

A Novel Assay to Measure Mitochondrial Dysfunction in Human Skeletal Muscle

Implications for the Diagnosis and Treatment of Mitochondrial Diseases

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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 of Neuroscience, Newcastle University, and is my own work. This research was carried out under the supervision of Professor D. M. Turnbull and Dr. Karolina Rygiel, between September 2012 and October 2015.

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.

Abstract

Mitochondrial dysfunction occurs in patients with mitochondrial disease, in neurodegenerative conditions and as part of the ageing process. It affects predominantly tissues with high metabolic requirements such as central nervous system and skeletal muscle. In patients with mitochondrial disease, both mitochondrial and nuclear genetic defects commonly cause a biochemical defect in muscle. However, due to the multi-copy nature of mitochondrial DNA, muscle displays a mosaic pattern of deficiency when the mitochondrial genome is affected. This particular pattern makes these defects challenging to quantify.

Current standard methods to diagnose and investigate mitochondrial disease in affected tissues present several limitations. Biochemical studies are only suitable for cases with a high proportion of cells with mitochondrial respiratory chain deficiency. Moreover, histochemical assays only provide qualitative assessment of complex II and IV activities and are not capable of evaluating other complexes, such as complex I - the commonest affected respiratory complex in mitochondrial pathology. This project aimed therefore at developing a novel assay to accurately quantify mitochondrial dysfunction in human skeletal muscle. Once optimised, this assay was further used to explore: the mechanisms underlying mitochondrial pathology, its potential in helping the current diagnostic setting, as well as its potential to assess the effectiveness of therapeutic approaches aimed at treating mitochondrial dysfunction.

This work described the development and validation of a novel quadruple immunofluorescent technique. This assay quantifies accurately key subunits of respiratory complexes I and IV together with mitochondrial mass, using a single 10µm section. The additional labelling of a cell membrane marker allows semi-automatic and computer-based sampling of large numbers of individual muscle fibres. Using this technique, this study characterised a variety of mitochondrial and nuclear genetic defects and demonstrated that specific genotypes exhibit distinct biochemical signatures in muscle. In patients with suspected mitochondrial disease, this assay provided clues on the possible genetic causes. Furthermore, this novel assay evaluated the effect of an endurance exercise program in patients with mitochondrial myopathy and was able to detect subtle changes in respiratory complexes levels.

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Abbreviations

AcetylCoA	AcetylCoenzyme A
Ach	Acetylcholine
AChRs	Acetylcholine receptors
ANT1	Adenine nucleotide translocator
ATP	Adenosine triphosphate
ATPase	ATP synthase
bp	Base pair
BSA	Bovin serum albumin
Ca ²⁺	Calcium
COX	Cytochrome <i>c</i> oxidase
CPEO	Chronic Progressive External Ophthalmoplegia
Cr	Creatine
CS	Citrate synthase
DAB	3,3 Diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DHPR	Dihydropyridine receptors
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
ELAC2	ElaC Ribonuclease Z 2
FADH ₂	Reduced flavin adenine dinucleotide
GFM2	G Elongation Factor, Mitochondrial 2
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
Hz	Hertz
K ⁺	Potassium
KSS	Kearns-Sayre syndrome
LHON	Leber's Hereditary Optic Neuropathy
LRPPRC	Leucine-Rich Pentatricopeptide Repeat Containing
MCU	Mitochondrial calcium uniporter
MELAS	Mitochondrial Encephalomyopathy, Lactic acidosis, and Stroke-like episodes
MERFF	Myoclonus Epilepsy with Ragged-red fibres
MILS	Maternally Inherited Leigh syndrome
MRC	Mitochondrial respiratory chain
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
mtEFG2	Mitochondrial elongation factor G2
MT-ND1	Mitochondrially Encoded NADH Dehydrogenase 1

mt-SSB	Mitochondrial single stranded binding protein
MT-TE	Mitochondrial-encoded glutamic acid
MT-TG	Mitochondrial-encoded glycine
MT-TL1	Mitochondrial-encoded leucine1 (UUA/G)
MT-TN	Mitochondrial-encoded asparagine
MT-TP	Mitochondrial-encoded proline
MT-TS1	Mitochondrial-encoded serine1
MT-TW	Mitochondrial-encoded tryptophan
Na⁺	Sodium
NAD^+	Nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
NGS	Normal goat serum
NRF-1	Nuclear respiratory factor 1
NRF-2	Nuclear respiratory factor 2
02. ⁻	Superoxide anion
OD	Optical density
OH [.]	Hydroxyl radical
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PEO1	Twinckle helicase gene
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1- α
PNPT1	Polynucleotide phosphorylase
POLG	Polymerase gamma
POLMRT	mtRNA polymerase
PS	Pearson's Syndrome
RNA	Ribonucleic acid
RNaseP	Mitochondrial ribonuclease P
ROI	Region of interest
ROS	Reactive oxygen species
RRF	Ragged-red fibres
RRM2B	Ribonucleotide Reductase M2 B (TP53 Inducible)
rRNA	Ribosomal RNA
RT	Room temperature
RyR	Ryanodine receptors
SDH	Succinate dehydrogenase
0011	
SERCA	Ca ²⁺ ATPase
SERCA SLC25A4	Ca ²⁺ ATPase Solute Carrier Family 25
SERCA SLC25A4 SOD1	Ca ²⁺ ATPase Solute Carrier Family 25 Copper/zinc superoxide dismutase
SERCA SLC25A4 SOD1 SOD2	Ca ²⁺ ATPase Solute Carrier Family 25 Copper/zinc superoxide dismutase Manganese superoxide dismutase

Tricarboxylic acid cycle
Mitochondrial transcription factor A
Mitochondrial transcription factor A
Mitochondrial transcription factor B1
Mitochondrial transcription factor B2
Tagged image file format
Translocase of the inner membrane
Translocase of the outer membrane
Transfer RNA
mitochondrial ribonuclease Z
voltage-gated ion channel

List of publications

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Rygiel KA, Miller J, Grady JP, Rocha MC, Taylor RW, Turnbull DM (2015) Mitochondrial and inflammatory changes in sporadic inclusion body myositis. Neuropathol Appl Neurobiol. (3):288-303. doi: 10.1111/nan.12149.

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The skeletal muscle is the most abundant tissue in vertebrates' body. It controls voluntary movement and its mass is a determinant factor of muscle strength, endurance and physical performance. Due to its high metabolic demand, skeletal muscle is highly dependent on mitochondria – the main producer of cellular energy.

During normal ageing, skeletal muscle undergoes an inevitable loss of muscle mass – also known as sarcopenia (Rosenberg, 1997); approximately 40% of muscle mass is lost between the ages of 20 and 80. This is responsible for a decline in muscle strength and fitness leading to varying degrees of restricted mobility, disability and loss of independence of the affected individual.

Since mitochondrial function and content in human muscle also declines with ageing, dysfunctional mitochondria are thought to play an important role in sarcopenia. In light of increasing life expectancy, understanding mitochondrial dysfunction in ageing skeletal muscle and finding strategies to reduce sarcopenia are important and relevant.

In trying to understand the role of mitochondrial dysfunction in sarcopenia, it became apparent to me that the methods used to assess mitochondria in human muscle sections had major limitations. My PhD studies therefore focused on developing improved methods to quantify respiratory chain deficiency in human skeletal muscle and for this I extensively used muscle from patients with mitochondrial disease. Mitochondrial diseases are a group of disorders that arise from defects of the mitochondrial oxidative phosphorylation system (OXPHOS). The skeletal muscle is one of the tissues frequently affected and remains a major target of clinical investigations, often used to confirm or refute a mitochondrial diagnosis.

This introduction provides an overview of (1) mitochondrial structure, functions and genetics and (2) the healthy skeletal muscle. This is important to understand the following sections covering mitochondrial damage accumulating in the skeletal muscle during (3) ageing and in (4) mitochondrial diseases.

1.1 Mitochondria structure and function

Mitochondria are vital cellular organelles with a predominant role in energy metabolism. They provide energy via the process of oxidative phosphorylation to produce ATP for the majority of cellular processes. In addition, mitochondria are also involved in regulation of cellular metabolism, redox homeostasis, (Ca²⁺) calcium signalling and programmed cell death.

1.1.1 Mitochondrial structure and organization

Mitochondria are highly dynamic organelles that form a complex network inside cells. Their organisation was first elucidated by Palade, which with the use of electron microscopy, identified a common mitochondrial structure irrespective of the species. These comprise of two separate membrane systems (the outer and the inner membrane), the mitochondrial matrix and a system of internal cristae. Both inner and outer membranes are separated by the intermembraneous space (Palade, 1953) (Figure 1.1). Later studies using 3D electron microscopic tomography provided a better resolution of mitochondrial structure. These described the cristae as separate tubular membranes bound to the inner mitochondrial membrane (Mannella *et al.*, 1994).

Both membranes are involved in the import and export of molecules. The outer membrane contains the voltage-gated ion channel porin (VDAC). This channel allows the diffusion of cations (such as Ca²⁺) and small molecules of low molecular weight (metabolites and nucleotides) (Szabo and Zoratti, 2014). Both inner and outer membranes contain the translocases TIM/TOM (translocase of the inner membrane/ translocase of the outer membrane), which mediate the transport of nuclear-encoded proteins across the membranes (Endo and Yamano, 2010). They also enclose proteins that are crucial for mitochondrial fusion, namely the mitofusins mfn1 and mfn2 (located in the outer membrane) and OPA1 (optic atrophy 1, located in the inner membrane) and also Drp1 (Dynamin related protein 1, located in the outer membrane) which is crucial for fission (Westermann, 2012). These act in concert to allow constant network remodelling - changes in mitochondria morphology, size, number and content – and therefore adapting to changing metabolic demand (Westermann, 2012). The inner mitochondrial membrane holds the mitochondrial respiratory chain system responsible for oxidative phosphorylation and energy generation.

Lastly, mitochondrial matrix houses the mitochondrial genome (mtDNA), all the biochemical reactions involved in energy production (such as the citric acid cycle and β -oxidation), as well as mitochondrial replication, transcription and translation.



Figure 1.1 Schematic illustration of mitochondrial structure and organisation.

Mitochondrial are ovoid double-bounded organelles. The inner and outer membranes are separated by the intermembrane space. The inner membrane harbours the oxidative phosphorylation system and mitochondrial matrix contains the mitochondrial genome.

1.1.2 Mitochondrial genome

1.1.2.1 Mitochondrial DNA

Mitochondria are the only cellular organelles holding their own genome in mammalian cells. They are maternally inherited (Giles *et al.*, 1980) and contain thousands of copies of mtDNA packaged in stable nucleoprotein complexes called nucleoids (Miyakawa *et al.*, 1987, Satoh and Kuroiwa, 1991). Nucleoids reside in the mitochondrial matrix in close association with the inner membrane (Albring *et al.*, 1977). Although each nucleoid was thought to contain 6-10 mtDNA copies (Legros *et al.*, 2004), more recent super-resolution microscopy revealed that it only contains 1-2 mtDNA copies (Kukat *et al.*, 2011). The number of mtDNA copies in cells is variable ranging from none (in erythrocytes) (Shuster *et al.*, 1988), to hundreds or thousands (Shoubridge and Wai, 2007), depending upon the tissue and cellular energy requirements.

Each mtDNA molecule measures 16,569 bp, is circular and double-stranded (Figure 1.2) (Anderson *et al.*, 1981, Andrews *et al.*, 1999). The mitochondrial genome is highly compact; there are no introns or intergenic non-coding regions except for the displacement loop (D-loop) - containing transcriptional promoters. Most genes are separated by only one or two non-coding base pairs, and two genes (*MT-ATP6* and *MT-ATP8*) have overlapping regions (Anderson *et al.*, 1981).

