using a Cryo-star HM 560M cryostat with the blade set at -21^{0}C.

2.2.3 Histology, histochemistry and immunohistochemistry

2.2.3.1 Haematoxylin and Eosin staining procedure

The haematoxylin and eosin (H & E) stain permits analysis of tissue and cellular morphology. Haematoxylin binds anionic tissues, such as nuclear chromatin, which are then stained by its oxidative derivative, haematein, providing a nuclear marker. Eosin acts a counter stain, indiscriminately staining the basic proteins of the cytoplasm.

Tissue sections were air-dried for one hour, then fixed in formal calcium for 15 minutes and rinsed in tap water. Sections were then immersed in Meyer's Haematoxylin solution for two minutes, then washed in tap water until the water ran clear, and immersed in Eosin solution (1% eosin yellowish, 0.4% erythrosine B, 0.2% phloxin B) for one minute, before final washing in tap water until the water ran clear again. Sections were dehydrated by passage through a graded ethanol series (70%, 95% and

100% ethanol) before immersion for ten minutes in 100% ethanol. Sections were then cleared in two changes of Histoclear and mounted in DPX with addition of a coverslip.

2.2.3.2 Sequential cytochrome c oxidase /succinate dehydrogenase histochemistry

Separate stains for the activities of cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) were performed in all tissues but sequential use of COX / SDH histochemistry was used to optimise identification of cells with histochemical evidence of respiratory chain abnormalities.

Histochemical analysis of functional complex IV activity is based on the oxidation of 3,3'-diaminobenzidine (DAB) tetrahydrochloride to produce a brown polymer precipitate as cytochrome *c* is reduced (Seligman *et al.*, 1968). Cardiac tissue sections were incubated for 40 minutes at 37°C with 100μM cytochrome *c*, 4mM DAB and liver catalase, all prepared in 0.1mM phosphate buffer at pH 7.0. Following incubation, sections were washed in two changes of phosphate buffered saline (PBS), each of five minutes' duration, before application of the SDH medium. Cardiac tissue sections were incubated for 30 minutes at 37°C with 1.5mM Nitro Blue Tetrazolium (NBT), 130mM sodium succinate, 0.2mM phenzine methosulphate (PMS) and 1mM sodium azide, all prepared in 0.1M phosphate buffer at pH 7.0. The histochemical analysis of functional complex II activity (entirely nuclear-encoded) aids identification of cells with evidence of respiratory chain deficiency as cells with a biochemical defect (no brown polymer precipitate) appear blue. With PMS acting as an electron carrier, SDH oxidises sodium succinate and reduces NBT, leading to the production of a blue precipitate. Sections were again washed in two changes of PBS, each of five minutes' duration, before being dehydrated thorough a graded ethanol series (as in Section 2.2.3.1), cleared in Histoclear, and mounted in DPX with addition of a coverslip.

2.2.3.3 Immunohistochemistry

Frozen cardiac tissue sections were air-dried for one hour then fixed in 4% paraformaldehyde in 0.1mM phosphate buffer at pH 7.4 for 10 minutes at room temperature. Sections were then washed for 10 minutes in tris-buffered saline (TBS) containing 0.1% Tween20 (TBST), before being permeabilised by passage through a graded methanol series. Sections were consecutively immersed in 70% methanol for

10 minutes, 95% methanol with 0.3% H_2O_2 for 10 minutes, and then 100% methanol for 20 minutes before returning through 95% and 70% methanol for the same periods. The addition of 0.3% H_2O_2 to the 95% methanol inhibited endogenous peroxidase activity. Sections were washed for 30 minutes in 5% bovine serum albumin (BSA) in TBST to block non-specific tissue binding and primary antibodies were diluted, to an appropriate concentration, in the same solution. All sections were incubated with primary antibodies for one hour at room temperature.

After incubation, sections were washed in two changes of TBS, each of 10 minutes' duration, and then for five minutes in TBST. The MenaPath X-Cell Plus HRP polymer detection kit was then used to detect the primary antibodies. Sections were incubated with the biotin-free universal probe for 30 minutes at room temperature and then washed in two changes of TBS, each of 10 minutes' duration, before being incubated for 30 minutes with the horseradish peroxidase polymer, which binds to the probe. Sections were then washed again in two changes of TBS, each of 10 minutes' duration, and then for five minutes in TBST. The MenaPath Liquid Stable DAB chromogen, which produces a brown precipitate in the presence of peroxidase enzyme, was then used to visualise staining. As per the manufacturer's instructions, one drop (32μl) of DAB chromogen was used per 1.0ml of the provided DAB substrate buffer. Sections were incubated in the DAB mixture for five minutes before being washed in tap water.

All tissue sections were counterstained with Meyer's Haematoxylin for 60 seconds to permit identification of nuclei and were then washed again in tap water. Sections were then dehydrated in a graded ethanol series (as in Section 2.2.3.1), cleared in Histoclear and mounted in DPX with addition of a coverslip.

2.2.3.3.1 OXPHOS antibodies

Monoclonal primary antibodies directed against complex subunits were obtained from a commercial supplier (MitoSciences, Oregon, USA) and were provided at a concentration of 1mg/ml in HEPES buffered saline. In addition to antibodies specific to subunits of the ETC, an antibody to porin (IgG2b) was also used as a non-specific marker of mitochondrial density as this channel protein is abundant in the mitochondrial outer membrane (see Section 1.1.2). Several antibodies for each OXPHOS complex under investigation (I, II and IV) were initially used with control

cardiac tissue to determine the antibody best suited to OXPHOS complex immunohistochemistry. Further details concerning this selection process and subsequent optimisation using control cardiac tissue to obtain the lowest concentration of dilution that delivered an identifiable staining pattern without non-specific staining in blood vessels or background are given in Section 3.2.4.3.

	Description	Isotype	Subunit	Details
Complex I	15 kDa	IgG1	NDUFB4	Nuclear-encoded subunit NDUFB4, which does not contribute to the catalytic function of complex I (Murray *et al.*, 2003).
	19 kDa	IgG2b	NDUFA13	The nuclear-encoded subunit NDUFA13, is also referred to as 'GRIM-19' (Gene associated with Retinoid IFN-induced Mortality) due to its association with programmed cell death.
	20 kDa	IgG1	NDUFB8	Nuclear-encoded subunit NDUFB8 (Fernandez-Moreira *et al.*, 2007). While NDUFB8 does not contribute to catalytic function of complex I, subunit entry into the assembling complex I is dependent on the mtDNA-encoded subunit, ND4 (Perales-Clemente *et al.*, 2010).
Complex II	70 kDa	IgG1	FP subunit	Flavoprotein subunit of complex II, but also described as subunit A (see Section 1.2.1.2)
Complex IV		IgG2a	COX I	The mtDNA-encoded subunit I of complex IV contains the active site of the enzyme complex and forms the core protein structure, together with subunits II and III.
		IgG2a	COX IV	The nuclear-encoded subunit IV of complex IV is detectable even cells that lack mtDNA, as discussed in chapter 4 (Marusich *et al*., 1997).

Table 2.1. Monoclonal OXPHOS antibodies. Structure and function of ETC complex subunits that were targeted through use of monoclonal antibodies in this study.

2.2.3.4 Quantification of respiratory chain deficiency

Respiratory chain deficiency was accurately quantified from all histochemical tissue sections using the Stereo Investigator Confocal SI System. Stereology is normally a tool used to quantify three-dimensional objects from two-dimensional sections through the object. However in this study, the Stereo Investigator System was used to count multiple histochemical cell categories (i.e. COX-positive, COX-intermediate, and COX-deficient) in a precise, unbiased manner (Murphy *et al*, 2012). Both COX-negative and COX-intermediate cells were considered abnormal in all patients (i.e. COX-deficient).

COX-positive (normal)	Normal activities for complex II and complex IV
COX-negative (abnormal)	Cells accumulate no brown precipitate during COX histochemistry and stain entirely blue during subsequent SDH histochemistry
COX-intermediate (abnormal)	Cells cannot be classified into either of the above categories and display an intermediate pattern

Under low magnification, a contour was drawn around the boundary of a tissue section; a suitable higher power objective was then selected for cell counting. Once a cell category had been identified, a marker was selected to represent this category and all cells within the field-of-view that corresponded to this category were labelled. Once all cells within a field-of-view had been thus categorised, the motorised stage of the Stereo Investigator System was used to move the field-of-view to an adjacent region of the outlined section and this approach was used to categorize all cells within the original outlined section. In tissue sections too large for all cells to be counted, a contour was drawn at low magnification either around an unbiased subset of cells, or around multiple random regions within a single tissue section.

To enable comparison of functional and structural ETC deficiencies detected with histochemical and immunohistochemical approaches respectively, an identical method of quantification with the Stereo Investigator System was used for tissue sections incubated with monoclonal antibodies to ETC complex subunits. Additionally to ensure appropriate recognition of the differential cellular staining in all immunohistochemical tissue sections, dedicated Zeiss KS 300 optical densitometry software was used to

quantify the density of the optical signal in an unbiased subset of cells selected at low magnification and photographed at high magnification. The default densitometry scale is an inverse linear scale ranging from 0 (black) to 255 (white). For all cells assessed the nuclear signal was excluded from the optical densitometry assessment, and the default scale was inverted and expressed as a percentage of 255 ranging from 0 (white) to 100 (black), and compared to post-mortem heart samples obtained from individuals without evidence or cardiac or metabolic disease.

2.2.4 Clinical assessment methods

2.2.4.1 Physical examinations

2.2.4.1.1 Body weight and composition

Participants' heights were recorded for body mass index (BMI) and body surface area (BSA) calculations. Body weights, fat percentages and lean body weights were measured using air displacement plethysmography.

2.2.4.1.2 Newcastle Mitochondrial Disease Adult Scale

All patients participating in the prospective exercise studies were examined and interviewed. Disease burden was assessed in these patients, in participants in the Medical Research Council Centre for Neuromuscular Diseases Mitochondrial Disease Patient Cohort (UK) study, and in attendees at the specialist mitochondrial clinic in Newcastle upon Tyne Hospitals NHS Foundation Trust using the Newcastle Mitochondrial Disease Adult Scale (NMDAS), a validated scoring system (Schaefer *et al.*, 2006).

2.2.4.1.3 Serum and urine laboratory analyses

Analyses of urinary epithelial cells for mtDNA mutation load (Whittaker *et al.*, 2009) and serum for biochemical markers of renal and metabolic function were performed using standard clinical protocols, available within the Newcastle upon Tyne Hospitals NHS Foundation Trust.

2.2.4.1.4 Fatigue and quality of life

Standard, validated self-completion questionnaires were used for the assessment of fatigue, symptoms of autonomic dysfunction and overall quality of life. The Fatigue

Impact Scale (FIS), Composite Autonomic Symptom Score (COMPASS) and the Short-Form 36 (SF-36) Health Survey are all international questionnaire with external validity, designed to record and investigate specific aspects of multi-system disease.

2.2.4.2 Cardio-pulmonary exercise testing

Cardiopulmonary exercise testing during the exercise study was performed on a calibrated, electro-magnetically braked stationary bicycle ergometer at a steady cadence between 60 and 80 bpm. A stepped incremental workload test (~10-20 W/min) was conducted to elicit a symptom-limited maximum oxygen uptake and heart rate response. Respiratory gas exchange data were collected continuously and the Borg Rating of Perceived Exertion (RPE) Scale was performed every 3 minutes. Exercise was terminated when subjects developed severe dyspnoea or peripheral muscle fatigue and they were physically exhausted (as indicated by respiratory exchange ratio (RER) >1.1, Borg RPE scale score >18, or the absence of rise in oxygen consumption with further increases in exercise intensity). In patients with mitochondrial disease, a blunted heart rate response was anticipated due to potential extra-cardiac manifestations of mitochondrial disease and concomitant medications, and this approach was taken to represent a maximal aerobic effort. Anaerobic threshold was determined using the V-slope method, as previously described (Beaver *et al.*, 1986) Baseline physical activity was assessed using an armband accelerometer.

2.2.4.3 Autonomic function testing

Numerous methodologies have been employed in attempts to quantify autonomic function (Parati *et al.*, 2001) Current assessments used in this study are non-invasive and based on fluctuations in the RR interval between consecutive QRS complexes (heart rate variability), and analogous changes in beat-to-beat blood pressure measurements (blood pressure variability).

2.2.4.3.1 Heart rate variability

Sympathetic and parasympathetic inputs to the cardiovascular system exert opposing influences on heart rate, with sinus rhythm oscillating around a mean heart rate; the dynamic interaction between these activity causes continuous oscillation of the heart rate and this feature is termed heart rate variability (HRV). Under resting conditions, parasympathetic activity dominates and fluctuations in heart rate are principally

determined by changes in parasympathetic activity (Levy and Pappano, 2007). The analysis of HRV can be used as a tool to assess sympatho-vagal balance in the autonomic nervous system.

HRV can be analysed using different techniques related to either the time domain or the frequency domain of the acquired data. Both forms of HRV analysis have been standardized in international guidelines (Taskforce of the ESC and NASPE, 1996) and are performed using RR interval time series obtained from continuous ECG recording and automated detection of QRS complexes (Figure 2.1).

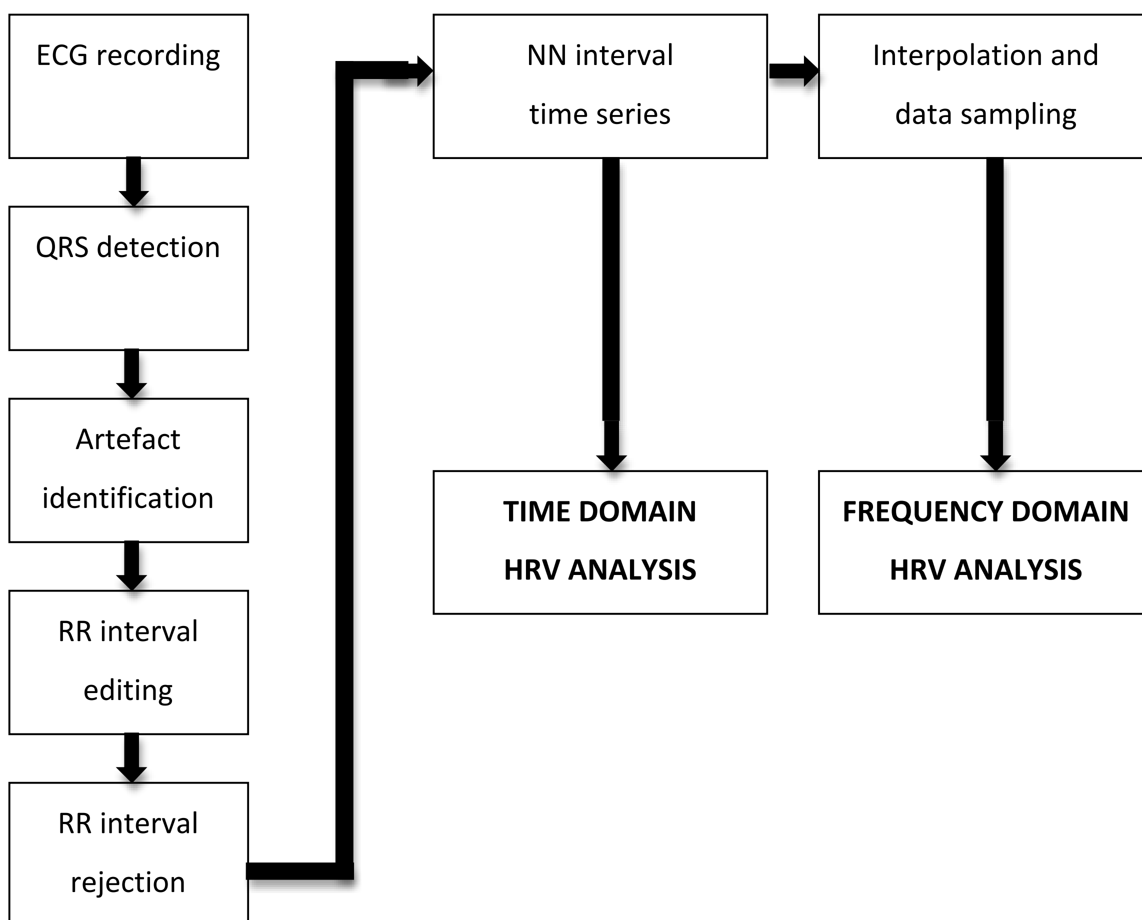


Figure 2.1 Flow diagram of the HRV data processing. Acquired ECG data is pre-processed to remove artefact and unreliable RR intervals with subsequent analysis of NN intervals directly and following interpolation. Flow diagram modified with permission from the original image (Taskforce of the ESC and NASPE, 1996). HRV = heart rate variability; ECG = electrocardiogram.

Consecutive RR intervals calculated over the duration of an ECG recording are referred to as an *RR interval time series* or an NN interval time series following pre-processing.

Although all time domain variables can be directly calculated the NN interval time series, such measures of HRV are most applicable over longer-term recordings (>24 hours). More theoretical knowledge and practical experience exists in the use of frequency domain analysis for autonomic function assessment.

Frequency domain measures are widely reported in the clinical literature and their use is recommended in all short-term recordings (<24 hours), as the most appropriate assessment of autonomic function. HRV analysis in the frequency domain in this study involved interpretation of the NN interval time series data as a spectrum in terms of how variance (or power) distributes as a function of frequency (Taskforce of the ESC and NASPE, 1996), using automated non-parametric methodologies.

2.2.4.3.2 Blood pressure variability

Reliable, non-invasive beat-to-beat blood pressure was measured using fingers plethysmography to detect arterial pressure waveforms, correcting values for oscillometric blood pressures recorded in the contralateral arm. Analogous to the situation with NN interval time series data, systolic and diastolic blood pressure time series data were analysed in the frequency domain. Power Spectral Density techniques enabled investigation of how variance changes as a function of frequency and provide LF (or sympathetic) and HF (or parasympathetic) assessments.

2.2.4.3.3 Apparatus

All autonomic function testing was performed in a dedicated cardiorespiratory laboratory in a quiet environment; all measurements were performed with the Task Force Monitor (TFM) – a dedicated device that provides automated and computed beat-to-beat analysis of heart rate and blood pressure. Biological inputs to the system are provided by: (i) 3-lead ECG; (ii) impedance cardiography; and (iii) continuous, non-invasive blood pressure, recorded using finger plethysmography with intermittently calibration to contralateral oscillometric blood pressure measurements. From these source data, the TFM calculates continuous, reliable and reproducible measurement of all haemodynamic and autonomic parameters (Gratze *et al.*, 1998; Parati *et al.*, 2003; Fortin *et al.*, 2006)).

2.2.4.3.4 Parameters

All cardiovascular parameters of HRV and blood pressure variability (BPV) were derived from continuous heart rate and beat-to-beat blood pressure time series data over 30 minutes using the TFM software. Power spectral analysis for HRV and BPV was conducted using an adaptive auto-regressive (AAR) model as previously described (Bianchi *et al.*, 1997). In addition to total Power Spectral Density (PSD), all three individual frequency band components of the signal were automatically calculated using the TFM [i.e. Very Low Frequency (VLF), Low Frequency (LF) ad High Frequency (HF)] but, since the relevance of the VLF band in short-term recordings is controversial, only two of these values were reported in the study of autonomic regulation of RR interval (RRI), systolic blood pressure (SBP) and diastolic blood pressure (DBP).

LF (0.05 – 0.17Hz) and HF (0.17 – 0.4Hz) components were reported in normalized units (nu) for HRV and BPV (LFnu-RRI, HFnu-RRI, LFnu-SBP, HFnu-SBP, LFnu-DBP, and HFnu-DBP) together with the respective ratios of LF and HF components (LF:HF-RRI, LF:HF-SBP, and LF:HF-DBP) to provide a comprehensive and reliable assessment of autonomic regulation. LF components refer to sympathetic modulation of sinus node activity and vasomotor function, while HF components refer to parasympathetic modulation of cardiovascular activity. PSD, LF and HF were recorded as quantitative indicators of autonomic regulation, while LF:HF ratio represented sympatho-vagal balance (Akselrod *et al.*, 1985; Taskforce of the ESC and NASPE, 1996; Stauss, 2003). The AAR model may produce outliers when analyzing RRI data. All RRI time series data were therefore filtered using Grubb's test for outlier elimination, a well-documented and accepted methodology that has a strong mathematical background (*NIST/SEMATECH e-Handbook of Statistical Methods*, 2012).

2.2.5 Cardiac imaging techniques

2.2.5.1 Transthoracic echocardiography

Using a Vivid 7 ultrasound machine, transthoracic echocardiography was performed by a single, experienced British Society of Echocardiography (BSE) accredited echocardiographer in the Newcastle upon Tyne Hospitals NHS Foundation Trust. A standard BSE template of views was acquired using two-dimensional, Doppler and

tissue Doppler techniques. All measurements and analyses of data were performed offline using dedicated cardiac echocardiography software.

2.2.5.2 Cardiac magnetic resonance imaging

Using a 3-Tesla scanner at the Newcastle Magnetic Resonance Centre, cardiac MRI was performed by an experienced radiographer to include: (i) Phosporus-31 (^{31}P) magnetic resonance spectroscopy (MRS), (ii) cine imaging, (ii) cardiac tagging and (iv) late gadolinium enhancement (LGE) imaging.

2.2.5.2.1 Cardiac spectroscopy

All subjects were scanned prone, using a 10cm diameter ^{31}P surface coil. A cardiac gated 1-dimensional (1-D) chemical shift imaging (CSI) sequence was used with spatial pre-saturation of skeletal muscle. A 7cm slice was selected using a "spredrex"-type pulse of 2.3ms duration to eliminate liver contamination (Schar *et al.*, 2010). Negligible liver contamination was assured by 1-D foot-head oriented CSI experiments in phantoms: less than 1% of the total phosphorus signal originated from outside the prescribed volume. Sixteen coronal phase-encoding steps yielded spectra from 10mm slices (TR=heart rate, 192 averages, acquisition time approximately 20 minutes). The first spectrum arising entirely beyond the chest wall was analyzed using AMARES time domain fit (Vanhamme *et al.*, 1999) to quantify phosphocreatine (PCr), the γ resonance of ATP and 2,3-diphosphoglycerate (DPG). ATP peak area was corrected for blood contamination by 1/6th combined 2,3-DPG peak, and PCr/ATP ratios were corrected for T_1 saturation and local flip angle (Jones *et al.*, 2010).

2.2.5.2.2 Cine imaging

Subjects were scanned supine using a 6-channel cardiac coil and ECG gating. Short-axis balanced steady-state free precession images were obtained covering the entire left ventricle (field of view (FOV) 350 x 350mm^2, repetition time/echo time (TR/TE) = 3.7/1.9ms, turbo factor 17, flip angle (FA) 40°, slice thickness 8mm, 25 phases, resolution 1.37mm); long-axis images were acquired. Endocardial and epicardial borders were traced manually on short-axis slices throughout the cardiac cycle using the ViewForum workstation. LV mass, and systolic and diastolic parameters, including the ratio of early to late ventricular filling velocity (E/A ratio) and early filling percentage, were calculated as previously described (Jones *et al.*, 2010). The ratio of

LV mass to end-diastolic volume (M/V ratio) was calculated as an index of concentric hypertrophy (Cheng *et al.*, 2009). Longitudinal shortening was determined in the 4-chamber view as the percentage difference in distance from mitral valve plane to apex at end-systole and end-diastole. The myocardial wall thickness was determined at the same level as tagging, and the percentage increase from diastole to systole (radial thickening) was calculated.

2.2.5.2.3 Cardiac tagging

MR signal from myocardium in diastole was cancelled in a rectangular grid pattern and tags were tracked through the cardiac cycle (Lumens *et al.*, 2006). A multi-shot turbo-field echo sequence was used (TR/TE/FA/number of averages = 4.9/3.1/10°/1, turbo factor 9, SENSE factor 2, FOV 350 x 350mm^2, voxel size 1.37 mm, tag spacing 7mm, 12 phases). Two adjacent short-axis slices of 10mm thickness were acquired at mid-ventricle with a 2mm gap. The Auckland UniServices Cardiac Image Modeling package was used to align a mesh on the tags between endocardial and epicardial contours. Peak circumferential strain for both the whole myocardial wall and the endocardial third were calculated. Peak torsion between the two slices was calculated as the circumferential-longitudinal shear angle, defined on the epicardial surface (Lumens *et al.*, 2006) (Figure 2.2).

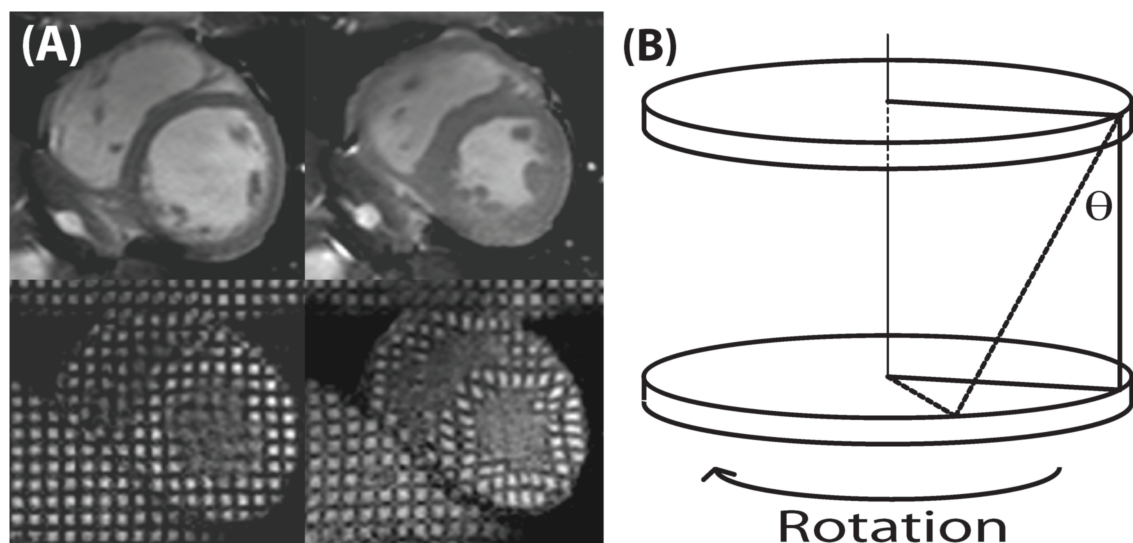


Figure 2.2. Cardiac tagging analysis. (A) Cine imaging (top panels) and tagging (bottom) at end-diastole (left panels) and end-systole (right). A rectangular grid of nulled myocardium applied in diastole enables tracking of myocardial deformation. (B) Tagging of 2 parallel short-axis slices allows calculation of torsion, the longitudinal-circumferential sheer angle (θ), as shown.

2.2.5.2.4 Late gadolinium enhancement imaging

Following completion of all other imaging protocols, 0.2mmol/kg of Gadolinium-DTPA was administered intravenously for assessment of late gadolinium enhancement (LGE). LGE images were obtained at 10 minutes using a breath-held, cardiac-triggered 3-dimensional phase-sensitive inversion recovery sequence (multi-shot gradient echo TR/TE = 5/2.4, FA = 15°/5°, acceleration factor 31, parallel imaging factor 2, 1.8mm resolution zero-filled to 1.3mm) with inversion time to null normal myocardium determined from a prior multi-slice 2D Look-Locker experiment (multishot EPI with EPI factor 5, acceleration factor 2, TR/TE = 7.3/2.8, 3mm resolution). Qualitative analysis for the presence and/or distribution of LGE in a 17-segment model was performed by consensus agreement of 2 experienced observers, blinded to MRI/MRS findings (Hundley *et al.*, 2009).

2.2.6 Statistical analysis

Unless otherwise stated, all data are presented as means ± SD for continuous data and as percentages and numbers for categorical data. Continuous data were tested for normality using the Shapiro-Wilk test, and group comparisons were made using two-tailed Student's *t*-tests or Mann-Whitney U tests, as appropriate. Categorical variables were compared using Fisher's exact test and correlations were executed using Pearson's method. All analysis was performed using SPSS version 17 (SPSS Inc, Chicago, Illinois). All tests were two-sided and statistical significance was assumed at $p<0.05$.

Chapter 3.

Cardiac disease in patients harbouring mt-tRNA point mutations: retrospective observational and cardiac tissue studies

3.1 Introduction

Although more than 250 different pathogenic mtDNA mutations have been reported in humans, and cardiac disease is accepted as a common manifestation of such mitochondrial disease, the true prevalence of mtDNA-related cardiomyopathy is unknown. Many factors have contributed to the relatively slow accumulation of knowledge in this specific area of mitochondrial research. Classical features of mitochondrial disease including the rarity of the condition, the complexity of achieving a definitive genetic diagnosis, and the weakness of the genotype:phenotype relationship are important but factors central to the nature of mtDNA-related cardiomyopathy may play a more critical role. Cardiomyopathy in mtDNA disease is a wide spectrum, with unpredictable progression and an extremely variable natural history; until recently, imaging techniques have not been sophisticated enough to suggest specific features of mtDNA-related cardiomyopathy creating clinical diagnostic uncertainty with potential phenocopies; and the importance of access to organ-specific tissue in mitochondrial disease has limited mechanistic study.

Consistent with the pattern of accumulation of knowledge in many newly-described conditions, initial reports of mtDNA-related cardiomyopathy included patients with particularly severe clinical phenotypes of limited relevance to the majority of patients with disease. Early cross-sectional studies were similarly limited by sample size and were not of sufficient duration to document robust clinical outcomes. Even in more recent studies, extremely restricted access to cardiac tissue from patients with mitochondrial disease has hampered progress. Given the rarity of mitochondrial disease, this lack of information about clinical outcomes and tissue mechanisms in patients with mtDNA-related cardiomyopathy, can only be addressed through collaborative, national or international cohort studies with comprehensive and standardized data acquisition. This prospective process is underway (Nesbitt *et al.*, 2013), but will take many years to report clinical outcomes of specific groups. Retrospective study of data acquired through recent clinical management of patients with mitochondrial disease therefore represents a valuable resource that can used to address some of the basic questions that remain unanswered regarding mtDNA-related cardiomyopathy.

3.1.1 Cardiac disease in patients with mt-tRNA mutations

As described earlier (see Section 1.4.4), the m.3243A>G mutation is the most common heteroplasmic, pathogenic point mutation in the mitochondrial genome (Elliott *et al.*, 2008). Although much rarer in clinical practice, the m.8344A>G mutation was one of the first heteroplasmic, pathogenic mtDNA mutations identified, following confirmation of its absence in control individuals and its presence in distinct pedigrees of patients with MERRF (Shoffner *et al.*, 1990; Yoneda *et al.*, 1990). Both the m.3243A>G and m.8344A>G mutations can cause classical clinical syndromes of mitochondrial disease, respectively MELAS and MERRF, and have been linked to the frequent development of cardiac involvement, and particularly cardiomyopathy (Majamaa-Voltti *et al.*, 2002; Wahbi *et al.*, 2010), but patterns of disease and potential mechanisms remain unclear.

The m.3243A>G mutation remains the most common pathogenic mutation in patients with MELAS, accounting for >80% cases (Goto *et al.*, 1990), and still frequently referred to as ‘the MELAS mutation’. Recent reports from a large cohort study have confirmed that the m.3243A>G mutation is associated with an extremely diverse range of phenotypes. The majority of patients do not fulfil diagnostic criteria for MELAS yet cardiomyopathy occurs in 19% individuals harbouring the mutation (Nesbitt *et al.*, 2013). The genotype:phenotype relationship is stronger between the m.8344A>G mutation and MERRF (Hammans *et al.*, 1993); it is reported rarely in association with phenotypes other than MERRF and accounts for >90% of cases, with prominent cardiomyopathy and an apparent association with ventricular pre-excitation and the Wolff-Parkinson-White syndrome.

Despite such associations, there are no imaging studies that report specific features of cardiomyopathy due to the m.3243A>G or m.8344A>G mutations, other than a predominant hypertrophic pattern of involvement. Notably studies reporting a deleterious effect of cardiac involvement on clinical outcomes largely arise from paediatric cohorts of patients with mitochondrial disease (Holmgren *et al.*, 2003; Scaglia *et al.*, 2004; Debray *et al.*, 2007), or implied conclusions from review of causes of death in adult populations (Majamaa-Voltti *et al.*, 2008). Robust data studying the nature, mechanisms and effects of cardiac involvement in adult patients with the m.3243A>G or m.8344A>G mutations are lacking.

3.1.2 Potential pathogenic mechanisms

Initial studies of the effects of mtDNA point mutations employed transmitochondrial cybrid cells and showed that the presence of the m.3243A>G mutation was associated with marked defects in mitochondrial protein synthesis and a reduction in cellular respiratory chain activity (Chomyn *et al.*, 1992; King *et al.*, 1992). The molecular mechanisms underlying these cellular defects, and therefore the occurrence of clinical mitochondrial disease, have been the subject of intense investigation.

3.1.2.1 Transcription of ribosomal RNAs

The m.3243A>G mutation is located in a 28-base pair region of *MTTL1* immediately downstream from the 16S rRNA, which functions as the binding site for mitochondrial transcription termination factor (mTERF) in humans (Kruse *et al.*, 1989). Although initial *in vitro* studies demonstrated that the m.3243A>G mutation altered binding at this site, causing dysfunctional synthesis of 12S and 16S rRNAs (Hess *et al.*, 1991), no defect in termination of transcription or processing of mitochondrial rRNAs was subsequently observed *in vivo* from analysis of size and steady-state level of relevant transcripts (Chomyn *et al.*, 1992; Koga *et al.*, 1993), suggesting that over-expression of mTERF or increased rates of transcription may ameliorate the effects of any reduced binding affinity. Uncertainty still surrounds the precise cellular role of human mTERF (Hyvarinen *et al.*, 2007), and, despite initial studies, these defects in protein synthesis, associated with the m.3243A>G mutation, appear not to result from dysfunctional transcription or processing of mitochondrial rRNAs.

3.1.2.2 Aminoacylation of mt-tRNAs

Clearly aminoaycl tRNA synthetases can play an important role in modulating the phenotype in patients with pathogenic homoplasmic mtDNA mutations (Perli *et al.*, 2012). Earlier studies of the levels of aminoacylated and total mt-tRNA$^{Leu(UUR)}$ and mt-tRNA^{Lys} cellular content revealed interesting results regarding pathogenesis of both the m.3243A>G and m.8344A>G heteroplasmic mtDNA point mutations.

For the m.3243A>G mutation, the percentage heteroplasmy was assessed in reverse transcript PCR products derived from total and aminoacylated mt-tRNA samples in individual patients (Borner *et al.*, 2000). Only one patient in this cohort showed no decrease in the level of the m.3243A>G mutation present in total or aminoacylated

tRNA compared to the overall mtDNA pool; all other patients showed a reduction in the mutation load in either the total tRNA pool, or the aminoacylated tRNA fraction, or both. This demonstration of differential effects on these measures in different patients supported the conclusion that the mutation had a variable effect on both the expression *and* aminoacylation of mt-tRNA$^{Leu(UUR)}$, such that an additional factor may be responsible for the pathogenicity of this mutation. Intriguingly, for the m.8344A>G mutation, the same methodology was utilised by the same group to demonstrate no significant effect on the mutation on either the expression or aminoacylation of mt-tRNA^{Lys}, suggesting that the two different mt-tRNA mutation exert their influence on protein synthesis and subsequent development of clinical mitochondrial disease via different molecular mechanisms (Borner *et al.*, 2000). It appears that the m.3243A> mutation is selected against, at least in some individuals, at a mt-tRNA level but that the m.8344A>G mutation is not (Enriquez *et al*., 1995).

3.1.2.3 Modification of post-transcriptional wobble

Both the m.3243A>G and the m.8344A>G mutation have been demonstrated to prevent post-transcriptional wobble modifications to the anticodon of their respective mutant mt-tRNAs (Yasukawa *et al.*, 2000a; Yasukawa *et al.*, 2000b). For mt-tRNA$^{Leu(UUR)}$, this modification concerned the presence of a 5-taurinomethyluridine (τm^5U) in the anticodon (Suzuki *et al.*, 2002), responsible for precise codon recognition (Figure 3.1). Constructed mt-tRNA$^{Leu(UUR)}$ molecules that do not include the m.3243A>G mutation but lack the τm^5U wobble modification are associated with a significant defect in translation of the UUG, but not the UUA, codon, confirming the codon-specific nature of dysfunction in the wobble modification (Kirino *et al.*, 2004). Additional constructs that lacked the wobble modification, but also contained the m.3243A>G mutation, were associated with a much more general defect in translation (Kirino *et al.*, 2004), affecting both UUG and UUA codons, perhaps due to an effect on the tertiary structure of molecule as the mutation directly disrupts the important U8:A14 reverse Hoogsteen base pair in mt-tRNA$^{Leu(UUR)}$ (Figure 3.1).

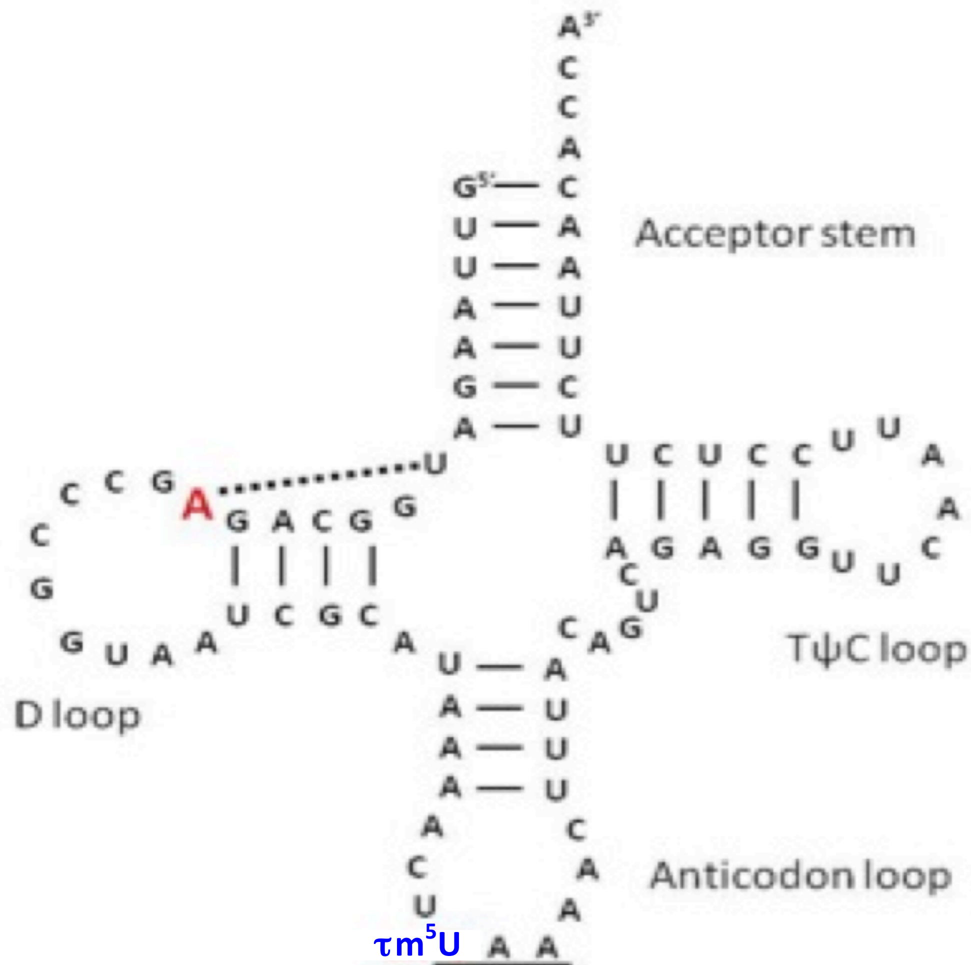


Figure 3.1 Tertiary structure of mt-tRNA$^{Leu(UUR)}$ and the m.3243A>G mutation. Mutation at the highly conserved A14 nucleotide site (red) in the dihydrouridine (D) loop affects a reverse Hoogsteen base pair (dotted line) with the U8 nucleotide that normally stabilises the tertiary structure of the tRNA. The anticodon corresponding to the UUR sequence of leucine is underlined (black), showing 5-taurinomethyluridine (τm^{5}U, blue) at the wobble position. Post-transcriptional modification of the iridine in the anticodon of mt-tRNA$^{Leu(UUR)}$ is implicated in pathogenesis and has a profound effect on translation of the UUG codon.

Reduction in complex I activity is a characteristic feature of the m.3243A>G mutation (Goto et al., 1992); notably the UUG codon is particularly common in the mitochondrial-encoded complex I ND6 gene, representing 42% leucine codons and 4% codons overall (Kirino et al., 2004). It has therefore been proposed that the primary pathogenetic mechanism of the m.3243A>G mutation is the UUG-specific translational defect caused by this defective wobble modification in the anticodon.

Further support for this theory is provided by the finding that the m.12300G>A mutation in the second mt-tRNA for leucine (mt-tRNA$^{Leu(CUN)}$) ameliorates the pathogenic effects of the m.3243A>G mutation (El Meziane *et al*., 1998). Although wild-type mt-tRNA$^{Leu(CUN)}$ has an unmodified uridine at the wobble position, the m.12300G>A mutation generates a mt-tRNA$^{Leu(CUN)}$ anticodon capable of recognising the UUG codon, thus reversing the defect of the m.3243A>G mutation (Kirino *et al*., 2006) and supporting the concept that the UUG-specific lesion is critical.

3.1.2.4 Formation of mt-tRNA dimers

The m.3243A>G mutation generates a palindromic, self-complimentary sequence in the D-loop of mt-tRNA$^{Leu(UUR)}$ that permits the formation of a stable mt-tRNA dimer, which is associated with markedly reduced aminoacylation (Wittenhagen and Kelley, 2002). Disruption of the palindromic sequence with a second point mutation, m.3246C>A, reduces formation of mt-tRNA dimers and lessens the defect in aminoacylation. However it is recognised that dimer formation cannot be the only pathogenic mechanism of the m.3243A>G mutation as aminoacylation was not *completely* rescued by this additional lesion, and the alternative, pathogenic m.3243A>T mutation (Alston *et al.*, 2010), which does *not* promote formation of a mt-tRNA dimer, is also associated with reduced aminoacylation. Both m.3243A>G and m.3243A>T mutations do alter the U8:A14 reverse Hoogsteen base pair and clearly this effect on the tertiary structure of mt-tRNA$^{Leu(UUR)}$ may again play an additional role.

3.1.3 Tissue specificity

Although initial reports in patients with mitochondrial disease suggested a relatively uniform distribution of mutation loads across diverse tissue types (Ciafaloni *et al.*, 1991), tissue-specific segregation of the mtDNA defect is now recognised as a characteristic feature of mtDNA disease; many groups have reported vastly different mutation loads in different tissues, with between-tissue ranges in excess of 70-80% (Kobayashi *et al.*, 1992; Shiraiwa *et al.*, 1993; Chinnery *et al.*, 1997b). Although individuals with a high mutation load for an individual heteroplasmic mtDNA mutation are more likely to express clinical disease and co-segregation of the mtDNA lesion and the cellular defect is a feature of mitochondrial disease, tissue segregation *alone* is not sufficient to explain the tissue specific nature of mitochondrial disease. For example,

while patients with gastro-intestinal involvement in mitochondrial disease have near homoplasmic levels of the m.3243A>G mutation in smooth muscle (Betts *et al.*, 2008), there are many reports of significant organ dysfunction in patients with mutation loads in the relevant organ well below the threshold for cellular respiratory chain dysfunction, and indeed comparable with control individuals (Lynn *et al.*, 2003). These observations support existing theories of variable tissue vulnerability to mtDNA mutations (Ciafaloni *et al.*, 1991), and selective removal of high mutation load cells from an affected tissue (Shiraiwa *et al.*, 1993).

Early investigation of a patient with a single, large scale mtDNA deletion revealed a significant disparity between low levels of the mutation in blood and higher levels in skeletal muscle (Moraes *et al.*, 1989). Later studies in patients with the m.3243A>G mutation demonstrated a similar observation (Hammans *et al.*, 1995), confirming previous suggestions that high cell turnover in such tissues could be linked to removal of cells with a higher mutation load (Shiraiwa *et al.*, 1993). Several groups subsequently demonstrated a progressive reduction in the m.3243A>G mutation load in blood with repeated sampling from the same individual (Poulton and Morten, 1993; Hammans *et al.*, 1995). This age-related phenomenon was not observed in patients harbouring the m.8344A>G mutation, suggesting that the selection pressures over time against the m.3243A>G mutation were more profound than those against the m.8344A>G mutation (Hammans *et al.*, 1995), and amounting to a progressive decline of ~1% per year in the mutation load in blood (t Hart *et al.*, 1996; Rahman *et al.*, 2001). Supporting this theory, foetal tissues show similar levels of heteroplasmy (Matthews *et al.*, 1994), and, while there is variation in the mutation level between individual cell types, there does appear to be a uniform distribution of mutant mtDNA throughout the three different embryonic germ cell layers (Frederiksen *et al.*, 2006). Taken together these findings suggest that selection pressures throughout adult life are the most important factor in explaining tissue differences in mtDNA heteroplasmy rather than random mitotic segregation during embryogenesis (Huang *et al.*, 1996).