The mitochondrial genome encodes 13 core subunits of the oxidative phosphorylation system (complex I, III, IV and V) as well as 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Anderson *et al.*, 1981, Andrews *et al.*, 1999) (Figure 1.2). Both tRNAs and rRNAs are required for the translation of mitochondrial mRNA transcripts. The remaining 79 subunits, over 35 assembly factors (Kadenbach, 2012) and proteins involved in mtDNA synthesis, mtDNA

transcription, mtDNA translation, RNA maturation, RNA stability and regulation, are encoded by the nuclear genome (Smits *et al.*, 2010). Following transcription, nuclear mRNAs are translated in the cytoplasm and then imported into the mitochondria where both mitochondrial-encoded and nuclear-encoded subunits are assembled into fully functional complexes (Tuppen *et al.*, 2010).



Figure 1.2 The human mitochondrial genome.

The mtDNA encodes 13 polypeptides of the OXPHOS system; genes encoding complex I subunits are shown in green, those encoding complex III in purple, complex IV in green and complex V in cyan. Additionally, it encodes two ribosomal RNA (shown in red) and 22tRNAs (denoted by their single amino acid code) required for mitochondrial protein synthesis. Image (adapted) with the courtesy of Dr Casey Wilson.

	Mitochondrial-encoded subunits	Nuclear-encoded Subunits	Nuclear-encoded assembly factors
Complex I	7 ND1-ND6, ND4L	37	11
Complex II	0	4	2
Complex III	1 Cytochrome <i>b</i>	10	2
Complex IV	3 MTCO1-MTCO3	11	18
Complex V	7 ATP6, ATP8	17	4

Table 1.1 Composition of OXPHOS complexes and factors required for the proper assembly.

1.1.2.2 Replication and maintenance of mitochondrial DNA

The replication of mtDNA relies entirely on the nuclear genome. It occurs continuously, independent of the cell cycle in replicative cells and in post-mitotic cells (Bogenhagen and Clayton, 1977). Korhonen *et al.* have reconstituted the minimum mitochondrial replisome *in*

vitro and identified three major nuclear-encoded proteins: the trimeric polymerase gamma (POLG), the mitochondrial single stranded binding protein (mt-SSB) and the helicase Twinkle (Korhonen *et al.*, 2004).

The heterotrimeric POLG (Lestienne, 1987) is made of a catalytic subunit (POLYA – encoded by *POLG1*) and two accessory subunits (POLYB – encoded by *POLG2*). It has polymerase activity, 3'-5' exonuclease/proofreading activity and 5'deoxyribose phosphate lyase activity (involved in mtDNA repair) (Gray and Wong, 1992). Twinkle has 5'-3' DNA helicase activity and facilitates mtDNA synthesis by unwinding the double stranded mtDNA (Spelbrink *et al.*, 2001) whereas the mt-SSB stabilises the single stranded regions of mtDNA at replication forks, and enhances POLG and Twinkle activities (Curth *et al.*, 1994, Korhonen *et al.*, 2003).

In addition, other nuclear proteins are indirectly involved in mtDNA maintenance by controlling the deoxynucleoside triphosphates (dNTP) pool, such as the cytosolic ribonucleotide reductase (p53R2 subunit, encoded by *RRM2B*) (Tanaka *et al.*, 2000) and the mitochondrial adenine nucleotide translocator (ANT1, encoded by *SLC25A4*) (Neckelmann *et al.*, 1987). The p53R2 subunit catalyses *de novo* synthesis of dNTPs whereas ANT1 mediates the exchange of ATP (out) for ADP - and by this it regulates the intramitochondrial concentration of adenine nucleotides. It was shown that a balanced proportion of the four nucleotides is essential for mtDNA and nDNA replication fidelity and repair (Mathews and Song, 2007).

1.1.2.3 Mitochondrial transcription and translation

Mitochondrial transcription is also operated by nuclear-encoded proteins; the basic machinery required for transcription initiation (mitochondrial transcriptional core) includes mtRNA polymerase (POLRMT) (Tiranti *et al.*, 1997), mitochondrial transcription factor A (TFAM) (Fisher and Clayton, 1988) and transcription factor B2 (TFB2M) (Falkenberg *et al.*, 2002, Sologub *et al.*, 2009). TFAM binds mtDNA upstream to the promotor site inducing a conformational change of the light-strand; this exposes the promotor region and activates transcription. Following this, POLRMT recognises the promotor and initiates RNA synthesis after forming a heterodimer with TFB2M (Rebelo *et al.*, 2011).

Transcription of both strands generates long polycistronic primary mRNA, which then undergo endonucleolytic processing. The mitochondrial ribonuclease P (RNase P) is responsible for the 5' end cleavage of mt-tRNAs precursors (Rossmanith and Karwan, 1998) whereas the tRNase Z

(encoded by *ELAC2*) is responsible for the 3' end cleavage (Schiffer *et al.*, 2002). Since most mRNAs and rRNAs are punctuated by tRNAs, this cleavage releases individual mRNAs, rRNAs and tRNAs (Ojala *et al.*, 1981).

Most excised mRNAs (except ND6 mRNA transcript) are polyadenylated on their 3'end and then released for translation (Van Haute *et al.*, 2015). By contrast, tRNAs follow extensive post-transcriptional modifications before being aminoacylated with the cognate amino acid (tRNAs). In that respect, the addition of CCA trinucleotide at the 3'end of tRNAs is essential for correct aminoacylation by the cognate tRNA-aminoacyl synthetases; the modifications of the wobble position (first position of the anticodon) are critical for accurate decoding and translation fidelity; as many as other modifications which are essential for structural stability, maturation and correct folding. Finally excised rRNAs (12S and 16S) undergo several nucleotide modifications required for proper folding, stability, and ribosome assembly and function (Van Haute *et al.*, 2015).

Translation of the 13 polypeptide subunits of OXPHOS complexes can then proceed. It requires several nuclear-encoded factors (involved in translation initiation, elongation, termination and ribosome recycling), assembled mitoribosomes (made up of 12S and 16S and further 81 nuclear-encoded mitochondrial ribosomal proteins) and aminoacylated tRNAs (Smits *et al.*, 2010).

1.1.3 Mitochondrial biogenesis

Mitochondrial biogenesis is the process by which mitochondrial number and activity increases in response to metabolic stress (such as exercise) or environmental stimuli (Kelly and Scarpulla, 2004). The peroxisome proliferator-activated receptor gamma co-activator $1-\alpha$ (PGC- 1α) plays a central role in this process through the co-activation of a great number of nuclear transcription factors, such as NRF-1 and NRF-2 (Wu *et al.*, 1999). The activation of the later, was shown to increase the expression of TFAM (Virbasius and Scarpulla, 1994), TFB2M (Gleyzer *et al.*, 2005) and several nuclear-encoded respiratory chain subunits (Evans and Scarpulla, 1990). Alongside, TFAM is imported into mitochondria were it up-regulates mitochondrial-encoded subunits such as COX-II (Puigserver *et al.*, 1998, Wu *et al.*, 1999). The resulting increased synthesis and incorporation of new mitochondrial proteins results in a higher overall mitochondrial activity and content (Kelly and Scarpulla, 2004).

1.1.4 Mitochondrial functions

1.1.4.1 Mitochondria oxidative metabolism

Mitochondria are the centre of oxidative metabolism - the major metabolic system to replenish ATP levels in cells. Production of ATP from both glucose and fatty acids occurs in four major steps: acetyl coenzyme A (acetyl-CoA) generation (the main substrate of the tricarboxylic acid cycle, TCA), electron transfer to electron carriers (TCA cycle), electron donation to the mitochondria respiratory chain and lastly ATP production by ATP synthase (MacIntosh *et al.*, 2006).

1.1.4.1.1 Acetyl-CoA and TCA cycle

Acetyl-CoA can be generated either through the oxidation of fatty acids or through the breakdown of pyruvate. Cytoplasmic fatty acids are transported into mitochondria via the carnitine shuttle (Bremer, 1983). Once in the matrix, these are sequentially broken into acetyl-CoA through the beta-oxidation (β oxidation) pathway. In parallel, pyruvate (a by-product of glycolysis) can also be imported into mitochondria - via the mitochondrial pyruvate carrier. There, pyruvate is converted by decarboxylation into acetyl-CoA by the pyruvate dehydrogenase. At this stage, acetyl-CoA derived from either glycolysis or β -oxidation enters TCA cycle (Baker *et al.*, 2010). Series of oxidative reactions transfer electrons to hydrogen from electron carriers - nicotinamide adenine dinucleotide (NADH⁺) and flavin monocleotide (FAD) - forming three NADH and one FADH₂ respectively (Krebs, 1940, Krebs and Eggleston, 1940).

1.1.4.1.2 The oxidative phosphorylation system

Oxidative phosphorylation (OXPHOS) is embedded in the inner mitochondrial membrane and consists of the mitochondrial respiratory chain (complexes I-IV) and ATP synthase (complex V). It is coupled to the oxidative pathway at complex I and complex II levels (Figure 1.3).

Complexes I and II are the first electron-acceptors of the respiratory chain, receiving the electrons derived from NADH and FADH₂ respectively. These are then transferred along the chain: to complex III, via coenzyme Q (or ubiquinone), and to complex IV through cytochrome *c*. The flow of electrons along the respiratory chain is coupled with the active pumping of protons into the intermembrane space (at complexes I, III and IV) building up the mitochondrial transmembrane potential. The generated electrochemical gradient is finally

used by the ATP synthase to drive ATP production upon the diffusion of protons back into the matrix (Mitchell, 1961).

The capacity of mitochondria to produce ATP under maximal or basal conditions (mitochondrial oxidative capacity) depends on the oxidative enzyme content and activity. These, together with mitochondrial density define muscle oxidative capacity (Schwerzmann *et al.*, 1989)





The respiratory chain (complexes I-IV) and ATP synthase (complex V) are responsible for ATP production. While electrons are transferred along the chain by diffusible electron carriers (coenzyme Q or ubiquinone and cytochrome c), protons are pumped into the intermembrane space via cl, cIII and cIV. The created proton gradient across the inner membrane is finally used by the complex V to drive ATP production. Black arrow indicate the direction of electron flow; Grey arrows represent the translocation of protons (H⁺). Keys: cl: complex I, clI: complex II, clII: complex III, cIV: complex IV, cV: complex V, CoQ: coenzyme Q, Cyt c: cytochrome c, e-: electron.

1.1.4.1.3 Components of the OXPHOS system

Complex I (NADH dehydrogenase) is the largest enzyme (~ 980 kDa) of the OXPHOS system and represents the major entry point of electrons. It consists of 45 structural subunits, of each seven are mitochondrial-encoded (ND1-ND6 and ND4L) and 38 are nuclear-encoded. The assembly of the structural subunits into a functional holocomplex requires additionally nuclear-encoded assembly factors; 10 have already been identified, but more are expected to be discovered (Kadenbach, 2012). The complex presents three functional modules. The N module where dehydrogenase oxidizes NADH, the Q module where hydrogenase reduces ubiquinone (lipid-soluble electron carrier) and the P module where protons are pumped into the intermembrane space by a proton translocase. The coordinated cross-talk between these modules allows the transfer of electrons from NADH to ubiquinone, and the translocation of

four protons across the inner membrane. The latter generates an internal negative membrane potential (Mimaki *et al.*, 2012).