Analysis of multiple tissue types from patients with the m.3243A>G mutation suggested an inverse relationship between the tissue specific mutation load and the rate of cell turnover for the tissue (Chinnery *et al.*, 1999). The mechanism by which the m.3243A>G mutation is gradually removed from blood, and other mitotic tissues, is

thought to relate to the existence of a negative selection pressure against stem cells that have accumulated high levels of mutation through random genetic drift (Rajasimha *et al.*, 2008). In post-mitotic tissues, such as cardiac and skeletal muscle, these mechanisms do not exist and the level of heteroplasmy can be high, exceeding the threshold for cellular respiratory chain deficiency.

Following the identification of specific patterns of abnormal expression of respiratory complex deficiency in patients with different mtDNA mutations (Tanji *et al.,* 1999), clear protocols have been developed to add immunohistochemical techniques to the routine histochemical and biochemical investigation of samples from patients with mitochondrial disease (Tanji *et al.,* 2008).

3.2 Aims

This study had two principal aims. Firstly, I sought to appraise retrospective clinical data in patients with mitochondrial disease, to describe the nature and frequency of cardiac involvement, focussing specifically on those patients harbouring mt-tRNA mutations including m.3243A>G and m.8344A>G. I aimed to identify any specific cardiac morphological features that may be characteristic of mitochondrial disease, and, by analysing data collected over many years, I sought to determine whether the presence of cardiomyopathy had any significant impact on clinical outcomes.

Secondly, given the unclear pathogenic mechanisms of the m.3243A>G and m.8344A>G mutations, as discussed above, and the tissue specific distribution of the m.3243A>G mutation, I sought to clarify the primary respiratory chain abnormality caused by these mt-tRNA mutations in cardiac muscle, and related this to proposed pathogenic mechanisms. Given the variable cardiac phenotype in patients harbouring these mutations, I hypothesized that the nature of any respiratory chain complex abnormalities in cardiac muscle may be related to the extent of cardiac involvement. I believed that comparison of cardiac tissue findings with more widely utilised diagnostic tissue samples in this cohort of patients could help improve the use of diagnostic testing and aid clinical decision-making.

3.3 Methods

3.3.1 Retrospective observational cohort study

3.3.1.1 Study design

I conducted a retrospective clinical cohort study to investigate the nature and frequency of cardiac involvement in patients with mitochondrial disease, and assess the impact of cardiomyopathy on clinical outcomes. All patients referred to Newcastle NCG Mitochondrial Diagnostic Service with confirmed mitochondrial disease from 1985-2010 and those patients participating in the MRC Centre for Neuromuscular Diseases Mitochondrial Disease Patient Cohort Study (UK) were included. Asymptomatic relatives were excluded from analysis, as were patients who had no pre- or post-mortem assessment of cardiac involvement. Following a review of all existing clinical and diagnostic information, a dedicated database was used to collate data concerning patient demographics, genotype, cardiac phenotype, age of onset of symptoms, disease burden, duration of follow-up and clinical outcome. The study had research ethical approval and complied with the Declaration of Helsinki.

3.3.1.2 Diagnostic evaluation

The diagnosis of mitochondrial disease in all patients was made according to international guidelines (*CMGS Practice Guidelines for the Molecular Diagnosis of Mitochondrial Diseases*, 2008), utilising muscle biopsy histochemistry, biochemical assessment of respiratory chain function and/or molecular genetic studies alongside clinical assessment. Genotype and mutation load, where available, from muscle, blood, urine, and/or buccal cells were extracted from diagnostic files.

3.3.1.3 Clinical assessment

Subjects underwent physical examination by an experienced clinician. The phenotypic pattern of disease involvement at the time of initial diagnostic evaluation and age of onset of symptoms were ascertained from the case notes. Where applicable, disease burden was assessed using NMDAS, a validated scoring system (Schaefer *et al.*, 2006). All follow-up data was reviewed from outpatient attendances, clinical investigations and correspondence. Clinical outcomes were determined in December 2011 by case

note review and/or additional direct contact with the patient, relatives or general practitioner.

3.3.1.4 Cardiac investigations

3.3.1.4.1 Electrocardiography

All available 12 lead ECGs from all study patients were reviewed. Voltage criteria for left ventricular hypertrophy were defined according to the Romhilt-Estes criteria (Romhilt *et al.*, 1969). Atrio-ventricular and intra-ventricular conduction and repolarisation abnormalities were defined by standard criteria (Surawicz *et al.*, 1978).

3.3.1.4.2 Transthoracic echocardiography

All available transthoracic echocardiographic two-dimensional, M-mode and Doppler images recorded in the Newcastle upon Tyne Hospitals NHS Foundation Trust were personally reviewed by the same investigator. Maximal left ventricular wall thicknesses and left-ventricular dimensions were assessed using standard long- and short-axis parasternal views. Where possible, left ventricular systolic function was assessed using the modified Simpson's method, and/or calculation of fractional shortening, rather than subjective assessment.

3.3.1.5 Statistical analysis

Data are presented as means ± SD for continuous data and as numbers or percentages for categorical data, unless stated otherwise. Group comparisons for continuous data were made using paired and unpaired Student *t*-tests or Mann-Whitney U tests, as appropriate, and categorical variables were compared using Fisher's exact test. Log rank analysis was used to assess survival. Seven factors were assessed for a potential impact on the presence of cardiac involvement: age, gender, genotype, skeletal muscle mutation load, clinical phenotype, disease burden, and age at symptom onset. Variables with a p value <0.10 in univariate analysis were entered into a multivariate model, and analysed using a logistic regression model. All analysis was performed using SPSS version 17 (SPSS Inc, Chicago, Illinois). Statistical significance was assumed at $p<0.05$.

3.3.2 Retrospective cardiac tissue study

3.3.2.1 Study design

I conducted a retrospective, comprehensive histochemical and immunohistochemical study of all stored, post-mortem cardiac tissue obtained from patients with known mitochondrial disease due to either the m.3243A>G or m.8344A>G mutations, referred to the Newcastle NCG Mitochondrial Diagnostic Service. Exclusion criteria included age <18 years (one patient), and post-mortem time before tissue processing ≥ 6 hours (two patients). The study had research ethics committee approval and all participants, or their families, had given written informed consent for the use of stored tissue samples in research studies.

3.3.2.2 Tissue samples

All cardiac tissue samples were obtained post-mortem and were analysed for the presence of mitochondrial disease using histochemical and immunohistochemical studies. Tissue samples had been previously investigated using molecular biological techniques to confirm the m.3243A>G or m.8344A>G mutation and quantify mutation load from tissue homogenates.

3.3.2.2.1 Tissue preparation

All tissues samples were mounted on filter paper and snap-frozen as previously described (see Section 2.2.2). Tissue sections of 10μm were obtained at -19^{0}C using a cryostat with the blade set at -21^{0}C. Where possible, tissue orientation was attempted to ensure longitudinal regions were used for quantification as the presence of intercalated discs allowed for more accurate identification of single cells.

3.3.2.2.2 Histochemistry

In all tissue samples, separate stains for the activities of COX and SDH were performed with sequential use of COX / SDH histochemistry to optimise identification of cells with histochemical evidence of respiratory chain abnormalities, as described (see Section 2.2.3.2). The stereo-investigator microscope was used to quantify any evidence of abnormal COX reactions in all cardiac samples, with cells classified as COX-positive, COX-intermediate and COX-negative, as previously described (see Section 2.2.3.4). Both COX-negative and COX-intermediate were considered abnormal or COX-deficient.

3.3.2.2.3 Immunohistochemistry

All left ventricular tissue samples were studied using a systematic approach with monoclonal OXPHOS antibodies directed against individual subunits of respiratory chain complexes I, II and IV as previously described (see Section 2.2.3.3).

Quantification of immunohistochemical defects was performed using both a subjective classification technique with the Stereo Investigator System, as performed for histochemical analyses, and an objective densitometric assessment of the cellular staining patterns (see Section 2.2.3.4). Using optical densitometry, direct comparison was made to control cardiac tissue, obtained from individuals without evidence of cardiac or metabolic disease, and processed and analysed alongside patient samples. For each sample ≥200 cardiomyocytes were analysed and a difference of two standard deviations (2SD) from the mean densitometry scale for control tissue was assumed to represent an abnormal cell. This technique permitted validation of the subjective classification technique to establish the proportion of cardiomyocytes deficient for individual respiratory chain complex subunits, and investigation of the pattern of this deficiency.

3.3.2.2.4 Molecular biology

No additional molecular biological investigations were performed. The results of previous investigations were reviewed from diagnostic files to confirm the presence of the m.3243A>G and m.8344A>G mutations and record the mutation load in a variety of post-mortem tissues.

3.3.2.3 Clinical disease

All patient records were reviewed retrospectively to obtain information pertaining to the nature and extent of clinical disease with particular reference to cardiac investigations and disease in life, and the probable cause(s) of death.

3.3.2.4 Statistical analysis

All data are presented as means ± SD for continuous data and as percentages and numbers for categorical data, unless stated otherwise. Given the small sample sizes involved, group comparisons for continuous data were made using Mann-Whitney U

tests, categorical variables were compared using Fisher's exact test, and Pearson's analysis was used to assess correlations. Statistical significance was assumed at $p<0.05$.

3.4 Results

3.4.1 Observational cohort study

A total of 430 patients (218 probands) with mitochondrial disease and evidence of pre or post-mortem assessment of cardiac involvement were identified (238 female, mean age 44.4 ± 20.7 years). Demographic and clinical characteristics are shown in Table 3.1, with genotypic groups containing ≥ 4 patients included in subsequent analysis. Although data were incomplete for some clinical parameters including NMDAS score and mutation load, a wide range of phenotypes were included in the cohort in terms of disease burden, age, and age at onset of symptoms.

Parameter	Patients with cardiac involvement (n=163)	Patients without cardiac involvement (n=267)	All patients (n=430)	*p* value
Male, n (%)	80 (49)	112 (42)	192 (45)	0.971
Age (years)	42.7 ± 18.1	45.1 ± 22.2	44.4 ± 20.7	0.683
Age of onset (years)	15.7 ± 8.1	22.9 ± 10.2	18.9 ± 11.3	<0.05
* SKM mutation load (%)	54 ± 18	49 ± 11	50 ± 19	0.091
† NMDAS score	16 ± 10	15 ± 7	16 ± 11	0.877
Genotypic group, n (%)				<0.05
m.3243A>G	53 (33)	76 (29)	129 (30)	
m.8344A>G	14 (9)	13 (5)	27 (6)	
Single mtDNA deletion	9 (6)	21 (8)	30 (7)	
Multiple mtDNA deletions	37 (23)	38 (14)	75 (17)	
OPA1 mutation	6 (4)	7 (3)	13 (3)	
PEO1 mutation	8 (5)	14 (5)	22 (5)	
m.14709T>C	2 (1)	9 (3)	11 (3)	
POLG1 mutation	3 (2)	12 (5)	15 (4)	
Unknown mutation	31 (19)	77 (29)	108 (25)	

Table 3.1 Retrospective cohort clinical and demographic parameters. SKM = skeletal muscle; NMDAS = Newcastle Mitochondrial Disease Adult Scale; * = data available from 122 patients (28%); † = data available from 313 (73%) patients.

3.4.1.1 Cardiac involvement

Cardiac involvement was detected in 163 patients (38%) overall with a significant difference in the proportion of affected individuals between different genotypic groups ($p<0.05$, Table 3.1). The group of patients harbouring multiple mtDNA deletions most frequently had evidence of cardiac involvement (49%), while this finding was rare in those patients harbouring the 14709T>C mutation (18%), with other genotypic groups between these two extremes (Figure 3.2).

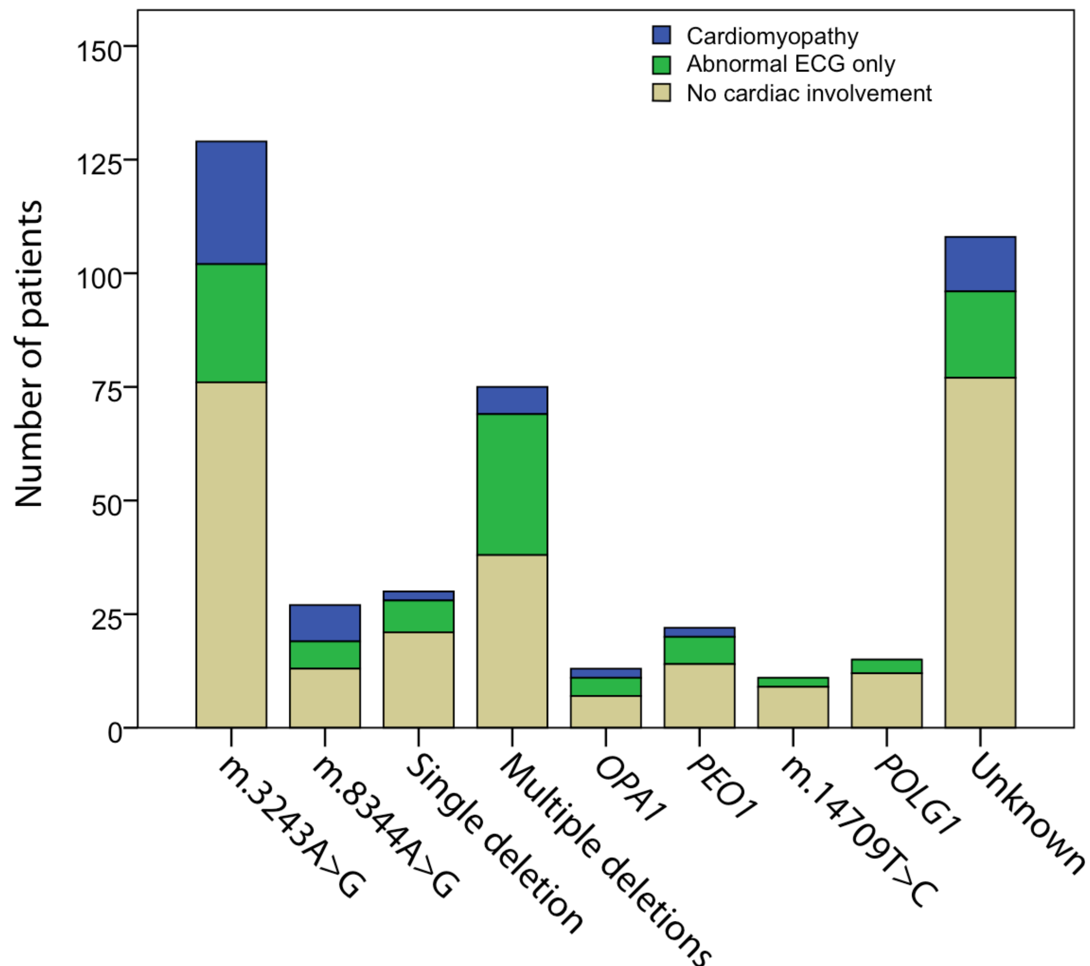


Figure 3.2 Frequency of cardiac involvement in genotypic groups. All genotypic groups with ≥4 individual patients were included in analysis. ECG abnormalities were the most common evidence of cardiac involvement in all groups, and the frequency of cardiac involvement ranged from 18-49%. Most patients with cardiomyopathy harboured the m.3243A>G or m.8344A>G mutations.

3.4.1.1.1 ECG abnormalities

ECG abnormalities dominated the clinical evidence of cardiac involvement in this cohort, with isolated ECG abnormalities present in 104 patients (64% of those with cardiac involvement), in addition to patients with evidence cardiomyopathy who also often had baseline ECG abnormalities. The majority of these abnormalities were relatively non-specific including minor P or T wave abnormalities (21 patients), sinus tachycardia (12), intra-ventricular conduction delay (7), and left or right axis deviation (3). Fourteen patients (3%) fulfilled voltage criteria for LVH, with left ventricular strain pattern evident in 3 patients. Five patients had undergone permanent pacemaker implantation (3 patients with single, large-scale deletions, and 2 patients with multiple deletions) but one ICD had been implanted in a patient undergoing cardiac resynchronization. Four patients (3 with m.8344A>G mutation, and 1 with multiple deletions) had had radiofrequency ablation for atrio-ventricular re-entrant tachycardia (3 patients) or atrio-ventricular junctional re-entrant tachycardia (1 patient).

3.4.1.1.2 Cardiomyopathy

Cardiomyopathy was evident in 59 patients (14%), with the majority of those identified though echocardiographic assessment rather than post-mortem. Patients harbouring the m.3243A>G and m.8344A>G mutations most frequently displayed evidence of cardiomyopathy (21% and 30% patients, respectively), while there were no patients with cardiomyopathy in several genotypic groups, including patients with the m.14709T>C mutation and *POLG1* mutations. The morphological pattern of cardiomyopathy was hypertrophic in the majority (63%), although several patients with hypertrophy initially appeared to develop systolic dysfunction and subsequent ventricular dilatation on subsequent assessments, where available. Six patients (1.4%) were listed for cardiac transplantation, with one patient harbouring the m.3243A>G mutation dying while on the urgent transplantation list. Five patients, all <18 years of age and with unknown genotypes at the time of transplantation, underwent successful orthotopic cardiac transplantation for end-stage cardiomyopathy (see Section 4.4.4).

3.4.1.1.3 Predictors of cardiac involvement

Univariate analysis of potential influencing factors for cardiac involvement in mitochondrial disease are summarised in Table 3.2. Three parameters with p <0.10 (genotype, age at symptom onset, and skeletal muscle mutation load) were included in

the multivariate analysis. Logistic regression revealed both genotype and age at symptom onset as the significant parameters in the prediction of cardiac involvement. The skeletal muscle mutation load had a potential considerable effect on the presence of cardiac involvement, although not statistically significant at the 5% level.

Parameter	**Univariate *p* value**	**Multivariate *p* value**	**Odds ratio**	**95% CI**
Genotype	0.027	0.044	2.23	1.2 – 3.7
Age at symptom onset	0.012	0.028	2.79	1.9 – 4.9
SKM mutation load	0.091	0.090	3.35	0.8 – 11.9
Age	0.683			
Gender	0.971			
Clinical phenotype	0.766			
Disease burden	0.877			

Table 3.2 Logistic regression for predictors of cardiac involvement. CI = confidence interval; SKM = skeletal muscle.

3.4.1.2 Clinical outcome

Survival from the time of symptom onset was significantly lower in those patients with evidence of cardiomyopathy compared to those without cardiomyopathy (Figure 3.3, p<0.01, median follow-up 22 years, range 0-57). This pattern was not evident when comparing those *with* and *without* evidence of cardiac involvement, including isolated ECG abnormalities only. Causes of death could be identified from the death certificate or clinical notes in 31 patients. Cardiovascular diseases were included in the immediate causes of death for 12 patients (39%) and in the underlying conditions for a further 4 patients (13%); for neurological diseases corresponding figures were 16 patients (52%) and 8 patients (26%). The cause of death could not be identified in 42 patients due to a lack of available documentation or a multitude of clinical possibilities that had been identified following case note review.

Eighteen subjects had died suddenly and unexpectedly. Cardiovascular diseases (n=6) were the most common cause of death in this group of patients, although the cause of death could not be identified in eight patients (44%). Six of these patients had diabetes mellitus, three had epilepsy, and two had cardiomyopathy.

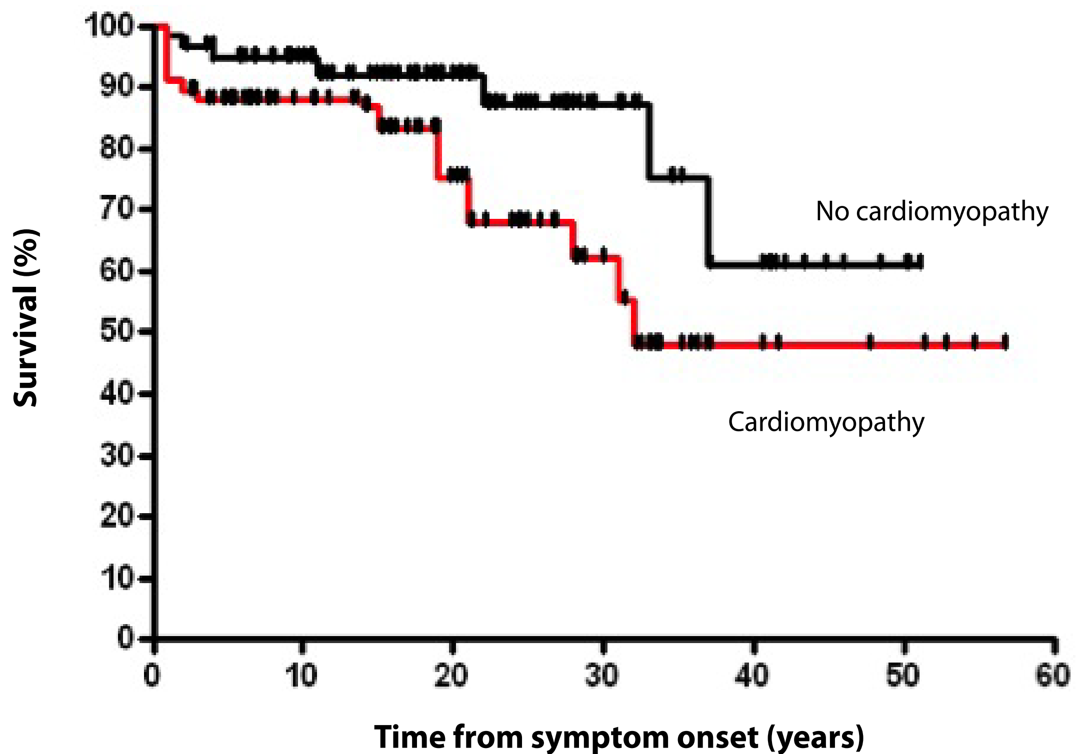


Figure 3.3 Patient survival from the time of symptom onset. There was a significant difference in patient survival between those *with* and *without* evidence of cardiomyopathy on echocardiography or post-mortem (log rank analysis $p<0.01$).

3.4.2 Cardiac tissue study

3.4.2.1 Study participants

Post-mortem cardiac tissue was available from ten patients (nine probands) with mitochondrial disease due to mt-tRNA mutations (five female, mean age 44.9 ± 10.5 years), including seven patients (three female) with the m.3243A>G mutation and three patients (two female) with the m.8344A>G mutation (Tables 3.3 and 3.4). There was considerable variation in tissue specific levels of heteroplasmy in patients with the range of maximum difference between post-mortem tissue mutation loads in an

individual varying from 4 – 58% (patient 8 all tissues 17-21%, patient 5 all tissues 25-83%).

Individual patient characteristics, including evidence of cardiac involvement, are reported in Table 3.3 and 3.4 for patients with m.3243A>G and m.8344A>G respectively. Seven patients (70%) had objective evidence of cardiac abnormalities in life: six patients (50%) had evidence of probable mtDNA-related cardiomyopathy, while one patient (patient 1) had an abnormal ECG with 1st degree atrio-ventricular block (AVB). One further patient (patient 10), who had a normal ECG and echocardiogram, died unexpectedly without identification of cause of death at post-mortem.

3.4.2.1.1 Patient 1

Patient 1 died aged 60 years from cardio-respiratory failure in the context of multi-organ failure secondary to pneumonia and these findings were confirmed at post-mortem. She had a strong positive family history with several affected relatives.

She had first presented in her twenties with cerebellar ataxia and proximal myopathy but had rapidly developed complex partial seizures, swallowing difficulties, weight loss, depression and diabetes mellitus. She had bilateral sensori-neural deafness and migraine but no history of ocular manifestations of mitochondrial disease. She had suffered stroke-like episodes and encephalopathy in the past and in the 12 months prior to death had developed significant cognitive impairment, with a profound functional decline, prominent gastro-intestinal tract involvement and dysphagia.

She had no symptoms suggestive of cardiac involvement. Despite the presence of conduction system disease on baseline ECG (1st degree AVB), repeated 24-hour Holter monitors failed to demonstrate any evidence of higher degree AVB. Transthoracic echocardiogram was within normal limits.

Mutation load was quantified in both blood and urinary epithelial cells, revealing levels of 5% and 72% respectively. Post-mortem levels ranged from 36-89% in diverse organs.

3.4.2.1.2 Patient 2

Patient 2 had died aged 57 years from congestive cardiac failure secondary to ischaemic heart disease. She had a strong family history of recurrent strokes and cardiomyopathy with a hypertrophic pattern due to mitochondrial disease, with the

proband for this diagnosis being her maternal niece, who also harboured the m.3243A>G mutation (patient 5).

She presented to medical attention aged 25 years with a large goitre and evidence of gastro-intestinal tract involvement with anorexia, recurrent constipation and vomiting. She underwent sub-total thyroidectomy with histology showing a colloid goitre. Subsequently she developed proximal myopathy, myalgia and a sensory neuropathy and had several strokes with partial recovery on each occasion. There was evidence of significant cerebrovascular disease on invasive angiography. Given her family history, echocardiography was performed and revealed left ventricular hypertrophy without evidence of outflow tract obstruction. She subsequently had several admissions with chest pain and suffered several myocardial infarctions despite aggressive medical therapy. She developed biventricular systolic dysfunction and clinical heart failure.

Cardiac disease was a prominent feature. She had a history of hypertension, left ventricular hypertrophy and ischaemic heart disease together with a family history of cardiac involvement in mitochondrial disease. The relative contributions of these two aetiological factors on cardiac dysfunction were not clear in life, or indeed at post-mortem. The most recent investigations prior to death showed evidence of moderate left ventricular systolic impairment (ejection fraction 40%), non-specific inter-ventricular conduction delay on ECG (QRS duration 120ms) and a dilated left ventricle. From a central nervous system viewpoint, she had evidence of multi-focal cerebellar and occipital ischaemic-like lesions on post-mortem brain examination. While she did have a history of hypertension and extensive cerebrovascular disease, the distribution of these lesions combined with evidence of COX-deficiency and other metabolic changes established the likely diagnosis of mitochondrial encephalopathy.

Post-mortem examination confirmed cause of death and diverse organ involvement. Mutation load was quantified in post-mortem tissues and was present at 40-84% in a wide range of tissues. Urinary mutation load in life was 72%.

3.4.2.1.3 Patient 3

Patient 3 died aged 45 years from mitochondrial encephalopathy due to the m.3243A>G mutation. He had no clear family history of mitochondrial disease.

He had a rapid deterioration from first symptoms and subsequent diagnosis to death over an 8-year period. He originally presented with migraine and a right homonymous hemianopia secondary to an occipital infarction aged 37 years. He had recurrent problems with seizures following his stroke, with only partial response to various anti-epileptic medications. He subsequently presented with status epilepticus, following a period of cognitive decline and depression with suicidal ideation. The m.3243A>G mutation was identified in urine following an abnormal muscle biopsy and the recognition of elevated lactate in both blood and cerebro-spinal fluid. He suffered multiple further stroke-like episodes, encephalopathy and cognitive decline.

There was no evidence of cardiac involvement on baseline screening investigations with ECG and echocardiography. There were no symptoms of cardiac disease.

Post-mortem examination confirmed evidence of prominent cerebral involvement and cortical atrophy. Cardiac examination was within normal, age-dependent limits with mild coronary and aortic atheroma. Mutation load was quantified in post-mortem tissues with levels 21-78%. No assessment of mutation load was made during life.

3.4.2.1.4 Patient 4

Patient 4 had died aged 35 years from aspiration pneumonia. He had had a positive family history of mitochondrial disease with his mother displaying an oligosymptomatic phenotype, and harbouring the m.3243A>G mutation.

Bilateral sensori-neural deafness had been noted in infancy, together with short stature and failure to thrive. He had reported fatigue and exercise intolerance in late childhood and had undergone surgical correction for ptosis aged 16 years. Progressive proximal myopathy, mild cerebellar ataxia combined with disabling fatigue and depression led to cessation of a relatively physical, unskilled job in his twenties. He developed further problems with impaired glucose tolerance and then diabetes mellitus, dysphagia and recurrent aspiration, dysphonia and dysarthria. Gastro-intestinal tract symptoms were prominent with regular use of laxative medications for significant constipation.

From a cardiac viewpoint, left ventricular hypertrophy was first noted in early adulthood and progressed over time, despite intermittent use of cardio-active

medications including calcium channel antagonists, ß-blockers and an ACE inhibitor at different times. Although there was evidence of impaired longitudinal and diastolic function, overall systolic function was maintained. The most recent screening investigations prior to death revealed a maximum wall thickness of 16mm (interventricular septum), with no arrhythmia on 24-hour Holter monitor and voltage criteria for LVH with strain pattern on ECG.

Post-mortem examination confirmed cause of death and diverse organ involvement. Biochemical analysis of cardiac muscle homogenate revealed low activity of complex I and complex IV. Mutation load was quantified in numerous post-mortem tissues and, although undetectable in blood, was present at 40-86% in a wide range of tissues.

3.4.2.1.5 Patient 5

Patient 5 died aged 36 years following a witnessed out-of-hospital cardiac arrest immediately outside the outpatient department; despite rapid assessment and intervention by medical staff, the resuscitation attempt was unsuccessful. The initial cardiac rhythm was asystole but ventricular fibrillation and pulseless electrical activity were also noted following the commencement of cardio-pulmonary resuscitation. There was no return of spontaneous circulation and the cause of the cardiac arrest remains unclear, but no formal post-mortem was performed at request of the family. Her sisters and several other maternal relatives, including her aunt (patient 2), harboured the m.3243A>G mutation and were affected by mitochondrial disease.

She had a history of short stature and failure to thrive in childhood associated with low body weight. Sensori-neural hearing loss was first noted in her teenage years and she developed diabetes mellitus in her twenties, with significant problems with bowel dysmotility. Seizures became a more prominent feature later and she developed encephalopathy six years prior to her death with frequent episodes resulting in notable and progressive cognitive decline.

Cardiac involvement was first noted three years prior to death, on screening investigations, with moderate left ventricular systolic dysfunction (EF 45%) with evidence of mild hypertrophic remodelling (posterior wall and inter-ventricular septum both 14mm) but no chamber dilatation. Left bundle branch block (LBBB) became evident on the baseline ECG and she received optimal medical therapy for clinical

heart failure, involving loop diuretics, ß-blockers and ACE inhibitors. At the time of death, she had NYHA class I symptoms with no clinical evidence of fluid overload. Recent 24-hour Holter monitor had been performed to investigate palpitation and had shown frequent atrial ectopics and brief episodes of a probable atrial tachycardia.

Mutation load was quantified during life in blood and urinary epithelial cells with heteroplasmy levels of 21% and 33% respectively. Post-mortem levels were 25-83%.

3.4.2.1.6 Patient 6

Patient 6 died from bronchopneumonia with multi-organ failure aged 45 years. He had a long history of mitochondrial disease with stable symptoms for many years but rapid physical and mental decline over several months prior to death. His father had cardiomyopathy but there was not other family history of mitochondrial disease.

He was assessed for failure to thrive as an infant, and several developmental milestones were delayed, although no specific diagnosis was initially reached. He developed bilateral sensori-neural deafness in his teenage years and, although attending mainstream school, he left with no qualifications aged 16 years. He developed a mild proximal myopathy, depression and significant gastro-intestinal tract involvement but did not achieve a diagnosis for his multisystem problems until his late thirties. He presented aged 39 years with status epilepticus. He had recurrent problems with stroke-like episodes, encephalopathy and epilepsy, and experienced profound cognitive decline. He had severe dysphagia and recurrent aspiration. He had significant psychological and behavioural problems associated with cognitive decline.

Cardiac involvement was evident on initial screening investigation – mild hypertrophic remodelling (posterior wall 14mm) with no chamber dilatation but moderate impairment of left ventricular systolic function (EF 35-40%). ECG showed voltage criteria for LVH with strain pattern. He was managed with optimal medical therapy including diuretics, an ACE inhibitor and spironolactone but failed to tolerate initiation of a ß-blocker. This led to some improvement in his systolic function (EF 40-45%) and he was euvolaemic and stable at cardiac assessment within 12 months of his death.

No assessment of mutation load was performed during life. Post-mortem levels from diverse organs were 71-96%.

3.4.2.1.7 Patient 7

Patient 7 had died aged 30 years from heart failure due to severe DCM. At the time of death, he had already been implanted with a combined biventricular pacemaker / implantable cardioverter defibrillator (ICD) and was listed for orthotropic cardiac transplantation but no suitable donor had been identified. He had a positive family history with an affected brother, also harbouring the m.3243A>G mutation, and his mother had diabetes mellitus but no other clinical features of mitochondrial disease.

Bilateral sensori-neural deafness had been noted in childhood, at 11 years of age, together with short stature and fatigue. He had been treated with growth hormone. He then developed diabetes mellitus at 22 years of age, CPEO and ptosis, and had symptoms of frequent constipation although no evidence of other bowel involvement. He developed mild ataxia and proximal myopathy with prominent fatigue limiting exercise tolerance. In the 12 months prior to death, he had had a pulmonary embolus and a transient ischaemic attack (TIA) affecting the left face secondary to an intramural left ventricular thrombus. Both these issues had been treated with anticoagulation.

Dilatation and impairment of systolic function of the left ventricle was first noted aged 25 years, but he had demonstrated progressive deterioration despite optimal medical therapy, and latterly initiation of a cardiac resynchronisation therapy with a defibrillator (CRT-D). A formal transplant assessment, completed six months prior to death, had shown marked chamber dilatation with severe impairment of left ventricular systolic dysfunction (EF 15%). Abnormal haemodynamics were evident at right heart catheterisation showing RA pressure 11mmHg, RV 24 mmHg with an end-diastolic pressure of 10mmHg and a trans-pulmonary gradient of 5 mmHg. Cardiac index was markedly reduced at 1.1 l/min/m^2 with pulmonary artery oxygen saturations of 50%. He was on the active orthotopic cardiac transplantation list.

During life, mutation load was quantified in skeletal muscle homogenate and urinary epithelial cells, revealing levels of 71% and 96% respectively.

3.4.2.1.8 Patient 8

Patient 8 died aged 40 years from aspiration pneumonia following a 22-year history of progressive mitochondrial disease due to the m.8344A>G mutation. No formal post-mortem was performed. She had a positive family history of mitochondrial disease.

She had presented in early adulthood with bilateral sensori-neural deafness, cerebellar ataxia, short stature, and a low body mass. She developed myoclonus and was diagnosed with epilepsy. She had a mild proximal myopathy with prominent CPEO and ptosis, for which she had undergone surgical correction. Fatigue, depression and gastro-intestinal tract involvement were all noted as particularly troublesome features. She had dysphagia and dysarthria and had had recurrent previous episodes of aspiration pneumonia. She had chronic kidney disease stage 3.

From a cardiac viewpoint, ECG and echocardiography screening investigation were within normal limits. She denied cardiac symptoms.

No assessment of mutation load was performed during life however post-mortem levels were 17-21% in diverse organs.

3.4.2.1.9 Patient 9

Patient 9 died aged 59 years with multi-organ failure and end-stage cognitive decline, with post-mortem confirming diverse organ involvement. He had no clear family history of mitochondrial disease, although his mother had had diabetes.

He originally presented in his thirties with cerebellar ataxia, myoclonus, epilepsy and mild proximal myopathy. He developed dysarthria, dysphasia and dysphagia but had no history of aspiration. Fatigue, depression and obstructive sleep apnoea contributed to his symptom burden significantly. He had recurrent strokes and episodes of encephalopathy ultimately resulting in significant and progressive cognitive decline over several years. At the time of his final admission to hospital, he had severe cognitive impairment, probable cortical blindness, and evidence of multi-organ failure.

His last cardiac assessment, performed nearly two years prior to his death, showed evidence of hypertrophic remodelling (interventricular septum 15mm) with mild impairment of left ventricular systolic function (EF 50%). ECG showed sinus rhythm with voltage criteria for LVH and strain. He had no cardiac symptoms.

Mutation load of the m.8344A>G mutation was quantified in blood and urinary epithelial cells with levels of 94% and 90%, respectively. Post-mortem levels were 70-91% in diverse organs.

3.4.2.1.10 Patient 10

Patient 10 died aged 42 years. She died both suddenly and unexpectedly without evidence of recent change in health status but no clear cause of death was identified at post-mortem. She had a strong family history of mitochondrial disease due to the m.8344A>G mutation with several affected maternal relatives.

She had first developed cerebellar ataxia and myoclonus in early adulthood and both features progressed gradually over time. She had a mild proximal myopathy, myalgia, bilateral sensori-neural deafness and bowel dysmotility. She was wheelchair dependent secondary to limiting ataxia and sensory neuropathy and had significant clinical depression. She received treatment with thyroxine for clinical hypothyroidism with normal thyroid hormones demonstrated on blood analyses, in the three months prior to death. She was agoraphobic and had resistant depression, avoiding medical contact.

From a cardiac viewpoint, ECG and echocardiography screening investigation were within normal limits. She denied cardiac symptoms.

Mutation load of the m.8344A>G mutation was quantified in blood with 92% heteroplasmy detected. Post-mortem levels were 76% in skeletal muscle and 82% in left ventricular tissue.

Patient number	1	2*	3	4	5*	6	7
Demographics							
Age (years)	60	57	45	35	36	45	30
Gender	F	F	M	M	F	M	M
Duration (years)	33	32	8	32	24	31	19
Mutation load (%)							
Blood	5			<5	21		
Urine	72	72			33		96
Muscle							71
Buccal							
Cardiac involvement							
Abnormal ECG	+	+		+		+	+
ECG details	1° AVB	IVCD	Normal	LVH	IVCD	LBBB	CRT-D
Arrhythmia				VE	SVT		NSVT
Cardiomyopathy		+		+	+	+	+
Morphology	Normal	DCM	Normal	HCM	HCM	HCM	DCM
EF (%)	>55	40	>55	50	45	35	15
Clinical Features							
Short Stature		+		+		+	
Low BMI	+	+	+	+	+		
Ataxia	+	+	+	+	+		+
Stroke	+	+	+			+	
Epilepsy	+		+		+	+	
Encephalopathy	+		+		+	+	
Migraine	+		+	+			
Neuropathy							+
Myopathy	+			+	+	+	+
Myoclonus							
Myalgia							
Sleep apnoea				+			
Fatigue	+			+			+
Depression	+	+		+		+	
Optic atrophy					+		
Retinopathy	+				+		
Ophthalmoplegia				+			+
Ptosis				+			+
Deafness	+			+	+	+	+
Dysarthria				+	+	+	
Dysphonia				+		+	
Dysphagia	+		+	+		+	
Constipation	+		+	+	+	+	+
Irritable bowel		+	+		+		+
Vomiting	+		+		+	+	+
Resp. failure		+					+
Diabetes mellitus	+	+		+	+		
Thyroid disease		+					
Other		IHD		CKD			CKD

Table 3.3 Clinical features of patients harbouring m.3243A>G mutation. ECG = electrocardiogram; EF = ejection fraction; BMI = body mass index; Resp. = Respiratory; IHD = ischaemic heart disease; CKD = chronic kidney disease; F = female; M = male; CRT-D = cardiac resynchronisation therapy with defibrillator; NSVT = non-sustained ventricular tachycardia; SVT = supraventricular tachycardia; VE = ventricular ectopics.

Patient number	1	2*	3
Demographics			
Age (years)	40	59	42
Gender	F	M	F
Duration (years)	18	22	22
Mutation load (%)			
Blood		94	92
Urine		90	
Muscle			
Buccal			
Cardiac involvement			
Abnormal ECG		+	
ECG details	Normal	LVH	Normal
Arrhythmia			
Cardiomyopathy		+	
Morphology	Normal	HCM	Normal
EF (%)	>55	50	>55
Clinical Features			
Short Stature	+		
Low BMI	+		
Ataxia	+	+	+
Stroke		+	
Epilepsy	+	+	+
Encephalopathy		+	
Migraine			
Neuropathy			+
Myoclonus	+	+	+
Myopathy	+	+	+
Myalgia			+
Sleep apnoea		+	
Fatigue	+	+	+
Depression	+	+	+
Optic atrophy	+		
Retinopathy			
Cataracts			
Ophthalmoplegia			
Deafness	+	+	+
Dysarthria	+	+	
Dysphonia	+	+	
Dysphagia		+	+
Constipation	+		
Irritable bowel	+		
Vomiting			
Resp. failure		+	
Diabetes mellitus			
Thyroid disease			+
Other			

Table 3.4 Clinical features of patients harbouring m.8344A>G mutation. ECG = electrocardiogram; EF = ejection fraction; BMI = body mass index; Resp. = Respiratory; F = female; M = male;

3.4.2.2 Mutation load

Post-mortem myocardial macrobiopsies were available from all ten patients (100%), and the availability of cardiac samples from different chambers is shown in Table 3.5, together with the chamber-specific mutation loads. There was a significant correlation between skeletal muscle mutation load and mean cardiac level (r=0.734, $p<0.01$, Figure 3.4), however individual patients displayed more marked tissue segregation. There was no significant difference in mutation load between cardiac chambers in any individual patient.

	Mutation load (%)				
Patient	**Skeletal muscle**	**Right atrium**	**Left atrium**	**Right ventricle**	**Left ventricle**
1	72	89	86	85	86
2					80
3	68			70	72
4	46	80	79	86	77
5	64			77	
6	85	88	93	94	92
7	72	90	93	92	89
8	20	21	17	20	19
9	91	89	87	83	85
10	70				75

Table 3.5 Mutation load in skeletal muscle and cardiac chambers. The mutation load of the patients harbouring the m.3243A>G (patients 1-7) and m.8344A>G (patients 8-10) mutations were calculated. Skeletal muscle refers to either psoas, or vastus lateralis (quadriceps) post-mortem sample. Blank boxes represent a lack of available post-mortem tissue.

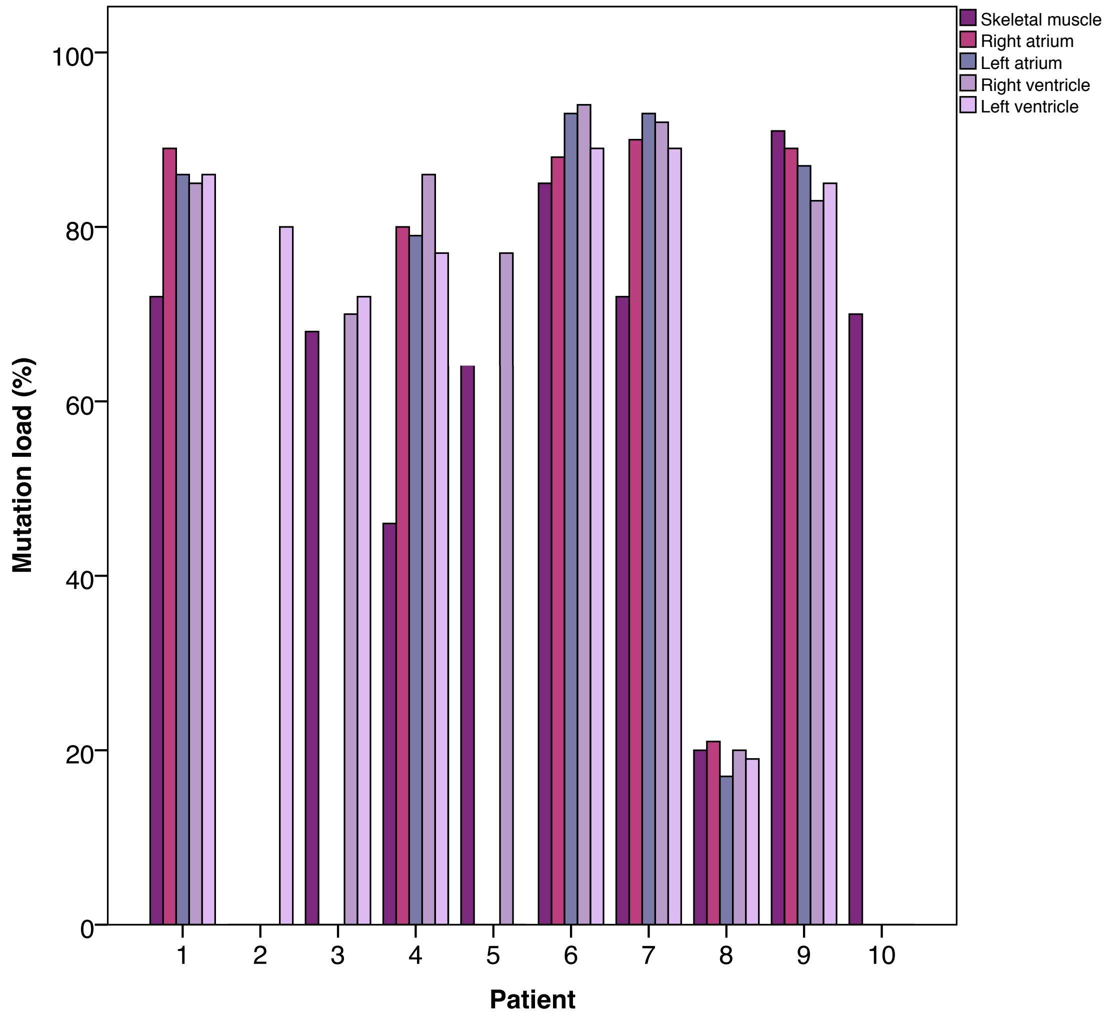


Figure 3.4 Tissue and chamber specific segregation of mtDNA point mutations. Overall there is a significant correlation between mutation loads in post-mortem skeletal muscle and cardiac tissue (r=0.734, $p<0.01$) in this patient group, with many patients displaying little variation between the different tissues (e.g. patient 8 with a maximum difference of 4% between any cardiac or skeletal muscle samples). However several patients showed more marked evidence of tissue segregation (e.g. patient 4 with 40% variation in mutation load between skeletal muscle 46% and right ventricle 86%). Interestingly, no patient displayed significant variation between different cardiac chambers (maximum difference of 7%), consistent with the common embryological origin of the atria and ventricles.

3.4.2.3 Optimisation

A standard protocol for sequential COX / SDH histochemistry, originally optimised for skeletal muscle, yielded satisfactory results for analysis in cardiac muscle tissue samples (Figure 3.5). Following a formal optimisation process in healthy control tissue, to enable clear classification of cells (Figure 3.), the optimised concentrations of different OXPHOS antibodies for the immunohistochemical analyses of left ventricular tissue are shown in Table 3.6.

	Description	Isotype	Subunit	Optimised concentration for cardiac muscle
Complex I	15 kDa	IgG1	NDUFB4	1:300
	19 kDa	IgG2b	NDUFA13	1:3000
	20 kDa	IgG1	NDUFB8	1:300
Complex II	70 kDa	IgG1	FP subunit	1:1000
Complex IV		IgG2a	COX I	1:3000
		IgG2a	COX IV	1:10000

Table 3.6 Immunohistochemistry optimisation. All studies in cardiac muscles were performed with the optimised dilution of specific antibodies.

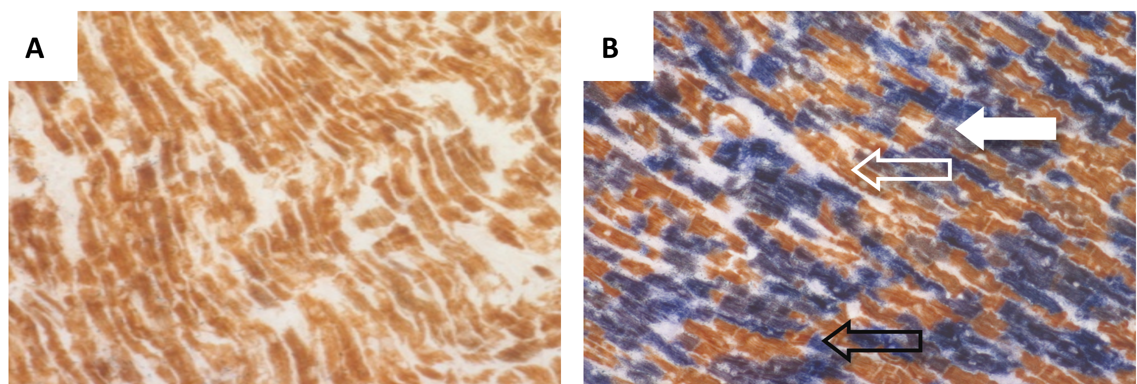


Figure 3.5 Sequential COX / SDH histochemistry. Cardiac samples from (A) a control patient without evidence of mitochondrial disease and (B) patient 10 showing COX-positive (open white arrow), COX-intermediate (closed white arrow) and COX-negative (open black arrow) cells.

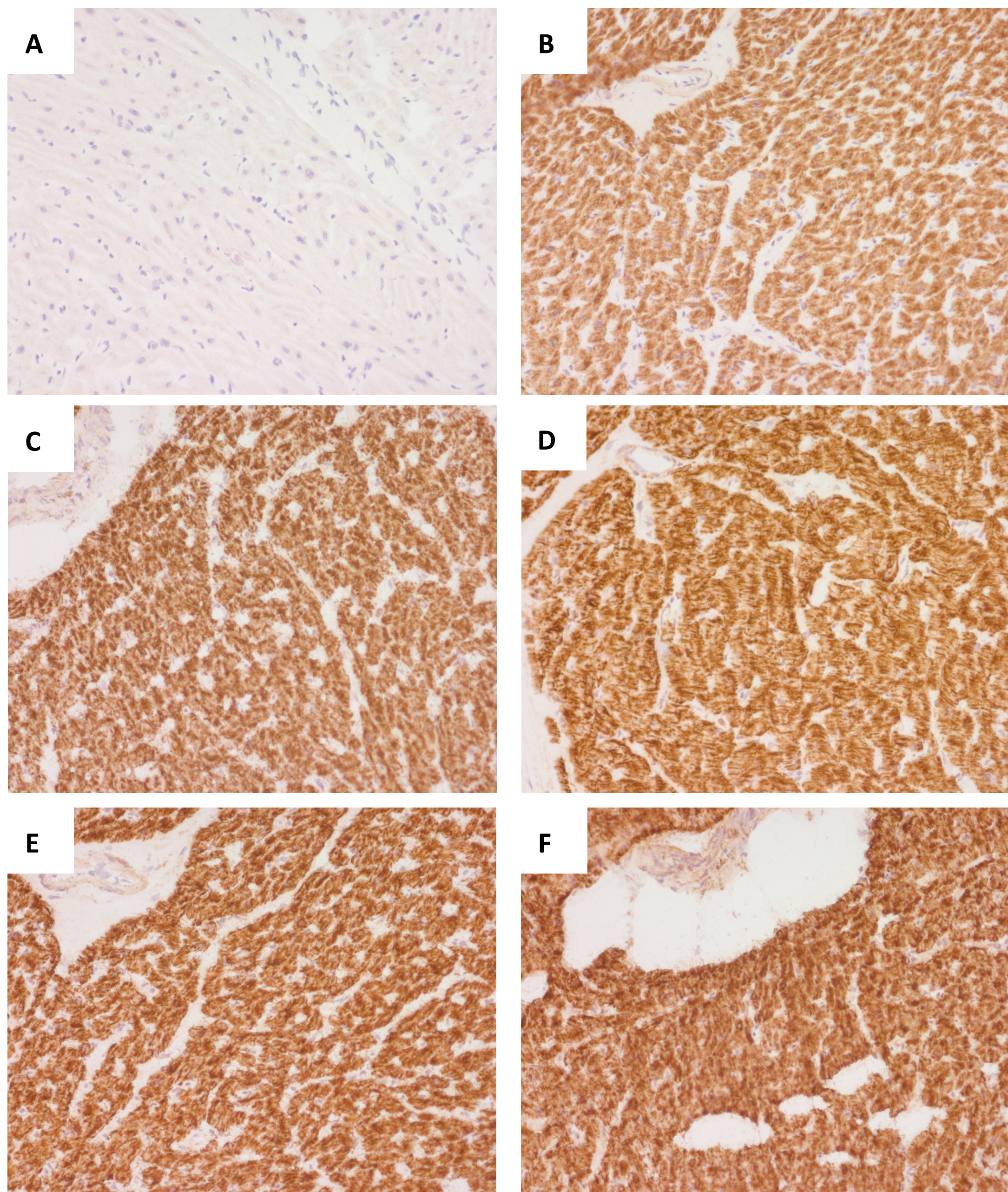


Figure 3.6 Optimisation of COX1 antibody. Immunohistochemistry was performed as described (see Chapter 2) in post-mortem cardiac tissue obtained from a healthy control with no evidence of cardiac disease (A) without primary antibody, and with antibody concentrations of (B) 1:10000, (C) 1:5000, (D) 1:3000, (E) 1:2000 and (F) 1:1000. A concentration of 1:3000 was selected on the basis that it provided a clear staining pattern with variation between cells without non-specific staining.

3.4.2.4 COX/SDH histochemistry

In patients in whom post-mortem cardiac samples were available form multiple cardiac chambers (Figure 3.7), there was a significant difference between the mean proportion of COX-deficient deficient cells in ventricles compared to atria (mean difference 31% $p<0.05$), and a trend towards a similar effect between left and right sided chambers (Figure 3.8), for both atria (mean difference 7%, $p=0.11$) and ventricles (mean difference 11%, $p=0.08$). Left ventricular samples were utilised for all subsequent analyses to maximise the detection of respiratory complex deficiencies.

All patients displayed evidence of COX-deficient cells on sequential COX/SDH histochemical analysis, although the proportion of such cells varied widely from 2-59% between different patients (Figure 3.9). The relative proportions of COX-negative and COX-intermediate cells were also inconsistent, with 10-80% of all COX-deficient cells being classified as COX-intermediate, in individual patients.

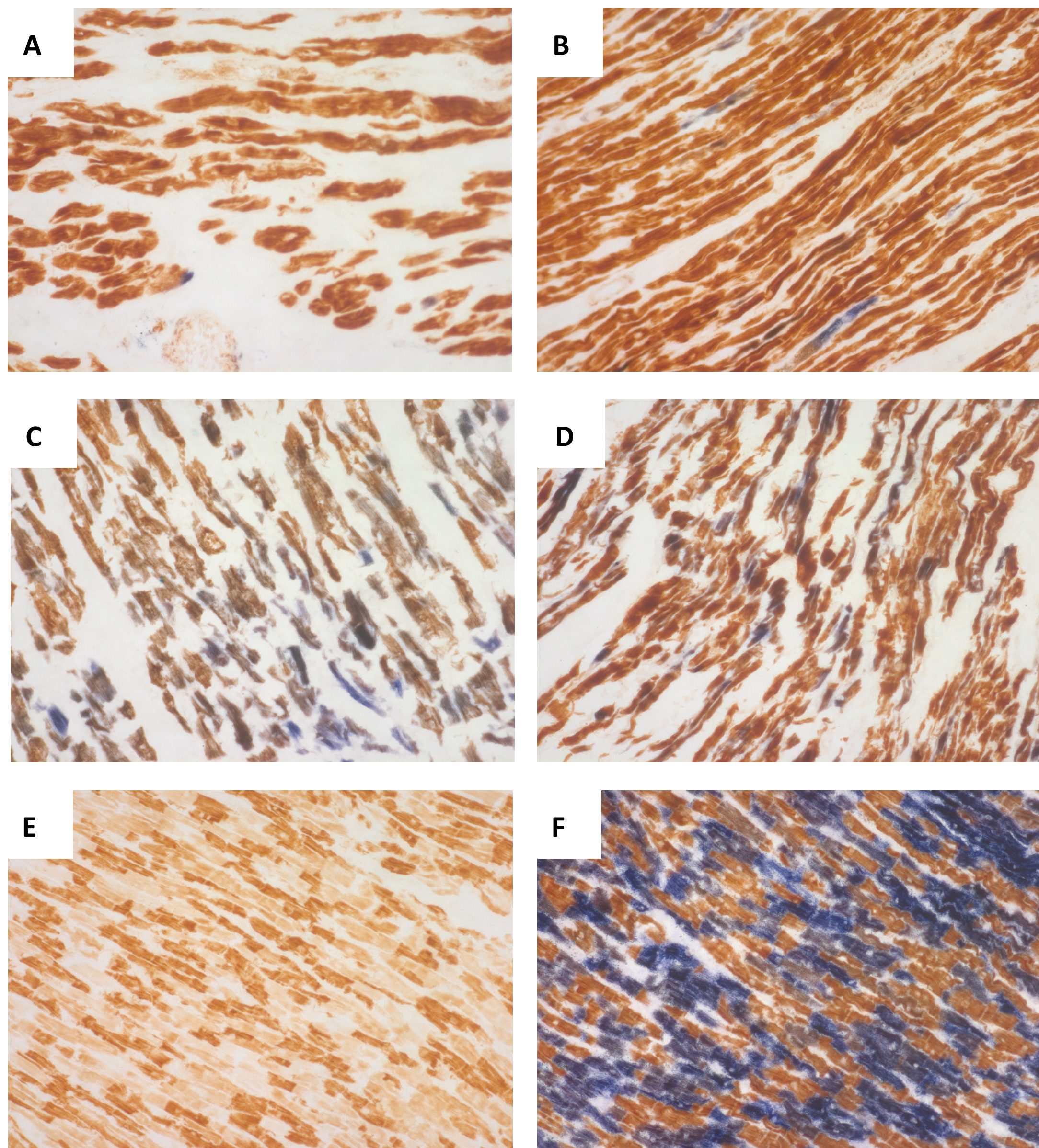


Figure 3.7 Histochemical analysis of cardiac tissue. Sequential COX/SDH histochemistry of tissue from patient 4, harbouring the m.3243A>G mutation, using post-mortem cardiac samples from (A) right atrium, (B) left atrium, (C) right ventricle, and (D) left ventricle showing a relatively low proportion of COX-deficient cells across these cardiac chambers, with a pattern of increased COX-deficiency in ventricles compared to atria. In contrast, left ventricular tissue samples from patient 10, harbouring the m.8344A>G mutation, showed a much higher percentage of COX-deficiency (57%) on (E) COX histochemistry, and (F) sequential COX/SDH analysis.

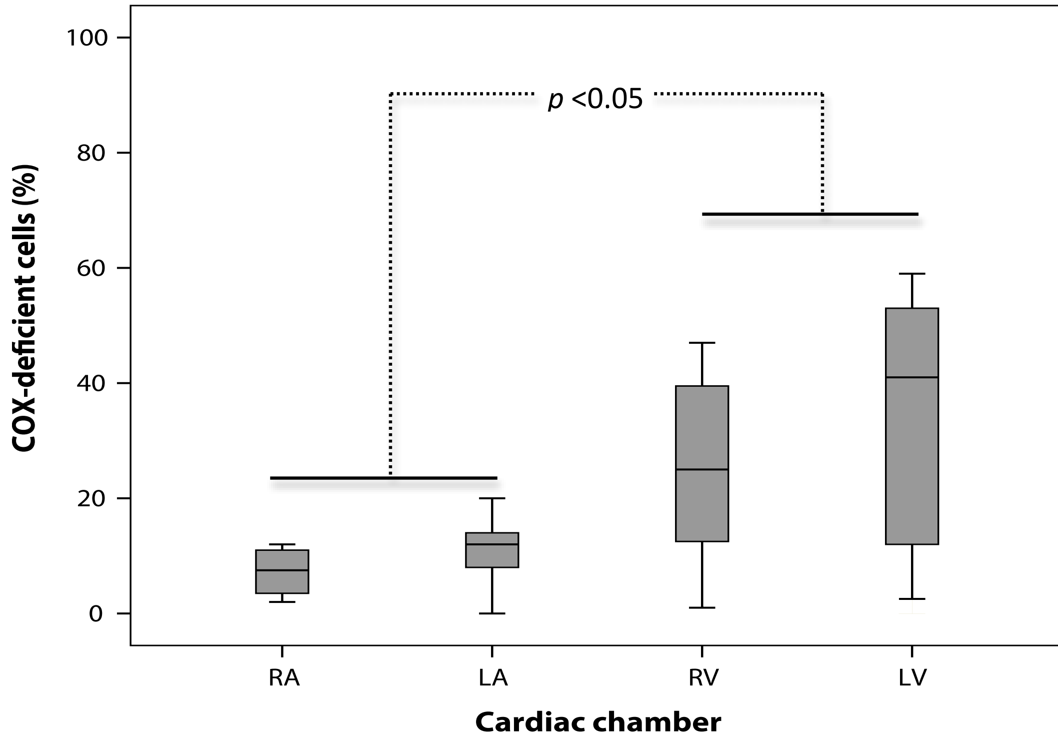


Figure 3.8 COX-deficiency and cardiac chambers. Box-plot of range and quartiles of the proportion of COX-deficient cardiomyocytes in samples from cardiac chambers. RA = right atrium; LA = left atrium, RV = right ventricle; LV = left ventricle.

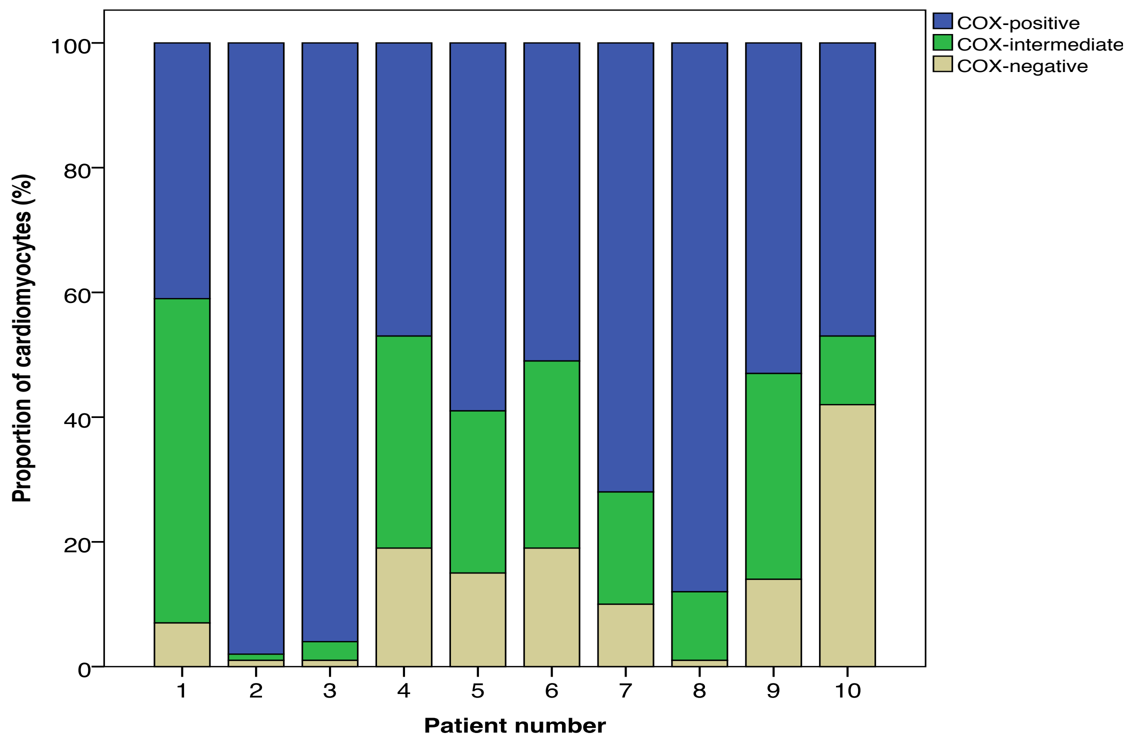


Figure 3.9 Bar chart of patterns of COX deficiency in patients. All patients showed a significant proportion of COX-intermediate cells amongst those cardiomyocytes that were assessed as showing evidence of COX-deficiency.

3.4.2.5 Immunohistochemistry

3.4.2.5.1 Visual analysis

All patients displayed evidence of immunohistochemical abnormalities on analysis with antibodies directly against the 19kDa subunit of complex I and/or the COX I subunit on complex IV (Figure 3.10). Immunohistochemical analysis with antibodies directed against the outer mitochondrial membrane polypeptide, porin, or the entirely nuclear-encoded 70kDa subunit of complex II revealed no abnormality of mitochondrial content in post-mortem cardiac tissue obtained from patients or controls (data not shown).

From visual observation, all patients harbouring the m.3243A>G or the m.8344A>G mutations displayed evidence of deficiency of complex I subunits. This was more marked in patients harbouring the m.3243A>G mutation (Figure 3.11) than the m.8344A>G mutation (Figure 3.12). A greater proportion of myocytes from all cardiac chambers appeared to be deficient for complex I subunits than deficient for complex IV subunits, or indeed complex IV activity on routine histochemistry (Figure 3.13), and this effect was particularly apparent in patients harbouring the m.3243A>G mutation.

Although serial section analysis was not possible in cardiac tissue, visual comparison of histochemistry and immunohistochemistry for patients harbouring both the m.3243A>G and m.8344A>G mutations suggested that there was a striking discrepancy between sequential COX/SDH histochemistry and immunohistochemistry for complex IV subunits. Many cells with evidence of COX-deficiency had *normal* or near normal staining patterns for the specific subunits of complex IV (COX I, COX II and COX IV). There appeared to be a closer relationship between abnormalities of complex I subunits (15, 19 and 20kDa subunits) and COX-deficiency on histochemistry across patients with both mutations (Figure 3.11 and Figure 3.12).

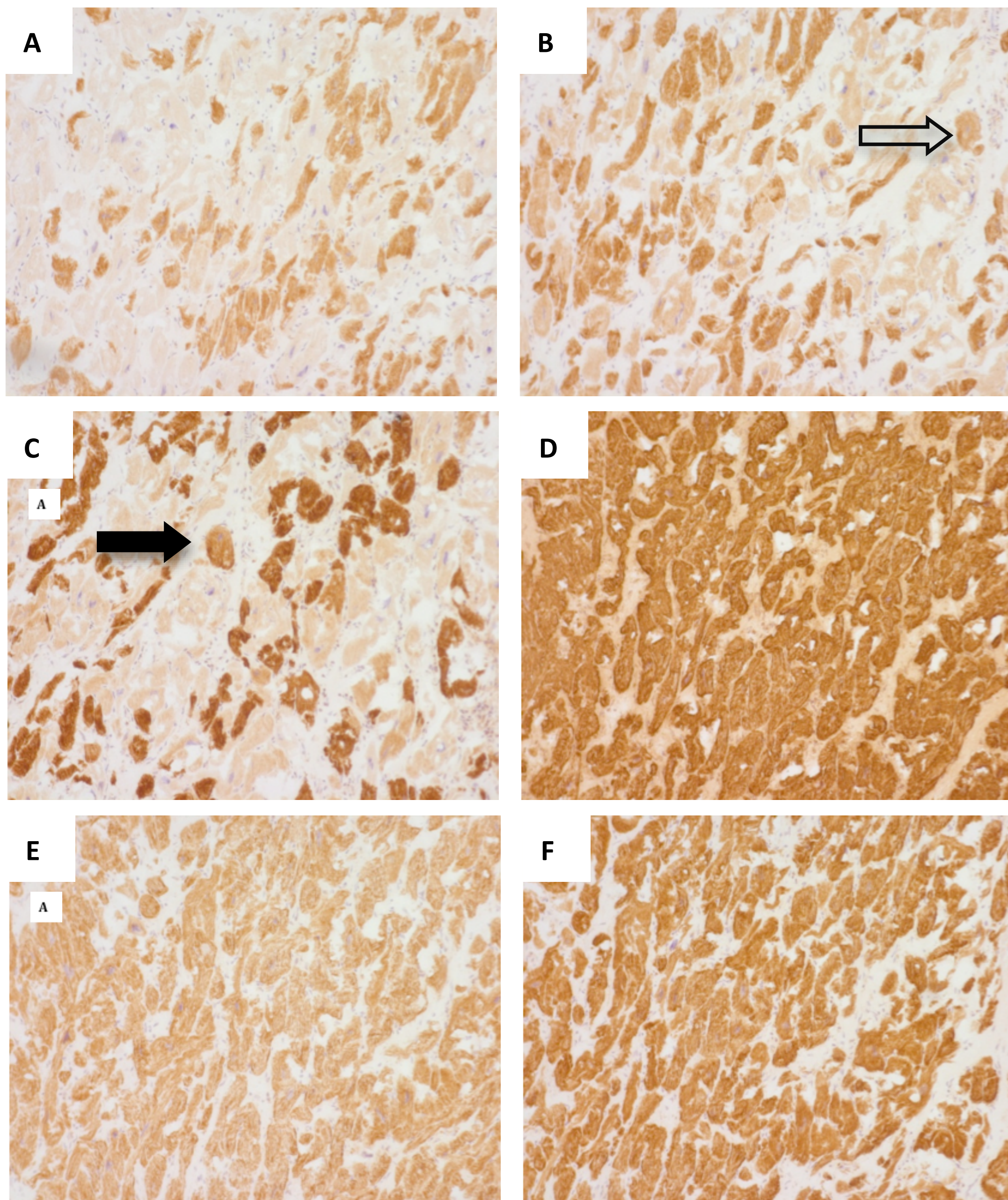


Figure 3.10 Immunohistochemistry using multiple subunit antibodies. To validate immunohistochemistry results, multiple antibodies were used against different subunits from individual complexes. All images are from patient 4 harbouring the m.3243A>G mutation. Complex I was analysed using antibodies to the (A) 15kDa, (B) 19kDa, and (C) 20kDa subunits. The 20kDa was selected as the preferential complex I subunit due to the unambiguous nature of the images produced, with better differentiation between positive cells – the same positive cell is demonstrated more clearly with the 20kDa antibody (closed arrow) compared to the 19kDa antibody (open arrow). From the same patient, (D) shows staining for the complex II 70kDa subunit, an entirely nuclear encoded subunit, emphasising the selective loss of complex I. Complex IV was analysed using antibodies to the (E) COX I and (F) COX IV subunits. COX I was selected as the preferred antibody due to greater differentiation between cells (COX IV showed uniform staining in all patients, whatever the defect apparent in COX I, as discussed in section 3.5.3.3).

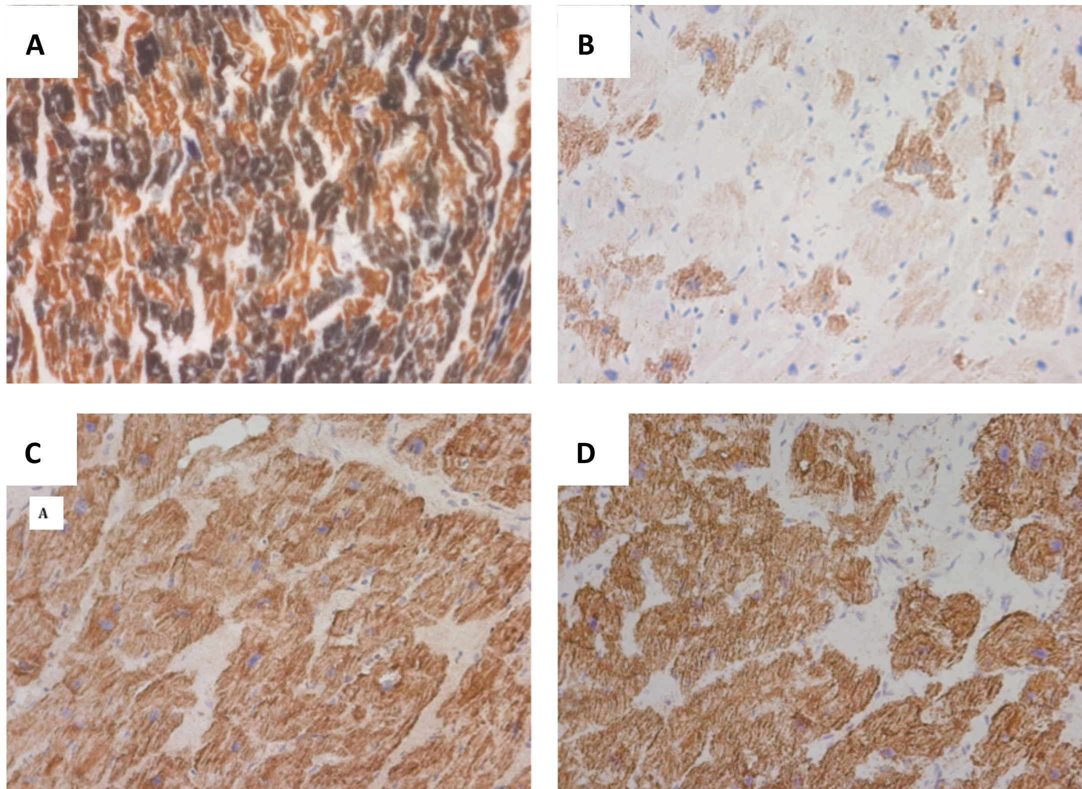


Figure 3.11 Histochemical and immunohistochemical analysis: patient 4 (m.3243A>G mutation). Using a post-mortem left ventricular cardiac sample from a patient harbouring the m.3243A>G mutation (A) sequential COX/SDH staining reveal 52% COX-deficient cells with a predominance of COX-intermediate cells. Immunohistochemical analysis of the same tissue sample using antibodies directed against (B) the 19kDa subunit of complex I, (C) the 70kDa subunit of complex II, and (D) the COX I subunit of complex IV shows a striking abnormality of 77% complex I deficiency with near normal staining patterns for complex II and complex IV (both <5% deficiency).

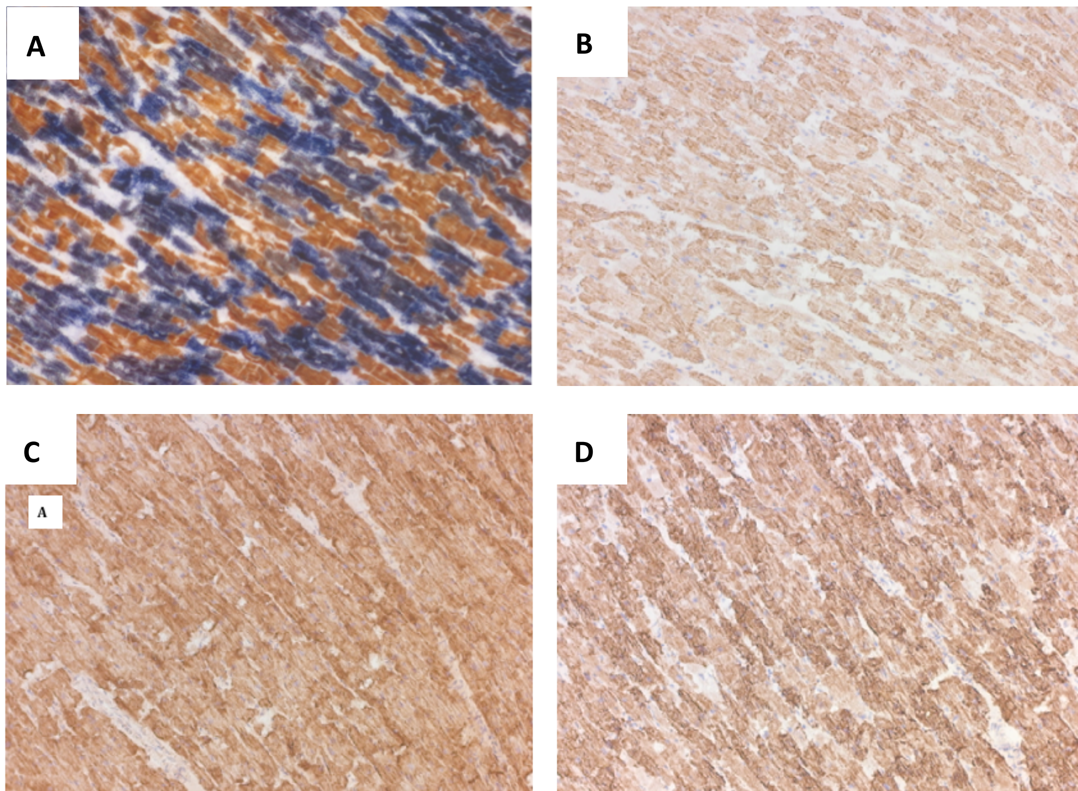


Figure 3.12 Histochemical and immunohistochemical analysis: patient 10 (m.8344A>G mutation). Using a post-mortem left ventricular cardiac sample from a patient harbouring the m.8344A>G mutation (A) sequential COX/SDH staining reveals 57% COX-deficient cells with a predominance of COX-intermediate cells. Immunohistochemical analysis of the same tissue sample using antibodies directed against (B) the 19kDa subunit of complex I, (C) the 70kDa subunit of complex II, and (D) the COX I subunit of complex IV, shows an abnormality of 38% complex I deficiency, a normal staining patterns for complex II (<5% deficiency) and 13% complex IV deficiency.

3.4.2.5.2 Quantitative analysis of immunohistochemistry

Quantitative analysis of immunohistochemistry confirmed the results of visual analysis with all patients displaying evidence of complex I and/or complex IV deficiency. There was no significant difference between the quantitative immunohistochemical results obtained via subjective visual inspection of counted cardiomyocytes or objective densitometry (Figure 3.). Both methods demonstrated a significant difference between the proportion of COX-deficient and complex IV deficient cardiomyocytes (35 ± 22% vs 8 ± 9%, $p<0.01$) but *no* difference in the proportions of COX-deficient and complex I deficient cardiomyocytes (35 ± 22% vs 39 ± 25%, $p=0.22$) (Figure 3.). This pattern was observed for all patients with both mutations.

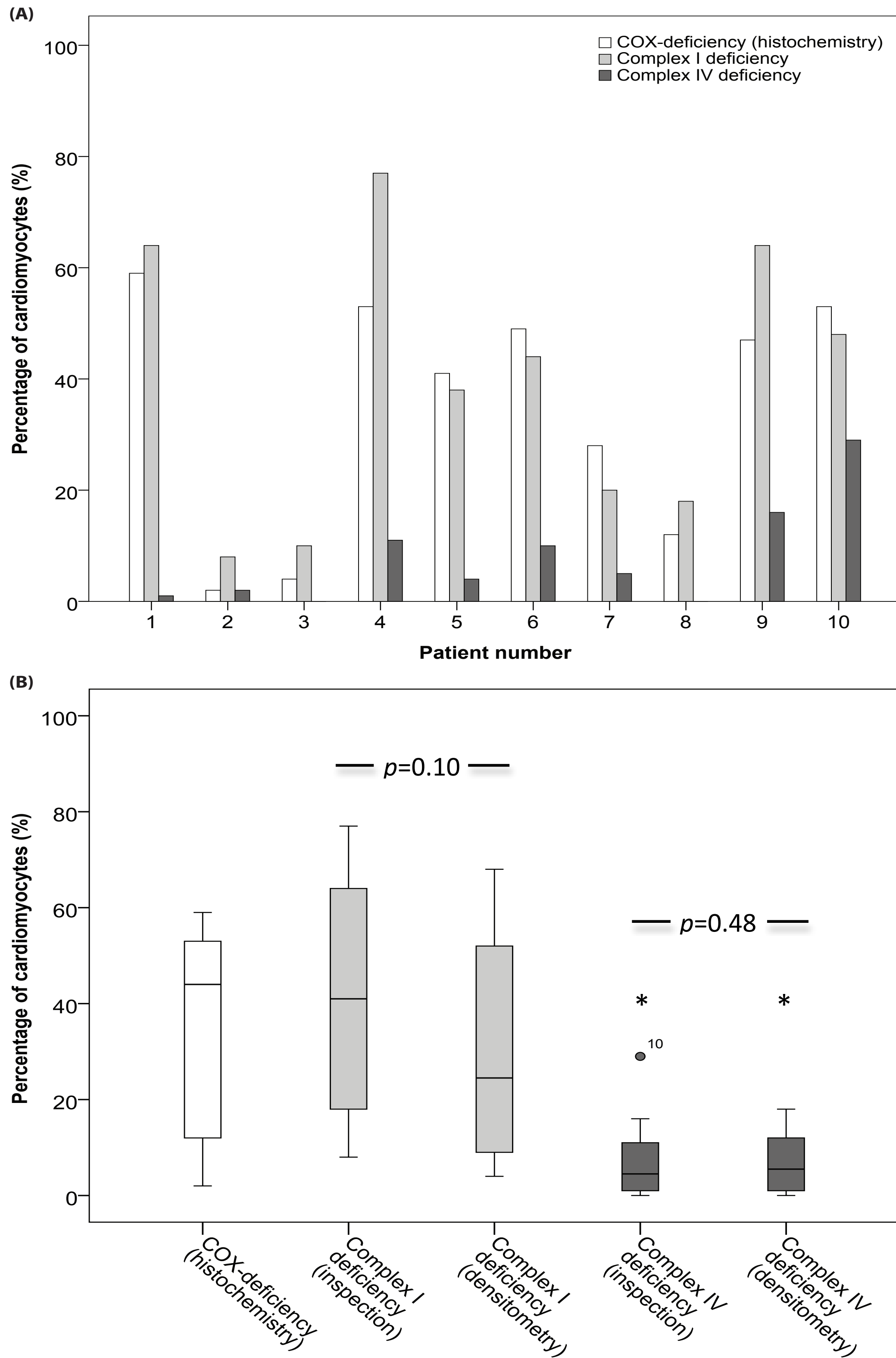


Figure 3.13 Quantitative immunohistochemistry and histochemistry. (A) Bar chart of the proportion of cardiomyocytes showing COX-deficiency, complex I deficiency (19kDa subunit), and complex IV deficiency (COX1), by visual inspection. (B) Box plots of the proportion of COX-deficiency by histochemistry with complex I and complex IV deficiency by two different methods of quantification: visual inspection and densitometry analyses. * = p<0.01 compared to COX-deficiency.

Comparison of the extent of COX-deficiency by histochemical analysis with the results of immunohistochemistry for left ventricular tissue showed a significant positive correlation between COX-deficiency and complex I deficiency (Figure 3.14), consistent with the observed results in individual patients (Figure 3.11 and 3.12). There was no clear relationship between COX-deficiency and complex IV deficiency. Conversely, comparison of the proportion of COX-negative cardiomyocytes *only* (i.e. excluding COX-intermediate cardiomyocytes) with complex IV deficiency showed a significant positive correlation that was not apparent with complex I deficiency (Figure 3.14).

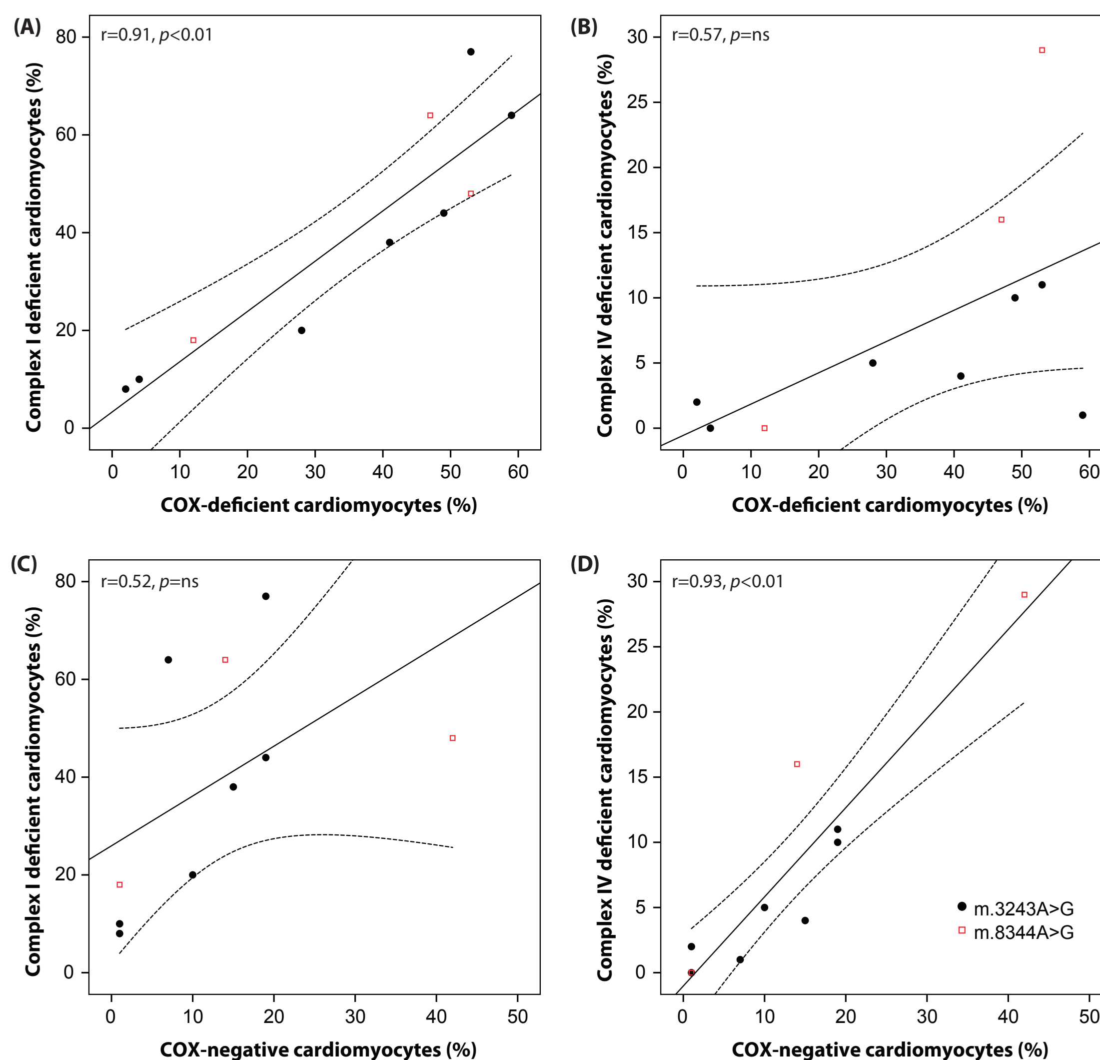


Figure 3.14 Scatter plots of histochemistry and immunohistochemistry. COX-deficiency showed a significant positive correlation with (A) complex I deficiency but not (B) complex IV deficiency. Conversely, the proportion of COX-negative cardiomyocytes showed no clear relationship to (C) complex I deficiency but a significant positive correlation with (D) complex IV deficiency.

3.4.2.5.3 Respiratory complex deficiencies and cardiomyopathy

In addition to validation of visual classification of the proportion of complex I and complex IV deficient cardiomyocytes, densitometric analysis of immunohistochemistry enabled assessment of the *pattern* of deficiency. For the whole group of patients, complex I deficiency was more profound by visual inspection, and displayed a more marked difference in density of the signal compared to control tissue – cells deficient for complex I displayed a signal almost reduced to background levels. This effect was most apparent for patients harbouring the m.3243A>G mutation, and particularly in those with evidence of cardiomyopathy (Figure 3.11).

Comparisons of immunohistochemistry for complex I (20kDa), complex II (70kDa) and complex IV (COX I) between control cardiac tissue and cardiac tissue from patients with the m.3243A>G mutation and the m.8344A>G mutation revealed distinct patterns of complex deficiencies (Figure 3.15). There was no difference between tissue samples in the signal density of immunohistochemical analysis directed against complex II. The signal densities of immunohistochemical analysis direct against both complex I and complex IV were significantly different to control cardiac tissue (Figure 3.15). However, while the complex deficiency for complex IV showed similar reduction in signal density for patients with both the m.3243A>G and m.8344A>G mutations, the situation was very different for complex I deficiency. A reduction in signal density was shown for all patients with regard to complex I deficiency, compared to control tissue but for those patients harbouring the m.3243A>G mutation, two distinct populations of cardiomyocytes were observed: complex I deficiency was more profound for these patients compared to those harbouring the m.8344A>G mutation with a reduction in signal density in affected cardiomyocytes to near background levels (Figure 3.15). Notably the comparison of patients by genotypic group displayed some overlap with a comparison of patients with and without cardiomyopathy (for patients harbouring the m.3243A>G mutation, 5/7 had evidence of cardiomyopathy while for those harbouring the m.8344A>G mutation 1/3 had cardiomyopathy).

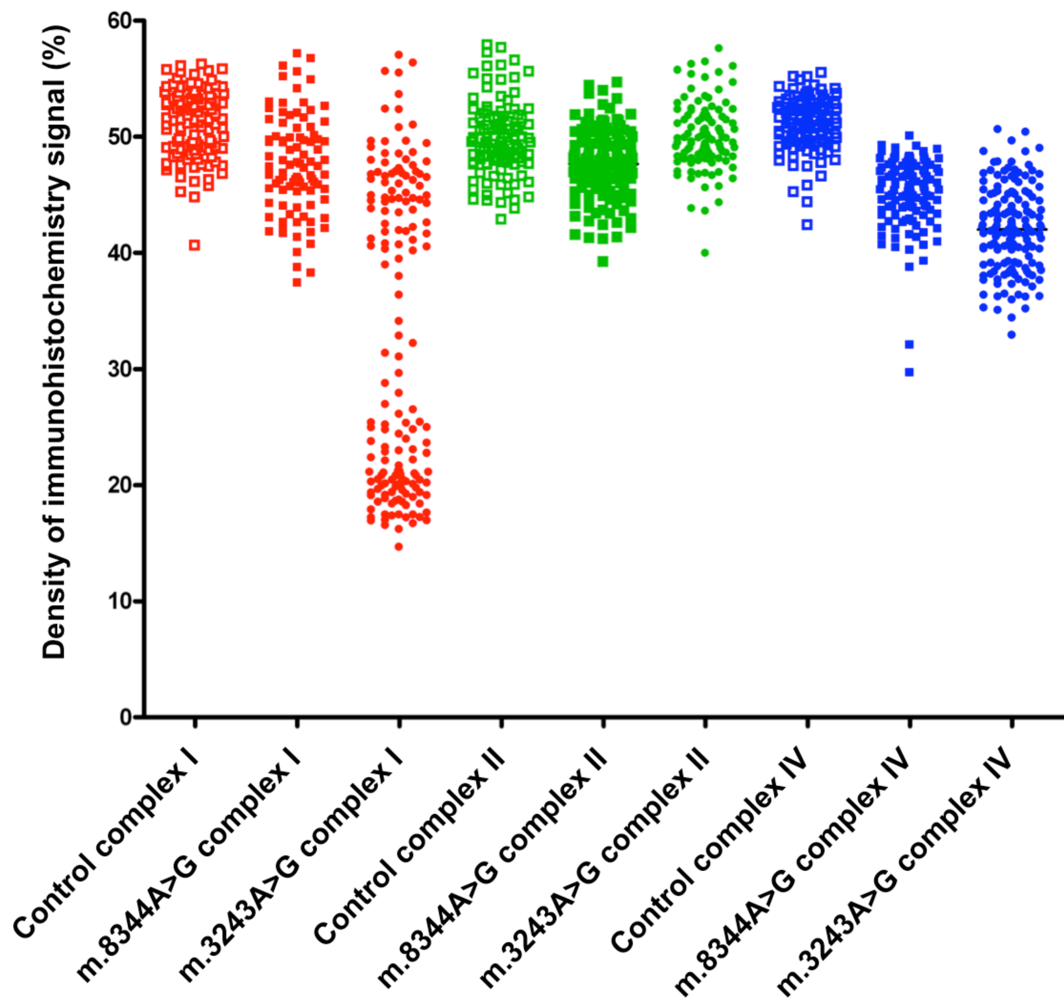


Figure 3.15 Distribution of signal density of individual cardiomyocytes. The density of the immunohistochemistry signal for cardiomyocytes from control cardiac tissue, and patients with mitochondrial disease harbouring the m.3243A>G and m.8344A>G mutation.