Complex II (succinate dehydrogenase) is the smallest complex (~123 kDa) of the OXPHOS system and is also a member of the TCA cycle. It consists of four structural subunits (SDHA, SDHB, SDHC and SDHD) all encoded by nuclear genes. SDHA and SDHB compose the catalytic core; SDHC and SDHD anchor the complex to the inner membrane. Only two assembly factors are presently known (Kadenbach, 2012). Complex II acts as a link between TCA cycle and the OXPHOS system. In the TCA cycle complex II catalyses oxidation of succinate to fumarate, producing FADH₂. The oxidation of FADH₂ releases electrons, which are transferred through several prosthetic groups and ultimately used to reduce ubiquinone to ubiquinol in the OXPHOS system (Hagerhall, 1997).

Complex III (cytochrome c reductase) consists of 11 subunits, of which one is mitochondrialencoded (cytochrome *b*). Two assembly factors have been identified. Complex III oxidizes ubiquinol (reduced by complex I and II) and transfers electrons to cytochrome *c* (a water soluble electron carrier located in the intermembrane space). This process is coupled with the translocation of two protons into the intermembrane space (Benit *et al.*, 2009).

Complex IV (cytochrome *c* oxidase, 200 kDa) is composed of 13 subunits, of which three (MTCOI, MTCO2, MTCO3) are mitochondrial-encoded. MTCOI and MTCO2 compose the catalytic core involved in the electron transfer whereas MTCO3 appears to be involved proton pumping. Complex IV mediates the transfer of electrons (through several prosthetic groups) from reduced cytochrome c to molecular oxygen. Reduction of oxygen to water is coupled with the translocation of four protons into the intermembrane space (Diaz, 2010).

Complex V (F_1F_0 ATP synthase, 550 kDa) consists of 16 subunits, two of which are mitochondrial-encoded (ATPase6 and ATPase8). It has two domains: the F_1 sector and F_0 sector. The F_1 sector is a soluble globular structure that extends into the matrix and composes the catalytic ATP synthase portion; the F_0 sector is cylindrical rotor-like structure embedded in the inner membrane, through which protons are translocated. Similarly, two assembly factors have been identified (ATPF1, ATPF2) (Kadenbach, 2012). Complex V uses the electrochemical proton gradient created by complexes I, III and IV to drive the synthesis of ATP. The diffusion of protons along F_0 creates a rotary motion which is transmitted to the catalytic domain F_1 that in turns drives the condensation of ADP and inorganic phosphate (Pi) into ATP molecules (Jonckheere *et al.*, 2012).

1.1.4.2 Calcium Handling

Mitochondria play a crucial role in calcium homeostasis due to their ability to uptake and buffer large quantities of Ca²⁺ (Rossi and Lehninger, 1963). Calcium crosses both outer and inner mitochondrial membranes mainly through VDAC and mitochondrial calcium uniporter (MCU) (Baughman *et al.*, 2011, De Stefani *et al.*, 2011), respectively, diffusing down its electrochemical gradient. This process reduces cytoplasmic calcium and is dependent on mitochondrial membrane potential. Extrusion occurs mainly through the 3Na²⁺/Ca²⁺ exchanger (Patergnani *et al.*, 2011).

Calcium is a major modulator of cellular function, with a crucial role in regulation of ATP production (Griffiths and Rutter, 2009) and apoptosis induction (Orrenius *et al.*, 2003). An increase in calcium uptake stimulates mitochondrial respiratory chain function (by stimulating the dehydrogenases and electron carriers) and hence ATP production (Denton *et al.*, 1980, Jouaville *et al.*, 1999). However, a sustained and excessive accumulation of calcium in mitochondria is toxic and can drive apoptosis (Mattson and Chan, 2003). Furthermore, in muscle, calcium is the signalling messenger that triggers all processes underlying muscle excitation-contraction. A tight regulation of calcium concentration is therefore crucial for proper muscle function (MacIntosh *et al.*, 2006).

1.1.4.3 ROS production

Mitochondrial OXPHOS system is a major source of ROS in cells (Jensen, 1966, Boveris *et al.*, 1972). Although historically they were seen as major cause of cell damage, it is now accepted that they are biologically important for several cellular functions such as adaptation to hypoxia and regulation of autophagy (Sena and Chandel, 2012).

During oxidative phosphorylation, some electrons leak from the transport chain, at complexes I and III (Loschen *et al.*, 1973, Cadenas *et al.*, 1977). These electrons can react with oxygen and convert it into superoxide anion (O_2^{--}), by partial reduction (Loschen *et al.*, 1974). O_2^{--} – the precursor of other ROS - is released either into the intermembrane space (O_2^{--} produced by complex III) or mitochondrial matrix (O_2^{--} produced by complex I), where it is converted into hydrogen peroxide (H_2O_2) by copper/zinc superoxide dismutase (SOD1) or manganese superoxide dismutase (SOD2), respectively. Subsequently, H_2O_2 diffuses into cytoplasm and is converted into water by glutathione peroxidase (Turrens, 2003). Although antioxidant enzymes work together to convert ROS into less harmful by-products, some H_2O_2 can be reduced to hydroxyl radical (OH⁻) by O_2^{--} and by reduced transition metals (Winterbourn, 1995).

When excessive ROS are produced, the cellular antioxidant system might not be sufficient to counteract. This results in damage of lipids, proteins and nucleotides and can ultimately lead to necrosis or apoptosis.

1.1.4.4 Apoptosis

Finally mitochondria have a pivotal role in programmed cell death (apoptosis) (Hockenbery *et al.*, 1990). Apoptosis can be triggered either extrinsically (by ligand-activation of death receptors on plasma membrane) or intrinsically (by DNA damage, oxidative stress, Ca^{2+} overload and endoplasmic reticulum stress). In the intrinsic pathway, intracellular signals facilitate mitochondrial inner membrane pemeabilisation and the consequent release of pro-apoptotic factors (proteins from the Bcl-2 family and cytochrome *c*) into the cytosol. This leads to the assembly of the apoptosome and further activation of caspases (Galluzzi *et al.*, 2007).

1.1.5 Mitochondrial genetics

1.1.5.1 Mitochondrial DNA mutations

The mutation rate of the mitochondrial genome is estimated to be 10 to 20-fold higher than the nuclear genome (Brown *et al.*, 1979). Somatic (*de novo*) mtDNA mutations are partly caused by reactive oxygen species (ROS) damage due to the close proximity of the mitochondrial genome with the source of mitochondrial ROS (complexes I and III) and the lack of protective histones (Ljungman and Hanawalt, 1992). Additionally, mutations can arise during mtDNA synthesis and/or during mtDNA repair processes (Krishnan *et al.*, 2008). POLG presents a high error rate (Kunkel and Loeb, 1981) and its exonuclease activity was shown to generate single-stranded regions of mtDNA at double strand beaks, thereby promoting mtDNA deletion formation (Krishnan *et al.*, 2008).

Mitochondrial DNA mutations can be grouped into two major classes, point mutations and rearrangements. The former arise from single base pair substitutions either in structural genes (coding for the respiratory chain complexes), rRNAs or tRNAs. They can be pathogenic or neutral polymorphisms (Tuppen *et al.*, 2010). Neutral polymorphisms arise from either synonymous mutation (mutations not causing changes in the amino acid) or neutral non-synonymous mutations (causing a change in the amino acid). Pathogenic mutations usually affect highly conserved amino acids/nucleotides and/or lead to a clear loss of function of the affected protein, among other features as revised by Taylor *et al.* (Taylor *et al.*, 2004).

Rearrangements include deletions (single or multiple), which are always pathogenic due to the partial removal of the mitochondrial genome, and duplications (Tuppen *et al.*, 2010).

1.1.5.2 Heteroplasmy

Due to the multi-copy nature of the mitochondrial genome, wild-type and mutant copies can co-exist in individual muscle fibres (Holt *et al.*, 1988, Zeviani *et al.*, 1988). Homoplasmy occurs when all mtDNA copies are identical (wild type or mutant) whereas heteroplasmy occurs when there is a mixture of more than one mtDNA genotype (wild type and mutant) (Taylor and Turnbull, 2005). The proportion between wild-type and mutated mtDNA copies – known as mutation load – can vary between muscle fibres, along muscle fibres length (Sciacco *et al.*, 1994) or among other tissues and organs within the same patient (Shanske *et al.*, 2004). Also, mutation load can change with time, decreasing in fast dividing tissues (blood) (Rahman *et al.*, 2001) and increasing in post-mitotic tissues (muscle and brain) (Larsson *et al.*, 1990, Weber *et al.*, 1997).

1.1.5.3 Threshold effect

MtDNA mutations are functionally recessive (with rare exceptions (Sacconi *et al.*, 2008)). The biochemical profile depends on the amount of mutated and wild type mtDNA (Figure 1.4); a muscle fibre segment shows respiratory chain dysfunction (Figure 1.4, blue segment of muscle fibre) when mutated mtDNA copies accumulate above a certain pathological threshold (Sciacco *et al.*, 1994, Elson *et al.*, 2002). Above this level, wild type mtDNA copies can no longer compensate for the mutation and support the normal mitochondrial function (Rossignol *et al.*, 2003).

The threshold level was shown to vary according to the tissue and the type of mutation. While single large-scale mtDNA deletions cause a biochemical defect when present at levels higher than 60% (Hayashi *et al.*, 1991), point mutations in tRNA genes only manifest a defect when present at levels above 90% (Chomyn *et al.*, 1992). Also, tissues which are highly dependent on OXPHOS system present a lower threshold level when compared to tissues relying on anaerobic glycolysis (Tuppen *et al.*, 2010). Deficient regions of muscle fibres are usually flanked by regions with intermediate activity progressing gradually into regions with normal mitochondrial function (Elson *et al.*, 2002, Murphy *et al.*, 2012).



Figure 1.4 Illustration of the threshold effect.

Mutated mtDNA can accumulate within cells up to a critical threshold with no impact on mitochondrial respiratory chain function (illustrated in the longitudinal fibre in beige); wild type molecules can complement the defect. When the percentage of mutated mtDNA relative to wilt type mtDNA (mutation load) exceeds the "threshold", mitochondrial respiratory chain function declines and the biochemical defect become apparent (illustrated in the longitudinal fibres in blue).

1.2 The healthy skeletal muscle

The skeletal muscle is formed during myogenesis through the proliferation, differentiation and fusion of mononuclear myoblasts into myotubes (MacIntosh *et al.*, 2006). These, group into cylindrical multinucleated syncytium called muscle fibres which are generally arranged in parallel along the longitudinal axis of the muscle (MacIntosh *et al.*, 2006). The number of muscle fibres increases until 25 weeks of gestational age. Beyond this age, cell proliferation is slowed (ceasing after birth) and muscle cross–sectional area increases through the hypertrophy of existing fibres (Stickland, 1981).

1.2.1 Muscle architecture

1.2.1.1 Extracellular matrix

The skeletal muscle is mechanically supported by the extracellular matrix organized into three interconnecting layers (Figure 1.5) (Borg and Caulfield, 1980). The endomysium covers individual muscle fibres and is crucial for force transmission to both tendons and adjacent fibres. The perimysium is a thicker layer that holds together muscle fibres into fascicules providing the pathway for the capillary network and innervating motor neurons. And finally,

the epimysium surrounds the entire muscle and is continuous with the outer layer of the tendon (Gillies and Lieber, 2011).

Each muscle fibre is additionally surrounded by a glycoprotein complex, called the basement membrane (Bowman, 1840), which lies between the endomysium and plasma membrane (or plasmalemma). It is involved in the organization of the neuromuscular junction, the termination of synaptic transmission and the connection between the plasmalemma and the endomysium (MacIntosh *et al.*, 2006). The plasmalemma and basement membrane are collectively referred to as sarcolemma (MacIntosh *et al.*, 2006).