3.5 Discussion

The principal findings of this study of the nature, frequency and pathogenic mechanisms of cardiac involvement in patients with mitochondrial disease due to the mt-tRNA mutations of m.3243A>G and m.8344A>G are: 1) cardiac involvement is frequently observed and, although restricted to isolated ECG abnormalities in many, includes cardiomyopathy in around ~1/7th patients; 2) the presence of cardiomyopathy is associated with decreased survival following symptom onset, in all patients with mitochondrial disease; 3) despite evidence of tissue segregation, the extent of heteroplasmy in skeletal muscle provides a reliable indicator of myocardial mutation load; 4) patients with an earlier age of onset, elevated skeletal muscle mutation load, and those within distinct genotypic groups, including mt-tRNA mutations and multiple mtDNA deletions, may be at increased risk of cardiac involvement; 5) although myocardial COX-deficiency is common in patients with mt-tRNA mutations, the majority of cardiomyocytes are COX-intermediate and, in isolation, histochemistry does not appear to explain differences in cardiac involvement; 6) patients with the m.3243A>G and m.8344A>G mutations display distinct differences in the pattern of immunohistochemical complex deficiencies that may be important in the development of cardiomyopathy. I have determined that cardiac involvement is more frequent in patients with mt-tRNA mutations and that the complex I deficiency may be important pathogenic factor in cardiomyopathy in patients harbouring the m.3243A>G mutation.

3.5.1 Cardiac involvement and clinical outcomes

Consistent with previous cohort studies, cardiac involvement was noted in a significant minority of patients with mitochondrial disease (Majamaa-Voltti *et al.*, 2002; Limongelli *et al.*, 2010; Wahbi *et al.*, 2010; Nesbitt *et al.*, 2013). The range of the cardiac phenotype observed was however very broad, extending from minor and non-specific ECG abnormalities to end-stage cardiomyopathy requiring cardiac transplantation. This diversity of phenotype is not surprising in a field of multisystem disease characterised by variability and unpredictability in the inheritance, nature and progression of clinical disease (Chinnery *et al.*, 2000; Majamaa-Voltti *et al.*, 2006; McFarland *et al.*, 2010). However some patters do emerge from analysis of a large cohort of patients, followed up over a number of years.

Firstly, electropathy was evident in the majority of patient with cardiac involvement in mitochondrial disease confirming the results of previous smaller studies. Minor ECG abnormalities were common (Limongelli *et al.*, 2010), but trends existed with regard to more significant electropathy in specific genotypic groups – permanent pacemaker implantation for conduction system disease was restricted to patients harbouring single, large scale or multiple mtDNA deletions (Roberts *et al.*, 1979); and patients harbouring the m.8344A>G mutation were over-represented among those undergoing invasive procedures for accessory pathway-mediated tachycardia (Wahbi *et al.*, 2010). Although mechanisms are unknown, differences in mutation load or in the threshold for cellular deficiency between cardiac cell types, or for different mtDNA mutations, may account for this phenotypic discrepancy (Muller-Hocker *et al.*, 1998). To differentiate between these possibilities would require an *in vitro* model, likely derived from inducible pluripotent stem cells from patients with disease, to study pathogenic mechanisms in specific populations of specialised cardiomyocytes, harbouring different mtDNA mutations.

Secondly, cardiomyopathy occurred in ~14% patients overall and predominantly had a hypertrophic pattern, broadly consistent with most previous smaller studies (Majamaa-Voltti *et al.*, 2002; Sorajja *et al.*, 2003; Vydt *et al.*, 2007; Limongelli *et al.*, 2010). However the frequency of cardiomyopathy observed in my retrospective study is towards the lower end of the range in the published literature. Consistently, earlier studies of newly recognised conditions tend to represent a more severely affected group of patients, with expansion of the clinical phenotype over time. This may have lead to initial over-estimation of the frequency of cardiac involvement in patients with mitochondrial disease. Additionally our cohort included patients with a wide range of genotypes, some of which have been less closely associated with clinical cardiac disease, and many individuals with oligosymptomatic status, rather than classical phenotypes. This is consistent with more recent attempts to describe clinical disease in comprehensive cohorts of patients with mitochondrial disease, to enable future prospective study (Nesbitt *et al.*, 2013). Data from our large retrospective cohort supported my decision to focus on patients with the m.3243A>G and m.8344A>G mutations for the remainder of this study, given the increased frequency of cardiomyopathy in these groups and the comparable baseline characteristics.

Thirdly, my data report a significant effect of cardiomyopathy on survival from the time of symptom onset in unselected patients with mitochondrial disease. Although previous natural history studies have demonstrated a deleterious effect of cardiac involvement on clinical outcome in patients with mitochondrial disease, these reports have largely focussed on paediatric populations (Holmgren *et al.*, 2003; Scaglia *et al.*, 2004; Debray *et al.*, 2007). The consistent message of my data showing cardiovascular disease as a common cause of death, significant interventions for cardiac involvement throughout life, and decreased survival in patients with cardiomyopathy is that cardiac involvement is an important cause of morbidity and early mortality in patients with mitochondrial disease. This is in keeping with previous reports (Anan *et al.*, 1995; Majamaa-Voltti *et al.*, 2008), and highlights the importance of identification of these vulnerable patients and attempts to institute early evidence-based treatment.

Finally, in light of recognition of the clinical importance of cardiomyopathy, and the potential diversity of phenotype in patients with mitochondrial disease, I sought to identify predictors of cardiac involvement. Consistent with clinical experience and previous case series (Anan *et al.*, 1995; Limongelli *et al.*, 2010), genotype is an important factor – patients with mt-tRNA mutations and multiple mtDNA deletions appeared to be at the highest risks of cardiac involvement. This justifies my clinical decision to focus on patients harbouring the m.3243A>G and m.8344A>G mutations as defined groups, and may have important implications for the nature and extent of clinical cardiac screening in these cohorts. Similarly, earlier age of onset of symptoms, which has previously been found to be a indicator of poor outcome in children (Holmgren *et al.*, 2003), was identified as a predictor of cardiac involvement. Interestingly, although identified through univariate analysis, skeletal muscle mutation load was not an independent predictor of cardiac involvement in this cohort. This is perhaps surprising given the significant correlation between the level of skeletal muscle and cardiac muscle heteroplasmy in this study, and suggests that factors other than cardiac heteroplasmy are more important in the expression of clinical cardiac disease. From a clinical viewpoint, urinary epithelial cells provide a reliable marker of both skeletal muscle mutation load and clinical outcome in patients with the m.3243A>G mutation (Whittaker *et al.*, 2009). While possession of such a non-invasive method of assessment of cardiac muscle heteroplasmy that could help predict cardiac

involvement would have clear clinical benefits, this does not appear to be a viable option in this cohort, although clearly a larger study utilising comprehensive urinary analysis would be needed to resolve this issue.

3.5.2 Tissue specific segregation

Using analysis of a variety of post-mortem tissues from patients with the m.3243A>G or m.8344A>G mutations, this study demonstrated significant variation in the level of heteroplasmy between different tissues. This finding was reflected in the limited number of samples that were analysed in these patients (from skeletal muscle, blood and urinary epithelial cells), and is consistent with previous studies (Shiraiwa *et al.*, 1993; Chinnery and Samuels, 1999; Frederiksen *et al.*, 2006).

Despite this evident tissue segregation, and consistent with a previous study analysing cardiac biopsies (Majamaa-Voltti *et al.*, 2002), there was a significant correlation between the levels of skeletal muscle and cardiac muscle heteroplasmy, with both tissues often displaying a high level of mutation load. Only patient 8 had a low level of heteroplasmy in cardiac muscle (19%) and suggestively, this patient had no pre-existing clinical evidence of cardiac involvement. Indeed the three patients in this study that had a normal ECG and echocardiogram documented during life (patients 3,8 and 10), all had the lowest levels of heteroplasmy in cardiac muscle of the entire group (72, 19, and 75% respectively); all patients with clinical cardiac involvement had a mutation load in the range of 77-92%. Although not reaching statistical significance on multivariate analysis, the retrospective cohort study also suggested skeletal muscle mutation load may have an influence on the presence of cardiac involvement and other studies have similarly suggested a link between heteroplasmy level and clinical disease (Betts *et al.*, 2008; Whittaker *et al.*, 2009). While intuitively attractive as a theory, the situation is undoubtedly more complex than these isolated results would suggest. Other groups, using analysis of affected tissues, have failed to document an association between mutation load and clinical organ involvement (Majamaa-Voltti *et al.*, 2002), and, despite a relatively low mutation level in cardiac muscle, patient 10 in fact displayed *significant* evidence of respiratory deficiency using histochemical and immunohistochemical analyses and died suddenly, with a possible cardiac aetiology. Equally, although it is well accepted that those with a very low mutation load are at less risk of clinical mitochondrial disease than those with a very high mutation load

(Taylor and Turnbull, 2005; McFarland *et al.*, 2010), it seems unlikely that the difference between 75% heteroplasmy (in patient 10 with no cardiac involvement) and 77% heteroplasmy (in patient 4, with hypertrophic cardiomyopathy) is sufficient to explain the difference in organ involvement. Levels of tissue heteroplasmy are derived from cardiac muscle homogenate and, as such, provide only a broad estimate of the average mutation. With uniform distribution of mutated mtDNA across a tissue, a homogenate mutation load could be quite high without exceeding the threshold for respiratory deficiency in any given cell. However it is much more likely that uneven cellular segregation *within* cardiac tissue means that, even for a lower homogenate mutation load, individual cells will contain extremely high levels of mutated mtDNA and express respiratory deficiency with clinical organ involvement.

Cardiac muscle is a tissue with high bioenergetic demands, and consequently has an exceptionally high concentration of mitochondria, with mtDNA content per diploid nucleus approximately twice that of skeletal muscle (Miller *et al.*, 2003). Segregation of mutated mtDNA between and within different tissues does likely play a role in the clinical phenotype but other pathogenic mechanisms may also have a significant impact. To investigate a link between cardiac muscle mutation load and phenotype would require a much larger study with access to biopsy tissue, and effective animal models would be required to explore the impact of heteroplasmy alone.

3.5.3 Cellular respiratory deficiency in cardiac muscle

3.5.3.1 COX-deficiency

Histochemistry of post-mortem cardiac tissue revealed a significant effect of cardiac chamber on the extent of COX-deficiency despite no difference in the level of heteroplasmy. In cardiac tissue, without single cell analysis using sequential samples, it was not possible to determine whether differences in the distribution of mutated mtDNA within the tissue may have explained this finding; a reliance on homogenised tissue to assess heteroplasmy prevented exploration of this concept, and notably other studies have documented significant differences in mutation load and respiratory deficiency in different cell types from close-related tissue areas (Betts *et al.*, 2008; Kazakos *et al.*, 2012). Yet the consistency of this observation of increased COX-deficiency across the patient group and the significance of the difference between

atria and ventricles suggests that and there may be an important interaction between chamber-specific environmental factors and mutation load. Indeed the concept of epigenetic factors influencing phenotypic expression has been recently reported for patients with lipomata and the m.8344A>G mutation (Kazakos *et al.*, 2012). Although previous studies have reported biochemical evidence of mitochondrial dysfunction in both atria and ventricles from patients with and without evidence of primary mitochondrial disease (Bonnet *et al.*, 2001; Majamaa-Voltti *et al.*, 2002; Montaigne *et al.*, 2013), no studies have previously reported comparative histochemical analysis of cardiac chambers in individual patients. Differences in COX-deficiencies between cardiac chambers highlighted potential difficulties in interpretation of invasive biopsy samples, most commonly performed from the right ventricle, in comparison to post-mortem samples (Bonnet *et al.*, 1998; Bonnet *et al.*, 2001). This finding however did enable suitable focus to be applied to left ventricular samples for subsequent investigations in this study, maximising the opportunity to investigate pathogenic mechanisms.

There appeared to be little clear relationship between the extent or pattern of COX-deficiency in cardiac muscle and clinical phenotype in patients with m.3243A>G and m.8344A>G mutations; even in patients harbouring the same m.8344A>G mutation, patient 10 had extensive histochemical evidence of COX-deficiency (57%) but no cardiac phenotype while patient 9 displayed 47% COX-deficiency and had hypertrophic cardiomyopathy; patient 7, who harboured the m.3243A>G and had severe cardiomyopathy awaiting cardiac transplantation at the time of death, had 28% COX-deficiency in cardiac muscle. In most patients, the major proportion of COX-deficient cardiomyocytes were in fact classified as COX-intermediate, rather than truly COX-negative, and this may at least partially explain the lack of correlation with clinical phenotype – other factors play a role in respiratory deficiency. In summary no clear pattern emerges with comparison of patients with mt-tRNA point mutations, and COX-deficiency does not appear isolation to explain the cardiac phenotype. Although early studies suggested characteristic patterns of skeletal muscle histochemical defects in phenotypic groups of patients harbouring the m.3243A>G mutation (Petruzzella *et al.*, 1994), this concept has recently been challenged and, aside from basic patterns such as a mosaic appearance suggesting the presence of an underlying heteroplasmic

mtDNA mutation, less emphasis is put on the precise nature of COX-deficiency in the diagnostic investigation of mitochondrial disease (McFarland *et al.*, 2010).

3.5.3.2 Complex I deficiency

In investigating the nature of the respiratory defect apparent in cardiac muscle in patients harbouring mt-tRNA mutations, I have demonstrated a significant correlation between COX-deficiency using histochemistry and complex I deficiency using immunohistochemistry. This relationship includes, in the definition of COX-deficient cells, all cardiomyocytes with any evidence of the abnormal activities of complex II and/or IV (i.e. COX-negative and COX-intermediate cells). Although systematic serial section analysis of cardiac muscle was not possible, certain sections did show the same cardiomyocytes to be both COX-deficient and complex I deficient. Indeed the level of complex I deficiency was generally more profound than COX-deficiency and cells with normal histochemistry yet abnormal immunohistochemistry for the complex I 20KDa subunit were certainly apparent in several patients, supporting loss of complex I subunits as an early or primary event in the pathogenesis of mt-tRNA mutations in cardiac muscle, as in other tissues (Goto *et al.*, 1992; Dunbar *et al.*, 1996).

Amongst respiratory chain enzyme complexes, complex I contains the highest proportion of mtDNA-encoded leucine (UUR) residues; subunits ND3, ND5 and ND6 contain 10, 14 and 9 such codons, respectively and it is therefore unsurprising that complex I would be sensitive to defective leucine translation. Several studies have reported that complex I activity is more frequently reduced than complex IV activity in patients with the m.3243A>G mutation (Goto *et al.*, 1992; Morgan-Hughes *et al.*, 1995), and immunohistochemistry has similarly suggested a difference between loss of expression of complex I in patients with m.3243A>G mutations and loss of expression of *both* complex I and IV in patients with the m.8344A>G mutation (De Paepe *et al.*, 2009). Importantly the ND6 subunit, which contains nine UUR residues amongst its fourteen UUG codons (see Section 3.1), plays a critical role in the integration of other subunits into complex I (Bai and Attardi, 1998; Ugalde *et al.*, 2003), and the rate of synthesis of ND6 polypeptides in myoblasts containing m.3243A>G is <10% that of control cells (Sasarman *et al.*, 2008).

The 20kDa subunit investigated in this study is not mtDNA-encoded but specific to the NDUFB8 subunit, located in the 1β subcomplex of complex I. In my study, the consistency of my finding of profound complex I deficiency using antibodies directed against the 15kDa, 19kDa, 20kDa subunits (respectively, the NDUFB4, NDUFA13 and NDUFB8 subunits) supports the notion of the m.3243A>G mutation causing a primary defect in translation of complex I as a whole. Such isolated defects can however have more wide-ranging effects on the respiratory chain. For example, abnormal complex II and III activity and/or evidence of COX-deficiency have been demonstrated in patients with mtDNA point mutations affecting complex I subunits in ND3, ND5 and ND6 (Santorelli *et al.*, 1997; V. Carelli *et al.*, 1998; Shanske *et al.*, 2008). Certainly my data support an association between any COX-deficiency and complex I deficiency. Although extensive further studies would be necessary to investigate this issue, it is possible that, in patients harbouring the m.3243A>G mutation, COX-deficiency is a downstream manifestation of abnormal complex I activity in cardiac muscle.

3.5.3.3 Complex IV deficiency

In cardiac muscle from patients with mt-tRNA point mutations, I noted a lack of correlation between COX-deficiency, reflecting the *activity* of complex IV, and expression of the COX I subunit, reflecting the *presence* of complex IV. For patients with either the m.3243A>G or m.8344A>G mutation, truly COX-negative cardiomyocytes did display a significant association with complex IV deficient cells. Although serial section analysis would be need to explore this concept further, studies in other tissues have reported that, while COX I expression was down-regulated in all aged colonic crypts that displayed no evidence of COX activity, it was also reduced in crypts that showed *any* level of respiratory defect (Taylor *et al.*, 2003a; Greaves *et al.*, 2010). I did not show such an association; while it is possible that COX I expression could have been reduced, in some cardiomyocytes, to a level that would not be apparent on visual analysis of the immunohistochemistry but nonetheless would results in dysfunction activity of complex IV, this is not a likely explanation of the lack of correlation in my data, particularly since visual analysis was in agreement with objective densitometry.

Previous studies in myoblasts containing homoplasmic levels of the m.3243A>G or m.8344A>G mutations have similarly demonstrated a discrepancy between complex IV

expression and activity (Sasarman *et al.*, 2008). In m.3243A>G myoblasts, the rate of synthesis of COX I, II and III was only marginally reduced compared to controls, whereas the rate of synthesis of these same subunits was <10%, <10% and 30% respectively, of control values, for m.8344A>G myoblasts. Despite this difference in expression, the activity of complex IV was severely reduced in myoblasts with either mutation, and BN-PAGE was used to demonstrate that a defect in full assembly of complex IV was critical in the respiratory dysfunction observed in m.3243A>G myoblasts (Sasarman *et al.*, 2008). Due to the quaternary structure of the holoenzyme, the COX I subunit is widely accepted as an accurate marker of fully assembled complex IV (Tsukihara *et al.*, 1996; Nijtmans *et al.*, 1998). Using immunohistochemical analysis In my study, however, COX IV showed a uniform pattern of staining for cardiac tissue with either mt-tRNA mutation, including cardiomyocytes which showed a defect for COX I, and indeed COX IV expression has been demonstrated in cells lacking mtDNA (Marusich *et al.*, 1997). It has been proposed that such expression of nuclear-encoded COX IV is explained through the generation of stable, partial versions of complex IV in which COX IV interacts directly with COX Va and other nuclear-encoded subunits (Marusich *et al.*, 1997; Rahman *et al.*, 2000).

Although the precise nature of the translational defect associated with mt-tRNA mutations is incompletely understood, recent evidence suggests that, for the m.3243A>G mutation, dysfunctional translation results in the formation of polypeptides that are either truncated or incorporate incorrect amino acid substitutions (Sasarman *et al.*, 2008). These mutant proteins may however be subject to rapid degradation as other groups have suggested, using mass spectrometric analysis of homoplasmic m.3243A>G cybrid cells, that complex IV subunits consisted only of wild-type polypeptides (Janssen *et al.*, 2007). Other studies have reported a tissue specific variability in the expression of respiratory complex subunits; a patient with 90% m.3243A>G heteroplasmy displayed an isolated complex I deficiency in skeletal muscle and cardiac tissue but significantly reduced expression of complexes I,III, and IV in frontal cortex (Fornuskova *et al.*, 2008). BN-PAGE was utilised to demonstrate that all complex IV assembly intermediates were found at significantly increased levels, suggesting that factors other than the availability of mtDNA-encoded complex IV subunits cause the reduction in functional complex IV holoenzyme.

Similarly, complexes I and III are known to form the stable core of the respirasome, and isolated complex I deficiency has been demonstrated to prevent super-complex formation, despite no effect on the expression of complex III (Schagger *et al.*, 2004). To differentiate between abnormal mtDNA-encoded subunit translation, tissue specific effects, and dysfunctional supercomplex formation would clearly require further studies of pathogenesis employing BN-PAGE technology in cardiac and other tissues but my results clearly support differences between complex IV expression and function in cardiac tissue in patients with mt-tRNA mutations.

3.5.4 Limitations

Although this study is the largest investigation of the frequency and nature of cardiac involvement performed to date in this patient group, it is clearly retrospective in nature and subject to the usual limitations of this methodology. Some data were incomplete, in particular assessment of heteroplasmy and cardiac investigations during life, and, although a single investigator reviewed all available echocardiographic images, there was considerable variability in the nature of original assessment. The cardiac tissue study, again by necessity, utilised a rare resource of stored post-mortem cardiac tissue and specifically excluded samples with a significant post-mortem delay but this is not an optimal tissue to investigate potential pathogenic mechanisms. This section of the study was relatively limited in sample size, and I recognise that my findings may not be generalisable to all patient with mt-tRNA point mutations. Finally I acknowledge that overlap between genotypic and cardiac phenotypic divisions of the group may have limited my ability to relate my tissue findings to clinical disease.

3.5.5 Conclusions

These studies have shown that cardiac involvement is common and varied in patients with mitochondrial disease, and that those harbouring mt-tRNA mutations and/or experiencing symptom onset at an early age appear at increased risk of cardiac involvement. Cardiomyopathy has a significant effect on survival from the time of symptom onset, and is therefore important to recognise and treat in all patients with mitochondrial disease. Furthermore, this study includes a comprehensive evaluation of respiratory complex deficiency in cardiac tissue from patients with m.3243A>G- and m.8344A>G-related mitochondrial disease. I have shown that complex I deficiency is a

near-universal finding in cardiomyocytes displaying any degree of COX-deficiency, and present also in some cells *without* evidence of COX deficiency, supporting loss of complex I as the primary event of cardiac disease in patients with mt-tRNA mutations, particularly m.3243A>G. Profound complex I deficiency in these patients may at least partially explain the difference in the incidence of cardiomyopathy in patients with m.3243A>G mutation, compared to those with the m.8344A>G mutation. COX deficiency however appears less important and is not always accompanied by complex IV deficiency in patients harbouring m.3243A>G or m.8344A>G mutations suggesting alternative pathogenic mechanisms in the aetiology of the defects associated with mt-tRNA mutations. While tissue specific segregation may play a role in the frequency and severity of cardiac involvement, skeletal muscle mutation level does not appear to be an appropriate marker of risk, with potential impact on patient screening strategies.

Chapter 4.

Mitochondrial respiratory chain disease in children undergoing cardiac transplantation: prospective cohort study

4.1 Introduction

4.1.1 Cardiomyopathy in children

Congestive heart failure due to cardiomyopathy is a leading cause of morbidity and early mortality in children and adults worldwide (Schocken *et al.*, 1992; O'Connell and Bristow, 1994). Clinical epidemiological studies have revealed that cardiomyopathy is less common in children than adults, where coronary artery disease accounts for 40-50% of cases of heart failure (O'Connell and Bristow, 1994), but displays greater aetiological diversity (Schocken *et al.*, 1992; Andrews *et al.*, 2008). In the UK and Ireland, the incidence of new onset heart failure in children <16 years of age, in the absence of congenital heart disease, is 0.87/100,000 population (Andrews *et al.*, 2008). The majority of children with heart failure (82%) present with New York Heart Association (NYHA) class III to IV symptoms, and a significant minority of patients (34%) die or undergo cardiac transplantation for end-stage disease within 1 year.

Cardiomyopathy in children can be caused by a variety of congenital, metabolic, or infective factors. However, despite extensive investigation of children with end-stage cardiomyopathy including metabolic screening, the underlying cause often remains unknown. A recent epidemiological survey in the UK and Ireland revealed the cause of new-onset heart failure in 104 children: idiopathic dilated cardiomyopathy (48%), probably myocarditis (22%), persistent arrhythmia (7%), anthracycline toxicity (5%), metabolic disease (4%) including 2 patients with mitochondrial disease, left ventricular non-compaction cardiomyopathy (3%), Duchenne muscular dystrophy (2%), restrictive cardiomyopathy (1%), and vitamin D deficiency (1%) (Andrews *et al.*, 2008). While traditional factors such as age at presentation and the extent of left ventricular systolic dysfunction were shown in this study to have an impact on clinical outcome, these findings have not been consistent (Chen *et al.*, 1990), and the impact of aetiology of cardiomyopathy on mortality remains controversial. Nonetheless screening tests are usually performed in children with end-stage cardiomyopathy, in an attempt to identify an underlying diagnosis that may alter management (Bonnet *et al.*, 1998). The maximum sensitivity for the diagnosis of metabolic disease is undoubtedly achieved with inclusion of invasive test including muscle and/or endomyocardial biopsy (EMB) but routine use of such investigations remains controversial. Importantly, the diagnosis of metabolic disease, with associated potential for life-limiting multisystem

involvement, is often regarded as a contraindication to consideration of cardiac transplantation in children, although robust data to support this viewpoint are lacking (Bonnet *et al.*, 2001).

4.1.2 Mitochondrial respiratory chain disease in children

Mitochondrial disease in children can cause single organ involvement, or more commonly multisystem disease. Cardiac involvement in present in 17-40% children with mitochondrial disease, and is most frequently reported as a hypertrophic phenotype (Guenthard *et al.*, 1995; Holmgren *et al.*, 2003; Scaglia *et al.*, 2004), although other morphological patterns are reported, including dilated cardiomyopathy (Santorelli *et al.*, 1999; Hirano *et al.*, 2001; Santorelli *et al.*, 2001). When present, cardiac involvement in mitochondrial disease is often severe with significant mortality. In a study of 101 children with mitochondrial disease (Holmgren *et al.*, 2003), the mortality rate in patients with cardiomyopathy was 71% compared to 26% in those without cardiomyopathy (p=0.001, mean follow up duration 5.6±4.3 years), and this result has been confirmed by other groups (Scaglia *et al.*, 2004).

Mitochondrial respiratory chain disease may mimic many other conditions, and the absence of a reliable non-invasive biomarker further complicates this situation, such that formal diagnosis remains a challenge even for experienced clinicians and laboratory teams. Currently, although molecular genetic testing may expedite the diagnosis of mitochondrial disease in some patients, this is rarely the case in children, where recessive nuclear mutations dominate and, in many patients, analysis of invasive biopsy tissue remains a mainstay in the diagnostic process. However in the presence of mitochondrial disease with prominent or isolated cardiac involvement, this presents a particular challenge.

International guidelines support the use of invasive EMB in patients with possible mitochondrial cardiomyopathy (Leone *et al.*, 2011), yet the technique is associated with ~1% risk of serious complications and use in this clinical scenario remains limited in the UK (From *et al.*, 2011). Despite studies emphasizing the efficiency of invasive cardiac and indeed skeletal muscle biopsy in children with idiopathic dilated cardiomyopathy (Bonnet *et al.*, 1998), EMB is not commonly part of the metabolic screening employed in this population. There are no reliable non-invasive tests for

mitochondrial disease. Given the complexities of tissue diagnosis, recent guidelines support the use of opportunistic biopsy of tissues that are difficult to access and this is particularly applicable to children with end-stage cardiomyopathy, who may have underlying mitochondrial respiratory chain disease (Stone *et al.*, 2012). In this regard, the time between cardiac biopsy and sample freezing to enable timely assessment of enzyme activities needs to be minimised. Initial reports of deficiencies in the activities of enzyme complexes in the explanted hearts of patients with idiopathic dilated cardiomyopathy due to a variety of pathologies, including ischaemic heart disease, raised concerns that such findings represented a non-specific response due to protein degradation (Quigley *et al.*, 2000). However tissues in these early studies were often obtained from samples with significant post-mortem delays, and with further elucidation of the molecular basis underlying the histochemical and biochemical abnormalities in children with end-stage cardiomyopathy, this technique remains central to the diagnostic process.

4.1.3 Mechanical circulatory support and cardiac transplantation

Lower peri-operative and early post-operative mortality combined with improved long term management including more effective use and monitoring of immunosuppressive medications have led to increased survival in children undergoing cardiac transplantation (Irving *et al.*, 2011). Technological developments, especially with regard to mechanical circulatory support from VADs, have allowed more patients to be supported for longer on waiting lists, increasing the chance of survival to transplantation. Thus paediatric cardiac transplantation is increasingly used to support patients with end-stage cardiomyopathy, and although it varies with age, median graft survival is now 18 years in the children < 2 years of age and 11 years in patients 13-18 years of age (Kirk *et al.*, 2009).

The Freeman Hospital within the Newcastle upon Tyne Hospitals NHS Foundation Trust is a quaternary referral centre for paediatric cardiac transplantation, accepting patients with cardiomyopathy and congenital heart disease from across the UK. Over a 22-year period from March 1987 to March 2009, 189 orthotopic cardiac transplants were performed in 182 children < 18 years of age (Irving *et al.*, 2011). A variety of mechanical support devices are routinely employed including extra-corporeal membrane oxygenation (ECMO) and VADs. The Berlin Heart Excor device has been in

use since November 2005 and the Levitronix VAD since 2007; the surgical implantation of both devices permits access to the myocardium and skeletal muscle for biopsy. However the clinical screening of patients with unexplained cardiomyopathy did not include any formal assessment of mitochondrial respiratory chain disease and the frequency of this diagnosis in this patient cohort was unknown.

4.2 Aims

A prospective cohort study was undertaken in children <18 years of age undergoing cardiac transplantation and/or implantation of a VAD for end-stage cardiomyopathy at a single UK centre, to describe the frequency of mitochondrial respiratory chain disease. The aims were to identify clinical features that might suggest the diagnosis of mitochondrial disease, and, given the controversial use of cardiac transplantation in patients with metabolic disease (due to the risk of potentially life-limiting multisystem involvement), to determine whether the identification of mitochondrial respiratory chain disease in children with end-stage cardiomyopathy had any significant impact on clinical outcomes post-transplantation.

The hypothesis was that mitochondrial respiratory chain disease would be detected in a significant minority of children with end-stage cardiomyopathy, and that there would be significant differences in clinical outcomes between patients with and without underlying mitochondrial disease, dependent on the extent of multisystem involvement at diagnosis. It was suggested that knowledge of such factors and of the frequency of mitochondrial respiratory chain disease in this cohort of children could help improve the use of diagnostic testing and aid clinical decision-making.

4.3 Methods

4.3.1 Study design and participants

A prospective observational cohort study was conducted including all children < 18 years of age with end-stage cardiomyopathy undergoing cardiac transplantation and/or implantation of a VAD within the Newcastle upon Tyne Hospitals NHS Foundation Trust. From January 2009 to December 2010, left ventricular macrobiopsies were obtained from consecutive children with idiopathic or familial cardiomyopathy, during VAD implantation or cardiac transplantation, with or without

skeletal muscle biopsy. Exclusion criteria were congenital structural heart disease including hypoplastic left heart syndrome and Fallot's Tetralogy, and the presence of a pre-existing aetiological diagnosis for end-stage cardiomyopathy. The study had research ethics committee approval and all parents gave written informed consent.

4.3.2 Protocol

4.3.2.1 Screening investigations

All children underwent detailed clinical and echocardiographic assessment by an experienced transplantation team, including a paediatric cardiologist, in addition to a routine panel of screening investigations to identify possible causative factors for the presentation end-stage cardiomyopathy (Table 4.1).

	Blood analyses	**Urinary analyses**
Routine	Full blood count	Amino acids
	Urea and electrolytes	Organic acids
	Liver function	
	Thyroid function	
	Oligosaccharides	
	Amino acids	
Optional	Viral serology	
	Acylcarnitines / carnitine	
	Intermediates metabolites	
	Non-esterified fatty acids	

Table 4.1 Screening investigations for end-stage cardiomyopathy in children.

4.3.2.2 Tissue samples

Matched macrobiopsies of left ventricular cardiac tissue and skeletal muscle were obtained from open surgical biopsy at the time of VAD implantation or cardiac transplantation and were then analysed for the presence of mitochondrial disease using histochemical and spectrophotometric studies. Tissue samples from all patients with evidence of mitochondrial disease were investigated using sequential molecular biological techniques to identify known or novel pathogenic abnormalities.

4.3.2.2.1 Tissue preparation

A 24-hour service for processing explanted cardiac tissue was established to ensure a delay of <60 minutes from biopsy to freezing and all tissues samples were mounted on filter paper and snap-frozen as previously described (see Section 2.2.2). Tissue sections of 10μm were obtained at -19°C using a cryostat with the blade set at -21°C.

4.3.2.2.2 Histochemistry

Separate stains for the activities of COX and SDH were performed with sequential use of COX / SDH histochemistry to optimise identification of cells with histochemical evidence of respiratory chain abnormalities as described (see Section 2.2.3.2).

4.3.2.2.3 Spectrophotometry

Spectrophotometric analysis of respiratory chain enzyme activities were performed on all biopsies, according to standard protocols (Kirby *et al.*, 2007).

4.3.2.2.4 Molecular biology

No additional molecular biological investigations were performed on any tissue samples from patients *without* evidence of respiratory chain abnormalities. In patients *with* histochemical or spectrophotometric evidence of respiratory chain abnormalities, sequential, focused molecular biological investigations were performed. Clinical experience was used to inform the cascade of investigations. In the presence of any evidence of respiratory chain disease, known pathogenic mutations of mtDNA commonly associated with cardiac involvement were assessed, prior to full sequencing of the mitochondrial genome and comparison to standard databases. Finally in patients with specific patterns of deficiency of isolated / combined respiratory chain complex activities, nuclear genes associated with these patterns were assessed in individual patients.

4.3.2.3 Clinical outcomes

All patients were followed up by routine clinical assessments to 12 months with all-cause mortality as the primary outcome. Secondary outcome measures included growth and quality of life, assessed by standardised 24-domain patient / parent questionnaires, designed to capture quality of life across specific categories.

4.3.2.4 Statistical analysis

All data are presented as means ± SD for continuous data and as percentages and numbers for categorical data, unless stated otherwise. Given the small sample sizes involved, group comparisons for continuous data were made using Mann-Whitney U tests, categorical variables were compared using Fisher's exact test, and log rank analysis was used to assess survival. Statistical significance was assumed at $p<0.05$.

4.4 Results

4.4.1 Study participants

Twenty-four children (14 female) underwent cardiac transplantation for cardiomyopathy (CM) of unknown cause with (n=13) or without (n=11) preceding VAD implantation, with one (female) undergoing VAD implantation only due to recovery of myocardial function (25 patients, median age 5.4 years, range 0.3-15.7).

Individual patient characteristics (Table 4.2), and a summary of the cardiomyopathic morphological presentations (Figure 4.1) are provided below. Sixteen patients (64%) had dilated cardiomyopathy (DCM), five (20%) had hypertrophic CM (HCM), three (12%) had restrictive CM, and one (4%) had CM secondary to persistent arrhythmias.

Myocardial macrobiopsies were available from 96% (24/25) patients with contemporaneous skeletal muscle biopsies available in five patients.

4.4.2 Quality of life

Quality of life assessments were completed post-transplantation by the families of four patients with mitochondrial disease (80%) and ten patients without mitochondrial disease (50%). There were no significant differences in scores (Table 4.3).

No.	Age	Cardiac disease	Additional feature(s)	Myocardial biopsy		Skeletal muscle biopsy	
				Histo	Bio	Histo	Bio
1	5y, 4m	DCM	Delayed speech Skeletal myopathy Growth failure	Normal	Normal	N/A	N/A
2	12y, 1m	RCM	-	Normal	Normal	N/A	N/A
3	11y, 1m	DCM	Diabetes mellitus Hypothyroidism	Normal	Normal	N/A	N/A
4	1y, 0m	DCM	-	Normal	Normal	N/A	N/A
5	6y, 2m	DCM	-	N/A	N/A	N/A	N/A
6	8y, 11m	DCM	Left MCA infarct	Normal	Low I	N/A	N/A
7	0y, 7m	DCM	Consanguinity	Normal	Low I	Normal	Low I
8	7y, 0m	DCM	FH dilated CM	Normal	Normal	Normal	Normal
9	0y, 4m	Tachy	-	Normal	Normal	N/A	N/A
10	0y, 7m	HCM	-	80% COX def	Low I and IV	Normal	Normal
11	15y, 7m	DCM	FH dilated CM	Normal	Normal	Normal	Normal
12	15y, 3m	RCM	-	Normal	Normal	N/A	N/A
13	0y, 3m	DCM	-	Normal	Normal	N/A	N/A
14	4y, 0m	DCM	-	Normal	Normal	N/A	N/A
15	14y, 1m	RCM	Immunodeficiency	Normal	Normal	N/A	N/A
16	0y, 11m	DCM	-	Normal	Normal	N/A	N/A
17	0y, 3m	DCM	Right MCA infarct	Normal	Normal	N/A	N/A
18	3y, 2m	DCM	-	Normal	Normal	N/A	N/A
19	13y, 8m	HCM	FH mito disease Bilateral cataracts Skeletal myopathy Multiple APs Autism	30% COX def	Low I and IV	N/A	N/A
20	1y, 9m	HCM	-	Normal	Normal	N/A	N/A
21	0y, 10m	DCM	-	Normal	Normal	Normal	Normal
22	15y, 2m	DCM	-	Normal	Normal	N/A	N/A
23	0y, 6m	HCM	-	Normal	Normal	N/A	N/A
24	14y, 7m	DCM	FH metabolic dis Consanguinity	Normal	Low I	N/A	N/A
25	12y, 7m	HCM	AV block	Normal	Normal	N/A	N/A

Table 4.2 Clinical features and biopsy results of individual children. No. = patient number; Histo = histochemistry; Bio = biochemistry; DCM = dilated cardiomyopathy; RCM = restrictive cardiomyopathy; N/A = not available; MCA = middle cerebral artery; FH = family history; CM = cardiomyopathy; Tachy = tachymyopathy; HCM = hypertrophic cardiomyopathy; mito disease = mitochondrial disease; APs = accessory pathways; metabolic dis = metabolic disease; AV = atrio-ventricular; y = years; m = months.

Component	**Patients with mitochondrial disease**	**Patients without mitochondrial disease**	***p* value**
Mobility	72 ± 10	72 ± 7	0.891
Sensory	90 ± 4	88 ± 3	0.799
Energy	52 ± 4	57 ± 7	0.213
Behaviour	83 ± 13	71 ± 11	0.081
Social	71 ± 7	73 ± 5	0.553
TOTAL	**80 ± 8**	**77 ± 8**	**0.719**

Table 4.2 Quality of life in patients with and without mitochondrial disease. Post-transplantation quality of life score are compared between patients with and without evidence of mitochondrial respiratory chain disease.

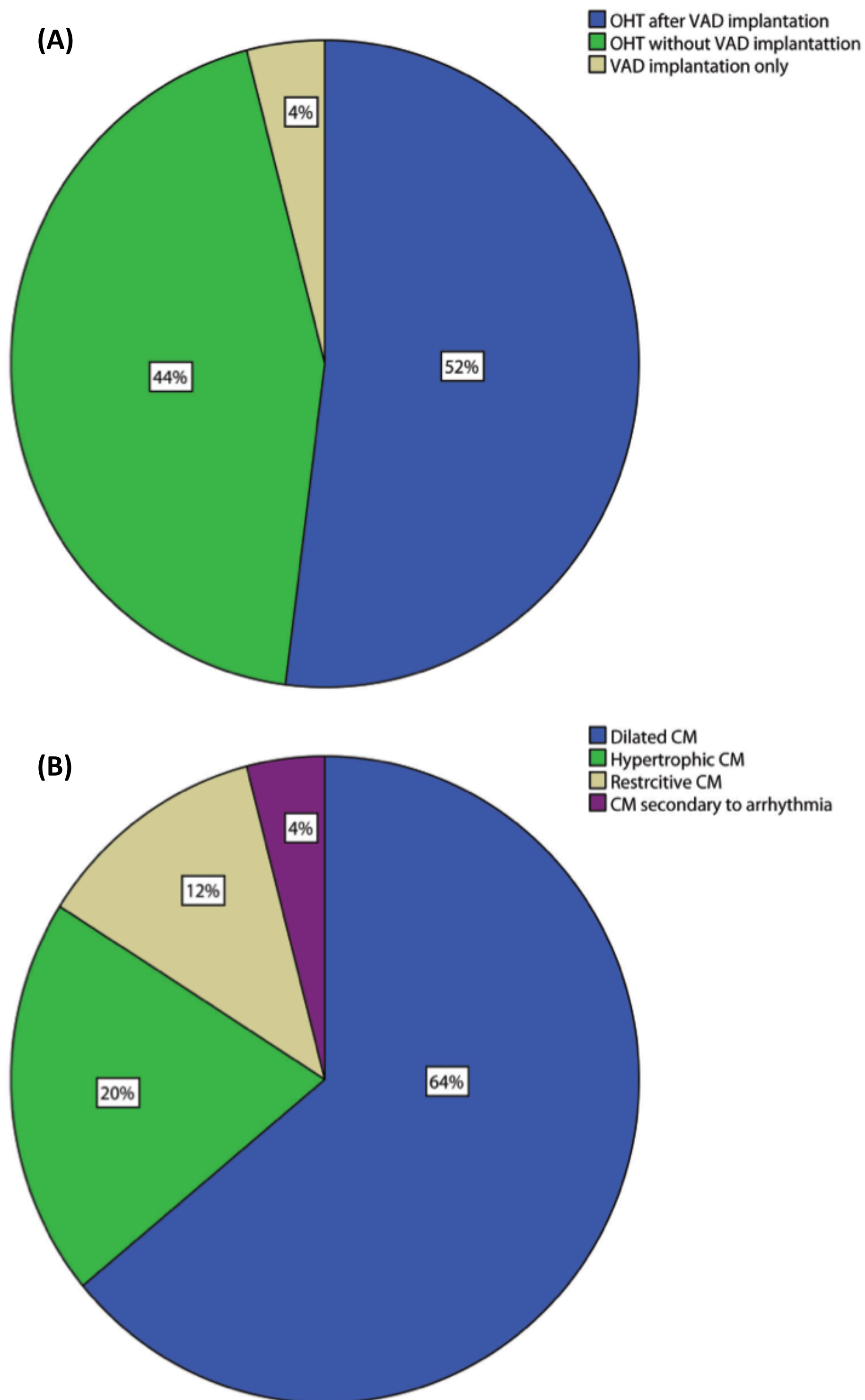


Figure 4.1 Requirements for support and cardiac morphology. (A) The majority of children (13 patients) underwent VAD implantation prior to transplantation, but several underwent transplantation directly (11 patients), with one patient requiring temporary VAD support only. (B) Children were commonly referred with dilated cardiomyopathy, although other presentations were seen. OHT = orthotopic heart transplantation; VAD = ventricular assist device; CM = cardiomyopathy.

4.4.3 Tissue analysis

4.4.3.1 Histochemistry and biochemistry

Histochemical and biochemical analyses of respiratory chain enzyme activities in cardiac muscle were abnormal in 21% (5/24) patients, including three with DCM and two with HCM. A mosaic pattern of COX-deficiency was apparent in the myocardium of two patients, quantified as 30% and 80% (Figure 4.2), respectively. Activities of enzyme complexes I and IV were abnormal in myocardial homogenates from both these patients, while isolated complex I deficiency was detected in three further patients who had normal histochemistry.

Paired skeletal muscle biopsies were available in 40% (2/5) patients who displayed evidence of a mitochondrial respiratory chain defect in cardiac muscle. Complex I activity was reduced in both skeletal and cardiac muscle homogenates in one patient, while a combined complex I and IV defect was restricted to cardiac muscle in a second patient.

4.4.3.2 Molecular biology

Pathogenic mtDNA mutations were excluded in all five patients who displayed evidence of abnormal respiratory chain enzyme activities, as were mutations in selected, nuclear-encoded genes including structural and assembly cofactor complex I genes and *ACAD9* in the three patients with isolated complex I deficiency. Recessive mutations in nuclear genes were assumed in all five patients. Recent exome sequencing has revealed a candidate gene in patient 10 and heterozygosity for two recessive *AGK* mutations, c.2972T>C and c.627C>T (p.Arg210X) in patient 19 (see section 4.4.4.4).

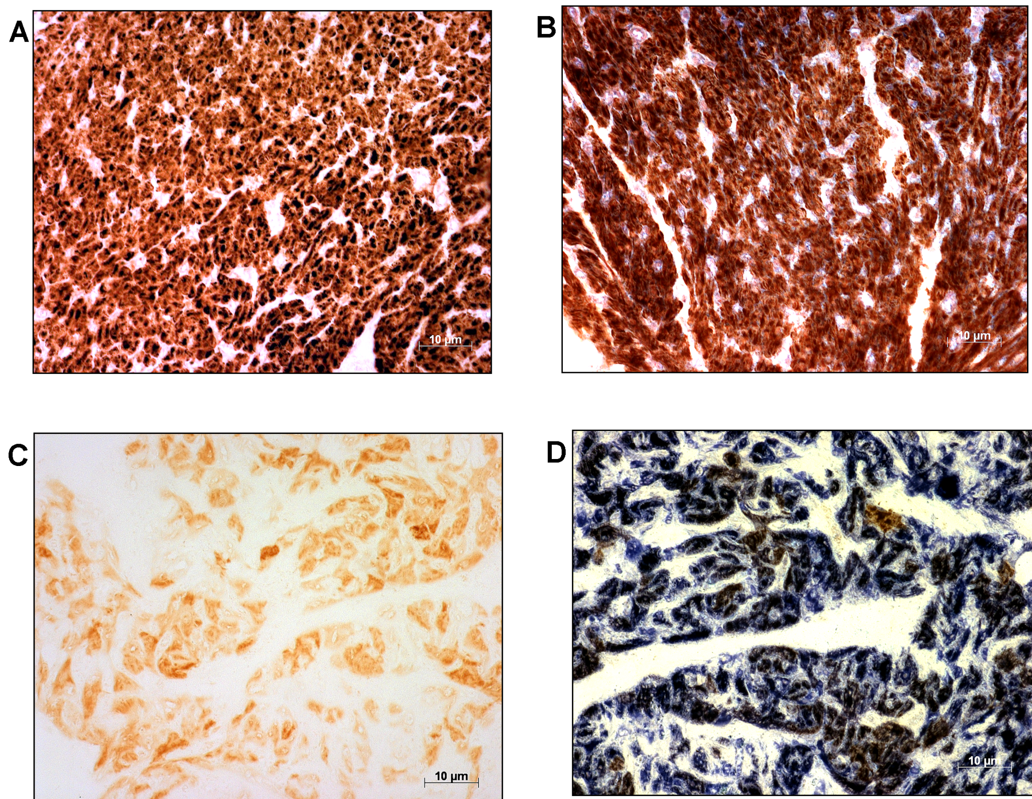


Figure 4.2 Mitochondrial histochemical analysis. Left ventricular samples from patient 1 without evidence of mitochondrial disease (A) reacted for cytochrome *c* oxidase (COX) activity, and (B) dual-reacted for COX and succinate dehydrogenase (SDH) activities, both showing normal staining patterns. Left ventricular samples from patient 10 with histochemical evidence of 80% COX-deficiency (C) reacted for COX activity alone, and (D) sequential COX and SDH activities, highlighting COX-deficient (blue) cells.

4.4.4 Clinical outcomes

Early post-transplant survival was not significantly different in those patients with mitochondrial respiratory chain defects compared to those without such defects with one early death in each group (Figure 4.3, *p*=0.27, median follow-up 551 days, range 71-970). Patient 10, with a mitochondrial respiratory chain defect identified in cardiac, but not skeletal, muscle died from multi-organ failure 2 months post-transplant. The four remaining patients with mitochondrial disease (patients 6, 7, 19 and 24) followed a normal post-transplant course with no clinical evidence of multi-system involvement, despite the presence of a skeletal muscle complex I defect in one patient at the time of transplantation (patient 7).

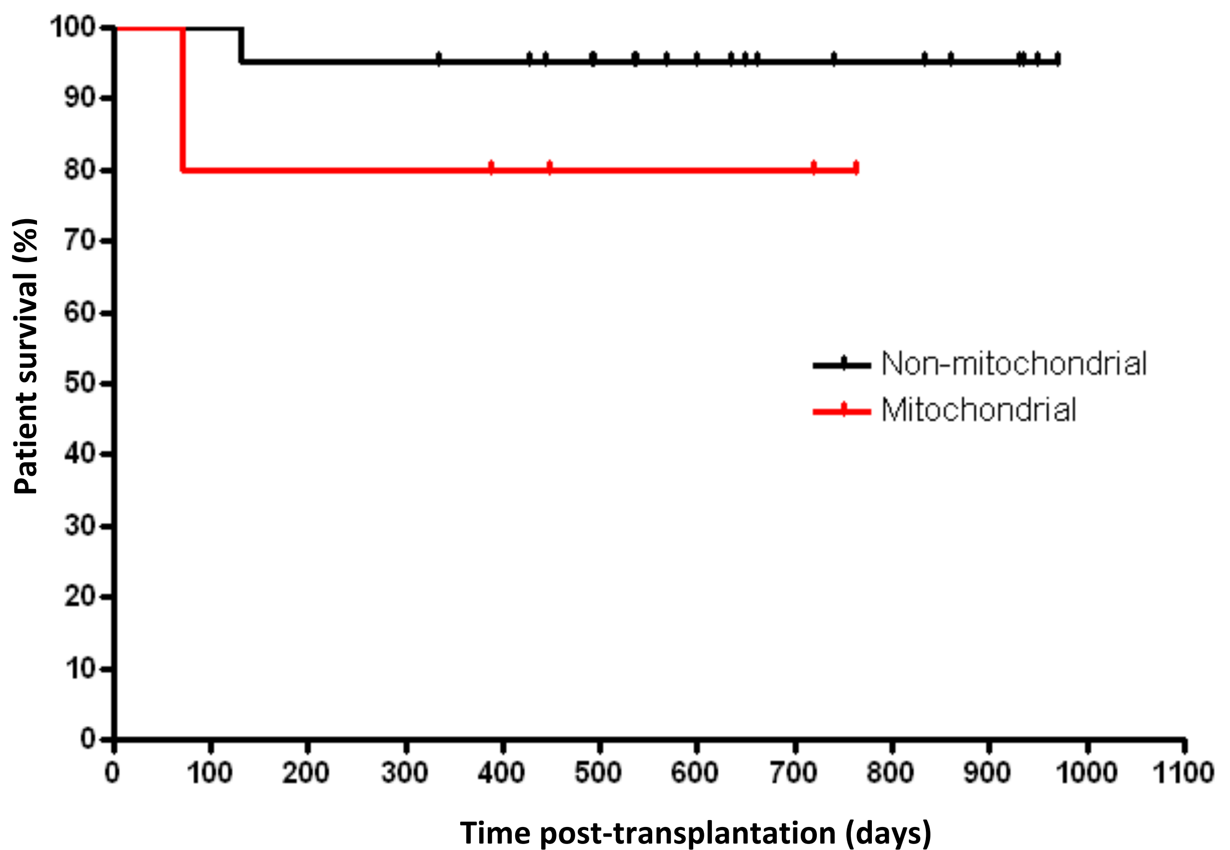


Figure 4.3 Patient survival post-transplantation. There was no significant difference in patient survival between children *with* and *without* evidence of mitochondrial respiratory chain enzyme defects (log rank analysis *p*=0.27).

Of the five patients with mitochondrial disease, four presented acutely in heart failure without prior cardiac diagnosis (80%), compared to 40% patients (5/20) in children without mitochondrial disease. None of these four patients displayed any additional clinical evidence of mitochondrial, or other metabolic multisystem, disease. The only patient with known cardiomyopathy (patient 19) already had a family history of mitochondrial disease, and a strong clinical suspicion of mitochondrial disease as a cause of cardiomyopathy. The diagnosis of metabolic disease more generally was considered on the basis of family history, prior to myocardial tissue analysis, in two further patients (patients 7 and 24), who were the children of consanguineous parents.

4.4.4.1 Patient 6

Patient 6, an 8 year old girl, born to non-consanguineous parents, had no significant antenatal or postnatal history, except for a previous episode of ophthalmic myositis with complete recovery. She presented acutely with a few weeks history leading to cardiogenic shock due to a dilated cardiomyopathy. She required respiratory and ionotropic support and was transferred for transplant assessment. A suitable cardiac donor became available and she was successfully transplanted but had developed a dense right hemiparesis due to a left middle cerebral artery occlusion pre-transplantation and required a contemporaneous left popliteal artery embolectomy at transplantation.

A screen for metabolic diseases was negative. Histochemical analysis of cardiac muscle was unremarkable however further biochemical analysis revealed a significant, isolated mitochondrial respiratory chain defect involving complex I. No paired skeletal muscle sample was obtained. No pathogenic mutation was identified in mtDNA, or in selected, nuclear-encoded genes including *ACAD9* and structural and assembly cofactor complex I genes. A recessive nuclear genetic defect is presumed, although not yet identified. She remained well at 12 month follow up and is developing appropriately for her age with no neurological concerns following her transplantation.

4.4.4.2 Patient 7

Patient 7, a girl born to consanguineous Asian parents (first cousins), had no significant antenatal or postnatal history. She presented acutely at 7 months of age with a short history of poor feeding and respiratory distress. Investigations revealed a dilated

cardiomyopathy with pulmonary oedema. She required respiratory and ionotropic support for cardiogenic shock but continued to deteriorate and was transferred for VAD implantation. A suitable cardiac donor became available and she was successfully transplanted 56 days after VAD insertion. A screen for metabolic diseases was negative, although a lactic acidosis was noted during her acute deterioration. Histochemical analysis of paired cardiac and skeletal muscle biopsies was unremarkable however further biochemical analysis revealed a significant, isolated mitochondrial respiratory chain defect involving complex I in both cardiac and skeletal muscle. No pathogenic mutation was identified in mtDNA, or in selected, nuclear-encoded genes including *ACAD9* and structural and assembly cofactor complex I genes. A recessive nuclear genetic defect is presumed, although not yet identified.

She suffered an episode of acute rejection (donor:recipient single HLA mismatch) that was successfully managed with altered immunomodulation. She remained well at 12 month follow up and is developing appropriately for her age with no neurological concerns following her transplantation.

4.4.4.3 Patient 10

Patient 10, a 7 month old boy, born to non-consanguineous parents after an uneventful pregnancy, had no significant medical history. He presented acutely with a hypertrophic cardiomyopathy and deteriorated rapidly, requiring biventricular assist device implantation within a few weeks. Metabolic screen was negative and other investigations revealed no significant distant organ involvement. He underwent successful cardiac transplantation with 4 months of presentation but initial post-transplantation course was not straightforward. He developed severe viral sepsis rapidly leading to multi-organ failure despite maximal support on the intensive care unit, including ionotropes, high frequency ventilation, and the use of nitric oxide and surfactant. Despite this support, he continued to deteriorate inexorably and died with institution of a palliative pathway 2 months post-transplantation.

The cardiac biopsy at VAD implantation had shown 80% COX deficiency with profound reduction in the activities of enzyme complexes I and IV on biochemical analysis. Mitochondrial disease was not considered likely prior to this unexpected result. The defect was restricted to cardiac muscle, with no histochemical or biochemical evidence

of skeletal muscle involvement. No pathogenic mtDNA mutation was identified and a recessive nuclear genetic defect is presumed, with exome sequencing revealing *AARS2,* encoding an alanyl-tRNA synthetase, as a candidate gene. Family studies are awaited.

4.4.4.4 Patient 19

Patient 19, a boy and the second child of non-consanguineous parents, was born at 29 weeks gestation by Caesarean section for maternal pregnancy induced hypertension. His older brother had died suddenly at the age of 7 years: he had had congenital cataracts, failure to thrive and developmental delay and severe left ventricular hypertrophy was noted at post-mortem. Respiratory chain enzymes activities from the brother's skin and skeletal muscle biopsies were normal. Cardiac biopsy however showed deficiencies in the activities of enzyme complexes I and IV. There were no pathogenic changes consistent with Barth Syndrome and sequencing of the mitochondrial genome did not reveal any pathogenic changes. An additional female pregnancy had resulted in intra-uterine death at 32 weeks gestation with left ventricular hypertrophy noted on antenatal ultrasound scan. Given the family history of established mitochondrial disease affecting siblings, this diagnosis was strongly suspected in patient 19, although no diagnostic biopsies had been performed.

Patient 19 had suffered acute intestinal perforation at two days of age, and was noted to have neonatal neutropaenia. He developed necrotizing enterocolitis at two months of age, which was medically managed. Screening for retinopathy of prematurity was negative at seven weeks of age, but he had developed cataracts by age four months. Echocardiography was normal in the neonatal period but left ventricular hypertrophy was noted at one year of age. By the age of four years, he severe biventricular hypertrophy and evidence of ventricular pre-excitation on electrocardiography, and a mild proximal myopathy. He underwent successful radio-frequency ablation of a manifest left lateral accessory pathway, with short anterograde refractory period, aged four years, and further ablation of a concealed left posteroseptal accessory pathway aged seven years, following re-presentation with atrio-ventricular re-entry tachycardia. He remained medically stable for several years with learning difficulties and autistic features, prior to rapid development of severe left ventricular systolic impairment at the age of 13 years, requiring VAD implantation and subsequent cardiac

transplantation eight months later, following an acute deterioration precipitated by sepsis. Post-transplantation course was initially eventful, complicated by coagulopathy, peri-operative atrial fibrillation, focal seizures secondary to a small cerebral infarction, and acute kidney injury requiring haemofiltration and then intermittent haemodialysis. He remained well at 12 months follow up.

Sengers' syndrome had already been considered in this family with a suggestive, although not typical, presentation involving cardiomyopathy, congenital cataracts and skeletal myopathy (Sengers *et al.*, 1975). Contemporaneous with the identification of causative mutations in Sengers' syndrome within *AGK*, the gene encoding mitochondrial acylglycerol kinase, patient 19 was confirmed to be heterozygous for two recessive *AGK* mutations, c.2972T>C and c.627C>T (p.Arg210X).

4.4.4.5 Patient 24

Patient 24, a 15 year old boy and fourth child of consanguineous Asian parents (first cousins), was born at term via vaginal delivery. An older sister (21 years) was well, but he had two sisters (20 and 13 years) both with evidence of methylmalonic acidaemia, delayed speech and impaired vision, and there has also been an additional male pregnancy in the family that had resulted in intra-uterine death at 34 weeks gestation and two further early miscarriages.

Patient 24 had avoided physical exercise as a younger child, and had been slow to talk although this was felt to be within the limits of normality by his bilingual parents. He developed palpitations and was found to have a dilated cardiomyopathy, with rapid deterioration resulting in referral for consideration of cardiac transplantation. A suitable cardiac donor rapidly became available and he was successfully transplanted 32 days after referral. A screen for metabolic diseases, including lactate, was negative. Although a paired skeletal muscle biopsy sample was not available, cardiac muscle biochemistry showed an isolated enzyme complex I defect with normal histochemistry. No pathogenic mtDNA mutation was identified and there were no mutations in selected, nuclear-encoded genes including *ACAD9* and structural and assembly cofactor complex I genes. Given the sibling history *SUCLA2* and *SUCLG1* gene mutations, that can cause mitochondrial disease with mtDNA depletion, were also

sequenced but no pathogenic mutation was found. Post-transplantation course was uneventful and he remains well at 12 months follow up.

4.5 Discussion

The principal findings of this study of mitochondrial respiratory chain disease in children with cardiomyopathy of unknown cause undergoing cardiac transplantation and/or VAD implantation are: 1) mitochondrial disease is common in children with idiopathic or familial cardiomyopathy undergoing cardiac transplantation or VAD implantation; 2) patients with mitochondrial disease can present acutely in heart failure and the majority have neither a previous history of cardiac disease, nor clear evidence of multisystem disease; 3) routine investigation of myocardial tissue for respiratory chain defects should be considered in all such children undergoing transplantation or VAD implantation; 4) combined histochemical and biochemical analysis of respiratory chain enzyme activities is essential to identify all patients with mitochondrial respiratory chain disease in this cohort; 5) there appears to no significant impact of mitochondrial disease on early post-transplantation survival, or quality of life, however there are important implications for subsequent management of patients and families, including molecular biological screening.

4.5.1 Patient cohort

This study was the first prospective examination of the nature and frequency of mitochondrial respiratory chain disease in children with cardiomyopathy of unknown cause undergoing cardiac transplantation and/or VAD implantation. Although a relatively small sample size due to the single centre time-limited nature of the study, the age range, gender distribution, and cardiac morphological presentation were consistent with contemporary, national and international databases of paediatric cardiac transplantation (Irving *et al.*, 2011; Dipchand *et al.*, 2013). Critically the ages of presentation of the patients in my cohort with evidence of mitochondrial respiratory chain disease span the age range of the paediatric population (ages 7 months – 15 years) and 80% (4/5 patients) had no preceding evidence of cardiac disease or clinical multi-system involvement, emphasising the need for a high index of clinical suspicion and/or the use of routine screening practices to make a diagnosis of mitochondrial disease in this population.

Previous studies in populations of children with mitochondrial disease have suggested that a hypertrophic phenotype is the commonest presentation of cardiac involvement (Holmgren *et al.*, 2003; Scaglia *et al.*, 2004), and indeed the most frequent presentation in all forms of cardiomyopathy due to inborn errors of metabolism. However the distribution of cardiac morphologies in these patients in this study, analysing a cohort of children with end-stage cardiomyopathy of *unknown* aetiology, is more consistent with the general population of children undergoing cardiac transplantation and/or VAD implantation both locally (Irving *et al.*, 2011), and internationally (Dipchand *et al.*, 2013). This likely reflects both the relative weakness of putative genotype:phenotype relationships in children with respect to cardiac involvement , and the end-stage nature of the cardiomyopathy in this study. Notably idiopathic dilated cardiomyopathy is the commonest cause of clinical heart failure and the commonest indication for cardiac transplantation in all international databases, suggesting that a significant number of patients may have undiagnosed underlying evidence of mitochondrial respiratory chain disease. Investigations for organ-specific respiratory chain defects have not featured in previous studies to identify an aetiology in children with idiopathic DCM (Towbin *et al.*, 2006). Explanted cardiac tissue at the time of VAD implantation or transplantation represents an ideal opportunity for improved diagnosis of mitochondrial disease (Stone *et al.*, 2012). Although International guidelines support consideration of EMB in the investigation of possible mtDNA-related cardiomyopathy (Leone *et al.*, 2011), the complications of routine EMB in the broader cohort of children with heart failure are often felt to outweigh any diagnostic advantage.

4.5.2 Clinical characteristics

Although small and not specifically designed to identify predictors of mitochondrial disease in children with end-stage cardiomyopathy, this study inevitable provides an opportunity to reflect on the clinical characteristics of patients that did and did not have evidence of a respiratory chain defect. Interestingly this clinical approach was challenged by recognition that mitochondrial disease was not considered in differential diagnosis, prior to examination of cardiac biopsy tissue, in 40% patients (2/5). In fact mitochondrial disease was considered very likely in only one patient with a positive family history of documented mitochondrial disease and a similar clinical presentation.

In two other families, mitochondrial disease was including in the differential diagnosis only as part of the broader categories of metabolic disease and/or recessive nuclear gene mutations, due to the consanguineous nature of the parental relationships. This was in fact the *only* feature present exclusively in patients with objective evidence of mitochondrial disease in this study and should raise a high index of clinical suspicion even in the absence of other suggestive features. A clinical history of skeletal myopathy, endocrine organ involvement, familial (dilated) cardiomyopathy, or cardiac conduction disease were unhelpful in differentiating patients as all these features, classically reported in patients with mitochondrial disease, were present in this study in patients without evidence of mitochondrial respiratory chain disease.

Four of the five patients (80%) with mitochondrial disease in this study presented acutely with no previous evidence of cardiac disease, and progressed rapidly to an end-stage requiring referral for organ support. This is a higher proportion than in those patients in this study who did not have evidence of mitochondrial disease (40%), and may reflect underlying abnormalities of myocardial bioenergetics in patients with mitochondrial disease, although clearly mechanistic studies would be required to investigate this issue further.

4.5.3 Tissue analysis

Although the clinical tissue handling protocol established in this study demonstrated a 96% success rate in obtaining myocardial biopsies from eligible patients, the completion of *paired* biopsy samples from skeletal muscle was poor. The reasons for this failure are unclear but may relate to a lack of cardiothoracic surgical involvement in the subsequent tissue analysis and investigations. There was no reported operative reason to justify omission of skeletal muscle biopsy on clinical grounds and this may merely reflect a lack of familiarity with a newly established investigational algorithm. Consistent with a previous study of metabolic screening in paediatric cardiomyopathy (Bonnet *et al.*, 1998), children with evidence of mitochondrial respiratory chain disease, who *did* undergo paired cardiac and skeletal muscle sampling in this study, showed that the defect was restricted to myocardial tissue in 50% (1/2 patients). While increasingly recognised that mitochondrial disease can cause isolated organ involvement (Taniike *et al.*, 1992; Santorelli *et al.*, 1999; Ware *et al.*, 2009), this isolated finding does support the use of opportunistic biopsies of myocardial tissues in

this population (Stone *et al.*, 2012), and may support wider use of diagnostic EMB in children in whom mitochondrial cardiomyopathy is considered (Leone *et al.*, 2011). The diagnosis is difficult in the absence of appropriate tissue. Interestingly the patient in whom there was least clinical suspicion of mitochondrial disease had the most significant evidence of complex I and IV deficiency, further emphasising the importance of universal application of screening and the lack of utility of clinical features to tailor investigations in this cohort.

Metabolic screening tests, including serum lactate, did not provide reliable identification of patients with mitochondrial disease in this cohort. This may reflect the fact that many patients in this study had evidence of cardiogenic shock and consequent tissue hypoperfusion with transient elevation of serum lactate alongside the confirmed lack of utility of this test as a discriminating feature in patients with mitochondrial disease (McFarland *et al.*, 2010). Novel biomarkers are being established that may help assist in the diagnosis of mitochondrial disease in patients with skeletal muscle involvement (Suomalainen *et al.*, 2011), and could prove particular useful in children where the use of open muscle biopsy requires general anaesthesia, but no biomarkers for mitochondrial cardiomyopathy current exist.

It is notable that despite histochemical and/or biochemical identification of respiratory chain disease in five patients in this cohort, successful establishment of a causative genetic mutation has to date only been achieved in one patient (20%), in whom there was already a strong clinical suspicion of an established mitochondrial syndrome and a preceding affected sibling, with an established biochemical diagnosis. This finding, in a paediatric population where recessive nuclear mutations are the commonest cause of mitochondrial disease, is not unexpected and reflects a time of flux in the molecular biological investigation of mitochondrial disease due to nuclear mutations. Next-generation exome sequencing techniques will lead to advances in the time taken to achieve a genetic diagnosis in such disorders (Calvo *et al.*, 2006), as has already been established in further patients in this study. These technological advances, however, remain reliant on preliminary investigation by alert clinicians, who are able to recognise the possibility of mitochondrial disease, and the participation of families in further molecular biological investigation.

4.5.4 Clinical outcomes

Cardiac transplantation was initially contra-indicated in metabolic diseases with ubiquitous enzyme defects and the potential for multisystem disease. However, early case series of the use of cardiac transplantation in patients with mitochondrial cardiomyopathy revealed no significant difference in clinical outcomes compared to children without mitochondrial disease (Adwani *et al.*, 1997; Bonnet *et al.*, 2001). This study is supportive of these findings, with importantly no significant differences in 12-month mortality, early post-transplantation clinical course, or any parameter of quality of life. Cardiac transplantation should be considered in patients with end-stage cardiomyopathy and evidence of mitochondrial respiratory chain disease with the expectation that outcomes will be dependent on complications of surgical intervention and the extent of clinically manifest mitochondrial disease. In this study the patient with contemporary evidence of skeletal muscle involvement in mitochondrial disease, has not developed any clinical myopathy and has had a normal post-transplantation course suggesting that histochemical or biochemical involvement of skeletal muscle should not preclude consideration of cardiac transplantation in this cohort (Bonnet *et al.*, 2001). Furthermore the absence of any significant difference in the mobility component of quality of life scores between patients with and without mitochondrial disease provides suggestive evidence that skeletal myopathy was not an important feature in these patients.

The post-transplantation death in a single patient with mitochondrial disease was explained by multi-organ failure due to overwhelming sepsis. On review of this case with all involved clinical and laboratory staff, no clear cause of this death that was felt to be specific to the underlying condition was identified. It was suggested that the severity of his mitochondrial respiratory chain defect (80% COX deficiency on initial cardiac biopsy) may have been a contributory factor but the 80% survival at 12 months that this represents, in this group, remains compatible with current international databases of paediatric cardiac transplantation (Dipchand *et al.*, 2013), and viral sepsis is a recognised complication of immunosuppressive therapy.

4.5.5 Limitations

Although this study is the largest prospective study performed in this patient group, it remains limited in sample size and, as an observational study, was not designed to investigate clinical or biochemical markers of mitochondrial respiratory chain disease. Except in one patient, in whom a molecular biological diagnosis was established, I cannot confirm the *causative* nature of identified mitochondrial respiratory chain enzyme defects in these children with end-stage cardiomyopathy, although clinical suspicion remains high in all reported patients. Paired skeletal muscle biopsies were not available in the majority of patients, due to study protocol non-adherence at the time of transplantation or VAD implantation. Finally, data regarding quality of life scores were not collected before transplantation as recruitment to the study occurred at the point of transplantation or VAD implantation, and not all families returned the self-completion questionnaires.

4.5.6 Conclusions

Mitochondrial respiratory chain disease is common in children with cardiomyopathy of unknown cause undergoing cardiac transplantation and/or VAD implantation. Routine histochemical and biochemical analysis of respiratory chain enzyme activities is achievable in clinical practice and can identify patients with unexpected, underlying mitochondrial respiratory chain disease, with important implications for the future management of individuals and families. Although parental consanguinity may be suggestive, the potential tissue-specific nature of mitochondrial disease, and lack of appropriate clinical biomarkers supports routine use of diagnostic testing in myocardial tissue, obtained opportunistically, in all children with idiopathic or familial cardiomyopathy undergoing cardiac transplantation or VAD implantation.

Chapter 5.

Cardiac structure and function in patients with m.3243A>G- and m.8344A>G-related mitochondrial disease without clinical cardiac involvement: cross-sectional study

5.1 Introduction

5.1.1 Mitochondrial tRNA mutations and cardiomyopathy

As previously discussed, cardiomyopathy is a frequent manifestation of mtDNA disease in both adults and children. Commonly presenting with a hypertrophic phenotype, cardiomyopathy occurs in 20-40% of patients carrying the specific m.3243A>G mutation (Hirano and Pavlakis, 1994; Anan *et al.*, 1995; Majamaa-Voltti *et al.*, 2002; Holmgren *et al.*, 2003; Vydt *et al.*, 2007), and is an independent predictor of morbidity and early mortality (Holmgren *et al.*, 2003; Majamaa-Voltti *et al.*, 2008); many patients die from cardiac arrhythmias or congestive heart failure (Majamaa-Voltti *et al.*, 2002). Cardiac involvement, in the form of cardiomyopathy with both a dilated and hypertrophic phenotype, has also been reported in patients with the m.8344A>G mutation (Limongelli *et al.*, 2010; Wahbi *et al.*, 2010).

5.1.2 Pathogenetic mechanisms

Although significant progress has been made in elucidation of the molecular basis of mitochondrial disease due to diverse mtDNA mutations, data remain scarce concerning the pathophysiological mechanisms underlying many common disease features, and this is particularly true with regard to mtDNA-related cardiomyopathy. Previous studies have used simple transthoracic echocardiography to document the frequency and nature of cardiomyopathic involvement in mtDNA disease (Majamaa-Voltti *et al.*, 2002; Wahbi *et al.*, 2010) but, while relatively straightforward to administer and widely available, this technique lacks the sensitivity to detect early manifestations of cardiac involvement. Moreover routine, clinical echocardiography can provide limited insights into myocardial tissue characterization, cardiac bioenergetics and abnormalities of myocardial deformation.

Accurate phenotypic characterization of the cardiac phenotype in patients with mtDNA disease is essential to enable progress of clinical care. Knowledge of the specific features and natural history of cardiac involvement in mtDNA disease will permit the development of tailored screening programmes; the initiation of robust clinical trails of therapies with relevant clinical endpoints; and the potential for establishment of novel genotype:phenotype relationships that may provide pathogenetic insights.

5.1.3 Screening strategies for cardiac involvement

Echocardiography and 12-lead ECG are recommended screening strategies as early identification of asymptomatic cardiac structural defects (stage B cardiomyopathy) allows initiation of cardioprotective therapies (Hunt *et al.*, 2009). In other neuromuscular diseases associated with cardiomyopathy, such interventions slow cardiac remodelling and reduce symptoms (Duboc *et al.*, 2005). Two-dimensional echocardiography has limited sensitivity to detect small changes in left ventricular (LV) mass, particularly in asymptomatic cases (Myerson *et al.*, 2002), and previous studies used normal reference ranges, rather than comparison with age- and gender-matched healthy controls, further decreasing sensitivity (Anan *et al.*, 1995; Holmgren *et al.*, 2003; Vydt *et al.*, 2007). MRI may reveal cardiac involvement in multisystem disease when standard evaluation is unremarkable (Yilmaz *et al.*, 2008), and may provide novel therapeutic targets, where the efficacy of early intervention remains to be determined (Pfeffer *et al.*, 2012).

5.1.4 Cardiac magnetic resonance imaging

MRI is the gold standard investigation of cardiac morphology and function. Cardiac tagging enables detection of early defects in myocardial deformation by analysis of circumferential strain and torsion (Lumens *et al.*, 2006). Torsion describes the twisting motion of the heart due to opposite rotation of base and apex, and maintains homogeneity of strain across the myocardial wall. The torsion to endocardial circumferential strain ratio (TSR), a sensitive marker of altered epicardial-endocardial interactions, is constant among healthy individuals of the same age but increases with normal ageing (Lumens *et al.*, 2006). Elevated torsion and/or TSR have been demonstrated in patients with LVH caused by increased hemodynamic loading (Van Der Toorn *et al.*, 2002), in hypertrophic cardiomyopathy (HCM) patients (Young *et al.*, 1994), and recently in HCM mutations carriers without hypertrophy, perhaps providing an early phenotypic marker (Russel *et al.*, 2011).

Phosphorus-31 (^{31}P) MRS permits evaluation of myocardial bioenergetics by calculation of the phosphocreatine (PCr)/ATP ratio (Crilley *et al.*, 2003). PCr/ATP ratio is reduced in systolic dysfunction and in HCM with normal systolic function (Neubauer *et al.*, 1992). Impaired cardiac bioenergetics occur in mutation carriers of both HCM (Crilley *et al.*,

2003), and mitochondrial disease (Lodi *et al.*, 2004), without echocardiographic evidence of hypertrophy, suggesting a potential role in early detection of disease.

5.2 Aims

Using the advanced modalities of cardiac magnetic resonance imaging and spectroscopy, I sought to characterize the cardiac phenotype in a clinically and genetically well-characterized cohort of patients with mtDNA disease with reference to age- and gender-matched healthy controls. Based on studies in patients with mitochondrial disease (Lodi *et al.*, 2004), and in HCM mutation carriers without hypertrophy (Crilley *et al.*, 2003; Russel *et al.*, 2011), my hypotheses were that abnormalities of left ventricular mechanics and bioenergetics would be detectable in patients carrying the m.3243A>G or the m.8344A>G mutation *without* known cardiac involvement, and that such abnormalities would be related to markers of disease burden. I provide a comprehensive MRI / ^{31}P MRS evaluation of cardiac changes in this population, with important implications for future screening and management strategies.

5.3 Methods

5.3.1 Participants

Twenty-two adult patients with mitochondrial disease due to either the m.3243A>G or the m.8344A>G mutation, but with no known cardiac involvement, were recruited from a mitochondrial disease specialist clinic. The absence of cardiac involvement was determined using screening strategies commonly employed in patients with mitochondrial disease, namely clinical history and examination with normal ECG, echocardiogram (documenting no significant valvular disease, maximum LV wall thickness ≤12mm, ejection fraction ≥55%, and LV end-diastolic dimension ≤32mm/m^2), and exercise stress ECG (documenting no symptoms or ECG changes suggestive of underlying coronary artery disease); patients were excluded using these criteria (n=3) and the presence of contra-indications to MRI (n=1; claustrophobia). All 22 patients were matched with respect to age and gender with healthy controls, with normal ECG and no history of cardiovascular or metabolic disease, recruited through advertisement. Institutional ethical approval and informed consent were obtained and the study complied with the Declaration of Helsinki.

5.3.2 Clinical assessment

Subjects underwent physical examination and electrocardiography by an experienced clinician. Disease burden was assessed using NMDAS, a validated scoring system (Schaefer *et al.*, 2006). Heteroplasmy level of the m.3243A>G mutation was determined in urinary epithelial cells (Whittaker *et al.*, 2009). The estimated glomerular filtration rate (eGFR) was calculated in all patients using the Modified Diet in Renal Disease (MDRD) equation, prior to administration of gadolinium.

5.3.3 Transthoracic echocardiography

Using a Vivid 7 ultrasound machine, transthoracic echocardiographic imaging was performed as previously described (see Section 2.2.5.1) in all participants.

5.3.4 Cardiac magnetic resonance imaging

Using a 3-Tesla scanner, cardiac magnetic resonance imaging was performed including (i) ^{31}P MRS, (ii) cine imaging, (iii) cardiac tagging, and (iv) late gadolinium enhancement (LGE) imaging as previously described (see Section 2.2.5.2) in all participants.

5.3.5 Statistical analysis

Statistical analysis was performed as previously described (see Section 2.2.6). Bonferroni adjustment for multiple comparisons was used for both group comparisons and correlations. The reliability of MRI measures of myocardial strains and diastolic function, and echocardiographic measures of systolic and diastolic function, were assessed with Bland-Altman analysis by comparing values derived from contours redrawn after one month, and by two independent observers, in four randomly selected patients and four controls.

5.4 <u>Results</u>

5.4.1 Patient population

Baseline characteristics of 22 patients (18 probands and 4 individual family members, unrelated to each other) and 22 control subjects, matched for age and gender, are presented in Table 5.1. Cardiovascular disease features and relevant medications are also included: nine patients had diabetes mellitus and one had treated hypertension. There were no significant differences in current blood pressures.

Patient details including specific clinical features, mutation loads and NMDAS scores are presented in Table 5.2. Disease burden was mild or moderate in most patients with clinical phenotypes consistent with MIDD (eight patients), myopathy (eight patients), MERRF (four patients), MELAS (one patient) and oligosymptomatic status (one patient). All patients were compared to controls as a group as exclusion of any single phenotypic group, including patients with MERRF, harbouring the m.8344A>G mutation, did not alter the presence and magnitude of differences. The frequencies of specific clinical features are summarized in Table 5.3. The eGFR was >60ml/min/1.73m^2 in all patients.

Characteristic	**Patients (n=22)**	**Controls (n=22)**	***p* value**
Age (years)	38.5 ± 14.1	38.2 ± 10.1	0.891
Male sex, n (%)	12 (55)	12 (55)	1.000
Height (cm)	168 ± 12	171 ± 12	0.761
Weight (kg)	66.7 ± 14.2	75.1 ± 16.4	0.293
Body mass index (kg/ m^2)	22.3 ± 5.1	25.6 ± 5.2	0.212
Body surface area (m^2)	1.72 ± 0.20	1.82 ± 0.18	0.198
Diabetes mellitus, n (%)	11 (50)	0 (0)	N/A
Hypertension, n (%)	5 (23)	0 (0)	N/A
Cardiac clinical parameters			
Sinus rhythm, n (%)	24 (100)	24 (100)	1.000
Heart rate (min^{-1})	77 ± 13	59 ± 9	<0.0001
SBP (mmHg)	116 ± 13	119 ± 12	0.807
DBP (mmHg)	76 ± 8	75 ± 9	0.665
Selected medications			
ACE inhibitor / ARB	9 (41)	0 (0)	N/A
Beta-blocker	2 (9)	0 (0)	N/A
Calcium channel blocker	2 (9)	0 (0)	N/A
Statin	8 (36)	0 (0)	N/A
Insulin	6 (27)	0 (0)	N/A

Table 5.1 Baseline characteristics. SBP = systolic blood pressure; DBP = diastolic blood pressure; ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker.

Patient	Age	Sex	mtDNA mutation	Urinary mutation load (%)	Phenotype	Clinical features	NMDAS score
1*	39	M	m.3243A>G	80	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, migraine, GIT, fatigue, hypothyroidism	17
2	58	F	m.3243A>G	59	Myopathic	SNHL, exercise intolerance, mild ataxia, proximal muscle weakness, GIT, low BMI, myalgia	12
3*	42	M	m.3243A>G	82	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, migraine, depression	12
4	47	M	m.3243A>G	63	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, proximal and distal muscle weakness, depression, fatigue, myalgia, PEO, ptosis, sensory neuropathy	28
5	37	F	m.3243A>G	48	MIDD	SNHL, diabetes mellitus, exercise intolerance, mild ataxia, mild dysarthria, asthma	10
6	38	F	m.3243A>G	53	Myopathic	SNHL, exercise intolerance, proximal muscle weakness, fatigue, migraine, GIT, asthma	13
7	22	M	m.3243A>G	89	Myopathic	SNHL, exercise intolerance, ataxia, fatigue, migraine, GIT, low BMI, epilepsy	18
8	36	M	m.3243A>G	80	Oligosymptomatic	Exercise intolerance, migraine	4
9	50	M	m.3243A>G	87	MELAS	SNHL, exercise intolerance, ataxia, proximal and distal muscle weakness, fatigue, depression, retinopathy, epilepsy, encephalopathy, cognitive decline, stroke-like episodes	23
10	55	F	m.3243A>G	68	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, proximal muscle weakness, GIT, depression, retinopathy, PEO, ptosis, short stature, mild dysphagia, hypertension	25

11	29	M	m.8344A>G	95	MERRF	SNHL, exercise intolerance, ataxia, GIT, fatigue, proximal and distal muscle weakness, epilepsy, retinopathy, low BMI, dysarthria, sensory neuropathy, myoclonus	55
12~	25	M	m.8344A>G	94	MERRF	Exercise intolerance, ataxia, fatigue, proximal and distal muscle weakness, epilepsy, depression, retinopathy, dysarthria, myoclonus	48
13^	50	F	m.3243A>G	34	Myopathic	Migraine, GIT, fatigue, myalgia, hypothyroidism	2
14^	58	M	m.3243A>G	66	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, GIT, proximal and distal muscle weakness, retinopathy, dysarthria, sensory neuropathy	28
15	53	F	m.3243A>G	22	Myopathic	SNHL, exercise intolerance, ataxia, GIT, proximal myopathy, retinopathy	16
16~	59	F	m.8344A>G	75	MERRF	SNHL, diabetes mellitus, proximal and distal muscle weakness, myoclonus, lipomata	19
17”	18	F	m.3243A>G	59	Myopathic	SNHL, exercise intolerance, proximal muscle weakness, retinopathy, short stature, low BMI	8
18”	42	F	m.3243A>G	43	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, GIT, depression, short stature	14
19	25	M	m.3243A>G	90	Myopathic	SNHL, exercise intolerance, ataxia, migraine, GIT, proximal and distal muscle weakness, depression, dysarthria, retinopathy, short stature	26
20	25	F	m.3243A>G	72	Myopathic	SNHL, exercise intolerance, ataxia, migraine, GIT, proximal muscle weakness, depression, dysarthria, retinopathy, short stature	27
21	28	M	m.8344A>G	93	MERRF	SNHL, exercise intolerance, ataxia, migraine, epilepsy, proximal muscle weakness, GIT, depression, low BMI, dysarthria, sensory neuropathy	35
22	55	M	m.3243A>G	76	MIDD	SNHL, diabetes mellitus, exercise intolerance, ataxia, proximal muscle weakness, depression	20

Table 5.2 Individual patient characteristics. mtDNA = mitochondrial DNA; NMDAS = Newcastle Mitochondrial Disease Adult Scale; MIDD = maternally inherited diabetes and deafness; MELAS = mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MERRF = myoclonic epilepsy with ragged red fibres; SNHL = sensori-neural hearing loss; GIT = gastro-intestinal tract involvement; BMI = body mass index; PEO = progressive external ophthalmoparesis; M = male; F = female; * = siblings; ~ = mother and son; ^ = siblings; “ = mother and daughter.

Clinical feature	Number of patients (n=22)	Frequency of patients (%)
Exercise intolerance	20	91
Sensori-neural hearing loss	19	86
Ataxia	17	77
Muscle weakness	14	64
GIT	13	59
Depression	10	45
Diabetes mellitus	9	41
Migraine	9	41
Retinopathy	9	41
Fatigue	8	36
Dysarthria	7	32
Epilepsy	5	23
Underweight (BMI <18.5)	5	23
Short stature	5	23
Sensory neuropathy	4	18
Myalgia	3	14
Myoclonus	3	14
Ophthalmoparesis	2	9
Ptosis	2	9
Hypothyroidism	2	9
Asthma	2	9
Cognitive decline	1	5
Dysphagia	1	5
Encephalopathy	1	5
Hypertension	1	5
Lipomata	1	5
Stroke-like episodes	1	5

Table 5.3 Frequency of clinical features. GIT = gastro-intestinal tract involvement, frequently constipation or irritable bowel symptoms; BMI = body mass index.

5.4.2 Echocardiography and magnetic resonance imaging

5.4.2.1 Reliability and reproducibility

Intra-observer and inter-observer variability for selected magnetic resonance measures of myocardial strain, function, and bioenergetics and echocardiographic measures of function, were determined as co-efficients of variation and limits of agreement (Table 5.4).

Cardiac parameter	**Intra-observer**		**Inter-observer**	
	Co-efficient of variation (%)	**Limits of agreement (%)**	**Co-efficient of variation (%)**	**Limits of agreement (%)**
MRI				
Ejection fraction (%)	2.81	-1.02 ± 3.30	4.11	2.24 ± 4.16
E/A ratio	5.32	0.08 ± 0.16	1.90	0.001 ± 0.11
EFP (%)	2.01	0.68 ± 2.84	1.89	-0.54 ± 1.58
Torsion (°)	3.22	0.06 ± 0.51	6.32	0.19 ± 0.31
Endocardial strain (%)	4.54	1.53 ± 1.08	5.31	0.74 ± 1.31
PCr/ATP ratio	8.73	0.18 ± 0.32	12.2	0.24 ± 0.28
Echocardiography				
Ejection fraction (%)	3.19	0.82 ± 5.34	6.54	-1.22 ± 7.89
E/A ratio	1.94	0.01 ± 0.13	4.39	0.12 ± 0.52

Table 5.4 Intra- and inter-observer variability for MRI and echocardiography. MRI = magnetic resonance imaging; E/A ratio = ratio of early to late diastolic filling; EFP = early filling percentage; PCr = phosphocreatine; ATP = adenosine triphosphate.

5.4.2.2 Comparison of imaging modalities

Equivalent measures of global systolic and diastolic function, obtained from echocardiography or magnetic resonance imaging, were significantly correlated (Figure 5.1). Derived values for both ejection fraction (r=0.527, $p<0.001$) and E/A ratio (r=0.915, $p<0.001$) were in good agreement between imaging modalities. Equivalent measures of LV mass and wall thickness in diastole also displayed a significant correlation (r=0.816, $p<0.001$ and r=0.656, $p<0.001$, respectively), but there was a significant decrease (11%, $p<0.05$) in LV mass from the value calculated using magnetic resonance imaging to that derived from echocardiography (Figure 5.1).