Figure 1.5 Schematic illustration of skeletal muscle organisation.

The skeletal muscle consists of muscle fibres encapsulated within epimysium, grouped by the perimysium, and covered by the endomysium. Each muscle fibre is composed of myofibrils. Image (adapted) with the courtesy of Professor Richard L. Lieber.

1.2.1.2 Muscle fibres ultrastructure

Muscle fibres are formed by myofibrils arranged in parallel, and each myofibril is formed by the repetition of sarcomeres. Sarcomeres represent the contractile unit of the muscle (Hopkins, 2006). They are separated from each other at Z line and are made up of actin (thin myofilaments), myosin (thick myofilaments) and associated proteins (troponins and tropomyosin) (Hanson and Huxley, 1953, Huxley, 1953).

Each myofibril is surrounded by two networks of tubular structures: T tubules and sarcoplasmatic reticulum (Franzini-Armstrong and Porter, 1964). T tubules are invaginations of the plasmalemma that run transversely across the fibre, encircling myofibrils. Its branched arrangement allows propagation of the action potentials into the interior of muscle fibre. T tubules harbour dihydropyridine receptors (DHPR, voltage-gated Ca²⁺ channels) involved in the transduction of excitation into contraction. The sarcoplasmatic reticulum derives from the outer nuclear envelope and forms a network of longitudinal channels and chambers. They house ryanodine receptors (RyR) and Ca²⁺ ATPase (SERCA). RyR is a channel responsible for Ca²⁺ release whereas SERCA is involved in Ca²⁺ sequestering (MacIntosh *et al.*, 2006).

In mature muscle fibres, nuclei accumulate in the periphery of individual fibres adjacent to the plasmalemma. According to the myonuclear domain theory, each nucleus controls the surrounding area of cytoplasm by transcribing mRNAs locally and incorporating proteins in the myonuclear domain (Hall and Ralston, 1989, Pavlath *et al.*, 1989).

1.2.1.3 Motor endplate

The motor endplate is the muscle region contacting with motor nerve terminals. It is characterized by a deep folding of the sarcolemma; the crests of the folds accumulate nicotinic acetylcholine (ACh) receptors (AChRs) (Fertuck and Salpeter, 1974) while the depths contain voltage-gated sodium (Na⁺) channels. Nicotinic AChRs are non-selective cations channels activated by ACh and mediate the depolarization of the plasmalemma. When the ACh-mediated depolarization reaches a certain threshold, voltage-gated Na⁺ channels are activated leading to the genereation of a muscular action potential (Kandel *et al.*, 2000).

1.2.2 Muscle fibre types

The growth and differentiation of muscle fibres is known to be tightly controlled by the nervous system. Motor neurons influence the phenotype of muscle fibres through the frequency of electrical activity (Buller et al., 1960). Discharge rates at 10 and 100Hz are decoded by the transcriptional machinery into different patterns of gene expression triggering slow and fast- phenotypes respectively (Schiaffino et al., 1999).

Muscle fibres can be grouped into different classes according to morphological, metabolic and contractile characteristics (Table 1.2) (Burke *et al.*, 1971, Peter *et al.*, 1972, Reiser *et al.*, 1985). Briefly, type I fibres present a high oxidative capacity, their contraction and relaxation times are longer, they generate less power (mechanical energy released per unit of time) and require less ATP. Type II present elevated glycolytic capacity, less mitochondria, high ATP consumption rates and they fatigue more rapidly in response to sustained activity. These differences between muscle fibres extend further to motor neuron properties as well as to the structure of contractile elements. Most skeletal muscles have a mixture of different fibre types with the ratio between type I to II varying with the muscle.

Several studies have shown marked differences in mitochondria between fast and slow fibre types. In type II fibres, the Ca^{2+} buffering capacity per mitochondria is lower, the threshold for Ca^{2+} induced apoptosis is 3-fold higher and they produce 3-fold more ROS (Picard *et al.*, 2012).

1.2.3 Mitochondrial subpopulations in muscle fibres

Skeletal muscle fibres contain two subpopulations of mitochondria that differ essentially in their subcellular localization, structure, bioenergy, activity, adaptive capacity and susceptibility to apoptosis (Palmer *et al.*, 1977, Elander *et al.*, 1985, Cogswell *et al.*, 1993, Bizeau *et al.*, 1998, Adhihetty *et al.*, 2005, Mollica *et al.*, 2006). In brief, 20% of mitochondria accumulate around the nuclei in sub-sarcolemmal regions (called sub-sarcolemmal mitochondria) whereas the remaining accumulate in parallel rows between myofibrils. These inter-myofibrillar mitochondria are involved in energy production for muscle contraction. They are smaller and more compact, have a higher oxidative capacity (efficiency in ATP production), produce more reactive oxygen species (ROS) and are less sensitive to Ca²⁺ induced cell death.

	Type I Slow	Type Ila Fast-resistant	Type IIb Fast-fatigable
Morphological properties			
Fibre diameter	Small	Medium	Large
Capillary density	Rich	Rich/ medium	Sparse
Mitochondria	Rich	Rich	Poor
Oxidative activity	High	High	Low
Glycolytic activity	Low	High/ intermediate	High
Myoglobin	High	High/ Intermediate	Low
Myosin heavy chain	Type I	Туре ІІа	Type IIb
Functional properties			
Recruitment	1	2	4
Ca ²⁺ sequestration (SR)	Slow	Slow/ rapid	Rapid
ATP consumption	Low	High	High
Contraction speed	Slow	Fast	Fast
Twitch force	Small	Intermediate	Large
Fatigues resistance	High	Low/ Intermediate	Low

Table 1.2 Muscle fibre type enzymes, function and contractile characteristics.

Key: SR: sarcoplasmic reticulum. Adapted from (MacIntosh et al., 2006) and (Hopkins, 2006).

1.2.4 Muscle contraction

Muscle contraction is initiated with synaptic transmission at the neuromuscular junction. The propagation of an action potential into the pre-synaptic terminals causes the opening of voltage-gated Ca²⁺ channels. This increases cytoplasmic Ca²⁺ concentrations which in turn activates the exocytosis machinery. As a result, ACh is released into the synaptic cleft. On the post-synaptic side, ACh binds to nicotinic AChRs. This interaction triggers the influx of cations into muscle fibres depolarizing the surrounding plasmalemma. Consequently, the activation of voltage-gated Na⁺ channels generates a muscular action potential that spreads bi-directionally along plasmalemma (Kandel *et al.*, 2000).

The transduction of depolarization into contraction (excitation-contraction coupling) is facilitated by DHPR receptors. While depolarization spreads down t-tubular membranes, DHPR interact with RyR receptors present in sarcoplasmatic reticulum. As a result, Ca²⁺ is released into cytoplasm initiating the interaction of actin and myosin (cross-bridges formation). Multiple cycles of actin-myosin interaction occur (increasing or shortening the muscle) (Huxley and Niedergerke, 1954a, Huxley and Niedergerke, 1954b) until Ca²⁺ sequestering by SERCA exceeds release. As a result, the sarcomere returns to normal length (Hopkins, 2006).

The activation of muscle fibres can result in three types of contractions: isometric, shortening and lengthening (Faulkner, 2003). Isometric contractions occur when the external load is equal to the force developed (or immovable load) and fibre length remains unchanged. When the load is lower, the muscle shortens (concentric contractions), and when it is greater the muscle lengthens (eccentric contractions). During shortening contractions muscle performs work and the velocity is load-dependent. By contrast, during lengthening contractions the energy expenditure drops and the intensity of muscular effort is lower (Faulkner, 2003).

1.2.5 Muscle metabolism

Muscle contraction requires a lot of energy. Cross-bridge movement is dependent on myosin ATPase, Ca^{2+} sequestering relies on SERCA and the restoration of Na⁺ and potassium (K⁺) concentrations across the plasmalemma on Na⁺/K⁺ ATPase (MacIntosh *et al.*, 2006). During short-term intense exercise, energy consumption can increase up to 1000-fold (Baker *et al.*, 2010). The energy expenditure rate (which is a function of the load and velocity) is dependent on the breakdown ATP. Since muscle fibres (like all cells) do not store ATP in large quantity, they rely mainly on three metabolic systems to replenish ATP levels (MacIntosh *et al.*, 2006, Baker *et al.*, 2010).

The immediate energy system relies on the form of phosphocreatine (PCr). ATP is derived from phosphocreatine by creatine (Cr) kinase (ADP + PCr = ATP + Cr) sustaining intense muscle contraction for brief periods of time (10-15 seconds). This system regenerates ATP at an extremely rapid rate (Baker *et al.*, 2010). The short-term energy system relies on the anaerobic metabolism of glucose. It is activated during more prolonged activity and starts with an increase in glucose levels through blood uptake and glycogenolysis (mobilisation of muscle glycogen). Glucose is cleaved into pyruvate by a series of enzymes (glycolysis) leading to a rapid ATP formation close to the required sites (MacIntosh *et al.*, 2006). Pyruvate is subsequently converted into lactate (NADH + H⁺ + pyruvate = lactate + NAD⁺), by lactate dehydrogenase; this reaction is essential to remove excessive pyruvate, regenerate nicotinamide adenine dinucleotide (NAD⁺) and fuel further glycolysis . Both Cr and pyruvic acid end-products stimulate mitochondrial oxidative metabolism, the long-term energy system (covered in detailed in 1.1.4.1 Mitochondria oxidative metabolism) (Baker *et al.*, 2010).

Any reduction in muscle ATP or ATP turnover results in the development of fatigue. This is characterised by decreased force production, slower force development and slower relaxation of the muscle (Baker *et al.*, 2010).
1.3 The ageing skeletal muscle

During ageing (between the 20s and 70s), skeletal muscle undergoes a decline in total muscle mass by 25-40% (Young *et al.*, 1984, Young *et al.*, 1985, Lexell *et al.*, 1988). This reduction has been associated with a preferential atrophy of type II fibres (Grimby *et al.*, 1984, Lexell *et al.*, 1988) and a decline in the number of muscle fibres (Lexell *et al.*, 1988, Lexell, 1993), with the number of both type I and II fibres being reduced by 50% between the ages of 20 and 90 years old. Functionally, muscle strength was shown to decrease by 25-40% during both isometric and shortening contractions in 70-80 aged individuals falling to 50% after 90 years of age (Larsson *et al.*, 1979, Young *et al.*, 1984, Young *et al.*, 1985, Vandervoort and McComas, 1986). Also, shortening velocities were shown to become higher, leading to slower movements and reduced power (Bassey *et al.*, 1992)

Due to the high metabolic demand of skeletal muscle, dysfunctional muscle mitochondria are thought to be an important driver of muscle functional decline with age. First evidence of mitochondrial involvement in ageing muscle arose with studies using electron microscopy which demonstrated a decrease in the total number of mitochondria, in the number of cristae and increase in size of individual organelles in aged mice and rats myocardium (Miquel et al., 1980). Subsequent histochemical, biochemical, and molecular studies provided more evidence for the role of mitochondria in ageing (Hiona and Leeuwenburgh, 2008). The next section highlights a selection of important studies performed in this field which support the association of mitochondrial dysfunction and muscle functional decline with ageing.

1.3.1 Biochemical and *in vivo* and *in vitro* studies

Several studies performed in healthy ageing individuals have shown a decline in muscle mitochondrial bioenergetics. Trounce *et al.* were the first to report a reduction in mitochondrial respiratory chain function with age in human muscle (16-92 years old) (Trounce *et al.*, 1989). Subsequently, Cooper et *al.* showed that complex I and IV activities decline by 59% and 47% between the 20-30 and 60-90 age ranges, respectively (Cooper *et al.*, 1992). These findings are consistent with reductions in ATP flux. In that respect, the *in vitro* ATP synthesis measured from isolated mitochondria was shown to decline at a rate of 5% per decade between 18 and 78 years old (Short *et al.*, 2005). Also, further *in vivo* studies using phosphorous magnetic resonance spectroscopy indicated a 50% reduction in muscle oxidative capacity of aged individuals (69 years of age), which was related to a decline of mitochondrial density by 30%, and a reduction in mitochondrial oxidative capacity by 50% (Conley *et al.*,

2000). Besides the bioenergetic decline, *in vivo* rates of mitochondrial protein synthesis were also shown to be reduced by 40% in middle-aged (\approx 54) and in older (\approx 72) individuals (Rooyackers *et al.*, 1996). These results support the functional decline observed in the ageing muscle – which include a reduction in force production and increased fatigue.

1.3.2 Histochemical studies

In 1990, Muller-Hocker *et al.* demonstrated that segments of fibres showing no COX activity were randomly distributed in ageing muscle creating a mosaic appearance (Muller-Hocker, 1990). This results from the focal accumulation of mutated mtDNA within individual muscle fibres, as demonstrated by cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) histochemistry (Figure 1.6) (Old and Johnson, 1989); fibres appearing brown show normal COX activity whereas those appearing blue are devoid of COX activity despite normal levels of mitochondria.



Figure 1.6 Mosaic deficiency of the mitochondrial respiratory chain in ageing muscle. Left panel shows young skeletal muscle; all muscle fibres show normal COX activity. Right panel shows an aged muscle section; some fibres show normal COX activity (brown fibres), whereas some fibres present no COX activity (blue fibres).

COX deficient fibres are a hallmark of mitochondrial dysfunction and were later shown to accumulate with age in several species. In rhesus monkeys *vastus lateralis*, the percentage of COX deficient fibres was shown to increase from 4% up to 60% between 11 and 34 years of age (Lopez *et al.*, 2000). Also, some abnormal fibres exhibiting subsarcolemmal accumulation of mitochondria (called ragged-red fibres, RRF (Egger *et al.*, 1981)) were observed. Similar results were observed in rat's *rectus femoris* and human *vastus lateralis*. In rats, abnormal fibres showing RRF phenotype increased from non-detectable levels up to 42% between 5 and 38 months (Wanagat *et al.*, 2001); in humans, they increased from 6% to 31% between the ages

of 49 and 92 years (Bua *et al.*, 2006). The increased overall mitochondrial mass in RRF is associated with the activation of sub-sarcolemma mitochondrial biogenesis as a compensatory response to the respiratory chain defect and energy failure (Moraes *et al.*, 1992).

1.3.3 Molecular studies

Mutations in mtDNA were shown to accumulate with age in diaphragm muscle (Hayakawa *et al.*, 1991) and cardiac muscle (Cortopassi and Arnheim, 1990), among other tissues (Yen *et al.*, 1991, Taylor *et al.*, 2003). Although deletions, point mutations and depletion of mtDNA have been reported (Fayet *et al.*, 2002, Welle *et al.*, 2003, Short *et al.*, 2005) the ageing muscle predominantly accumulates deletions (Cortopassi and Arnheim, 1990, Cortopassi *et al.*, 1992).

Initial studies performed by Simonetti et al. in human muscle homogenates underestimated the impact of mtDNA deletions. These assessed mtDNA mutation load in homogenised muscle and found that mutated mtDNA constituted only up to 0.1% of total mtDNA (Simonetti et al., 1992). Later studies analysing individual muscle fibres provided evidence that mtDNA deletions accumulated focally to high levels within a subset of muscle fibres. This was observed in laser microdissected fibres displaying mitochondrial dysfunction from aged rhesus monkey (34 years old) (Schwarze et al., 1995), humans (75-90 years old) (Brierley et al., 1998) (49-92 years old) (Bua et al., 2006) and rats (38 months old) (Cao et al., 2001, Wanagat et al., 2001). Further quantitative PCR analysis in both aged human vastus lateralis and rat quadriceps (36 months) showed that mutated mtDNA accumulate to detrimental levels, higher than 90% (Bua et al., 2006, Herbst et al., 2007). Interestingly, studies performed in rodents revealed that regions accumulating high levels of mtDNA deletions co-localize with dysfunctional cellular phenotypes such as atrophic fibres, and fibres displaying splitting and breakage (Wanagat et al., 2001, Herbst et al., 2007). Also, RRF were shown to be more frequent in rat type II fibres (Wanagat et al., 2001) and in muscles showing a higher muscle mass loss, such as vastus lateralis when compared to the soleus or adductor longus muscles (Bua et al., 2002). These results suggest that accumulation of mtDNA mutations in ageing muscle fibres correlates with fibre atrophy and degeneration.

1.3.4 Studies in animal models

In 2004, Trifunovic *et al.* provided more evidence for the association between mitochondria and ageing with the generation of the mutator mice. These mice harbour a knock-in mutation

in the proofreading region of mtDNA polymerase gamma which impairs the fidelity of mtDNA replication (Trifunovic *et al.*, 2004). Consequently, the mutator mice accumulate high levels of mtDNA point mutations (3 to 5 fold higher, (Trifunovic *et al.*, 2004)) and deletions (7 to 11 fold, (Vermulst *et al.*, 2008)), and interestingly, exhibit a premature ageing phenotype and sarcopenia (Trifunovic *et al.*, 2004, Hiona *et al.*, 2010).

In this mouse model, mtDNA mutations accumulating in muscle fibres were shown to induce a decline in total content of complexes I, III and IV and a severe respiratory chain dysfunction. Also, both oxygen consumption and ATP production were shown to be reduced (Trifunovic *et al.*, 2004, Trifunovic *et al.*, 2005, Hiona *et al.*, 2010). Due to the impaired oxidative metabolism these mice present a higher reliance on glycolysis as a mean of energy production (Saleem *et al.*, 2015).

Although some studies did not find correlations between mtDNA mutations and enhanced ROS production, mtDNA oxidative damage and up-regulation of the antioxidant system (Kujoth *et al.*, 2005, Hiona *et al.*, 2010) some contradictory results were recently published. Kolesar *et al.* using a newly developed method to measure mtDNA replication and oxidative damage in intact mtDNA, found that the mutator skeletal muscle had lower levels of mitochondrial proteins and increased levels of 8-OHdG (marker of oxidative damage) within the mtDNA of muscle (Kolesar *et al.*, 2014). Also, Logan *et al.* used a new method (mitochondria-targeted mass spectrometry probe MitoB) to measure H₂O₂ within mitochondria in living mice. They found an increased production of ROS in skeletal muscle of the aged mutator group (35-42 weeks) when compared to the young mutator group (6-20 weeks) - a trend not observed in ageing control mice (Logan *et al.*, 2014).

Finally mtDNA mutations in cardiac and skeletal muscle were shown to trigger apoptosis (Zhang *et al.*, 2003, Kujoth *et al.*, 2005, Hiona *et al.*, 2010). This suggests that mtDNA mutations, and the consequent decrease in respiratory chain function, may lead to energy defects and ultimately to programmed cell death, with fibre loss being the phenotypic hallmark.

Although many studies have already documented the accumulation of mitochondrial dysfunction in ageing muscle, a more rigorous study using accurate quantification tools was needed. For that purpose, a novel assay was developed and extensively optimised using muscle samples from patients with mitochondrial disease. The following section gives an overview of mitochondrial diseases

1.4 The skeletal muscle in mitochondrial diseases

Mitochondrial diseases are a group of heterogeneous disorders that arise from defects of the OXPHOS system. The prevalence of 1 in 4300 people has been reported for the North East of England (Schaefer *et al.*, 2008). These diseases are caused by mutations in either mitochondrial or nuclear genomes. Mitochondrial DNA mutations were shown to account for 9.6 in 100,000 and nuclear mutations to account for 2.9 in 100,000 of the adult cases in the North East of England (Gorman *et al.*, 2015). This section will overview the most common mitochondrial and nuclear defects - which are relevant to this research. Rare defects will only be covered in chapter 5.

1.4.1 Clinical presentations

Mitochondrial disorders are characterised by a wide spectrum of clinical presentations: they can affect either a single structure such as the optic nerve in Leber's hereditary optic neuropathy, a whole organ such as seen in myopathies, cardiomyopathies and encephalopathies or multiple organs (Chinnery, 2014). Due to its high metabolic demand, muscle is commonly affected, either exclusively or predominantly in patients with a multi-systemic phenotype. Common clinical features include ptosis, proximal myopathy and exercise intolerance, cardiomyopathy, external ophthalmoplegia, deafness, optic atrophy and diabetes (Chinnery and Hudson, 2013).

Despite the heterogeneity of mitochondrial disorders, some well-defined syndromes have been characterised such as Kearns-Sayre syndrome (KSS), chronic progressive ophthalmoplegia (CPEO), Pearson syndrome (PS), mitochondrial encephalomyopathy, lactic acidosis and strokelike episodes (MELAS), myoclonus epilepsy with ragged-red fibres (MERRF), Leber's hereditary optic neuropathy (LHON), neuropathy, ataxia and retinitis pigmentosa (NARP) and maternally inherited Leigh syndrome (MILS) (Chinnery, 2014). Description of these symptoms are summarised in Table 1.3 and Table 1.4. It is important to mention that not all patients fall neatly into just one category.

Table 1.3 Common syndromes caused by mutations in mitochondrial DNA.

Syndrome	Common genotypes	Affected organs		
Point mutations				
Mitochondrial encephalopathy, lactic acidosis and stroke-like episode (MELAS)	m.3243A>G <i>MT-TL1</i> m.3271T>C <i>MT-TL1</i> m.9957T>C <i>MT-CO3</i> m.12770A>G <i>MT-ND5</i>	Brain (stroke-like episodes, encephalopathy, migraines), skeletal muscle (myopathy, lactic acidosis), heart, pancreas, ear and gut Onset in late childhood or adult life. Characterised by stroke – like episodes with seizures commonly leading to visual fields defects.		
Myoclonic epilepsy and ragged red fibres (MERRF)	m.8344A>G <i>MT-TK</i> m.8356T>C <i>MT-TK</i>	Skeletal muscle (myoclonus and myopathy), brain (cerebellar ataxia, deafness and dementia), heart Onset in late childhood or adult life. Progressive neurodegenerative disease characterised mainly by myoclonus (involuntary twitching of muscle), myopathy, proximal muscle wasting and neurological features (generalised epilepsy, cerebellar ataxia, optic atrophy, hearing loss)		
Neuropathy, ataxia and retinitis pigmentosa (NARP)	m.8993T>G <i>MT-ATP6</i>	Brain (ataxia), eye Onset in late childhood or adult life. Characterised mainly by peripheral neuropathy. Development delay, seizures and dementia may occur.		
		Eye (central vision loss), heart		
Leber's hereditary optic neuropathy (LHON)	m.3460G>A <i>MT-ND1</i> m.11778G>A <i>MT-ND4</i> m.14484T>C <i>MT-ND6</i>	Onset in late childhood or adult life (around 24years). Most common mtDNA-related disorder, affecting predominantly men. Organ-specific disease characterised by acute visual loss (unilateral and progressing to bilateral). Mutations are usually homonlasmic		
		Brain (brainstem, diencephalon and basal ganglia), peripheral		
Leigh syndrome	m.10158T>C <i>MT-ND3</i>	nerve, skeletal muscle Onset in early infancy. Neurodegenerative condition affecting mainly brainstem, diencephalon and basal ganglia. Common clinical features include: development delay, respiratory abnormalities, ataxia, dystonia and early death		
Single deletions				
Kearns-Sayre Syndrome (KSS)	Single large-scale mtDNA deletion	Extra-ocular skeletal muscle (progressive paralysis) heart (cardiomyopathy), brain (cerebellar ataxia, deafness), eye Onset in early infancy/childhood: <20years. Characterised by retinitis pigmentosa and PEO before age of twenty.		
Pearson's Syndrome (PS)	Single large-scale mtDNA deletion	Bone-marrow, exocrine pancreas, skeletal muscle and brain. Onset in early infancy. A blood disorder characterised by sideroblastic anemia and exocrine pancreatic failure. It causes either early death or development of KSS clinical feature in patients that survive.		
Chronic progressive external ophthalmoplegia (CPEO)	Single large-scale mtDNA deletion m.3243A>G <i>MT-TL1</i>	Extra-ocular skeletal muscle (progressive weakness/paralysis and ptosis), proximal skeletal muscles (muscle weakness and wasting), exercise intolerance, heart Onset in late childhood or adult life (most common presentation of mtDNA disease in adults). Characterised by a progressive paralysis of eye muscles causing impaired eye movement and ptosis (asymmetrical or bilateral)		

(Zeviani and Di Donato, 2004, Tuppen et al., 2010, Ylikallio and Suomalainen, 2012)

Table 1.4 Common syndromes caused by mutations in nuclear DNA.