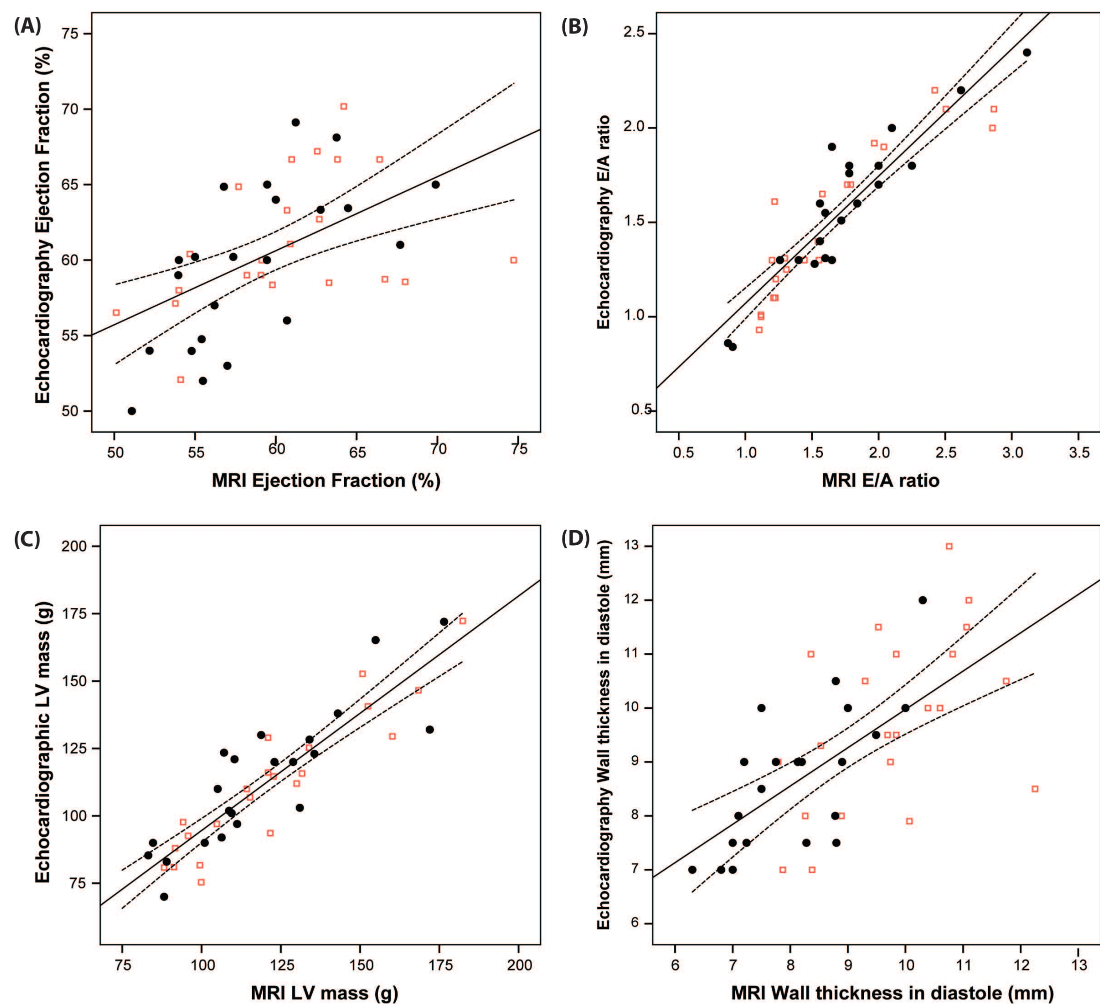


Figure 5.1 MRI and echocardiography measures. There were significant correlations between values from different imaging modalities both controls (black circles) and patients (red squares) for (A) ejection fraction, (B) E/A ratio, (C) LV mass, and (D) wall thickness. E/A ratio = ratio of early to late filling; MRI = magnetic resonance imaging; LV = left ventricular.

5.4.3 Cardiac morphology and global function

Table 5.5 summarizes the morphological and functional parameters for patient and control groups with all subjects completing the scan protocol (total duration 77 ± 13 minutes). The means and ranges of control group parameters are in agreement with a large cohort study using quantitative cardiac MRI (Alfakih *et al.*, 2003).

End-systolic and end-diastolic volumes, both as raw values and when indexed to body surface area (BSA), were significantly decreased in patients compared to controls (Table 5.5). A proportional decrease in these parameters (33% and 37% respectively) ensured no significant difference in ejection fraction. Stroke volume and stroke index were significantly decreased in patients: this occurred in association with a significant increase in heart rate ($r=-0.65$, $p<0.01$) with no significant difference in cardiac output or cardiac index between the groups.

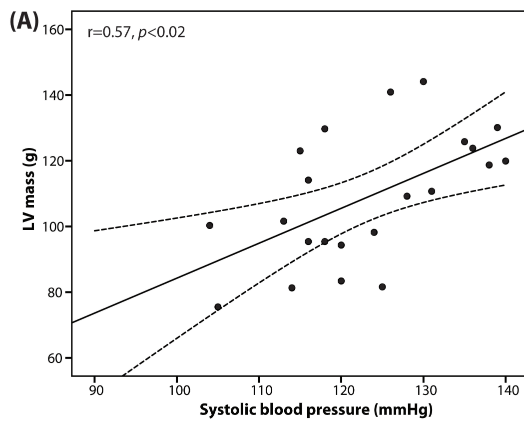
LV mass was not significantly different between patient and control groups (table 5.5). However, when indexed to BSA, LV mass index (LVMI) was significantly increased in patients. A significant increase in M/V ratio (58%) suggested concentric remodelling had occurred. Consistent with this, radial wall thicknesses in both diastole and systole were significantly increased in patients. Within the control group, there were significant correlations between systolic blood pressure and both LV mass ($r=0.57$, $p<0.02$) and radial wall thickness in diastole ($r=0.54$, $p<0.03$). Within the patient group, no significant correlations were shown between systolic or diastolic blood pressure and any markers of LV mass (**Figure 5.2**). Similarly fasting blood glucose and HbA1C in patients did not correlate significantly with LV mass, LVMI, radial wall thicknesses or M/V ratio. Exclusion of diabetic or treated hypertensive patients from statistical analyses did not affect the significance of increases in LVMI or radial wall thickness.

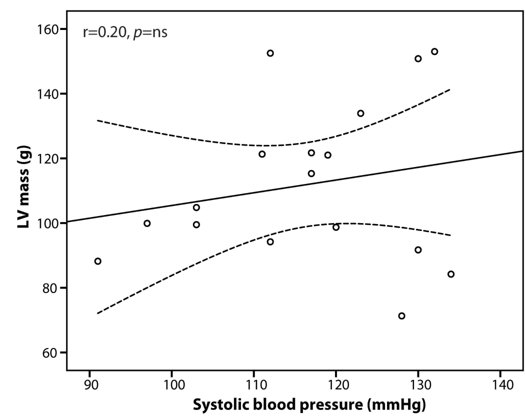
Both independent observers were in agreement that no patients displayed evidence of focal intramyocardial fibrosis on LGE imaging.

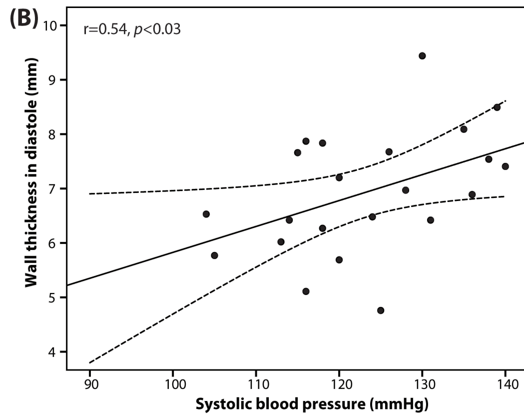
Characteristic	**Patients (n=22)**	**Controls (n=22)**	***p* value**
End-Diastolic Volume (ml)	93 ± 17	138 ± 26	<0.0001
End-Diastolic Index (ml/m^2)	54 ± 9	73 ± 15	<0.0001
End-Systolic Volume (ml)	36 ± 10	57 ± 15	<0.0001
End-Systolic Index (ml/m^2)	21 ± 6	31 ± 8	<0.001
Stroke Volume (ml)	57 ± 10	82 ± 15	<0.0001
Stroke Index (ml/m^2)	33 ± 5	45 ± 8	<0.0001
Cardiac Output (l/min)	4.4 ± 1.0	4.7 ± 0.6	0.89
Cardiac Index (l/min/m^2)	2.5 ± 0.5	2.6 ± 0.4	0.92
Ejection fraction (%)	62 ± 7	59 ± 6	0.73
Left Ventricular Mass (g)	119 ± 28	109 ± 20	0.21
Wall thickness in systole (mm)	13.5 ± 3.1	10.9 ± 1.9	<0.01
Wall thickness in diastole (mm)	9.8 ± 2.9	6.9 ± 1.1	<0.001
LVMI [LV mass / BSA] (g/m^2)	70 ± 12	59 ± 7	<0.01
M/V ratio [LV mass / EDV] (g/ml)	1.28 ± 0.33	0.81 ± 0.14	<0.0001

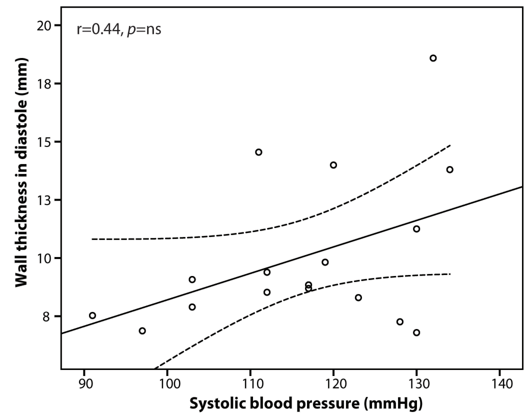
Table 5.5 Cardiac morphology and function. LV = left ventricular; LVMI = left ventricular mass index; BSA = body surface area; M/V = left ventricular mass / end-diastolic volume ratio.

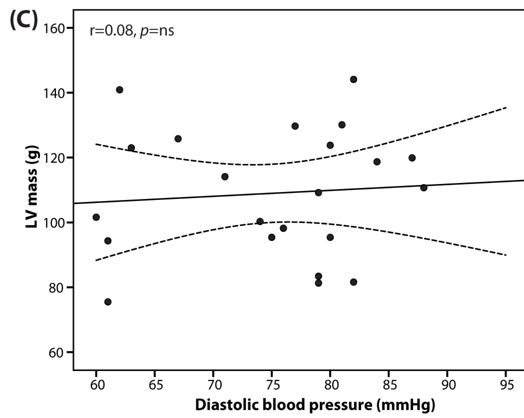
Figure 5.2 Left ventricular mass, wall thicknesses and blood pressures (overleaf). In the control group (left panels, closed circles), systolic blood pressure correlated with (A) LV mass and (B) wall thickness, but diastolic blood pressure did not correlate with either parameter (C and D, respectively). In contrast, in the patient group (right panels, open circles), there were no significant correlations between systolic or diastolic blood pressures and LV mass or wall thickness in diastole.

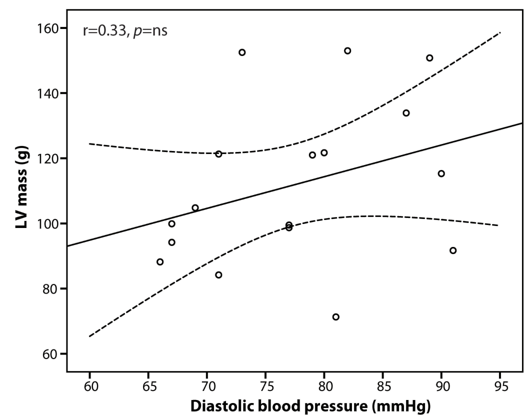

(A)
r=0.57, p<0.02
LV mass (g)
160
140
120
100
80
60
90
100
110
120
130
140
Systolic blood pressure (mmHg)

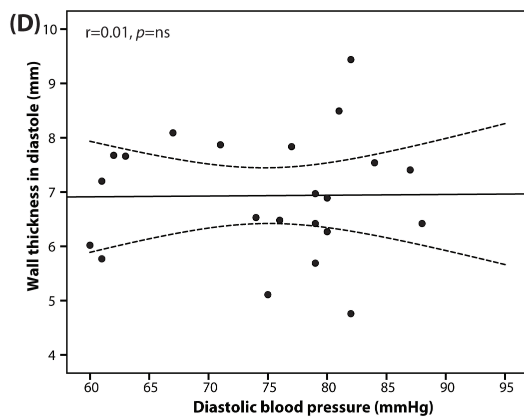

r=0.20, p=ns
LV mass (g)
160
140
120
100
80
60
90
100
110
120
130
140
Systolic blood pressure (mmHg)

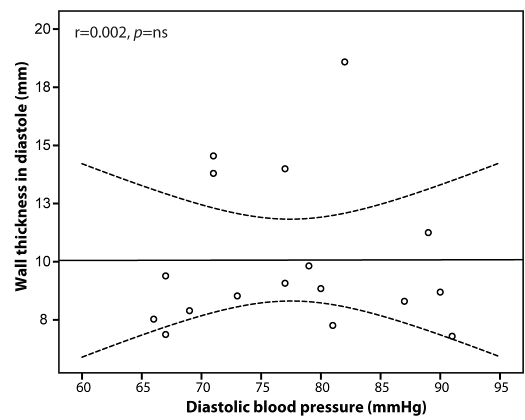

(B)
r=0.54, p<0.03
Wall thickness in diastole (mm)
10
9
8
7
6
5
4
90
100
110
120
130
140
Systolic blood pressure (mmHg)


r=0.44, p=ns
Wall thickness in diastole (mm)
20
18
15
13
10
8
90
100
110
120
130
140
Systolic blood pressure (mmHg)


(C)
r=0.08, p=ns
LV mass (g)
160
140
120
100
80
60
60
65
70
75
80
85
90
95
Diastolic blood pressure (mmHg)


r=0.33, p=ns
LV mass (g)
160
140
120
100
80
60
60
65
70
75
80
85
90
95
Diastolic blood pressure (mmHg)


(D)
r=0.01, p=ns
Wall thickness in diastole (mm)
10
9
8
7
6
5
4
60
65
70
75
80
85
90
95
Diastolic blood pressure (mmHg)


r=0.002, p=ns
Wall thickness in diastole (mm)
20
18
15
13
10
8
60
65
70
75
80
85
90
95
Diastolic blood pressure (mmHg)

5.4.4 Cardiac tagging and myocardial strains

Longitudinal shortening was significantly decreased (17%) in patients (Table 5.6) and correlated significantly with increased LVMI (r=-0.52, *p*<0.03). Peak torsion (36%) and TSR (35%) were significantly increased in patients. No significant differences were detected in rates of systolic and diastolic torsion, after correction for peak torsion, or in diastolic function represented by E/A ratio and early filling percentage.

No significant differences were found in radial thickening, or in circumferential whole wall or endocardial strain (Table 5.6).

Characteristic	Patients (n=22)	Controls (n=22)	*p* value
Longitudinal shortening (%)	15.1 ± 1.5	18.2 ± 2.3	<0.0001
Radial wall thickening (%)	65.6 ± 17.0	60.1 ± 16.0	0.12
Peak torsion (°)	8.0 ± 2.7	5.9 ± 1.4	<0.03
Systolic torsion rate (°/s)	37 ± 13	24 ± 10	<0.02
Diastolic torsion rate (°/s)	-23 ± 10	-17 ± 10	0.33
Systolic torsion rate/peak torsion (s^{-1})	4.7 ± 1.3	4.1 ± 1.8	0.21
Diastolic torsion rate/peak torsion (s^{-1})	3.0 ± 1.3	2.8 ± 1.6	0.40
Whole wall circumferential strain (%)	16.7 ± 2.2	17.8 ± 2.5	0.19
Endocardial circumferential strain (%)	22.6 ± 2.6	24.4 ± 2.5	0.41
Torsion to endocardial strain ratio (rad)	0.62 ± 0.21	0.46 ± 0.10	<0.03
E/A ratio	1.53 ± 0.55	1.75 ± 0.62	0.08
Early filling percentage (%)	72.4 ± 8.9	73.2 ± 8.3	0.56

Table 5.6 Cardiac tagging and diastolic function. E/A ratio = ratio of early to late ventricular filling velocity.

5.4.5 Mutation load and clinical status

There was a significant correlation between urinary mutation load (mean 62 ± 20%, range 22-90%) and disease burden (NMDAS mean score 18 ± 11, range 2-42) among patients (r=0.59, *p*<0.02). Both these clinical markers displayed significant correlations

with LVMI (respectively r=0.71 and r=0.79, both *p*<0.001), and peak endocardial circumferential strain (r=-0.59 and r=-0.57, both *p*<0.03, Figure 5.3).

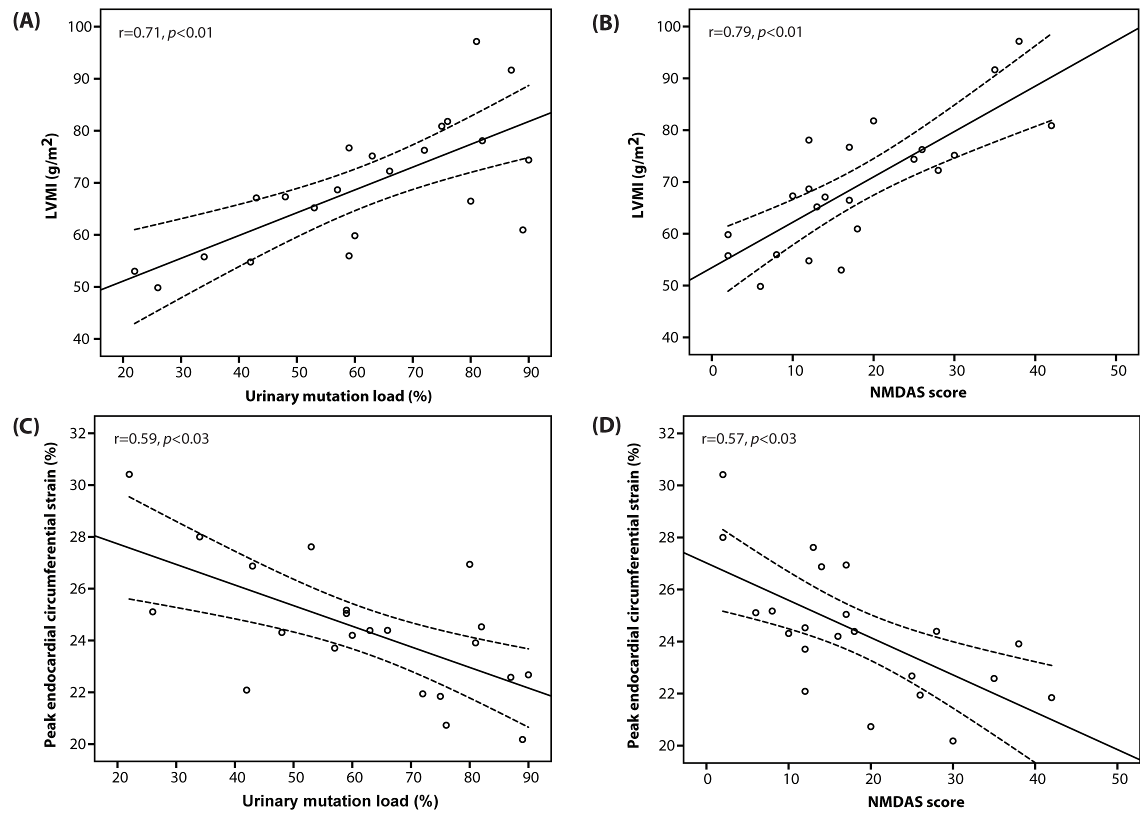


Figure 5.3 LVMI and clinical markers. Among patients with the m.3243A>G and m.8344A>G mutations, LVMI correlated positively with both (A) urinary mutation load and (B) NMDAS score, while peak endocardial circumferential strain correlated negatively with (C) urinary mutation load and (D) NMDAS score. LVMI = left ventricular mass index; NMDAS = Newcastle Mitochondrial Disease Adult Scale.

5.4.6 Myocardial bioenergetics

PCr/ATP ratio was decreased (Figure 5.4, mean decrease 21%, $p<0.001$) in patients (1.51 ± 0.34) compared to controls (1.92 ± 0.20). There were no significant correlations between PCr/ATP ratio and markers of disease burden or myocardial mass or function. Thirteen patients (59%) had an abnormal PCr/ATP ratio (<1.6) but there were no significant differences in markers of disease burden, cardiac morphology or function between patients with PCr/ATP ratio >1.6 and those <1.6 (Neubauer *et al.*, 1992).

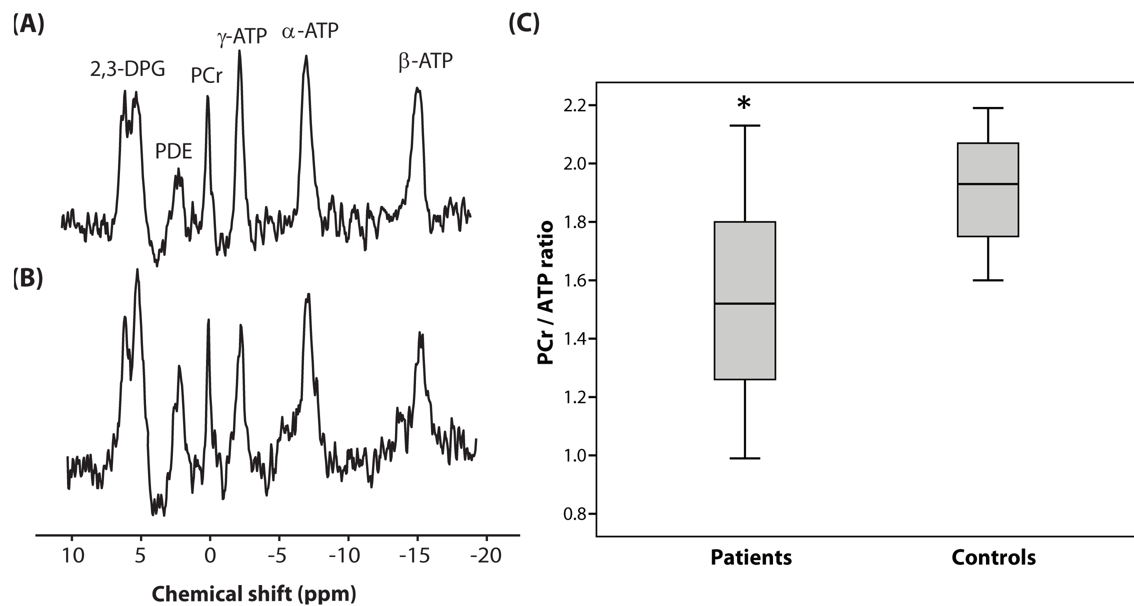


Figure 5.4 Phosphorus-31 magnetic resonance spectroscopy. Representative spectra from (A) a patient carrying m.3243A>G, with PCr/ATP ratio 1.23, and (B) a matched control subject, with PCr/ATP ratio 2.10, showing a difference in PCr concentration. Spectra are presented as acquired before correction for heart rate, flip angle and blood content. (C) Box-plot of range and quartiles of the PCr/ATP ratio in patient and control groups. 2,3DPG = 2,3-disphosphoglycerate; PDE = phosphodiesters; PCr = phosphocreatine; ATP = adenosine triphosphate; ppm = parts per million; * = $p<0.001$ compared to controls.

5.5 Discussion

This study used a combined approach of comprehensive cardiac MRI and ^{31}P MRS to examine myocardial morphology, function, and bioenergetics in 22 patients harbouring either the m.3243A>G or m.8344A>G mutations without clinical cardiac involvement. The major findings in these patients compared with age- and gender-matched controls are: 1) LVMI is greater and is a more sensitive indicator of subtle cardiac hypertrophy than LV mass; 2) concentric remodelling occurs in the absence of hypertension or diabetes mellitus; 3) altered systolic myocardial strains occur, with reduced longitudinal shortening and increased peak torsion, in the absence of global systolic or diastolic dysfunction; 4) early changes in cardiac morphology and strains are associated with increased mtDNA mutation load and NMDAS score; and 5) PCr/ATP ratio is reduced, but does not correlate with structural or functional cardiac indices or markers of disease burden.

5.5.1 Cardiac morphology and function

Patients in this study displayed concentric hypertrophic remodelling, as evidenced by increased M/V ratio and wall thicknesses, which were independent of diabetic or hypertensive status. Reductions in end-systolic and end-diastolic blood pool volumes, consistent with concentric remodelling, resulted in decreased stroke volume and index in this study. The finding of significantly elevated heart rate, that ensured no difference in cardiac output or index, is however intriguing and not previously reported in this patient group. No likely culprit medications were identified in the patient group as a whole and the possibility of a relationship to the underlying disease process remains to be explored (see Section 6.4.10). The pattern of concentric hypertrophic remodelling observed in this study is similar to that in normal ageing where reduced ventricular volumes and increased M/V ratio with minimal change in LV mass have been linked to adverse cardiac outcomes, particularly when present in those <65 years of age (Cheng *et al.*, 2009).

Small studies have suggested differing estimates of the prevalence of LVH in m.3243A>G mutation carriers (Majamaa-Voltti *et al.*, 2002; Vydt *et al.*, 2007). This study, performed in patients without preceding evidence of cardiac involvement, demonstrates the critical importance of referencing LV mass to body surface area.

Although not reaching statistical significance, patients had smaller body mass, height, BMI and BSA compared with controls. LV mass was significantly increased in patients, but only when indexed to BSA. Taken together, these findings suggest that previous cohort studies that used absolute measures of LV mass and did not employ indexation, may have underestimated the number of patients with increased indexed LV mass. While standard definitions of LVH should be used, this implies that the frequency of LVH in patients, which is potentially amenable to treatment, may be higher than previously indicated (Majamaa-Voltti *et al.*, 2002). A larger cross-sectional study would be required to investigate this issue.

This study used reliable and reproducible measures of magnetic resonance imaging to investigate early cardiac involvement in patients with mtDNA disease. All participants had the absence of *known* cardiac involvement demonstrated using a normal clinical echocardiogram, in addition to other investigations, prior to study entry. There was generally good agreement between equivalent measures of global systolic and diastolic function derived from echocardiography and magnetic resonance imaging, consistent with published guidelines and clinical studies (Thomson *et al.*, 2001; Nagueh *et al.*, 2011). While reassuring from a clinical standpoint, where echocardiography is the mainstay of clinical cardiac screening in patients with mtDNA disease, a notable underestimation of LV mass by echocardiography in comparison to the gold standard of MRI was evident in this study. This may have further limited the ability of previous studies to detect early hypertrophic left ventricular remodelling, again underestimating the prevalence of cardiomyopathy in this patient cohort.

5.5.2 Myocardial strains and torsion

Consistent with LVH in other clinical contexts (Young *et al.*, 1994; Fonseca *et al.*, 2004), concentric remodelling in this study was associated with reduced longitudinal shortening. In healthy ageing and patients with diabetes mellitus or neuromuscular diseases, circumferential strain is reduced, and associated with reduced longitudinal shortening (Fonseca *et al.*, 2004; Cheng *et al.*, 2009; Hor *et al.*, 2009). In this study, endocardial and whole wall circumferential strains tended to be reduced in patients without reaching statistical significance. Higher mutation load and NMDAS score, indicative of greater disease burden, did however correlate with increased LVMI and reduced endocardial circumferential strain.

Increased torsion and/or TSR, often with reduced longitudinal shortening, have been reported in patients with LVH secondary to HCM (Young *et al.*, 1994; Russel *et al.*, 2011), or conditions of increased afterload including aortic stenosis and hypertension (Van Der Toorn *et al.*, 2002; Kang *et al.*, 2008). In all cases, such changes in torsion, TSR and longitudinal shortening are believed to be due to a reduction in contractile function in the subendocardium compared to subepicardium (Van Der Toorn *et al.*, 2002). Recently Chung et al demonstrated increased torsion in the absence of morphological cardiac disease in diabetic patients (Chung *et al.*, 2006). Although the pathophysiology of diabetes in mitochondrial disease is distinct (Maassen *et al.*, 2004), my results of increased torsion and TSR without significant change in circumferential strains concur with these findings and those reported in HCM mutation carriers without LVH (Russel *et al.*, 2011). Additionally, as in my cohort, both these studies reported isolated systolic abnormalities of torsion with no difference to controls in rate of torsion dissipation during diastole, or basic measures of diastolic function. Myocardial perfusion defects could contribute to this increased torsion, with abnormalities of subendocardial arterioles in HCM hearts (Schwartzkopff *et al.*, 1998), and small vessel disease in diabetes. Such abnormalities could similarly be responsible for the differences in myocardial deformation in my study. However my patients also demonstrated significant reductions in end-diastolic and end-systolic volumes, yielding smaller radii for the myocardium. Increased torsion could result from the additional dominance this gives to the subepicardium (Arts *et al.*, 1979). To distinguish between these explanations would require a measure of perfusion at the subendocardium.

5.5.3 Disease burden

Cardiac involvement is an important prognostic factor in mitochondrial disease since complications of cardiomyopathy are a frequent cause of premature death. Yet the pathophysiological mechanisms linking the m.3243A>G mutation to LVH and cardiomyopathy remain unknown. It has previously been shown that urinary mutation load is the best predictor of overall clinical outcome in m.3243A>G mutation carriers (Whittaker *et al.*, 2009). In this study, I report for the first time a correlation between urinary mutation load and cardiac involvement specifically, as evidenced by increased LVMI. NMDAS score correlated strongly with both urinary mutation load and LVMI. These important findings support the primary importance of mtDNA mutations in the

changes observed in cardiac morphology, and may support more intensive cardiac evaluation of patients with higher mutation loads and/or NMDAS scores.

5.5.4 Cardiac bioenergetics

Reductions of PCr/ATP ratio, assessed non-invasively using ^{31}P MRS, have prognostic importance in diverse forms of cardiomyopathy (Neubauer *et al.*, 1992). Indeed myocardial energy depletion has been proposed as a critical mechanism linking sarcomeric defects to hypertrophy in HCM (Crilley *et al.*, 2003). The present study confirms the findings of a previous study in m.3243A>G mutation carriers, which found that the PCr/ATP ratio was significantly reduced (Lodi *et al.*, 2004). However I was unable to detect any correlation between the cardiac bioenergetic defect and MRI-based parameters of myocardial structure or function, or markers of disease burden in patients without clinical cardiac disease. In different forms of inherited cardiomyopathy, several groups have suggested the primacy of bioenergetic defects by detection of abnormalities in mutation carriers without evidence of LVH (Lodi *et al.*, 2001; Crilley *et al.*, 2003). However I detected significant differences in cardiac remodelling, known itself to cause a reduction in PCr/ATP ratio and potentially explaining the lack of correlation with other parameters. PCr/ATP ratio does not in isolation appear to have prognostic value in detection of early cardiac remodelling in patients harbouring the m.3243A>G mutation, but a larger natural history study would be required to confirm this suggestion.

5.5.5 Clinical implications

There are several clinical implications from my findings. Firstly the indexing of measures of LV mass to body surface area or end-diastolic volume is essential to detect early concentric remodelling in patients harbouring the m.3243A>G mutation. Cardiac involvement may be more prevalent than previously suspected. Secondly patients with a higher urinary mutation load, or NMDAS score, may be at an increased risk of developing cardiomyopathy, supporting more frequent cardiac screening. Finally natural history studies of pathogenesis and eventual clinical therapeutic trials are dependent on an ability to identify the earliest biomechanical changes attributable to the m.3243A>G mutation. I have shown for the first time increased torsion and abnormal myocardial strains in this cohort, and suggest that measurement of LV

mechanics may be useful in assessing disease progression and response to intervention.

5.5.6 Limitations

Although this study is the largest cardiac MRI-based investigation performed to date in this patient group, it remains limited in sample size and was not designed to investigate pathogenetic mechanisms or disease progression. Cardiac involvement in mitochondrial disease is linked to clinical outcomes, yet I acknowledge that the prognostic importance of the changes I describe must be determined through longitudinal studies. I studied a relatively homogenous cohort of patients, harbouring the single commonest mtDNA point mutation, without known cardiac involvement; such patients account for ~25% of specialist clinic attendees yet I recognize that these findings may not be generalizable to all patients with mtDNA point mutations. Longitudinal shortening was used to provide a global measure of long axis function rather than longitudinal strain, which would require additional tagged long axis slices. Similarly, in an already extensive MRI protocol, I did not study flow-based analyses of diastolic function or first-pass perfusion. Finally I did not perform LGE imaging in controls and cannot exclude the presence of focal fibrosis in these individuals although the probability of this is very low.

5.6 Conclusions

Concentric remodelling is prevalent in patients harbouring the m.3243A>G or m.8344A>G mutations and occurs in association with characteristic changes in systolic intramyocardial strains and torsion. These findings, which are closely related to urinary mutation load and disease burden, occur in patients without existing evidence of cardiac involvement, and may provide an early marker of myocardial pathology, enabling future studies of pathogenesis and intervention. Magnetic resonance imaging may be more sensitive than echocardiography in detection of cardiac involvement.

Chapter 6.

Effects of endurance training on cardiac parameters and autonomic function in patients with m.3243A>G- and m.8344A>G-related mitochondrial disease: case-control exercise interventional study

6.1 Introduction

6.1.1 Exercise training and mitochondrial DNA disease

There are currently limited therapeutic options for patients with mitochondrial disease (Pfeffer *et al.*, 2012), with the majority of interventions focussed on symptomatic improvement through multi-disciplinary team management. As progress is made in investigating the underlying pathophysiological mechanisms of mitochondrial disease, novel therapeutic targets will emerge. However, in a progressive and multisystem condition, the clinical management of prominent disease features has already stimulated the development of a *potential* treatment for patients with mtDNA disease.

Despite phenotypic variability, exercise intolerance and fatigue are particularly common features and debilitating symptoms in patients with mitochondrial disease. Aerobic, endurance exercise training has, however, been demonstrated to improve exercise tolerance, quality of life and skeletal muscle oxidative capacity, with reversal of baseline de-conditioning, in patients with mtDNA point mutations including the m.3243A>G mutation (Taivassalo *et al.*, 1998; Cejudo *et al.*, 2005; Jeppesen *et al.*, 2006; Taivassalo *et al.*, 2006). The effects on fatigue in this patient group are not clear. Similarly, resistance exercise training has been linked to clinical improvements in patients with mitochondrial disease (Murphy *et al.*, 2008). While the cellular mechanisms underlying the effects of exercise training in general and endurance training specifically remain unclear, no deleterious clinical outcomes related to skeletal muscle function, quality of life or disease progression have been reported in studies from diverse genotypic groups. Moreover such training programmes can reduce mtDNA mutation load and reverse the clinical phenotype in an animal model of progeroid ageing associated with the accumulation of somatic mtDNA mutations (Safdar *et al.*, 2011), providing supportive molecular biological evidence for a cellular benefit of this approach.

6.1.2 Cardiac involvement in mitochondrial DNA disease

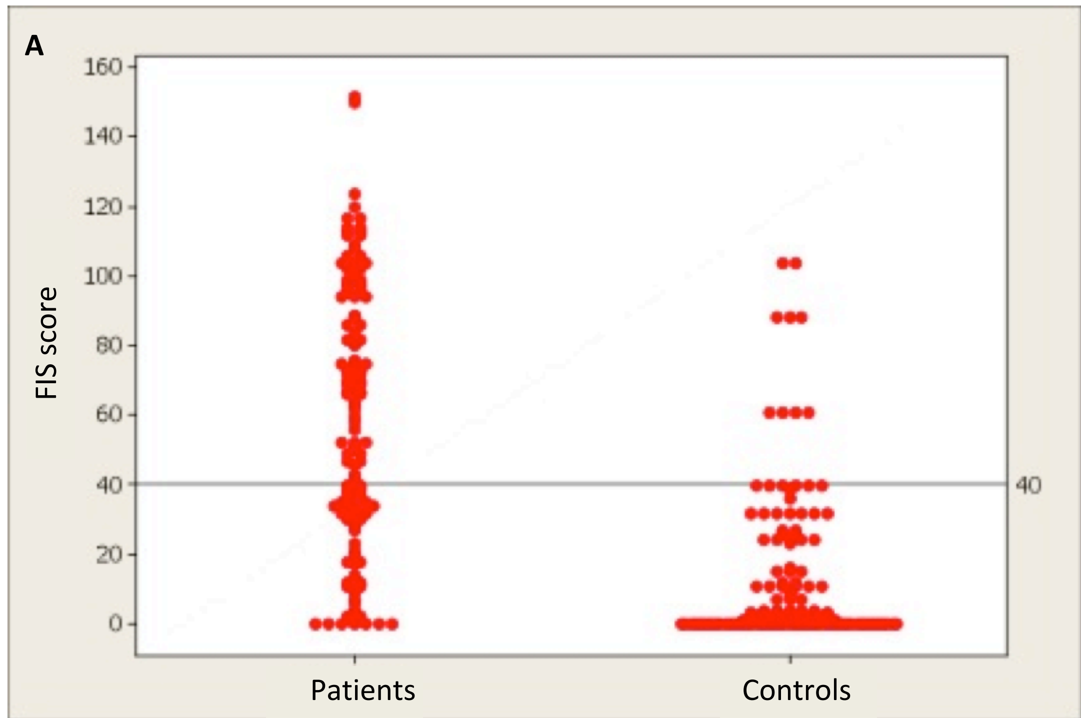
Cardiomyopathy, most commonly with a hypertrophic phenotype, occurs in 20-40% of patients harbouring the m.3243A>G mutation (Hirano and Pavlakis, 1994; Anan *et al.*, 1995; Majamaa-Voltti *et al.*, 2002; Holmgren *et al.*, 2003; Vydt *et al.*, 2007), and is an independent predictor of morbidity and early mortality (Holmgren *et al.*, 2003;

Majamaa-Voltti *et al.*, 2008). Impaired cardiac bioenergetics also occur in patients with m.3243A>G-related mitochondrial disease (Lodi *et al.*, 2004; Bates *et al.*, 2013), and are predictive of prognosis in diverse forms of cardiomyopathy (Neubauer *et al.*, 1992). Cardiovascular autonomic dysfunction has been demonstrated in patients harbouring the m.3243A>G mutation (Momiyama *et al.*, 2002; Majamaa-Voltti *et al.*, 2004), and is independently associated with an increased risk of sudden death in the general population, after myocardial infarction (Bigger *et al.*, 1992).

While an appreciation of the potential risk of cardiac involvement exists amongst medical professionals caring for patients with mitochondrial disease, specific predictors of cardiac involvement in mtDNA disease remain unknown and data are scarce concerning the physiological factors that may contribute to cardiac disease progression. Understandable concerns of adverse cardiac remodelling in a cohort of patients prone to the development of LVH has undoubtedly restricted the widespread uptake of therapeutic endurance exercise training in patients with mitochondrial disease, hampering clinical care.

6.1.3 Fatigue and mitochondrial DNA disease

A further conceptual limitation on the use of exercise as a therapeutic intervention in patients with mitochondrial disease has been the unknown impact on the important symptom of fatigue. Clinical fatigue is undoubtedly a complex and multifaceted problem, encompassing both physical and psychological features, but it is perhaps best defined as an overwhelming sense of tiredness, lack or energy and feeling of general exhaustion (Krupp and Pollina, 1996). A recent qualitative assessment of 132 patients in the MRC Mitochondrial Disease Patient Cohort (UK), using the validated FIS questionnaire (Fisk *et al.*, 1994), suggested that 62% patients had clinically-relevant fatigue, with 64% rating their fatigue symptoms as moderate to very severe (personal communication from Dr Grainne Gorman, Figure 6.1).



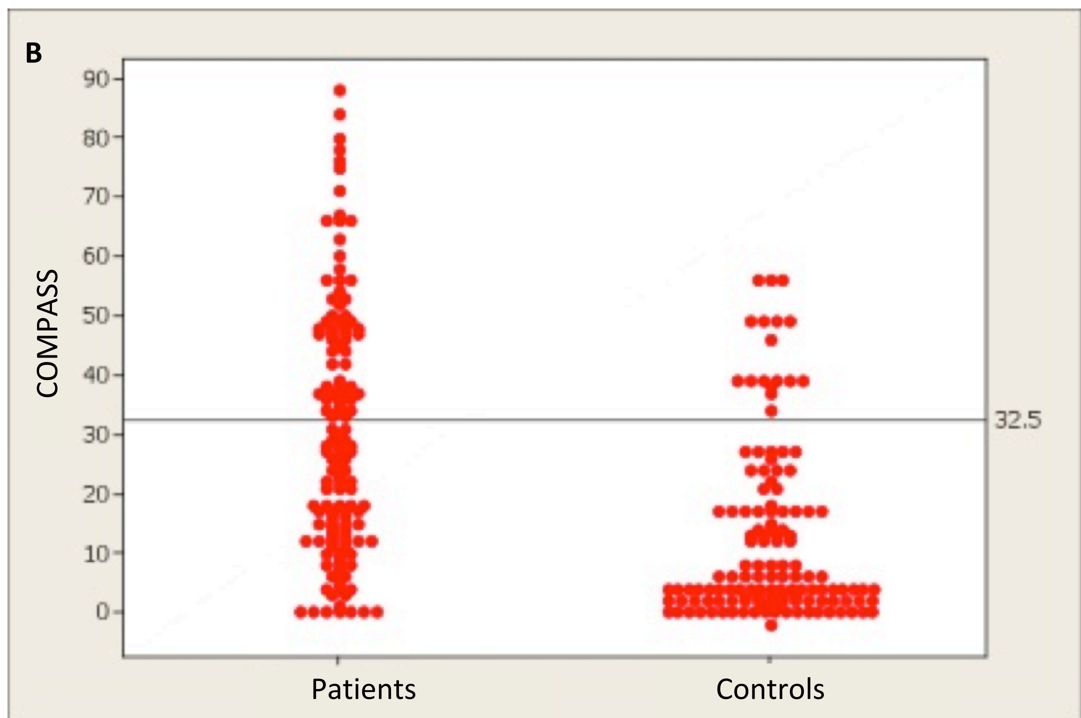


Figure 6.1 Fatigue and symptoms of autonomic dysfunction. Scatter plots of (A) Fatigue Impact Scale (FIS) score and (B) Composite Autonomic Symptom Score (COMPASS) in 132 patients with different forms of mitochondrial disease and age- and gender-matched healthy controls. The majority of patients (62%) had clinically relevant fatigue (FIS score >40) while a significant minority (43%) reported COMPASS >32.5 suggesting prominent symptoms of autonomic dysfunction (Newton *et al.*, 2007b).

These findings were consistent across ages, genotypes and genders, and the use of COMPASS, an international questionnaire validated against quantitative measures of autonomic function, revealed a similar frequency and severity of autonomic symptoms with 43% patients affected. There was a significant correlation between fatigue and autonomic symptoms, suggesting that autonomic dysfunction may be an important factor in fatigue (Figure 6.2).

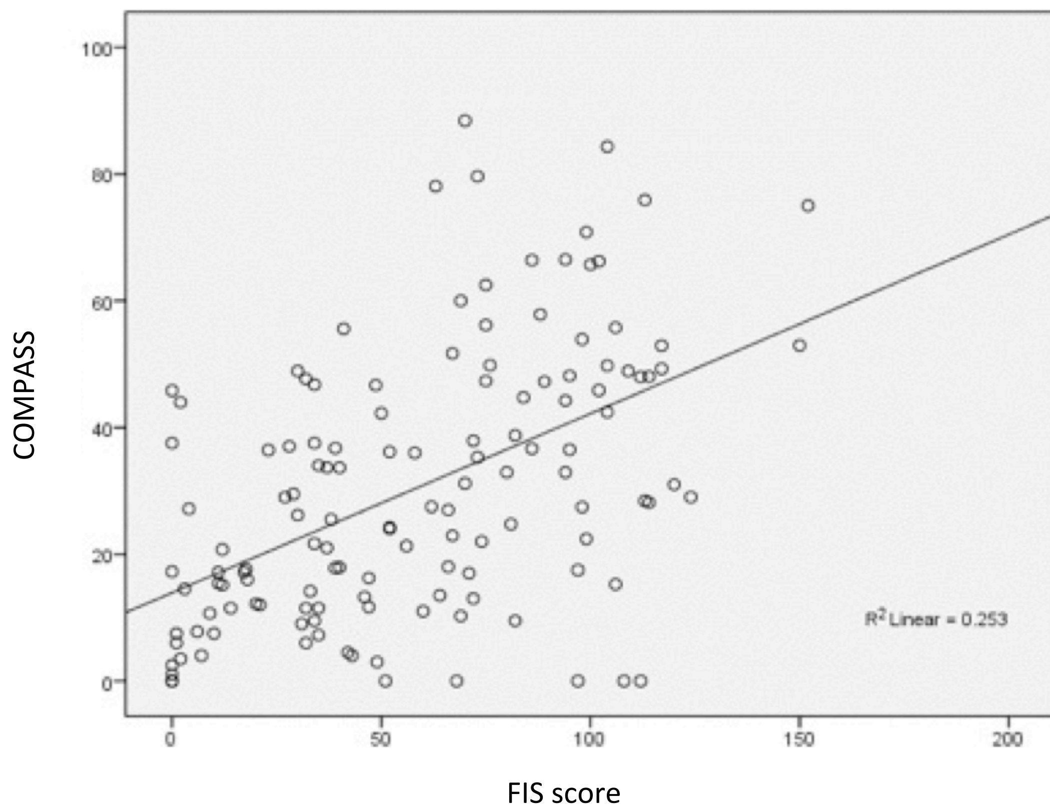


Figure 6.2 Scatter plot of COMPASS and FIS scores. Scatter plot of Composite Autonomic Symptoms Score (COMPASS) and Fatigue Impact Scale (FIS) score in patients with mitochondrial disease (r=0.50, p<0.01), demonstrating the positive correlation between these two parameters.

6.1.4 Autonomic dysfunction in mitochondrial DNA disease

Multiple factors have been implicated in the pathogenesis of fatigue both in the general population and in patients with neurological diseases. While disorders of sleep (Attarian *et al.*, 2004), mood (Krupp *et al.*, 2010), and metabolism (Roelcke *et al.*, 1997) may represent important aetiological factors or merely prominent confounders in fatigue, recent data support a central role for autonomic dysfunction in different clinical scenarios.

In patients with neurodegenerative diseases, including multiple sclerosis (Flachenecker *et al.*, 2003) and Parkinson's disease (Nakamura *et al.*, 2011), or multisystem disorders with prominent fatigue, such as chronic fatigue syndrome (Freeman and Komaroff, 1997) and primary biliary cirrhosis (Newton *et al.*, 2007a), autonomic dysfunction is common and implicated in the aetiology of fatigue. These conditions share some features with mtDNA disease and importantly, there are several lines of evidence that further support a role for autonomic dysfunction in this patient group. Firstly, symptoms of autonomic dysfunction occur more frequently in patients with the m.3243A>G mutation than healthy controls (Parsons *et al.*, 2010), and are widely recognised as an important feature of specific presentations of mitochondrial disease including MNGIE (Hirano *et al.*, 1994). Secondly, autonomic dysfunction has been demonstrated in a small cohort of exclusively diabetic patients carrying the m.3243A>G mutation (Ueno and Shiotani, 1999; Momiyama *et al.*, 2002), and in a larger heterogenous cohort of patients with diverse mtDNA mutations (Di Leo *et al.*, 2007). Thirdly, distinct clinical observations in patients with mtDNA disease suggest that autonomic dysfunction may make an important contribution to morbidity: in addition to fatigue and exercise intolerance, peripheral neuropathy is a well-recognized clinical feature (Karppa *et al.*, 2003), and I have already demonstrated an elevation in resting heart rate in this patient group (see Section 5.4.1) (Bates *et al.*, 2013). Finally sudden and unexplained death is not uncommon in patients carrying the m.3243A>G mutation (Majamaa-Voltti *et al.*, 2006); while known neurological or cardiovascular involvement may at least partly explain this phenomenon, autonomic dysfunction may be an important contributory factor in patients with mtDNA disease. Autonomic dysfunction is independently associated with an increased risk of sudden death in the general population after myocardial infarction (Bigger *et al.*, 1992) and

has been linked to unexplained excess non-liver-related mortality in patients with liver disease (Newton *et al.*, 2006). In addition to any putative effect on mortality, autonomic dysfunction can certainly be a major cause of morbidity: in patients with paroxysmal atrial fibrillation (AF), autonomic symptoms are predictive of poor quality of life, independent of the arrhythmia itself (van den Berg *et al.*, 2001), and patients with postural orthostatic tachycardia syndrome (POTS) report equivalent symptom burden to those with heart failure or chronic obstructive pulmonary disease (Benrud-Larson *et al.*, 2002).

6.1.5 Effects of exercise on the heart and autonomic function

In untrained individuals *without* mitochondrial disease, endurance exercise training increases left ventricular mass, ventricular cavity dimensions and haemodynamic parameters of cardiac function (Douglas *et al.*, 1997; Pelliccia *et al.*, 1999; Scharhag *et al.*, 2002). All these changes are regarded as physiological, representing appropriate cardiac adaptation to changes in the loading conditions and metabolic demands of endurance exercise. Moreover, such training programmes can also attenuate the effects of deleterious insults to the myocardium including ischaemia-reperfusion injury (Ascensao *et al.*, 2007), ageing (Starnes *et al.*, 2003), and diabetes (Lumini *et al.*, 2008). There is growing evidence to suggest a pivotal role for mitochondria in modulating many cellular aspects of this apparent cardioprotection (Starnes and Taylor, 2007). Endurance exercise appears to have many beneficial effects on cardiovascular function, in untrained individuals, through physiological adaption of mitochondrial function.

Exercise capacity is dependent on the ability of the heart to increase cardiac output and on efficient use of oxygen and substrates by both cardiac and skeletal muscle (Pina *et al.*, 2003). In patients with heart failure, exercise limitation results from a combination of cardiac and peripheral muscle factors, including altered substrate utilization and reduced mitochondrial copy number, with impaired skeletal and cardiac muscle energy metabolism and function (Pina *et al.*, 2003). Exercise as an adjuvant therapy in patients with established heart failure reduces symptoms and improves cardiac function, exercise tolerance, quality of life and daily activity levels, *without* a significant deleterious effect on impaired cardiac bioenergetics (Belardinelli *et al.*, 1999; Stolen *et al.*, 2003; Beer *et al.*, 2008). Moreover these beneficial effects of

exercise in this population have been particularly attributed to peripheral skeletal muscle adaptations, including increased mitochondrial oxidative capacity, and correction of endothelial dysfunction (Piepoli *et al.*, 2010). Only relatively modest improvements in left ventricular systolic and diastolic function and no absolute change in resting high energy phosphate metabolites have been described after endurance exercise training (Stolen *et al.*, 2003; Beer *et al.*, 2008), suggesting cardiac adaptations play an insignificant role in the benefits of exercise training in heart failure. Certainly no *detrimental* effects of exercise on cardiac structure and function in patients with heart failure have been reported. Whether this holds true in patients with mitochondrial disease is currently unknown.

Autonomic control of the cardiovascular system is of critical importance in the maintenance of cardiovascular homeostasis and dysfunction plays an important role in diverse diseases (Levy and Pappano, 2007). Exercise has a beneficial physiological role in adaptation of components of cardiovascular autonomic function in the general population. The baroreceptor reflex, that modulates heart rate and blood pressure, displays an inverse relationship with sudden death in patients following myocardial infarction (Bigger *et al.*, 1992; La Rovere *et al.*, 1998). Yet endurance exercise training in this patient group has a similar effect to ß blockade therapy, improving baroreceptor sensitivity, and having a significant beneficial impact on prognosis (Hull *et al.*, 1994; La Rovere *et al.*, 1998). As described above, cardiovascular autonomic dysfunction may contribute to both morbidity and mortality in patients harbouring the m.3243A>G or m.8344A>G mutations, yet the effects of exercise training on fatigue, autonomic function and disease progression are unknown in this patient group.

6.2 Aims

The aim of this study was to characterise the effects of endurance exercise training on disease burden, resting cardiac function, high energy phosphate metabolism, cardiovascular autonomic function, fatigue and quality of life in a clinically and genetically well-characterized cohort of patients with m.3243A>G- or m.8344A>G-related mitochondrial disease with reference to age- and gender- and habitual physical activity level-matched controls.

The principal hypothesis was that there would be no differences in cardio-pulmonary responses to exercise between the groups - analysis of any differential effects could help elucidate the mechanisms of cardiac dysfunction in patients with mitochondrial disease; conversely, demonstration of proportional effects in patients and controls would help facilitate the prescription of efficacious exercise therapy in this patients with mitochondrial disease due to the m.3243A>G or m.8344A>G mutations.

6.3 Methods

6.3.1 Participants

Twelve patients with mitochondrial disease due to either the m.3243A>G or the m.8344A>G mutation, but without clinical cardiac involvement (assessed using history, examination, ECG and echocardiogram), were recruited from consecutive participants in the cross-sectional study (see Section 5.3). All eligible patients involved in the baseline study were verbally invited to participate in this further study based on the following clinical inclusion criteria: (i) clinical stability for >6 months; (ii) ability to use a semi-recumbent stationary bicycle ergometer; and (iii) no current participation in regular physical activity (≥1 weekly session). Exclusion criteria were again the presence of known cardiac involvement, comorbidities precluding exercise training (e.g. osteoarthritis), and contra-indications to MRI, including the presence of a pacemaker or implantable cardioverter-defibrillator, abnormal renal function (eGFR <60ml/min/1.73m^2) or claustrophobia. All 12 patients were matched with respect to age, gender and physical activity level with untrained healthy controls with normal ECG and no history of cardiovascular or metabolic disease, recruited through local advertisement. Institutional ethical approval and written informed consent were obtained.

6.3.2 Protocol

All baseline and 16 week follow-up assessments were completed in Newcastle upon Tyne on a single day at both the Newcastle Magnetic Resonance Centre and the NIHR Clinical Research Facility, Royal Victoria Infirmary. Assessments of exercise parameters, autonomic function, resting venous lactate and creatine kinase (CK) were performed between 0800 and 1000 following an overnight fast >10 hours, and cardiac MRI took place at the same time of day throughout the study for each subject. All subjects were

asked to refrain from smoking, alcohol ingestion, physical exertion and medications that could influence haemodynamic, exercise or autonomic parameters (including ß blockers, calcium channel blockers, anti-depressants) for 24 hours prior to assessment.

6.3.3 Assessments

6.3.3.1 Exercise testing

Cardiopulmonary exercise testing was performed using analysis of expired gases and non-invasive cardiac output on a stationary bicycle ergometer as previously described (see Section 2.2.4.2).

Cardiac power output (in Watts) was calculated as the product of mean arterial pressure (in mmHg), cardiac output (in litres/min) and the conversion factor 2.22×10^{-3} (Williams *et al.*, 2001). Arterial-venous oxygen difference, expressed in ml 0_2/100ml of blood, was calculated as the ratio of peak oxygen consumption and cardiac output.

6.3.3.2 Disease burden

All subjects underwent physical examination. Disease burden in patients was assessed using NMDAS, as previously described (Schaefer *et al.*, 2006), whilst mutation load was determined in urinary epithelial cells by the NSCT Mitochondrial Diagnostic Laboratory (Whittaker *et al.*, 2009).

6.3.3.3 Body weight and composition

Subjects' heights, weights and body compositions were calculated, as previously described (see Section 2.2.4.1.1).

6.3.3.4 Cardiac magnetic resonance imaging

Cardiac magnetic resonance imaging was performed including (i) ^{31}P MRS (ii) cine imaging, and (ii) cardiac tagging, as previously described (see Section 2.2.5.2).

6.3.3.5 Autonomic function

All participants underwent assessment of autonomic function over 30 minutes at rest in a dedicated cardio-respiratory laboratory using the TFM, as previously described. Power spectral analysis of HRV and BPV was conducted to provide component markers of sympatho-vagal balance (see Section 2.2.4.3).

6.3.3.6 Fatigue and quality of life

Standard, validated self-completion questionnaires were used for the assessment of fatigue (Fatigue Impact Scale, FIS) and quality of life (Short Form 12, SF-12).

6.3.4 Exercise intervention

Participants were instructed to perform 30 minutes of cycling (excluding 5 minutes of warming-up and cooling-down) unsupervised on an upright, stationary bicycle ergometer, three times per week, for 16 weeks at a self-regulated workload that achieved a heart rate corresponding to 70-80% of the symptom-limited maximum oxygen uptake, and a Borg RPE score 12-14. Target heart rate for all subjects was monitored using an RS100 watch (Polar Electro, Finland) and adjusted at 8 weeks following completion of a maximal graded cardiopulmonary exercise test. Workload was adjusted continuously during all training sessions to achieve target heart rates. Participants were asked to limit training to the prescribed program, and, although exercise training was unsupervised, all subjects received weekly phone calls to ensure compliance with exercise training, and completed exercise diaries that were regularly reviewed by study investigators.

6.3.5 Statistical analysis

Data are presented as means ± SD for continuous data and as numbers or percentages for categorical data. Continuous data were tested for normality, linearity and homogeneity of covariance matrices, as previously described (see Section 2.2.6). *A priori* power calculation, based on previous implementations within our group of endurance exercise training, with a type I error of 5%, showed that this study had 80% power to detect a clinically significant difference of 10% in the relative change of peak VO_2 following exercise training with a sample size of 10 in each group.

A mixed model multivariate analysis of variance was performed between patients and controls, before and after completion of the exercise intervention. Group (patient or control) and time (baseline or follow up) were respected as factors and physiological parameters were taken as the dependent variables. The interaction between group and time conditions permitted detection of any differences in the response to exercise. Where the mixed model main factors or interaction were statistically significant,

subsequent between group comparisons were made using unpaired Student's *t*-tests or Mann-Whitney U tests and within group comparisons using paired Student's *t*-tests or Wilcoxon signed rank tests. Categorical variables were compared using Fisher's exact test and correlations were executed using Pearson's method.

6.4 Results

6.4.1 Patient group characteristics

The baseline characteristics of 12 patients (11 probands) and 12 control subjects, matched for age, gender and habitual physical activity, are presented in Table 6.1. Although well matched for these characteristics, weight, BMI and BSA were significantly lower in patients than controls. Cardiovascular disease features and relevant medications are included in Table 6.1: five patients had diabetes mellitus and one had treated hypertension. There were no significant differences in current systolic or diastolic blood pressures.

Patient details including specific clinical features, mutation loads and baseline NMDAS, FIS and SF-12 scores are presented in Table 6.2. Disease burden was mild or moderate in all patients with phenotypes consistent with MIDD (five patients), myopathy (three patients), MERRF (two patients), MELAS (one patient) and oligosymptomatic status (one patient). All patients were compared to controls as a group as exclusion of any single phenotypic group, including the two patients with MERRF, harbouring the m.8344A>G mutation, did not alter the presence and magnitude of differences.

The overall frequency of specific clinical features in the patient group are presented in Table 6.3. Fatigue was a clinical feature of mitochondrial disease in seven patients (58%): three patients reported excessive fatigue (FIS score ≥ 40) and three further patients reported severe fatigue (FIS score ≥ 80). No control participants reported clinical fatigue (FIS score <40 in all controls).

Characteristic	Patients (n=12)	Controls (n=12)	*p* value
Age (years)	39.8 ± 11.5	38.0 ± 12.2	0.781
Male sex, n (%)	8 (67)	8 (67)	1.000
Height (cm)	170 ± 10	171 ± 10	0.707
Weight (kg)	60.9 ± 10.2	78.2 ± 13.9	0.003
Body mass index (kg/ m^2)	21.1 ± 3.5	26.7 ± 5.5	0.004
Body surface area (m^2)	1.71 ± 0.17	1.94 ± 0.18	0.010
Habitual physical activity			
Daily energy expenditure (kCal)	2234 ± 675	2567 ± 381	0.119
Daily steps (n)	8112 ± 3768	9090 ± 2508	0.217
Diabetes mellitus, n (%)	5 (42)	0 (0)	N/A
Hypertension, n (%)	1 (8)	0 (0)	N/A
Cardiac clinical parameters			
Heart rate (min^{-1})	75 ± 11	62 ± 12	0.023
SBP (mmHg)	117 ± 12	115 ± 9	0.431
DBP (mmHg)	79 ± 9	77 ± 8	0.184
Selected medications			
ACE inhibitor / ARB	4 (33)	0 (0)	N/A
Beta-blocker	0 (0)	0 (0)	N/A
Calcium channel blocker	1 (8)	0 (0)	N/A
Insulin	3 (25)	0 (0)	N/A
Metformin	2 (17)	0 (0)	N/A
Statin	4 (33)	0 (0)	N/A
Antidepressant	2 (17)	0 (0)	N/A
Co-enzyme Q10	5 (42)	0 (0)	N/A

Table 6.1 Baseline characteristics. SBP systolic blood pressure; DBP = diastolic blood pressure; ACE = angiotensin-converting enzyme; ARB = angiotensin receptor; N/A = not applicable.

Patient	Age	Sex	mtDNA mutation	Urinary mutation load (%)	Clinical features	Clinical scores			
						NMDAS	FIS	SF-12 PHC	SF-12 MHC
1*	39	M	m.3243A>G	80	SNHL, DM, exercise intolerance, ataxia, migraine, GIT, fatigue, hypothyroidism	17	44	38	37
2	58	F	m.3243A>G	59	SNHL, exercise intolerance, ataxia, muscle weakness, GIT, low BMI, myalgia	12	2	56	58
3*	42	M	m.3243A>G	82	SNHL, DM, exercise intolerance, ataxia, migraine, depression	12	9	50	59
4	47	M	m.3243A>G	63	SNHL, DM, exercise intolerance, ataxia, muscle weakness, depression, fatigue, myalgia, PEO, ptosis, neuropathy	28	109	23	29
5	37	F	m.3243A>G	48	SNHL, DM, exercise intolerance, ataxia, mild dysarthria, asthma	10	13	52	53
6	38	F	m.3243A>G	53	SNHL, exercise intolerance, muscle weakness, fatigue, migraine, GIT, asthma	13	29	32	55
7	22	M	m.3243A>G	89	SNHL, exercise intolerance, ataxia, fatigue, migraine, GIT, low BMI, epilepsy	18	62	40	46
8	36	M	m.3243A>G	80	Exercise intolerance, migraine	4	9	57	54
9	50	M	m.3243A>G	87	SNHL, exercise intolerance, ataxia, muscle weakness, fatigue, depression, retinopathy, epilepsy, encephalopathy, cognitive decline, stroke-like episodes	23	68	57	46
10	55	F	m.3243A>G	68	SNHL, DM, exercise intolerance, ataxia, muscle weakness, GIT, depression, retinopathy, PEO, ptosis, short stature, dysphagia, hypertension	25	23	36	49
11	29	M	m.8344A>G	95	SNHL, exercise intolerance, ataxia, GIT, fatigue, muscle weakness, epilepsy, retinopathy, low BMI, dysarthria, neuropathy, myoclonus	55	123	27	25
12	25	M	m.8344A>G	94	Exercise intolerance, ataxia, fatigue, muscle weakness, epilepsy, depression, retinopathy, dysarthria, myoclonus	48	85	28	25

Table 6.2 Disease features of patients. NMDAS = Newcastle Mitochondrial Disease Adult Scale; M = male; F = female; SNHL = sensori-neural hearing loss; DM = diabetes mellitus; PEO = progressive external ophthalmoplegia; GIT = gastro-intestinal tract; BMI = body mass index; FIS = Fatigue Impact Scale; SF-12 = Short-Form 12 Health Survey; MHC = mental health component; PHC = physical health component; * = sibling.

Clinical feature	Number of patients (n=12)
Exercise intolerance	12
Ataxia	10
Sensori-neural hearing loss	10
Fatigue	7
Muscle weakness	7
GIT	6
Depression	5
Diabetes mellitus	5
Migraine	5
Epilepsy	4
Retinopathy	4
Dysarthria	3
Underweight (BMI <18.5)	3
Asthma	2
Myalgia	2
Myoclonus	2
Sensory neuropathy	2
Ophthalmoparesis	2
Ptosis	2
Cognitive decline	1
Dysphagia	1
Encephalopathy	1
Hypertension	1
Hypothyroidism	1
Short stature	1
Stroke-like episodes	1

Table 6.3 Frequency of clinical features. GIT – gastro-intestinal tract involvement; BMI – body mass index

6.4.2 Exercise training

All patients and control subjects completed ≥ 80% of the 48 scheduled training sessions, and no adverse events were reported. All patients remained clinically stable throughout the study, and there were no changes in medication.

Cardio-pulmonary exercise testing responses for patients and controls before and after completion of the exercise training programme are presented in Table 6.4. There was no significant effect of subject status (patient or control) on the response to endurance exercise in any haemodynamic, exercise physiology or cardiac parameter.

6.4.3 Haemodynamic parameters

At peak exercise stroke volume, heart rate, cardiac output, and cardiac index, were all decreased in patients compared to controls (Table 6.4), achieving statistical significance at either baseline or follow up or both time-points for each of these parameters. There were no significant differences between the groups in peak systolic, diastolic or mean arterial blood pressures or in cardiac power output (CPO), expressed either as an absolute value or when indexed to BSA (CPO index). Although there was a trend towards reduction in peak heart rate following exercise training in both groups, this only achieved statistical significance in controls (mean decrease 7 beat per minute, p=0.001).

6.4.4 Exercise physiology

At baseline, peak VO_2, peak arterial-venous oxygen difference (A-VO_2 diff), and peak power were all significantly decreased in patients compared to controls (mean decreases 22%, 30%, 23% and 77% respectively). The anaerobic threshold (AT) at baseline, when expressed as a percentage of predicted peak VO_2, was significantly decreased in patients compared to controls, while absolute values showed a supportive trend in the same direction (Table 6.4). In both patient and control groups, sixteen weeks of endurance exercise training significantly increased peak work capacity (power, 12% and 13% respectively), peak oxygen uptake (VO_2, 16% and 10%), and anaerobic threshold (AT, 23% and 23%) without a significant change in CPO or CPOI. In patients, there was a non-significant trend towards an increase in peak capacity for oxygen extraction (A-VO_2 diff, 12%, p=0.059). Resting venous lactate and creatine kinase were not significantly different at baseline between patients and controls and there was no significant effect of endurance exercise training on these blood parameters, in either group (Table 6.5).

Parameter	Patients			Controls			Interaction
	Baseline	Follow up	*p* value[a]	Baseline	Follow up	*p* value[a]	*p* value[b]
Peak exercise							
Heart rate	167 ± 21	161 ± 18	0.299	184 ± 17	178 ± 14*	0.001	0.921
SBP (mmHg)	183 ± 24	193 ± 21	0.082	188 ± 18	194 ± 16	0.183	0.744
DBP (mmHg)	94 ± 17	93 ± 15	0.880	99 ± 12	89 ± 17	0.069	0.222
MAP (mmHg)	124 ± 15	126 ± 13	0.535	129 ± 10	124 ± 13	0.260	0.258
Stroke volume (ml)	91 ± 23	95 ± 29	0.532	116 ± 20**	118 ± 26	0.774	0.786
Stroke index (ml/m^2)	53± 9	54± 12	0.687	59 ± 9	60 ± 9	0.832	0.695
Cardiac output (l/min)	14.5 ± 3.9	15.1 ± 4.8	0.398	21.3 ± 4.1**	21.0 ± 4.8*	0.946	0.643
Cardiac index ($l/min/m^2$)	8.3 ± 1.5	8.6 ± 2.1	0.681	10.6 ± 1.2**	10.4 ± 1.5	0.828	0.517
VO_2 (ml/min)	1382 ± 625	1596 ± 726	0.030	2276 ± 670**	2525 ± 562*	0.049	0.983
VO_2 (ml/kg/min)	21.4 ± 6.3	24.8 ± 8.9	0.009	27.6 ± 6.3*	30.4 ± 5.9	0.047	0.965
VO_2 (% predicted VO_2)	61 ± 20	72 ± 25	0.011	94 ± 21**	106 ± 26**	0.032	0.882
CPO	4.0 ± 1.3	4.0 ± 1.2	0.708	5.7 ± 1.0**	5.4 ± 1.2*	0.408	0.413
CPOI	2.3 ± 0.5	2.3 ± 0.5	0.732	2.7 ± 0.4	2.8 ± 0.5	0.396	0.294
Power (W)	100 ± 45	112 ± 55	0.044	177 ± 40**	200 ± 39**	0.001	0.621
A-VO_2 diff (ml O_2/dl)	9.4 ± 3.0	10.5 ± 3.1	0.059	10.9 ± 2.9*	11.8 ± 1.9*	0.242	0.736
Anaerobic threshold							
$ATVO_2$ (ml/kg/min)	13.4 ± 5.0	16.5 ± 6.4	0.014	15.4 ± 4.1	18.8 ± 4.1	0.017	0.772
$ATVO_2$ (% predicted peak VO_2)	38 ± 14	47 ± 18	0.221	53 ± 14*	65 ± 19**	0.032	0.665
$ATVO_2$ (% recorded peak VO_2)	61 ± 8	66 ± 5	0.128	56 ± 7	63 ± 5	0.049	0.758

Table 6.4 Cardio-pulmonary exercise parameters before and after exercise training. BMI = body mass index; [a] *p* value represents the within group comparison of paired before and follow=-up time points; [b] *p* value represents group by time interaction from mixed model repeated measures analysis; * $p<0.05$ and ** $p<0.01$ for patients vs controls at equivalent time points.

Parameter	Patients			Controls			Interaction *p* value[b]
	Baseline	Follow up	*p* value[a]	Baseline	Follow up	*p* value[a]	
Body composition							
Weight (kg)	62.1 ± 12.2	62.8 ± 11.8	0.150	81.9 ± 12.9**	81.6 ± 12.8**	0.777	0.909
BMI	21.3 ± 3.4	21.5 ± 3.3	0.154	27.7 ± 4.7**	27.6 ± 4.4**	0.670	0.907
Lean body weight (kg)	46.6 ± 12.7	47.6 ± 12.4	0.056	55.7 ± 9.2	55.8 ± 8.8	0.728	0.944
Fat (% of body weight)	25.7 ± 8.9	25.1 ± 7.6	0.509	31.7 ± 11.1	31.0 ± 10.0	0.421	0.993
Blood analyses							
Lactate	2.1 ± 0.5	2.6 ± 0.9	0.132	2.3 ± 1.0	1.9 ± 0.8	0.648	0.202
Creatine kinase	121 ± 46	143 ± 77	0.404	125 ± 49	124 ± 55	0.958	0.833

Table 6.5 Body composition and blood analyses before and after exercise training. BMI = body mass index; [a] *p* value represents the within group comparison of paired before and follow=-up time points; [b] *p* value represents group by time interaction from mixed model repeated measures analysis; * $p<0.05$ and ** $p<0.01$ for patients vs controls at equivalent time points.

6.4.5 Body composition

Although significant between group differences existed at baseline, there were no significant changes in body weight or BMI in patient or control groups (Table 6.5) in response to exercise training. In patients, there was a non-significant trend towards an increase in lean body weight (mean increase 1.0 kg, p=0.056) following the endurance exercise training intervention.

6.4.6 Cardiac structure and function

Table 6.6 summarizes the cardiac MRI structural and functional parameters for patient and control groups. The means and ranges of control group parameters are in agreement with a large cohort study using quantitative cardiac MRI (Alfakih *et al.*, 2003). Similarly, the descriptive statistics of the patient data concur with the baseline data from the larger group of patients with mitochondrial disease (see Section 5.4.3).

At baseline, end-systolic and end-diastolic cardiac volumes were proportionally decreased in patients compared to controls, with no difference in ejection fraction (Table 6.6). Stroke volume was also decreased in patients: this occurred in association with an increase in heart rate (r=-0.71, p=0.021), with no difference in cardiac output. Endurance exercise had no significant effect on cardiac volumes or global systolic or diastolic function in patients or controls.

LV mass index (LVMI) was significantly increased in patients compared to controls (Table 6.6) at baseline, but remained within the normal range, and LVMI did not fulfil the definition of left ventricular hypertrophy (LVH) in any patient. A significant increase in M/V ratio (57%, p=0.001) suggested this subclinical difference represented concentric remodelling. Cardiac mass increased significantly in both patient and control groups following endurance exercise, with similar proportional sizes of effect: LVM (mean increases 13% and 16% respectively, Figure 6.3), LVMI (13% and 17%), and M/V ratio (5% and 10%). Subject status (patient or control) had no significant effect on the effect of exercise on LVMI (Figure 6.4).

Parameter	Patients			Controls			Interaction p value[b]
	Baseline	Follow up	p value[a]	Baseline	Follow up	p value[a]	
Structure and systolic function							
EDV (ml)	96 ± 21	101 ± 17	0.120	136 ± 25**	139 ± 20**	0.305	0.737
EDI (ml/m^2)	55 ± 8	58 ± 6	0.138	69 ± 10**	71 ± 8**	0.224	0.992
ESV (ml)	38 ± 13	41 ± 13	0.158	56 ± 13**	56 ± 14*	0.972	0.551
ESI (ml/m^2)	22 ± 6	23 ± 6	0.184	29 ± 5*	29 ± 6	0.898	0.646
SV (ml)	58 ± 9	60 ± 6	0.342	79 ± 14**	83 ± 10**	0.354	0.926
SI (ml/m^2)	33 ± 3	35 ± 3	0.340	41 ± 6**	43 ± 5**	0.281	0.537
CO (l/min)	4.3 ± 0.7	4.2 ± 0.7	0.502	5.0 ± 1.0	5.1 ± 1.0	0.755	0.843
CI (l/min/m^2)	2.5 ± 0.2	2.4 ± 0.4	0.419	2.6 ± 0.4	2.7 ± 0.5	0.689	0.528
EF (%)	61 ± 5	60 ± 7	0.515	59 ± 4	60 ± 6	0.489	0.410
LS (%)	14.8 ± 0.2	13.2 ± 0.3	0.213	18.1 ± 0.2**	17.3 ± 0.3*	0.553	0.573
LVM (g)	124 ± 20	140 ± 21	0.003	116 ± 20	134 ± 26	0.001	0.989
LVMI (g/m^2)	72 ± 13	81 ± 10	0.004	59 ± 8*	69 ± 11*	0.002	0.666
M/V ratio (g/ml)	1.35 ± 0.40	1.41 ± 0.26	0.302	0.86 ± 0.07**	0.97 ± 0.11**	0.003	0.620
Diastolic function							
E/A ratio	1.65 ± 0.62	1.45 ± 0.57	0.387	1.65 ± 0.40	1.83 ± 0.43	0.320	0.451
EFP (%)	67.7 ± 6.7	70.7 ± 14.1	0.468	72.7 ± 7.6	73.9 ± 6.4	0.698	0.557
Tagging and strains							
Torsion (°)	9.1 ± 3.5	7.9 ± 1.0	0.337	5.9 ± 1.9*	6.7 ± 1.5*	0.057	0.292
Whole wall circumferential strain (%)	16.4 ± 1.9	16.7 ± 1.4	0.624	17.8 ± 1.8	18.6 ± 3.4	0.435	0.714
Endocardial circumferential strain (%)	20.6 ± 2.0	20.0 ± 2.0	0.353	24.7 ± 2.3**	25.9 ± 5.2*	0.367	0.713
TSR (rad)	0.80 ± 0.38	0.71 ± 0.10	0.555	0.41 ± 0.12**	0.46 ± 0.09**	0.325	0.292
Cardiac high energy phosphates							
PCr/ATP ratio	1.45 ± 0.42	1.61 ± 0.39	0.260	1.95 ± 0.34**	1.97 ± 0.37*	0.907	0.749

Table 6.6 (overleaf) Cardiac parameters before and after exercise training. EDV = end-diastolic volume; ESI = end-diastolic index; ESV = end-systolic volume; ESI = end-systolic index; SV = stroke volume; SI = stroke index; CO = cardiac output; CI = cardiac index; EF = ejection fraction; LS = longitudinal shortening; LVM = left ventricular mass; LVMI = left ventricular mass index; M/V ratio = ratio of left ventricular mass to volume; E/A ratio = ratio of early to late ventricular filling velocity; EFP = early filling percentage; TSR = torsion to (endocardial) strain ratio; PCr = phosphocreatine; ATP = adenosine triphosphate; [a] *p* value represents the within group comparison of paired before and follow=-up time points; [b] *p* value represents group by time interaction from mixed model repeated measures analysis; * $p<0.05$ and ** $p<0.01$ for patients vs controls at equivalent time points.

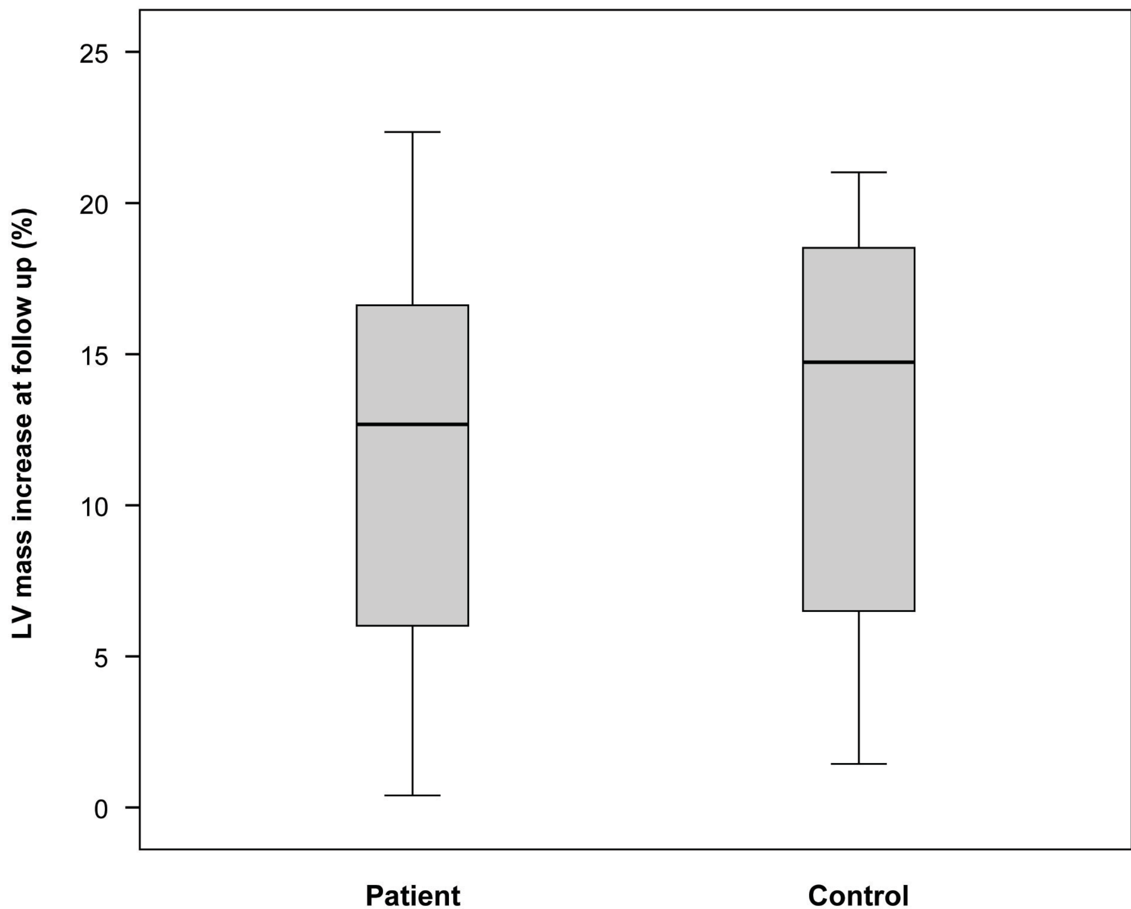


Figure 6.3 Box plot of the proportional increase in LV mass with exercise training. Box-plot showing the range and quartiles of the increase in LV mass between baseline and 16-week follow up cardiac MRI assessment, after endurance exercise training, expressed as a percentage of the baseline assessment in patient and control groups. LV = left ventricular.

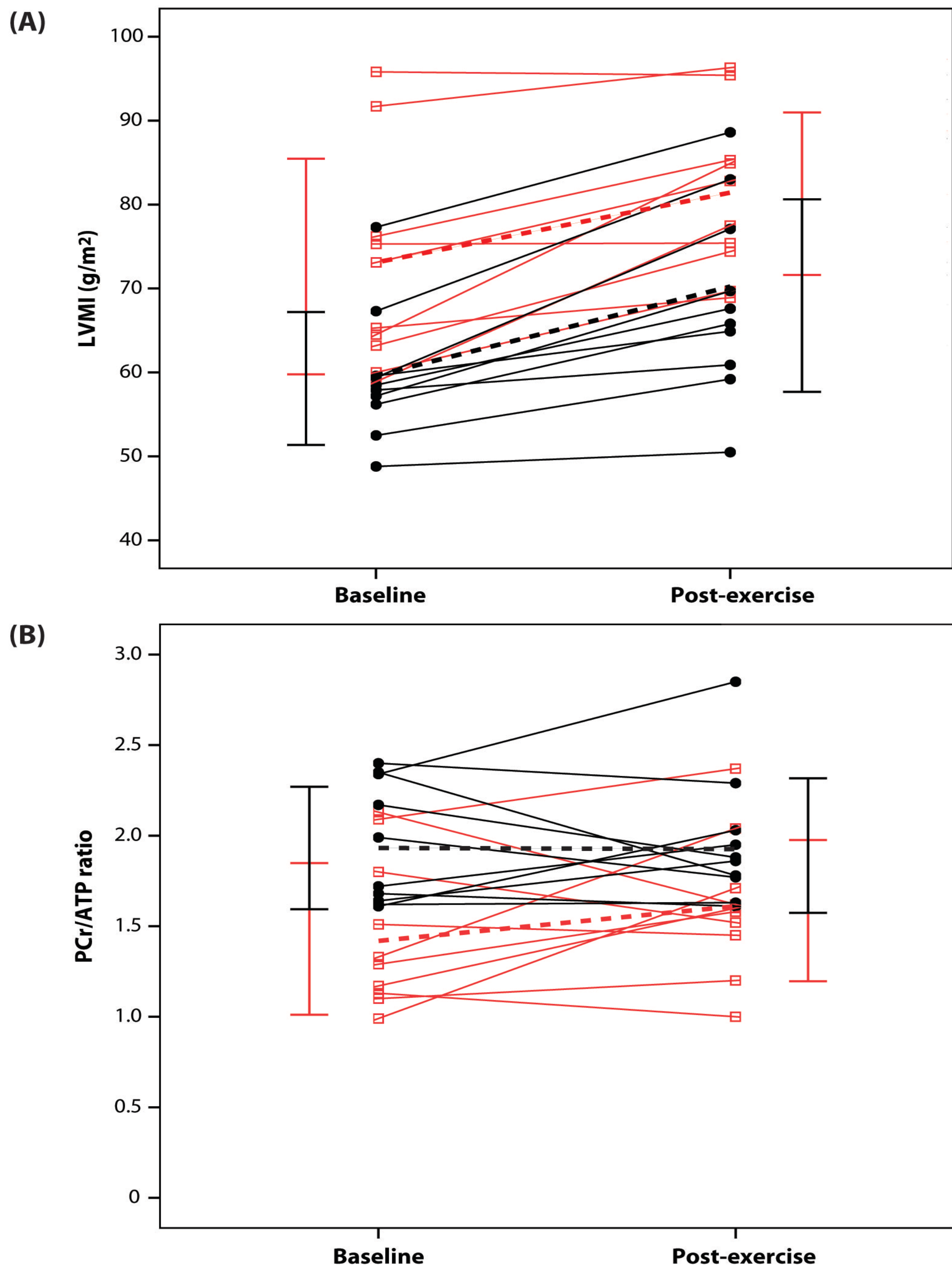


Figure 6.4 Spaghetti plot of the effects of exercise training on LV mass and bioenergetics. The change in (A) LVMI and (B) PCr/ATP ratio in patients (red, open squares) and controls (black, closed circles) are shown. Solid lines represent participants; dotted lines represent mean group values; vertical bars show standard deviation at baseline (left) and following exercise training (right). LVMI = left ventricular mass index; PCr = phosphocreatine; ATP = adenosine triphosphate; baseline = initial assessment; post-exercise = follow up assessment after completion of 16 weeks' exercise training; LV = left ventricular.

6.4.7 Cardiac tagging and strains

At baseline, there was evidence of altered myocardial strains in patients compared to controls, consistent with previous results (see Section 5.4.4) and the presence of subclinical concentric remodelling (Table 6.6): longitudinal shortening was significantly decreased (18%) in patients and correlated significantly with increased LVMI ($r=-0.61$, $p=0.03$). Peak torsion was increased (54%) and endocardial circumferential strain was decreased (17%) in patients compared to controls, with a subsequent significant increase in their ratio, the TSR (95%).

No significant differences were detected between groups in diastolic function represented by E/A ratio and the early filling percentage. There were no significant effects of exercise on myocardial strains or torsion in either patients or control.

6.4.8 Myocardial bioenergetics

At baseline, PCr/ATP ratio was decreased in patients compared to controls (mean decrease 26%, $p=0.002$, Table 6.6). There were no significant correlations between PCr/ATP ratio and markers of disease burden, myocardial mass or cardiac function. Seven patients (58%) but no controls had an abnormal PCr/ATP ratio (<1.6) (Neubauer *et al.*, 1992); there was no significant difference in markers of disease burden, cardiac morphology or function between patients with PCr/ATP ratio >1.6 and those <1.6.

There was no significant effect of exercise training on the PCr/ATP ratio in patients or controls (Figure 6.4), and no significant difference in the response to exercise between the groups (Table 6.6). There was a trend towards an increase in the PCr/ATP ratio in patients following exercise training (mean increase 11%, $p=0.260$), such that the lower PCr/ATP values in patients compared to controls were less evident following exercise training (mean decrease 18%, $p=0.048$, Table 6.6) than at baseline. PCr/ATP ratio increased in six patients and decreased in four patient following exercise training, such that at follow-up four patients (40%) had an a ratio <1.6 (Figure 6.4). Representative examples of ^{31}P MR spectra are shown in Figure 6.5.

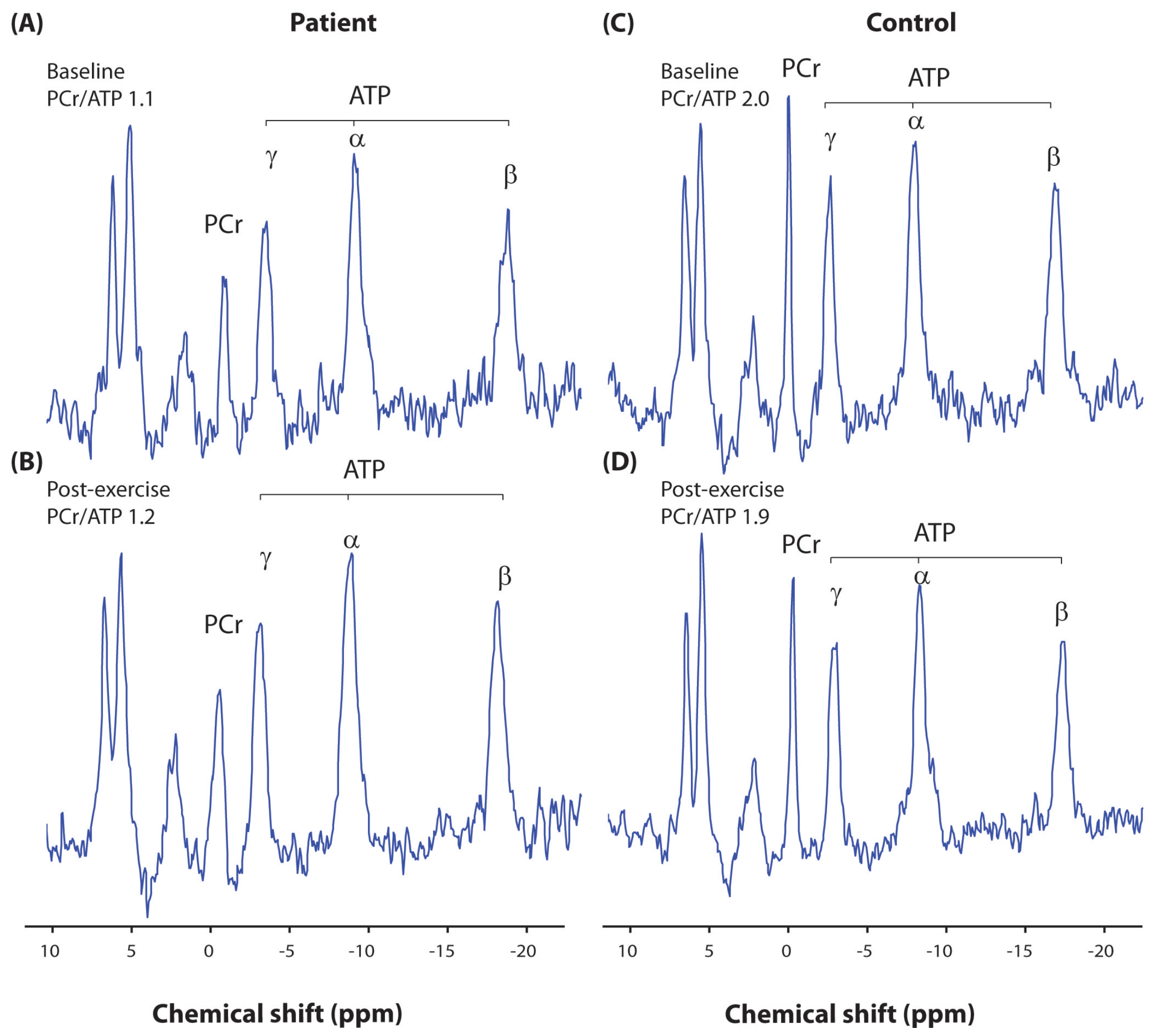


Figure 6.5 Phosphorus-31 magnetic resonance spectroscopy. Representative spectra from a patient carrying the m.3243A>G mutation (left) and a matched control subject (right) at baseline (upper panels) and following completion of 16 weeks' exercise training (lower panels) showing a difference in PCr concentration that is unaffected by exercise. Spectra from patient 3 (A) at baseline with PCr/ATP ratio 1.1, and (B) following exercise training with PCr/ATP 1.2. are displayed alongside spectra from the matched control participant (C) at baseline with PCr/ATP 2.0, and (D) following exercise training with PCr/ATP 1.9. Spectra are presented as acquired before correction for heart rate, flip angle and blood content. PCr = phosphocreatine; ATP = adenosine triphosphate; ppm = parts per million.