Syndrome	Gene mutated	Affected organs			
Genes encoding structural and assembly factors					
Leigh syndrome	NDUFS1-4, NDUSFS6- 8, NDUFV1-2, NDUFA1 (Complex I subunits) SURF1, SCO1, LRPPRC (Complex IV assembly factors)	Brain (brainstem, diencephalon and basal ganglia), peripheral nerve, skeletal muscle Onset in early infancy/childhood. Neurodegenerative condition affecting mainly brainstem, diencephalon and basal ganglia. Common clinical features include: development delay, respiratory abnormalities, ataxia, dystonia and early death			
Genes involved in r	ntDNA maintenance				
mtDNA depletion syndrome	POLG, PEO1 (replisome machinery) SLC25A4, RRM2B (dNTR: matchalism)	Skeletal muscle (muscle weakness), progressive encephalopathy or liver failure. Onset in early infancy/childhood. Clinical features depend on the organ affected by mtDNA depletion.			
Progressive external ophthalmoplegia (PEO)	POLG, PEO1 (replisome machinery) SLC25A4, RRM2B (dNTPs metabolism)	Extra-ocular skeletal muscle (progressive weakness/paralysis and ptosis), proximal skeletal muscles (muscle weakness and wasting), exercise intolerance, heart Onset in late childhood or adult life. Characterised by a progressive paralysis of eye muscles causing impaired eye movement and ptosic (asymmetrical or bilateral)			
Genes involved in t	ranscript maturation and	mtDNA translation			
Myopathy, lactic acidosis, sideroblastic anemia	PUS1 (tRNA modification)	Skeletal muscle, bone-marrow Onset in early infancy/childhood. Characterised by mitochondrial myopathy and sideroblastic anemia			
Infantile hepatocerebral syndrome	GFM1 (Elongation factor)	Brain, liver Onset in early infancy. Characterised by severe lactic acidosis and rapidly progressive, fatal encephalopathy.			
Fatal neonatal lactic acidosis	MRPS16, MRPS22 (Ribosomal proteins)	MRPS16: brain (corpus callosum) MRPS22: heart, kidney Onset in early infancy. Characterised by severe, infantile, lactic acidosis, development defects in the brain and hipotonia. MRPS16: facial dimorphisms; MRPS22: fatal neonatal hypertrophic cardiomyopathy and kidney tubulopathy.			
Leukoencephalop athy, brainstem and spinal cord involvement, lactic acidosis	DARS2 (Amynoacyl-tRNA synthase)	Brain Onset in childhood disorder. Characterized by slowly progressive pyramidal, cerebellar and dorsal column dysfunction. Some affected children have learning problems, and some affected adults experience mild mental decline.			
Genes involved in p	protein and RNA import				
Progressive mitochondrial myopathy	<i>GFER</i> (protein import into IMS)	Skeletal muscle (progressive myopathy), eye Onset in early infancy. Characterised by congenital cataract, progressive muscular hypotonia, sensorineural hearing loss, and developmental delay.			
Deafness	PNPT1	Brain (severe hearing impairment) Onset in early childhood. Characterised by a severe hearing impairment and consequently inability to acquire normal speech			
Genes involved in mtDNA dynamics					
Dominant optic atrophy	Brain (optic atrophy, hearing loss), Extra-ocular skeletal muDominant opticAutosomal-dominantatrophyOPA1Characterised by optic atrophy and visual failure. Sensoring hearing loss, and ophthalmoplegia can also occur.				

Data shown here serves merrily to illustrate the variety of symptoms caused by secondary mitochondrial defects. ((Zeviani and Di Donato, 2004, Tuppen *et al.*, 2010, Ylikallio and Suomalainen, 2012)

1.4.2 Hallmarks of mitochondrial disease

Patients with OXPHOS defects can present with increased lactic acid production. This can be detected in blood and cerebrospinal fluid (Jackson *et al.*, 1995) in severely affected patients. This increased production results from the up-regulation of glycolysis and the accumulation of pyruvate; due to the OXPHOS impairment, pyruvate is no longer imported into mitochondria to feed the TCA cycle after its decarboxylation. Instead it remains in the cytoplasm where it is converted into lactic acid. This overproduction is commonly associated with lactic acidosis – a condition of global low pH in blood and body tissues (Schon *et al.*, 2012).



Figure 1.7 Mosaic deficiency of the mitochondrial respiratory chain in muscle from patients with mitochondrial disease.

Left panel shows a muscle section from a patient with multiple deletions in muscle. Right panel shows a muscle section from a patient with a single large-scale mtDNA deletion. Brown fibres show normal COX activity (brown fibres), blue fibres are devoid of COX activity.

Additionally, patients usually display other biochemical and morphological features. The activity of one or more complexes is commonly reduced (Taylor *et al.*, 2004). Similar to what occurs in the ageing muscle, involvement of mtDNA (as point mutations in tRNAs, single or multiple DNA deletions) lead to a mosaic pattern of COX deficiency in muscle (Johnson *et al.*, 1983, Sciacco *et al.*, 1994) (Figure 1.7). RRF are also common, however these can either be COX normal (in MELAS) or COX deficient (in CPEO, KSS or MERRF) (Petruzzella *et al.*, 1994). Involvement of nuclear DNA (other than mutations in genes leading to multiple deletions) can only be detected using COX/SDH histochemistry if the defect impairs complex IV activity and in this case, the defect is usually uniformly expressed across all muscle fibres. The impairment of remaining complexes can be detected by performing biochemical studies on muscle homogenates (Taylor *et al.*, 2004).

Most of the functional and cellular consequences of specific mutations have been studied using transmitochondrial cytoplasmic cybrid cells; these are immortalised human cell lines depleted of their own mtDNA that are repopulated with patients' mutated mtDNA (King and Attardi, 1989). This system allows testing of different levels of heteroplasmy and the resulting impact on ATP production, ROS production and calcium homeostasis. It was shown that when one or more respiratory chain complexes are compromised, oxygen consumption and ATP production are usually decreased (Trounce et al., 1994, Hofhaus et al., 1996, Pallotti et al., 2004, Gong et al., 2014). This could explain why mitochondrial patients commonly experience fatigue, muscle weakness and exercise intolerance, which is characterised by incapacity to produce force during sustained muscle contraction (Haller et al., 1978). Additionally, other mitochondrial functions may be impaired. For instance ROS production and cell death were shown to be upregulated in cybrid cells containing specific pathogenic mutations in complex I and V (Wong et al., 2002, Mattiazzi et al., 2004). A perturbation of calcium homeostasis, as reported by elevated cytosolic Ca²⁺ due to a reduced capacity of calcium sequestering, was also detected in fibroblasts from patients with MELAS and complex I deficiencies caused by nuclear mutations (Mattiazzi et al., 2004, Willems et al., 2008).

1.4.3 Mitochondrial defects

First pathogenic mutations in mtDNA were identified in 1988. Single large-scale mtDNA deletions of up to 7kb were detected in patients with encephalomyopathy (mitochondrial myopathy and KSS) (Holt *et al.*, 1988, Zeviani *et al.*, 1988) and a single base alteration in the mitochondrial gene encoding ND4 was found in patients with LHON (Wallace *et al.*, 1988a). Since then many more mutations and rearrangements of mitochondrial genome have been reported (Mitomap). Table 1.3 summarises and characterises common syndromes observed in patients with mitochondrial defects.

1.4.3.1 Point mutations

Pathogenic point mutations are present in the adult population with a prevalence of 1 in 5000 (Schaefer *et al.*, 2008, Gorman *et al.*, 2015). They are typically maternally inherited and most of them (50%) have been reported in tRNAs genes; 40% were located in structural genes and only 2% within rRNAs genes (Schon *et al.*, 2012).

Point mutations are usually heteroplasmic and require high thresholds (80-90%) to express mitochondrial dysfunction (Chomyn *et al.*, 1992, Mariotti *et al.*, 1994). However, some exceptions have been found with some mutations causing disease at low threshold. Two studies detected: a 25% mutation load in clinically affected tissues of patients with the m.5545C>T within the tryptophan tRNA (*MT-TW*) gene (Sacconi *et al.*, 2008) and 7% mutation load in patients with the m.14723T>C in the glutamic acid tRNA (*MT-TE*) gene (Alston *et al.*, 2010). Also, some cases of homoplasmy have been reported in structural genes of complex I (Wallace *et al.*, 1988a) and within the mitochondrial serine 1 tRNA gene (m. 7445T>C *MT-TS1*) (Reid *et al.*, 1994); these mutations were associated with organ-specific symptoms such as blindness and deafness, respectively.

Common point mutations include those occurring within mitochondrial lysine (m.8344A>G *MT*-*TK*) and leucine (3243 A>G *MT*-*TL1*) tRNAs – first identified in patients with MERRF (Shoffner *et al.*, 1990) and MELAS (Goto *et al.*, 1990, Kobayashi *et al.*, 1990), respectively. The m.3243A>G *MT*-*TL1* mutation is the most prevalent (7.8 in 100,000) point mutation in the North East of England (Gorman *et al.*, 2015). Althought 80% of the patients display a MELAS syndrome (Goto et al., 1990), CPEO (Moraes *et al.*, 1993) and myopathy (Karppa *et al.*, 2005) are also frequent. The m.8344A>G *MT*-*TK* mutation associated with MERFF (Shoffner *et al.*, 1990) is rarer in the North East of England (0.7 in 100,000) (Gorman *et al.*, 2015). Mutations in tRNAs can affect mitochondrial protein synthesis through several mechanism such as altered aminoacylation, altered three-dimensional structure and incorrect folding or decoding. Regardless of the underlying mechanism, mutations in tRNAs commonly lead to a decline in oxygen consumption rate, decreased activity of multiple OXPHOS complexes (such as I and IV) and increased lactate production (Mariotti *et al.*, 1994).

Pathogenic point mutations in all mitochondrial subunits of complex I, IV and V genes have been associated with disease (Ylikallio and Suomalainen, 2012). For instance, three common mutations in mitochondrial *ND1* (m.3460G>A), *ND4* (m.11778G>A) and *ND6* (m.14484T>C) were shown to cause LHON (Wallace *et al.*, 1988b, Howell *et al.*, 1991, Johns *et al.*, 1992).