6.4.9 Quality of life and disease burden

At baseline, patients had NMDAS scores averaging 16 ± 7, which correlated with FIS score (r=0.784, *p*=0.007), but not urinary m.3243A>G mutation load (r=0.465, *p*=0.176) or SF-12 scores. (r=0.234, *p*=0.477). After exercise training these was no significant change in NMDAS or SF-12 quality-of-life scores in patients (Table 6.7).

Score	Baseline	Follow-up	*p* value
NMDAS	16 ± 7	15 ± 7	0.269
SF-12 MHC	50 ± 9	48 ± 14	0.673
SF-12 PHC	45 ± 12	44 ± 12	0.434

Table 6.7 Disease burden and quality of life before and after exercise training. NMDAS = Newcastle Mitochondrial Disease Adult Scale; SF-12 = Short Form 12 Health Questionnaire; MHC = mental health component; PHC = physical health component.

6.4.10 Fatigue and autonomic function

FIS scores, both at baseline and after exercise training, were significantly increased in patients compared to controls (Table 6.8), but there was no significant effect of exercise on FIS score in either group; the proportion of patients reporting excessive or severe fatigue at baseline (40%) was unchanged following exercise training.

Consistent with the significant elevation in resting heart rate, mean RR interval was significantly decreased in patients compared to controls (Table 6.8). LF:HF-RRI was significantly increased (mean increase 79%, *p*=0.03) in patients compared to controls, providing evidence of a shift in sympatho-vagal balance, with effects predominantly driven through an increase in sympathetic function (LFnu-RRI). Total diastolic BPV (PSD-DBP) was significant decreased in patients compared to controls both before and after exercise training (Table 6.8). A significant shift in sympatho-vagal balance was again evident in patients compared to controls (LF:HF-DPB), but driven here by a decrease in high frequency components, representing parasympathetic function (HFnu-DBP). There was no significant effect of exercise training on any HRV or BPV parameter in either group.

Parameter	Patients			Controls			Interaction *p* value[b]
	Baseline	Follow up	*p* value[a]	Baseline	Follow up	*p* value[a]	
Heart rate variability							
Mean RRI (ms)	808 ± 101	854 ± 112	0.220	891 ± 102*	948 ± 119*	0.128	0.568
LFnu-RRI	68 ± 18	64 ± 19	0.711	54 ± 12**	48 ± 17*	0.086	0.591
HFnu-RRI	46 ± 18	48 ± 19	0.711	49 ± 22	58 ± 25	0.086	0.591
PSD-RRI	1472 ± 923	1976 ± 979	0.083	1248 ± 824	2084 ± 1094	0.051	0.769
LF:HF-RRI	3.4 ± 0.9	2.8 ± 1.5	0.249	1.9 ± 1.7*	1.9 ± 1.8	0.994	0.380
Blood pressure variability							
LFnu-SBP	47 ± 9	44 ± 14	0.613	42 ± 13	38 ± 19	0.274	0.843
HFnu-SBP	18 ± 10	20 ± 15	0.729	26 ± 8	28 ± 11	0.950	0.852
PSD-SBP	9.0 ± 2.7	11.8 ± 7.1	0.169	10.9 ± 6.3	12.5 ± 9.8	0.716	0.543
LF:HF-SBP	3.9 ± 1.8	3.7 ± 2.7	0.330	3.0 ± 2.6	2.8 ± 3.5	0.830	0.503
LFnu-DBP	51 ± 14	47 ± 15	0.317	48 ± 15	43 ± 17	0.382	0.969
HFnu-DBP	13 ± 9	18 ± 11	0.081	20 ± 11*	29 ± 13**	0.006	0.767
PSD-DBP	4.9 ± 2.0	6.0 ± 3.7	0.088	7.1 ± 2.4*	10.6 ± 5.6**	0.341	0.430
LF:HF-DBP	6.1 ± 2.5	5.6 ± 2.1	0.278	4.2 ± 2.1*	3.9 ± 2.5	0.278	0.127
Fatigue							
FIS score	37 ± 34	34 ± 37	0.350	9 ± 11*	5 ± 8**	0.087	0.928

Table 6.8 Autonomic parameters and fatigue before and after exercise training. FIS = Fatigue Impact Scale; LF = low frequency; HF = high frequency; SBP / DBP = systolic / diastolic blood pressure; nu = normalized units; PSD = power spectral density; RRI = RR interval.

6.5 Discussion

The principal findings of this study of the effects of 16 weeks' endurance exercise training in patients harbouring either the m.3243A>G or the m.8344A>G mutation compared to controls are: 1) reduced exercise capacity at baseline, with predominate limitations mediated through peripheral skeletal muscle, rather than central cardiac, factors; 2) similar proportional benefits of training on cardio-pulmonary and haemodynamic parameters including peak work capacity, peak oxygen uptake and anaerobic threshold, with no evidence of disease progression or increased fatigue; 3) similar physiological changes in cardiac mass following exercise training, with no deterioration of global systolic or diastolic dysfunction or myocardial bioenergetics, despite the presence of subclinical concentric remodelling at baseline; 4) increased sympathetic and decreased parasympathetic cardiovascular autonomic activity pre- and post-exercise training suggesting a role for sympathetic over-activation in disease pathogenesis. I have determined that endurance exercise training is safe in patients harbouring the m.3243A>G or m.8344A>G mutations, leading to improvements in clinical parameters of a similar magnitude and direction to those observed in untrained, sedentary controls.

6.5.1 Exercise capacity and skeletal muscle

Consistent with a previous case-control study of patients with mitochondrial disease (Jeppesen *et al.*, 2006), peak oxygen consumption, peak arterial-venous oxygen difference and peak work capacity at baseline were significantly decreased in patients compared to untrained, sedentary controls, with proportional reductions in peak exercise haemodynamic parameters. Despite these facts, all patients completed the 16-week endurance exercise training intervention (≥ 80% of the 48 scheduled sessions) and no adverse events were reported. Participants remained medically stable throughout the study with no deleterious changes in markers of disease burden, skeletal muscle dysfunction or quality of life.

Limitations in exercise parameters at baseline in patients were not reflected in indexed peak cardiac power output, which was not statistically different to controls. This suggests that the reduction in maximal exercise capacity and the associated symptom

of exercise intolerance in patients with the m.3243A>G or the m.8344A>G mutation is related to reduced peripheral ability of the skeletal muscle to extract oxygen during exercise, due to mitochondrial impairment, rather than to a central cardiac limitation. This potential mechanism is consistent with previous studies that have reported preservation, or even elevation, of skeletal muscle oxygen delivery (Haller *et al.*, 1989; Bank and Chance, 1994; Ozawa *et al.*, 1995; Vissing *et al.*, 1996; Abe *et al.*, 1997; Bank and Chance, 1997; Taivassalo *et al.*, 2001; Jensen *et al.*, 2002; Taivassalo *et al.*, 2002; Taivassalo *et al.*, 2003; Jeppesen and Kiens, 2012), endothelial function, and oxygen unloading mechanisms (Jeppesen and Kiens, 2012) in similar cohorts of patients with mitochondrial disease. A similar blunted response to exercise resulting from limited peripheral muscle oxygen extraction has been reported in other disease states, where skeletal muscle dysfunction, and indeed peripheral mitochondrial abnormalities, have been implicated in the aetiology of reduced exercise capacity, including stroke (Jakovljevic *et al.*, 2012), heart failure (Mancini *et al.*, 1989; Mancini *et al.*, 1992; Harrington *et al.*, 1997), chronic obstructive pulmonary disease (COPD) and chronic renal failure (Troosters *et al.*, 2004).

Interestingly, while the anaerobic threshold of patients in my study was reduced compared to controls when expressed relative to *predicted* peak oxygen consumption, there was no significant difference in the absolute values or those relative to the *actual* peak oxygen consumption between the groups. This implies that skeletal muscle oxidative consumption may be similar between the groups, suggesting that peripheral factors other than mitochondrial oxidative metabolism may contribute to the reduced exercise tolerance seen in patients with mitochondrial disease. Biopsy studies have previously demonstrated an increase in skeletal muscle oxidative capacity and respiratory chain enzymatic complex activities with exercise training in patients with mitochondrial disease (Jeppesen *et al.*, 2006; Murphy *et al.*, 2008), but further interventional studies would be necessary to examine other skeletal muscle factors in this patient group.

Endurance exercise training improved peak work capacity, peak oxygen consumption and anaerobic threshold in both patient and control groups. This expected result is similar to previous aerobic exercise training studies in patients with mitochondrial disease (Cejudo *et al.*, 2005; Jeppesen *et al.*, 2006; Taivassalo *et al.*, 2006; Murphy *et*

al., 2008), and occurred without an improvement in CPO or indexed CPO. This further suggests that training improvements in exercise capacity, as well as the baseline restrictions in these parameters, are manifestations of skeletal muscle rather than cardiac involvement in mitochondrial disease. The trend towards an improvement in peak arterio-venous oxygen difference at peak exercise in patients following a 16 week aerobic exercise training intervention suggests an increased ability of the skeletal muscle to extract and utilise oxygen during aerobic exercise as a result of the training programme. The ability of skeletal muscle to extract oxygen during exercise represents an important and physiologically-relevant surrogate marker of skeletal muscle mitochondrial function, and my data suggest that although this parameter is blunted in patients with mitochondrial disease, it can be improved by exercise training and that the magnitude of this improvement is similar to that in untrained, sedentary controls. Similarly, despite a much lower weight at baseline, patients showed a trend towards an increase in lean body mass following exercise training, which may represent morphological changes in skeletal muscle. Importantly, although all patients in this study reported the clinical feature of exercise intolerance, and fatigue was noted in half, there was no deleterious effect of exercise training on fatigue or reported quality of life. This finding occurred despite an *increase* in exercise capacity in patients, suggesting a clinical benefit in this group.

6.5.2 Endurance exercise and cardiac remodelling

As already demonstrated in this thesis, a similar cohort of patients harbouring the m.3243A>G or m.8344A>G mutations, without known cardiac involvement on standard screening, displayed evidence of sub-clinical hypertrophic remodelling compared to age- and gender-matched controls (Bates *et al.*, 2013; Hollingsworth *et al.*, 2012). The baseline results in the current smaller study group support these findings with decreased blood pool volumes, increased LVMI and M/V ratio, and subtle abnormalities of systolic myocardial strains in patients compared to controls (see Section 5.4). The direction and magnitude of exercise-induced changes in LVM, LVMI and M/V ratio in both patient and control groups are consistent with normal physiological responses to aerobic exercise in untrained healthy controls (Douglas *et al.*, 1997; Pelliccia *et al.*, 1999; Scharhag *et al.*, 2002). Importantly, there was no significant effect of group status on the proportional degree of hypertrophic

remodelling observed: patients and controls had similar responses to exercise. I found no significant effect of a 16-week endurance exercise training programme on left ventricular volumes or cardiac systolic or diastolic function, but acknowledge that these additional physiological changes may not have been appreciated in this time frame.

Consistent with the larger study group in this thesis (see Section 5.4.6), abnormal cardiac bioenergetics at baseline were present in patients harbouring the m.3243A>G and m.8344A>G mutations (Bates *et al.*, 2013). In mitochondrial disease and other forms of inherited hypertrophic cardiomyopathy, several groups have suggested the primacy of bioenergetic defects by detection of abnormalities in mutation carriers without evidence of LVH (Ashrafian *et al.*, 2003; Crilley *et al.*, 2003; Lodi *et al.*, 2004). Given that I also detected significant differences in cardiac remodelling, known itself to cause a reduction in PCr/ATP ratio, I cannot comment on the temporal relationship of these findings, but a reduced ratio has already been demonstrated to have prognostic importance in a variety of forms of cardiomyopathy (Neubauer *et al.*, 1992). Importantly therefore, I was able to demonstrate, using ^{31}P cardiac MRS, that endurance exercise training had no further deleterious effect on the abnormal myocardial bioenergetics in my patients, despite the expected and modest physiological increase in LVMI. Indeed, following aerobic exercise training, *fewer* patients had an abnormal PCr/ATP ratio and the decreased mean ratio of the patient group was less evident compared to controls. Taken together, these morphological, functional and bioenergetic results suggest that 16 weeks' endurance exercise training has no deleterious effects on the heart in patients with mitochondrial disease due to the m.3243A>G mutation. Given previous demonstrations of the beneficial effects of endurance exercise on exercise tolerance, quality of life and skeletal muscle function in this cohort of patients, this important result should enable more widespread adoption of a proven treatment in a population with currently limited therapeutic options.

6.5.3 Skeletal muscle oxidative capacity and sympathetic activation

Significant differences between patients and controls in resting heart rate and mean RR interval together with directionally opposing changes in low and high frequency components of both HRV and diastolic BPV provide evidence of a shift in the

sympatho-vagal balance in patients harbouring mt-tRNA mutations. This finding is supported by an increased LF:HF ratio for both RR interval and diastolic blood pressure, suggesting increased sympathetic activity and a parallel reduction in parasympathetic function in patients. This is consistent with previous observations of resting sympathetic over-activity both in small studies of patients with mitochondrial disease and in larger cohorts of patients with other chronic diseases characterised by similar skeletal muscle dysfunction and exercise intolerance, such as heart failure, essential hypertension, COPD, end-stage renal disease and obesity (Converse *et al.*, 1992; Middlekauff, 2010).

Chronic sympathetic activation is recognised as detrimental contributing to vasoconstriction, tissue hypoxia, inflammation, oxidative stress, and impairment of muscle proton homeostasis; all factors that may lead to increased skeletal myopathy, hypertension and elevated cardiovascular risk (Syme *et al.*, 1991; Jones *et al.*, 2010; Middlekauff, 2010). The mechanisms underlying the effects of chronic sympathetic activation are largely unknown, but sympathetic outflow to skeletal muscle, which is dependent on both baroreceptor and chemoreceptor regulation, is heightened selectively in patients with other chronic diseases (Middlekauff *et al.*, 1994; Grassi *et al.*, 1998; Hering *et al.*, 2007; Park *et al.*, 2008). A hyperadrenergic state in patients with mitochondrial disease has been indicated by increased plasma concentration of noradrenaline in patients (Jeppesen and Kiens, 2012), and the prevalence of sympathetic symptoms in this population (Parsons *et al.*, 2010). It has been postulated that over activity of skeletal muscle somatic afferents maintains an increased sympathetic drive both during exercise and at rest, contributing to skeletal myopathy (Clark *et al.*, 1996). These nerve fibres, which mediate the exercise pressor reflex, include metaboreceptors that are sensitive to ischaemic metabolites during exercise, including lactic acid, and mechanoreceptors that are primarily sensitive to stretch. Yet it has been shown that exercise intolerance and sympathetic activation are independent of lactic acidosis in patients with mitochondrial disease (Vissing *et al.*, 1998; Vissing *et al.*, 2001).

I propose that the increased sympathetic tone evident at baseline in patients, and the exaggerated neurovascular responses to exercise, with increased heart rate at equivalent cardiopulmonary work capacities and time intervals, may be at least partly

due to increased skeletal muscle afferent mechanoreceptor sensitivity as seen in other chronic diseases with sympathetic over activity and exercise intolerance (Smith *et al.*, 2006). Indeed my data also suggests a physiological up regulation of parasympathetic activity in patients in response to exercise training, similar in magnitude to the effect in control subjects. Further evaluation of the role of autonomic dysregulation and impact on disease burden, phenotypic expression and exercise intolerance in patients with mitochondrial disease is warranted. Such studies may help to discern the major limitations of exercise capacity in other chronic diseases associated with skeletal myopathy, in which mitochondrial dysfunction has been implicated, directing future therapeutic interventions.

6.5.4 Limitations

Although this study is the largest investigation of cardiac adaptations to exercise performed to date in this patient group, it remains limited in sample size, and duration, and was not designed to investigate pathogenetic mechanisms or disease progression. Cardiac involvement in mitochondrial disease is linked to clinical outcomes, yet I acknowledge that the prognostic importance of the changes I describe must be determined through longitudinal studies. I studied a relatively homogenous cohort of patients, harbouring common mtDNA point mutations, without known cardiac involvement: such patients account for ~25% of our specialist clinic attendees yet I recognize that my findings may not be generalizable to all patients with mtDNA point mutations. Finally, in an already extensive protocol investigating cardiovascular autonomic function, I did not assess baroreceptor sensitivity or the response to postural challenges.

6.5.5 Conclusions

These data represent the first comprehensive evaluation of the cardiac safety and efficacy profile of 16 weeks' aerobic, endurance exercise training in patients with m.3243A>G-and m.8344A>G-related mitochondrial disease. I have determined that cardiac and haemodynamic adaptations to exercise intervention do not relate to disease progression, but rather are comparable to physiological changes in untrained, sedentary controls and hold potential therapeutic benefits for patients with

mitochondrial disease. I also provide evidence of altered sympatho-vagal balance with sympathetic over-activation in patients harbouring common mt-tRNA mutations. This represents a potential therapeutic target for pharmacological intervention and improved vagal tone with exercise. Identification of an effective intervention that could slow or reverse progression of skeletal muscle involvement in mitochondrial disease has the potential to achieve significant health gains with a substantial impact on patient health and well being.

Chapter 7.

Synopsis

7.1 Introduction

The central theme of the studies comprising this thesis is accurate characterisation of the cardiac manifestations of mitochondrial disease. I have studied the cardiac phenotype in this cohort of patients to enable accurate diagnosis and evidence-based management of a condition that has been linked to significant morbidity and early mortality. In this synopsis, principal findings will be summarised, limitations will be acknowledged, and clinical relevance and future work will be discussed. A particular strength of this thesis is that I have employed a wide variety of research techniques to explore the cardiac manifestations of mitochondrial disease, applying both prospective and retrospective approaches, observational and interventional protocols, and clinical and tissue-based analyses. Information gathered from diverse sources enables important conclusions to be drawn. Using a comprehensive approach, I have demonstrated that:

1) cardiac involvement is common in patients with mitochondrial disease;

2) the cardiac phenotype is extremely variable, yet patterns of disease exist within genotypic groups;

3) cardiomyopathy has a significant effect on clinical outcomes, and individual patient factors can impact on the likelihood of cardiac involvement;

4) respiratory chain complex I deficiency may be the primary defect in cellular pathogenesis of mt-tRNA mutations;

5) altered sympatho-vagal balance with sympathetic over-activation provides a novel target for treatment in mitochondrial disease; and

6) early responses to endurance exercise training and cardiac transplantation are similar in patients with mitochondrial disease to those in the equivalent, general population.

7.2 Summary of major findings

7.2.1 Cardiac involvement in mitochondrial disease

Over three decades, isolated case reports, case series and cohort studies have identified extremely varied cardiac abnormalities in patients with mtDNA mutations. Using a variety of difference experimental approaches, my studies have confirmed the apparently unlimited diversity of the cardiac phenotype in this cohort of patients – from the absence of cardiac involvement to early mortality due to cardiovascular disease; from isolated, stable ECG abnormalities to a requirement for invasive procedures for brady- and tachy-arrhythmia; and from asymptomatic LVH to end-stage cardiomyopathy in patients undergoing transplantation. Indeed unpredictability both of morphology and natural history appeared initially to be the only defining characteristic of cardiac involvement in mtDNA disease. Yet patterns have emerged with supportive evidence from diverse aspects of this thesis.

7.2.1.1 Frequency of cardiac involvement

With robust assessment of clinical details in a large retrospective cohort, I have shown that cardiac involvement is present in ~14% patients with undifferentiated mitochondrial disease. Although lower than the reported incidence in earlier studies from specific genotypic groups or cohorts of more severely affected individuals (Anan *et al.*, 1995; Holmgren *et al.*, 2003; Limongelli *et al.*, 2010), this value is perhaps reflective of the broader spectrum of mitochondrial disease that is now seen among patients attending specialist clinics, following utilisation of a case-finding approach from probands. Such frequency mandates specific cardiovascular study of these patients, and highlights the importance of identification of features that are specific or predictive of cardiac involvement to tailor management and treatment decisions.

In discussing the frequency of any clinical finding, it is important to recognise the impact of external factors on natural history studies. With evolving understanding and definitions of mitochondrial disease, the severity and frequency of mtDNA-related cardiomyopathy may have been initially over-estimated from small case series. Similarly, modern imaging techniques have been instrumental in probing diverse forms of cardiomyopathy and have resulted in the identification of abnormalities in individuals *without* pre-existing cardiac phenotypes. Data from the magnetic

resonance imaging study (Chapter 5), conducted in a cross-sectional cohort of patients harbouring the m.3243A>G or m.8344A>G mutation, suggest that the retrospective study of cardiac involvement (Chapter 3), reliant as it was on echocardiographic imaging, potentially underestimated the true extent of cardiac involvement. Although this may be true in the broadest definition of cardiac involvement, and use of the most sensitive imaging modality at diagnosis may be relevant to phenotypic classification, patients are routinely followed up with echocardiography and this will remain the case in the future. Moreover early data from a coordinated, national prospective study of this patient group (Nesbitt *et al.*, 2013), and extensive clinical experience actually support my retrospective incidence data, and, in the real-life clinical management of a condition without uniformly present *or* predictable cardiac involvement, potential therapeutic interventions will only be used on recognition of disease using standard investigations. While modern imaging techniques and indexation of cardiac parameters will modify the cardiac phenotype in some patients and are important to use for accurate phenotypic descriptions, it is reassuring to recognise the general agreement between echocardiographic and MRI parameters in this patient group and the suitability of *indexed* echocardiography as a surveillance technique (Chapter 5).

7.2.1.2 Nature of cardiac involvement

Several different studies in this thesis have aimed to refine the cardiac phenotype in mitochondrial disease. While confirming the inevitably broad range of cardiac manifestations, some common themes do emerge from these diverse studies. For example, although clinical symptoms with a potential cardiac aetiology, such as fatigue, exercise intolerance, and lethargy do occur frequently in patients with mitochondrial disease, they are most often attributable to significant skeletal muscle involvement or manifest autonomic dysfunction. In children with end-stage cardiomyopathy (Chapter 4) and adult patients with more mild clinical cardiac disease (Chapter 3), classical symptoms of cardiomyopathy or arrhythmia such as chest pain, palpitation, presyncope, syncope or exertional dyspnoea occur infrequently. This is perhaps not unexpected in a condition with potential multisystem involvement, but many patients with isolated organ cardiac disease are also relatively oligosymptomatic from a cardiac viewpoint until late in disease severity and this highlights the importance of screening. Importantly in a paediatric population, I have demonstrated

mitochondrial respiratory chain disease in children from age 7 months to 15 years (Chapter 4), and in an adult population, subtle abnormalities of cardiac morphology in patients from 22 to 58 years of age (Chapter 5), emphasising the diverse age range of patients presenting with mitochondrial disease and the life-long need for screening investigations.

7.2.1.3 Electropathy

The 12-lead ECG is the most frequently encountered abnormal cardiac investigation in patients with mitochondrial disease but also, perhaps, one of the least specific for the condition. Minor abnormalities were very common and appeared of little significance. During the retrospective review of existing clinical cardiac data, for example, there were very few patients with evidence of significant progression over time; several patients had pre-existing abnormalities of atrio-ventricular or interventricular conduction but an extremely small number underwent interventions for high-grade AV block over a prolonged period of monitoring; similarly a minority of patients underwent radio-frequency ablation for re-entrant arrhythmia but only one patient developed more significant cardiac involvement during follow up (end-stage cardiomyopathy). While some of these findings may be a reflection of the retrospective nature of study and limitations of non-uniform data collection, the rate of progression of ECG abnormalities appears relatively slow and unlikely to have a significant impact on clinical outcomes for the cohort as a whole. Nevertheless an ECG is a simple investigation and, as with other multisystem neuromuscular conditions with prominent cardiac involvement, the rate of progression may be difficult or impossible to predict in individual patients and pragmatic clinical decisions should be made.

A relative sinus tachycardia (baseline heart rate >90bpm) in many patients with mitochondrial disease is a novel finding in this thesis, and as highlighted in Chapter 6, may point to an underlying aspect of disease pathogenesis. Both the retrospective data (Chapter 3) and baseline ECGs from the cross-sectional (Chapter 5) and interventional (Chapter 6) studies in patients with m.3243A>G or m.8344A>G mutations reported this finding. Confirmatory evidence from heart rate and blood pressure variability analysis supported an imbalance of sympatho-vagal control that is worthy of further investigation in terms of patient symptoms and the potential for therapeutic intervention. In terms of ventricular tachyarrhythmia, I found little

objective evidence of an increased predisposition, independent of co-existing cardiomyopathy in these studies. One patient, included in the post-mortem analysis of cardiac tissue (Chapter 3), had died suddenly and unexpectedly but had a significant history of epilepsy and no clinical cardiac disease despite significant abnormalities of respiratory chain enzyme complex in cardiac tissue. Similarly the retrospective study of cardiac data did not support a high risk of sudden cardiac death in patients with mitochondrial disease in the absence of cardiomyopathy. Clearly prospective studies will help to address this issue and future cellular models may enable investigation of the impact of mitochondrial respiratory chain disease on cardiac cellular electrophysiology, but the clinical risks of ventricular arrhythmia appear similar to the general population.

7.2.1.4 Cardiomyopathy

The presence of cardiomyopathy in patients with mitochondrial disease is associated with decreased survival from the time of symptom onset. This important finding from the retrospective cardiac data study (Chapter 3) was supported by other studies in this thesis: patients harbouring the m.3243A>G or m.8344A>G mutations had abnormal cardiac bioenergetics, displaying a reduction in the PCr/ATP ratio that has prognostic importance in diverse forms of cardiomyopathy (Chapter 5); the concentric remodelling evident in these patients was accompanied by subtle abnormalities of myocardial strain and function consistent with exaggerated cardiac ageing; and all patients in the post-mortem study had evidence of mitochondrial respiratory enzyme complex deficiency in cardiac muscle (Chapter 3). Mitochondrial cardiomyopathy is an entity worthy of further investigation to enable effective treatment for patients.

By focussing attention on well-defined and well-characterised genotypic groups, I have been able to confirm previous suggestions that patients harbouring mt-tRNA mutations most commonly display a hypertrophic phenotype of cardiomyopathy (Chapter 3) and, supporting this assertion, that additional features of abnormal strain and torsion occur with early hypertrophic remodelling in this group, even in patients without clinical evidence of cardiac involvement (Chapters 5 and 6). Unfortunately these features of subendocardial dysfunction appear consistent with other forms of hypertrophic remodelling in cardiomyopathy, and indeed normal ageing, and not specific to mitochondrial disease. Hypertrophic cardiomyopathy does appear, never

the less, to be an intrinsic feature of mitochondrial disease, unrelated to the presence of diabetes in the clinical phenotype or co-incident hypertension.

In contrast, in children with end-stage cardiomyopathy, dilated cardiomyopathy appeared a more common morphological manifestation of cardiac involvement in mitochondrial disease (Chapter 4). While admittedly a small sample size, this finding may be a reflection of the paediatric nature or disease severity of selected cohort of patients; it is a pertinent reminder that diverse forms of cardiomyopathy occur and both disease progression and cardiac morphology can be unpredictable. Previous studies had suggested that a hypertrophic pattern of cardiomyopathy was more common in children with mitochondrial disease (Holmgren *et al.*, 2003; Scaglia *et al.*, 2004).

7.2.1.5 Factors predictive of cardiac involvement

Univariate and multivariate analysis of the retrospective cohort revealed age of onset of symptoms, genotypic group and skeletal muscle mutation load to have a potential impact on the presence of cardiac involvement (Chapter 3). My prospective analysis of cardiac involvement in patients harbouring the m.3243A>G or m.8344A>G mutations suggested that concentric remodelling and abnormalities of myocardial strain and function were closely related to urinary mutation load and disease burden. These conclusions from different aspects of my work are particularly consistent as urinary mutation load is a significant predictor of clinical outcome and linked to skeletal muscle mutation load (Whittaker *et al.*, 2009), and an earlier age of onset of symptoms in a progressive condition, is compatible with an increased disease burden at any time point. Despite evidence of tissue segregation, in my studies cardiac muscle mutation load was related to skeletal muscle heteroplasmy for both the m.3243A>G and m.8344A> mutations. While the utility of a non-invasive technique, such as urinary mutation load, to focus the attention of screening investigations is undoubtedly attractive, doubt remains as to whether this would be practical since: the importance of skeletal muscle mutation load as an independent predictor of cardiac involvement was not borne out by multivariate analysis; the relationship of urinary mutation load to cardiac mtDNA heteroplasmy levels and clinical cardiac outcomes remains to be determined in prospective study; and, in any case, mutation load in cardiac muscle itself does not appear to be the only factor in dictating cardiac involvement. While

more effective non-invasive biomarkers may emerge for mitochondrial disease (Suomalainen *et al.*, 2011), no analyses for cardiac disease yet provide adequate sensitivity or specificity.

My contention that certain genotypic groups, particularly those patients harbouring mt-tRNA mutations generally, and the m.3243A>G mutation more specifically, appear at increased risk of the development of cardiac involvement (Chapter 3), is consistent with the finding of subclinical concentric remodelling in my prospective study of this group with cardiac MRI (Chapter 5). Certainly this group warrants further study to understand the mechanistic factors linking the mutation to increased prevalence of the cardiac phenotype. However data from my study of children with end-stage cardiomyopathy where, initially, no genetic diagnoses were made in patients with evidence of mitochondrial respiratory chain disease, is a reminder of the fact that a high index of suspicion is necessary to make the diagnosis of mitochondrial disease and predictive factors that suggest the underlying aetiology of disease are not always apparent. Indeed the majority of these patients with mitochondrial respiratory chain disease had no supportive family history, or pre-existing cardiac or multisystem disease. In this small cohort, parental consanguinity as a non-specific marker of metabolic disease caused by recessive nuclear mutations was the only frequent feature. It is acknowledged that the advent of exome sequencing may revolutionise this area of mitochondrial disease.

7.2.1.6 Impact on clinical outcomes

I have established that cardiomyopathy in patients with mitochondrial disease has a significant impact on clinical outcome – decreased survival was noted following symptom onset in those patients with cardiomyopathy. This intuitive observation is consistent with previous smaller or more selective studies (Holmgren *et al.*, 2003; Scaglia *et al.*, 2004; Majamaa-Voltti *et al.*, 2008), and mandates further diagnostic, mechanistic and therapeutic studies. However, I have also shown that mitochondrial disease limits exercise capacity through abnormalities of skeletal muscle, rather than central cardiac factors, albeit in a limited cohort of patient harbouring the m.3243A>G or m.8344A>G mutation with subclinical evidence of cardiac involvement (Chapter 6). Future studies should use such objective measures to assess treatment effects, and it should be remembered that those with minimal evidence of cardiac involvement in

mitochondrial disease may be symptomatically and prognostically more limited by other organ involvement.

7.2.2 Pathogenic mechanisms

The study of pathogenic mechanisms of cardiac involvement in mitochondrial disease is limited by the absence of effective animal or cellular models and difficulties with access to both patient and control cardiac biopsy tissue. Nonetheless my studies have been able to explore potential cellular mechanisms of mitochondrial cardiomyopathy, using diverse direct and indirect approaches.

Firstly, the comprehensive post-mortem study demonstrated consistent differential evidence of COX-deficiency in cardiac chambers, unrelated to differences in chamber-specific mutation load, suggesting a specific interaction between local environment and genotype. Importantly, complex I deficiency was more extensive and more profound than COX-deficiency, specifically in patients harbouring the m.3243A>G mutation, and associated with minimal evidence of complex IV deficiency. These finding are in keeping with *in vitro* studies exploring the pathogenic mechanisms underlying the translational defect associated with the m.3243A>G mutation, and warrant specific further study to investigate the importance of this respiratory chain defect in cardiac muscle. Supporting evidence for the role of both complex I and complex IV deficiency in cardiac involvement in mitochondrial disease was provided by my prospective study of children with end-stage cardiomyopathy – complex I deficiency was present in all, suggesting the primary importance of this defect in cardiac disease in patients with mitochondrial respiratory chain disease but *without* the m.3243A>G mutation. While cardiac muscle mutation load and the extent of histochemical COX-deficiency are important factors in the presence of cardiac involvement in mitochondrial disease, they are clearly not the full explanation. COX-intermediate cardiomyocytes were frequent in patients harbouring the m.3243A>G or m.8344A>G mutations suggesting distinct abnormalities in complex IV and/or complex II. Yet distinct differences were also apparent in the nature and extent of complex I and complex IV deficiency between those patients with the m.3243A>G and m.8344A>G mutations. Such differences may relate to different mechanisms of dysfunctional translation between the two mutations and also impact on the nature of cardiac involvement. Further studies in cardiomyocyte models will be necessary to

determine the primacy of complex I deficiency, the importance of complex IV deficiency and their combined relevance to cardiomyopathy.

Secondly, baseline and follow up data from my exercise intervention study demonstrated altered sympatho-vagal balance in patients with mitochondrial disease. Importantly these findings are consistent with other clinico-pathological findings in this group, from other studies in this thesis including the prevalence of a resting sinus tachycardia (Chapter 3) and prominent fatigue (Chapter 6). Indeed the exercise study itself, although not powered to investigate modification of sympatho-vagal balance demonstrated subtle but significant changes consistent with a normal physiological response to exercise, with an increased parasympathetic component. Identifying sympathetic over-activation as a potential pathological feature in mitochondrial disease aligns the conditions with other chronic diseases associated with fatigue and elevated resting heart rate and suggests a novel mechanism for therapeutic intervention.

7.2.3 Therapeutic options

There is no drug treatment that has shown clear clinical benefit in the primary outcome in patients with mtDNA disease (Pfeffer *et al.*, 2012). Resistance and endurance exercise training programmes can improve symptoms but they have not been widely used in clinical practice, at least in part due to limited appreciation of the potential benefits and concerns regarding the deleterious consequences on cardiac function in patients at significant risk of cardiac involvement. The presence of (potential) multisystem disease has been regarded as a relative contra-indication to organ transplantation. The treatment options for patients with mitochondrial disease are therefore restricted, and there are significant problems with the conduct of large-scale clinical trails in this patient group that will limit the rate of emergence of future, evidence-based therapies.

Given this lack of therapeutic options, my studies sought to determine the impact of mitochondrial disease itself as a factor in patients' responses to proven therapeutic interventions. Despite demonstrating the important prognostic effect of cardiomyopathy in this group of patients, our studies contain important findings that may lead to institution of clinical interventions in patients with mitochondrial disease.

For example, I have shown, that mitochondrial respiratory chain disease had no significant impact on early post-transplantation survival or quality of life in children with end-stage cardiomyopathy (Chapter 4). Furthermore, the one death in this group was related to a known complication of the immunosuppression required for transplantation and not mitochondrial disease specifically, despite evidence of distant organ involvement. These finding are perhaps surprising given the potential and indeed evidence, in some individuals in my study, of multisystem disease and it will require longer term follow up to determine formal clinical outcomes. Yet, as a preliminary finding, it serves to underline the importance of giving consideration to the full range of management options to alter the natural history of mitochondrial disease, particularly in patients with cardiac disease, where evidence-based treatments exist for phenotypically similar conditions.

Similarly, in the exercise intervention study, a 16-week aerobic exercise training programme resulted in similar cardio-pulmonary, cardiac morphological and haemodynamic improvements in patients with m.3243A>G- and m.8344A>G-related mitochondrial disease as sedentary controls. There was no deleterious effect on myocardial bioenergetics, patients' quality of life or disease burden. Endurance exercise training is beneficial in patients with mitochondrial disease and patients with heart failure and appears to have a real clinical role in those with cardiac involvement in mitochondrial disease, who respond in a predictable physiological manner; current therapeutic options should be considered in all patients with mitochondrial disease.

7.3 Limitations

Throughout this thesis, the use of contrasting study protocols has enabled detailed exploration of the cardiac manifestations of mitochondrial disease. In an area of study inherently limited by disease rarity, tissue availability and a lack of robust cellular and animal models, alternative techniques have largely provided mutually supportive evidence for the conclusion contained within this thesis. Nevertheless, each different type of study has been subject to different and important limitations, which have been acknowledged throughout the thesis, and which will be summarised in this synopsis.

7.3.1 Retrospective clinical study

To enable robust exploration, within the timeframe of this thesis, of the nature and frequency of a specific manifestation of a rare condition, I studied retrospective clinical data, gathered over decades through the clinical care of patients with mitochondrial disease. Although special measures were employed to attempt to standardise the interpretation of echocardiograms and ECGs, this study was inevitably limited by the basic nature and non-uniform collection of clinical data, missing data points and other potential biases due to the retrospective nature of analysis. I was unable to select an equivalent control population for comparison regarding clinical outcomes and did not assess the impact of changes in management on natural history. Nevertheless, basic conclusions regarding my study aims were achieved, justifying a focus on patients with mt-tRNA mutations in latter studies and providing data for the generation of hypotheses regarding the natural history of cardiac disease in this group.

7.3.2 Tissue-based studies

Both tissue-based studies reported in this thesis were limited in terms of sample size, albeit in the context of restricted access to a relatively inaccessible tissue in patients with a rare condition. All conclusions should be regarded as hypothesis-generating.

The observational study of children with end-stage cardiomyopathy (Chapter 4) was designed to investigate the frequency of mitochondrial respiratory chain disease in this cohort of patients, but not to explore clinical or biochemical markers of disease; conclusions in this regard are hypothesis-generating. Similarly, although there was a high suspicion of mitochondrial disease as the cause of the cardiac presentation in children with evidence of myocardial respiratory chain disease, a causative genetic mutation was confirmed in only one patient, limited conclusions regarding frequency of mitochondrial disease. Additionally, there were specific issues with the protocol in this study including non-adherence to the contemporaneous collection of skeletal muscle tissue at the time of cardiac transplantation, and the absence of pre-transplantation quality of life data due to the identification of cardiac transplantation as the index event. While understandable in the context of paediatric cardiac transplantation, the limitation of absent skeletal muscle tissue was a significant concern in patients with a multi-system condition.

Post-mortem tissue used in the retrospective cardiac tissue study is not an ideal medium for the investigation of cellular mechanisms. While reasonable quality control was employed in limiting tissue analysis to those samples without a long post-mortem delay, I cannot be certain that tissue degradation post-mortem did not have an impact on my findings. Reassuringly, however, all tissues including the ten patient and two control samples were processed by the same team in the same rapid fashion decreasing the likelihood of tissue processing factors having an impact on subsequent cellular analysis. I exclusively studied tissue from patients harbouring the m.3243A>G or m.8344A>G mutations, using available cardiac samples from related and relatively common genotypic groups to ensure a reasonable sample size. While useful for my study of potential cellular pathogenic mechanisms in patients with mt-tRNA mutations, this approach limited my ability to generalise my findings to other genotypic groups. Due to significant overlap between the groups of patients with and without cardiac involvement and those harbouring the m.3243A>G versus the m.8344A>G mutation, I experienced difficulties relating cellular findings to cardiac phenotype: 5/7 patients with the m.3243A>G mutation had cardiomyopathy while only 1/3 patients with the m.8344A>G mutation had cardiomyopathy, and no attempt was made to age- or gender-match control cardiac tissue due to profound difficulties with access to samples. In the absence of balanced groups, it is difficult to draw conclusions about differences *between* groups and results are hypothesis generating.

7.3.3 Cardiac imaging studies

Although again limited in sample size, all my cardiac MRI-based studies were amongst the largest reported in the clinical literature and focussed on well-characterized and homogenous groups of patients, representing ~25% current specialist clinic attendees. my data provide robust evidence of cardiac involvement in patients harbouring two common mt-tRNA point mutations, but may not be generalizable to wider groups patients with mitochondrial disease. Individual studies were appropriately powered for surrogate clinical endpoints but were not specifically designed to assess pathogenic mechanisms or disease progression. Additionally due to the constraints of protocol adherence and thesis duration, the cross-sectional analysis and exercise intervention were of limited duration. Although cardiac involvement in mitochondrial disease is associated with morbidity and early mortality, I did not formally assess the clinical

relevance of my findings of subtle hypertrophic remodelling in my cross-sectional study and this await further longitudinal outcome data. Similarly the 16 weeks' duration of the endurance exercise training study showed significant effects on a variety of cardiac, haemodynamic and cardio-pulmonary measures but may not have been long enough to investigate diastolic dysfunction, or clinical outcomes. Finally, while both cardiac imaging-based studies employed extensive protocols exploring different aspects of mitochondrial disease, both studies had limitations in data collection. I did not investigate first-pass perfusion or longitudinal tagging in the cross-sectional study, not bar-receptor sensitivity or challenges to sympatho-vagal control in the intervention study.

7.4 Clinical relevance and future directions

The combined studies of this thesis have already had a significant impact on the clinical management of patients with mitochondrial disease. Presented abstracts and publications of the data have increased awareness, among an international audience, of the cardiac manifestations of mitochondrial disease, and enabled the drafting of preliminary international guidelines (www.mitochondrialncg.nhs.uk), and algorithms for the recommended investigation and management of cardiac disease in this population (Bates *et al.*, 2012). The outcome of these studies should lead to further focussed research to address the hypotheses generated in this thesis, and active engagement with more detailed clinical data collection should facilitate prospective cohort studies.

Clearly cardiomyopathy has an important impact on survival in patients with mitochondrial disease and cardiac screening investigations are vital to detect cardiac involvement, given a lack of cardiovascular symptoms in many patients in my studies. These studies support cardiac MRI as a gold standard investigation to detect subtle hypertrophic remodelling, and yet also display good agreement between MRI and echocardiographic findings. Such data may enable adoption of cardiac MRI as a research tool in future interventional or longitudinal studies and as a baseline investigation in all newly-diagnosed individuals, who could be subsequently followed up with echocardiography. Large, national prospective cohort studies are already exploring the natural history of the abnormalities reported in my imaging studies.

The frequency of mitochondrial respiratory chain disease that I have reported in children with end-stage cardiomyopathy was surprising. My study protocol has already been adopted into clinical practice locally as a baseline investigation in all children undergoing cardiac transplantation or VAD implantation. Given the lack of clinical suspicion of mitochondrial disease as an aetiological factor in several patients in my study, this action will undoubtedly uncover novel patients with the condition, particularly amongst those with parental consanguinity. This diagnosis will have an important impact on family screening and genetic counselling. Combined histochemical and biochemical analysis of tissue is clearly necessary and, given tissue segregation, opportunistic investigation of affected cardiac tissue is justified. Moreover, by demonstrating that patients with mitochondrial respiratory chain disease can present acutely in heart failure, without multisystem disease or family or personal history of cardiomyopathy, I have highlighted the need to investigate routinely all children presenting with end-stage cardiomyopathy. Indeed due to the success of this project, local clinicians have begun tissue analysis for mitochondrial respiratory chain disease in adult patients, presenting with end-stage idiopathic dilated cardiomyopathy or unexplained hypertrophic cardiomyopathy undergoing VAD implantation or cardiac transplantation. While ischaemic heart disease accounts for the majority of patients undergoing cardiac transplantation in an adult population, this novel research direction may prove informative. Data collection regarding the individuals included in my study, and those subsequently identified, is already a priority for the local cardiac transplantation team to assess the impact of mitochondrial disease on long term clinical outcomes; quality of life measures are now being assessed pre-operative as a matter of clinical routine. Additionally molecular biological investigations are ongoing to determine genetic diagnoses in all affected patients, as experience with exome sequencing in this population increases.

The post-mortem studies defined the respiratory chain abnormalities of cardiac muscle in patients with and without clinical cardiac involvement harbouring either the m.3243A>G or m.8344A>G mutation. I have shown the critical importance of complex I deficiency, particularly in patients with the m.3243A>G mutation, and demonstrated novel relationships between complex IV deficiency or dysfunction and abnormalities of complex I. These findings may have direct relevance to the development of

cardiomyopathy in patients harbouring mt-tRNA mutations and in patients with isolated complex I abnormalities with other or unknown genotypes. However, the tissue used limits interpretation of my results, and further study of the impact of these mutations on the structure and function of cardiomyocytes is dependent on the development of isolated cardiomyocytes harbouring specific mutation, using inducible pluripotent stem cell technology. This process is in its infancy and remains time consuming with a successful conversion rate of <1%, even in patients with autosomal mutations. Undoubtedly, the successful development of cardiomyocytes harbouring significant levels of individual mt-tRNA mutations is some way off, but our group has started to explore this process that will underpin future studies of the structural and electrophysiological effects of mtDNA mutations. This will provide real insight into cellular pathogenic processes.

Finally, my studies have highlighted an important feature of different therapeutic interventions in different populations of patients with mitochondrial disease. In children, I have demonstrated that patients with end-stage cardiomyopathy due to mitochondrial respiratory chain disease have similar early post-transplantation survival to those without underlying mitochondrial disease. In adults, I have shown that aerobic, endurance exercise training has similar, beneficial cardiac, haemodynamic and cardio-pulmonary effects in patients with mitochondrial disease and sedentary control individuals. While interesting from a physiological point of view and supporting further tissue-based and clinical investigation of these patients' outcomes, these results have wider importance. Mitochondrial disease is often a severe, progressive condition, associated with significant morbidity and early mortality; there are few effective treatments. I have demonstrated to patients and professionals alike, that there may be effective therapeutic interventions for patients with mitochondrial disease, that an evidence base can be established to ensure adherence to proven therapies, and that hope exists for the future care of these patients, and their families.

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