1.4.3.2 Single deletions

Most rearrangements of mtDNA consist of single large-scale mtDNA deletions (1.5 in 100,000) (Gorman *et al.*, 2015). They typically occur sporadically (either in the mother's germ line or early embryogenesis) and can remove from 1.3 kb up to 10 kb of mtDNA, encompassing at least one tRNA gene (Pitceathly *et al.*, 2012). A common deletion of 4,977 bp (with

breakpoints at nucleotides: 8470 and 13477) is frequently reported (Schon *et al.*, 1989). Although individual single deletions may vary between patients, each patient harbours only one deleted genome species within affected tissues. These are heteroplasmic, and require lower pathological threshold (60%) to manifest dysfunction when compared to point mutations (Hayashi et al., 1991). Patients can present variable clinical phenotypes (PEO, KSS and PS) which can be predicted by the mtDNA deletion size and mutation load (Grady *et al.*, 2014).

1.4.3.3 Duplications

Duplications of mtDNA were first identified in 1989 in blood from patients with mitochondrial myopathy and a multi-systemic disorder (Poulton *et al.*, 1989). More cases have been reported since then (Rotig *et al.*, 1992, Poulton *et al.*, 1994). There is still some uncertainty whether these larger genomes result from the insertion of individual deletion genome into a wild type molecule, or they result from the combination of two partially deleted molecules (Poulton *et al.*, 1989). Also, their effects on mitochondrial functions are unclear since duplications do not lack any tRNAs, rRNAs or structural genes, and no oxidative impairment has been reported in these patients (Manfredi *et al.*, 1997, Odoardi *et al.*, 2003). Interestingly, mtDNA duplications are found with combination in single deletions in patients with an early-onset KSS disease, representing an hallmark of KSS (Poulton *et al.*, 1994).

1.4.4 Nuclear defects

The nuclear genome contributes to mitochondrial function in many different ways: through the structure or assembly of OXPHOS complexes, maintenance of mtDNA, transcription and translation of mtDNA, mitochondrial fusion and fission and last but not least, import of nuclear-encoded RNA and proteins into mitochondria (Schon *et al.*, 2012).

The first pathogenic mutation in a nuclear gene was identified in 1995. Bourgeron *et al.* reported a mutation in a nuclear-encoded subunit of the succinate dehydrogenase (SDH) in two sisters with complex II deficiency (Bourgeron *et al.*, 1995). Since then, molecular and sequencing approaches made the identification of novel mutant nuclear genes possible (Calvo *et al.*, 2012). Table 1.4 characterises syndromes observed in patents with nuclear defects; some of the defects are mentioned to illustrate the heterogeneity and complexity of this disease. As opposed to mitochondrial defects, nuclear mutations present a Mendelian inheritance.

1.4.4.1 Mutations in nuclear-encoded structural subunits or assembly factors

The nuclear genome encodes 70 (out of 83) polypeptides of the OXPHOS complexes. Mutations in nuclear-encoded subunits of all OXPHOS complexes have been identified (Schon et al., 2012). Those affecting complex I are most common, and have been described in 16 out of 45 nuclear structural genes. By contrast, mutations in nuclear-encoded subunits of complex IV are less frequent and have only been described in 2 out of the 11 nuclear structural genes. These mutations in nuclear-encoded structural genes usually cause severe disorder, sometimes are even lethal during infancy, and manifest at birth or soon after (DiMauro et al., 2013). Apart from structural genes, the nuclear genome encodes assembly factors which are strictly required for proper assembly and stability of the holocomplexes. Mutations in assembly factors of all OXPHOS complexes have also been reported (Tiranti et al., 1998, Zhu et al., 1998, Visapaa et al., 2002, Ghezzi et al., 2009, Calvo et al., 2010).

1.4.4.2 Mutations in genes involved in mtDNA maintenance

Several nuclear-encoded proteins are directly (POLG and Twinkle) (Lestienne, 1987, Spelbrink *et al.*, 2001) or indirectly (p53R2 subunit, and ANT1) (Neckelmann *et al.*, 1987, Tanaka *et al.*, 2000) responsible for mtDNA maintenance and regulation of mtDNA copy number - either by composing the replisome or by supplying dNTPs to mitochondria. Mutations in these genes are commonly associated with a severe onset multisystemic disease (mtDNA depletion syndrome), or with a mild adult-onset autosomal-recessive or autosomal-dominant PEO (with accumulation of multiple mtDNA deletions and no effect on mtDNA copy number) (Ylikallio and Suomalainen, 2012).

1.5 Aims

Due to the increasing prevalence of sarcopenia, with great impacts on both the quality of life and health care costs, there was a need to explore the biology of the ageing skeletal muscle. For this reason, this study was originally designed to investigate the following areas:

- 1. Changes occurring in selected skeletal muscles during ageing;
- 2. Mechanisms driving muscle wasting in ageing;
- 3. Potential therapeutic interventions which could be offered to the elderly.

COX/SDH histochemistry and immunohistochemistry were initially used to investigate the ageing muscle. However, soon after, I realised that these methods presented serious limitations. To overcome these problems, I developed a new method to accurately quantify the level of mitochondrial damage - initially using COX/SDH histochemistry and subsequentely using immunofluorescence. In order to optimise the new assay, I assessed patients with mitochondrial disease, and during this process, it became evident that this assay could greatly benefit patients with mitochondrial diseases. For this reason, this research was then redirected to meet the following aims:

- 1. Devise an assay that allows accurate quantification of mitochondrial dysfunction in diseased and healthy ageing muscle,
- 2. Improve diagnosis of patients and explore mitochondrial disease mechanisms,
- 3. Use the developed assay to investigate the effectiveness of potential therapeutic interventions which could be offered to patients with mitochondrial disease.

2.1 Materials

Equipment

Axio Imager M1 microscope
Apotome Axio Imager Z2 microscope
AxioCam MRm monochrome digital camera
AxioCam MRc colour digital camera
AxioVision (release.4.8.2) image capture software
Balance: sartorius basic
Binder general purpose incubator
CCD colour camera
Cyostat (Cryo-star HM 560M)
Dry heat block
Excel
Image J – image analysis software
Imaris - image analysis software
Laminar flow hood
Minitab 16
Olympus BX51 light microscope
Prims 5
3510 pH meter
Stereology software (Stereo Investigator, MBF)
Vortex Genie
Zen 2011 (blue edition) image capture software

Consumables

Aerosol resistant pipette tips Coverslips (22 x 22mm, 22 x 40mm, 22 x 50mm) Eppendorf tubes (0.6ml, 1.5ml, 2.0ml) Falcon tubes (15ml, 50ml) Gilson pipetteman (P2, P10, P20, P200, P1000) Pasteur pipettes Carl Zeiss Carl Zeiss Carl Zeiss Carl Zeiss Carl Zeiss Sartorius Philip Harris Olympus **Microm International** Techne Microsoft Public Domain, NIH Bitplane Jencons-PLS Minitab Inc. Olympus GraphPad Software Inc. Jenway Bioscience Scientific Industries Carl Zeiss

Star lab Merck Star lab BD Biosciences Anachem VWR

Superfrost slides	Merck
Slide tray and box	Thermo Scientific
Whatman filter paper	Fisher Scientific

Reagents: tissue preparation

Iso-pentane	Merck
Liquid Nitrogen	BOC
OCT cryo-embedding matrix	Raymond Lamb

Reagents: Histology and immunohistochemistry

Catalase
Cytochrome c
3,3 Diaminobenzidine tetrahydrochloride
3,3 Diaminobenzidine tetrahydrochloride tablets
DPX mounting media
Bovine serum albumin (BSA)
Ethanol
Haematoxylin
Histoclear™
Hydrochloric acid
Hydrogen peroxide 30%
Hydrophobic pen
Methanol analar
MenaPath detection system
Nitroblue tetrazolium
Normal goat serum (NGS)
4% Paraformaldehyde (PFA)
Phenazine methosulphate
Phosphate Buffered saline (PBS) tablets
Prolong gold mounting media
Sodium azide
Sodium succinate
Sodium chloride (NaCl)
Sudan black
Trizma base
Tween-20

Sigma-Aldrich Sigma Sigma Sigma BDH Sigma **Fisher Scientific** Raymond A Lamb **National Diagnostics** VWR Sigma Daido Sangyo Merck Menarini diagnostics Sigma-Aldrich Sigma Santa Cruz Biotechnology Sigma-Aldrich Sigma-Aldrich Life Technologies Sigma-Aldrich Sigma Sigma-Aldrich VWR International (342013F) Sigma-Aldrich Sigma-Aldrich

Reagents: Primary antibodies

Mouse IgG2a MTCOI	Abcam (Ab14705)
Mouse IgG2b NDUFA13	Abcam(Ab110240)
Mouse IgG1 SDHA	Abcam (Ab14715)
Mouse IgG1 NDUFB8	Abcam (Ab110242)
Mouse IgG2b Porin	Abcam (Ab14734)
Policlonal Rabbit IgG Laminin $lpha extsf{-1}$	Sigma-Aldrich (L9393)

Reagents: Secondary antibodies

Anti- mouse IgG2a Fluorescein – 488nm
Anti- mouse IgG2b Rhodamine – 546nm
Anti- mouse IgG1 Alexa fluor – 647nm
Anti- mouse IgG1 Rhodamine - 546nm
Anti- mouse IgG2b Alexa fluor – 647nm
Anti- mouse IgG1 Alexa fluor – 488nm
Anti- mouse IgG2a Alexa fluor – 546nm
Anti- mouse IgG2b Alexa fluor – 633nm
Anti- mouse IgG2b Alexa fluor – 488nm
Anti- mouse IgG1 Alexa fluor – 546nm
Anti- mouse IgG2a Alexa fluor – 647nm
Anti- mouse IgG2a Biotin
Streptavidin – 647nm
Anti-rabbit IgG Alexa fluor 405nm
Anti- mouse IgG2a Alexa fluor – 488nm
Anti- mouse IgG2b Alexa fluor – 546nm
Anti-mouse IgG1 Biotin

Solutions

Phosphate buffered saline (PBS)

Tris buffered saline with tween (concentrated 5x) (TBST, pH 7.6)

1 tablet 100ml distilled water 121g Trizma base 90g NaCl 50ml Tween-20

* Jackson IR Lab: Jackson ImmunoResearch Laboratories

Jackson IR Lab (115-095-206) Jackson IR Lab (115-295-207) Life Technologies (A21240) Jackson IR Lab (115-295-205) Life Technologies (A21242) Life Technologies (A21121) Life Technologies (A21133) Life Technologies (A21146) Life Technologies (A21141) Life Technologies (A21123) Life Technologies (A21241) Life Technologies (M32315) Life Technologies (S31556) Life Technologies (A31556) Life Technologies (A21131) Life Technologies (A21143) Jackson IR Lab (115-065-205)

2.2 Methods

2.2.1 Ethical approval

Ethical approval for this research was granted by the Newcastle and North Tyneside Local Research Ethics Committees (reference 09/H0906/75). Ethical approval for the use of postmortem tissue and surplus tissue taken for diagnostic purposes in research studies (through a written informed consent) was granted by Newcastle and North Tyneside 1 REC (reference 2002/205).

2.2.2 Muscle biopsies

Clinical muscle biopsies from cases of suspected mitochondrial disease were taken from the mid-portion of the *vastus lateralis* using the needle biopsy technique. *Post-mortem* tissue (biceps, *vastus laterali* and psoas muscle) from healthy aged controls were collected with a maximum delay of 72h and processed immediately. Transversely orientated muscle blocks were snap-frozen in an isopentane bath pre-cooled to -160°C in liquid nitrogen. Subsequently, frozen muscle was embed on Whatman filter paper using OCT cryo-embedding matrix and finally stored at -80°C.

2.2.3 Cryostat sections

Serial sections from transversely orientated muscle blocks were obtained using a Cryo-star HM 560M cryostat. Frozen muscle blocks were cut at -20°C into sections of 10µm-thick and collected onto superfrost slides (Merck). Sections were allowed to air dry at room temperature (RT) for 1h and then stored at -80°C for subsequent use.

2.2.4 Mayer's Haematoxylin counter-staining

Haematoxylin stain (Mayer 1904) was used to visualise muscle nuclei. Haemotoxylin is a compound that binds to arginine-rich nucleoproteins such as histones. This binding oxidises haematoxylin to hemalum, which has a blue colour.

2.2.5 Cytochrome *c* oxidase (COX)/ Succinate dehydrogenase (SDH)

Sequential cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) histochemistry is the gold standard methodology to assess mitochondrial respiratory chain activity in muscle cryo-sections (Old and Johnson, 1989). COX reaction measures complex IV activity and SDH reaction measures complex II activity, in individual muscle fibres. Since complex IV is partially mitochondrial encoded, mtDNA mutations will most likely affect COX activity. However, complex II is fully nuclear encoded and therefore it measures overall mitochondrial mass.

The reaction of COX relies on the oxidation of diaminobenzine (DAB) by active cytochrome. Active complex IV oxidises DAB which forms a brown precipitate that saturates cells. The reaction of SDH, on the other hand, relies on the reduction of nitroblue tetrazolium by succinate dehydrogenase (SDHA and SDHB) and the subsequent production of a blue precipitate. By combining both reactions in a single slide, identification of cells with mitochondrial dysfunction is easy. COX positive cells appear brown (due to the oxidation of DAB); COX deficient fibres appear blue (due to the absence of oxidised DAB during the incubation with COX reaction and formation of the blue precipitate during the incubation with SDH reaction).

For both COX and SDH solutions preparation, reagents were defrosted at 55°C. COX reaction was prepared by adding 800µl of 4mM 3,3'-diaminobenzidine tetrahydrochloride (DAB), 200µl of 100µM cytochrome c and a pinch of catalase, in 0.2M phosphate buffered saline solution (PBS, pH 7.0), and vortexed quickly. SDH was prepared by adding 800µl of 1.5mM nitrobluetetrazolium, 100µl of 130mM sodium succinate, 100µl of 0.2mM phenazine methosulfate and 10µl of 0.1mM sodium azide in 0.2M PBS (pH7.0), and vortexed quickly.

COX/SDH histochemistry was carried out on transverse muscle sections (10µm). Serial sections were taken from the freezer and allowed to air dry for 1h at RT. After rehydrating in PBS, sections were incubated with either 100µl of COX solution (for 45min at 37°C) or 100µl of SDH solution (for 40min at 37°C) in a humidified chamber. For the combined COX/SDH histochemistry a section was incubated with COX reaction for 45min followed by the SDH reaction for 40min with a brief wash in PBS in between. Following the staining procedure the sections were washed with PBS, dehydrated in ascending ethanol gradients (70%, 95%, 100%, 100%) and cleared in two washes in histoclear. Sections were mounted in DPX medium and covered with a cover slip.

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2.2.6 Immunohistochemistry

Immunohistochemistry was carried out on transverse muscle sections (10µm) using antibodies detecting subunits of mitochondrial respiratory chain complexes. Each method's chapter will provide information about the type of antibodies used, incubation times and conditions. Two systems were used to detect primary antibodies: chromogen and fluorescence. For both, the initial steps of the immunohistochemical protocol were similar, and as follow:

Briefly, muscle sections were taken from the freezer and allowed to dry for 1h at RT. Approximately 10 min before initiating the protocol, sections were encircled using a hydrophobic pen; this prevents the leakage of incubation medium from muscle sections. The sections were fixed in cold 4% paraformaldehyde (PFA) at room temperature (RT) and washed in tris buffered saline with tween (TBST, 1x). This fixation step is frequently essential because it cross-links proteins ensuring unaltered tissue morphology, and preserves tissue for further processing.

Sections were then permeabilised in a gradient of methanol as follows: 10min in 70% methanol, 10min in 95% methanol, 20min in 100% methanol, 10min in 95% methanol and 10min in 70% methanol. This step was required since most investigated antigens were intracellular proteins. The methanol gradient dissolves lipids from cell membranes, and therefore allows antibodies to access the inside of muscle fibres.

After washing in TBST (1x) the sections were incubated with protein blocking solution; this is a solution of normal serum from the species in which secondary antibodies were raised. This step prevents non-specific binding of secondary antibodies to endogenous antigens. Primary antibodies were diluted in the protein blocking solution to the correct concentrations and were applied onto the sections. Sections were incubated overnight at 4°C in a humidified chamber. Secondary antibodies/reagents incubation and the subsequent detection steps are explained in detail below.

2.2.6.1 Chromogen-based immunohistochemistry

For chromogen-based immunohistochemistry, the protocol was performed as described above with one incorporated change: 0.3% of H_2O_2 was added to the 95% methanol pot. Hydrogen peroxide allows blocking endogenous peroxidase activity. This is crucial since primary antibodies were detected using MenaPath kit (Menarini Diagnostics) which relies on horseradish peroxidase enzymatic detection. This system uses a specific universal probe to detect mouse antibodies and a second probe - a polymer coupled with horseradish peroxidase (polymer-HRP) - to increase labelling intensity and sensitivity. The hydrogen peroxide step prevents therefore non-specific background due to active endogenous peroxidase.

Following the primary antibodies incubation and subsequent washes in TBST (1x), the sections were incubated with universal probe (MenaPath) for 30 min. Having washed the slides in TBST (1x), polymer-HRP was applied onto the sections (MenaPath). Finally DAB was prepared by dissolving 1 silver and 1 gold tablet in 5ml of distilled water (according to the manufacturer's instructions, Sigma) and applied onto the sections; DAB is a substrate of HRP. The development of brown precipitate was monitored under the microscope until satisfactory signal appeared. Sections were counter-stained with Mayer's Haematoxylin (see 2.2.4 Mayer's Haematoxylin counter-staining) by immersing slides in haematoxylin for 10min. Subsequently, sections were washed in tap water until the water turned clear, dehydrated in in ascending ethanol gradients (70%, 95%, 100%, 100%) and cleared in two washes in histoclear. Sections were mounted in DPX medium and covered with a cover slip.

2.2.6.2 Fluorescence-based immunohistochemistry

For fluorescence-based immunohistochemistry, primary antibodies were detected using secondary antibodies coupled with fluorophores. An important aspect of this technique is the ability to visualise several epitopes at the same time, using a single sample. This is possible due to the multitude of: gamma Ig (IgG) isotypes (IgG1, IgG2a, IgG2b), of host species donating antibodies (mouse, rabbit, chicken, among others), and of fluorophores available.

Double, triple and quadruple immunofluorescence was performed in this study. In all, sections were incubated with a cocktail of diluted primary antibodies (of two, three or four primary antibodies). After washing in TBST (1x), the sections were incubated with a cocktail of diluted secondary antibodies (two, three or four secondary antibodies). The sections were washed in TBST (1x) and mounted in mounting medium and covered with a cover slip (further details will be provided in chapters 3 and 4).

2.2.7 Imaging

Brightfield and fluorescent images were acquired at 10x or 20x magnification using Zeiss Microscope (Axio Imager M1) and Zen 2011 (blue edition) software. The microscope was equipped with both colour and monochrome Digital Cameras (AxioCam MRc, AxioCam MRm) and appropriate filter cubes that allow detection of 488nm, 546nm and 647nm fluorescence. The exposure times for each channel were set to avoid pixel saturation and kept constant between cases (unless stated otherwise). Sections were imaged (with a safety gap between scanned areas to avoid overlapping) and stored as 16-bit fluorescent tagged image file format (TIFF) and czi files.

3.1 Introduction

Mitochondrial dysfunction occurs in patients with mitochondrial disease and as part of the ageing process, predominantly affecting tissues with high metabolic requirements such as brain and muscle (Muller-Hocker, 1989, Cortopassi and Arnheim, 1990, Chinnery *et al.*, 2002). Mutations in the mtDNA usually lead to mosaic deficiency of the mitochondrial respiratory chain that results from the accumulation of mutated mtDNA in a subset of muscle fibres (Taylor *et al.*, 2004).

COX/SDH histochemistry is currently the standard method to assess mitochondrial respiratory chain function in muscle cryo-sections (Old and Johnson, 1989, Sciacco and Bonilla, 1996). This technique interrogates complex IV (COX) and II (SDH) activities. Since COX activity is proportional to mitochondrial mass in a healthy cell, any reduction or loss of COX activity with preserved SDH activity indicates mtDNA damage. This is a particularly valuable method for evaluating cases with mosaic pattern of COX deficiency. In these instances, biochemical assays in muscle homogenates are not sensitive enough to detect subtle respiratory chain deficiency especially when only a few muscle fibres are involved (Taylor *et al.*, 2004).

Conventional analysis of COX/SDH histochemistry relies on visual classification of muscle fibres by a researcher based on the colour intensity. This makes the assessment subjective and inconsistent, and therefore prone to inter- and intra-researcher variability (Taylor and Levenson, 2006, Choudhury *et al.*, 2010, Rizzardi *et al.*, 2012, Chatterjee *et al.*, 2013). Also it makes the assessment inaccurate; whereas a complete loss of COX reactivity is easily detected, subtle changes may not be visible to the human eye (Arechavala-Gomeza *et al.*, 2010). This can lead to miss-classification (Ellis *et al.*, 2005) and ultimately to under- or over-estimation of mitochondrial dysfunction. Last but not least, COX/SDH histochemistry is limited to measuring complex IV activity and it does not provide any information about other complexes of the OXPHOS system.

Recently, an objective assessment based on a combination of COX/SDH histochemistry and NDUFB8 immunohistochemistry was developed. The intensities of individual COX, SDH and NDUFB8 reactivity from single muscle fibres were used to determine the respiratory status of

muscle fibres ((COX*NDUFB8)/SDH)/100 (Murphy *et al.*, 2012). This allows differentiating fibres with normal COX-I activity and NDUFB8 levels from those with: a progressive loss of COX and NDUFB8 and preserved SDH, or from those exhibiting: a complete loss of both COX-I and NDUFB8 and high levels of mitochondrial mass (judged by SDH activity).

Other groups have also explored the use of immunohistochemical techniques to detect mitochondrial respiratory chain deficiency. Mahad *et al.* developed a new method to quantify COX-I and porin levels following COX histochemistry in single cells of the central nervous system and muscle. This provided insights on the functional and structural impairment of mitochondrial dysfunction (Mahad *et al.*, 2009). De Paepe *et al.* developed a chromogen-based immunohistochemical approach to assess other OXPHOS complexes (I, III and V) in serial muscle sections. This was particularly valuable to detect complex I deficiency in single fibres which is not possible using COX/SDH histochemistry (De Paepe *et al.*, 2009). Similarly, Hanson *et al.* set out a double fluorescent-based immunohistochemistry to assess levels of OXPHOS complexes (I, II, III, IV and V) in relation to porin levels, in cultured fibroblasts (Hanson *et al.*, 2002). Although very useful, all these studies present some limitations: immunohistochemistry was either performed in serial muscle sections (Rahman *et al.*, 2000, De Paepe *et al.*, 2009) and/or immunoreactivity of cells was qualitatively assessed (Rahman *et al.*, 2000, Hanson *et al.*, 2002, De Paepe *et al.*, 2009).

3.2 Aim

The aim of this chapter was to develop an objective assessment of mitochondrial respiratory chain function in single muscle fibres that would allow quantifying accurately mitochondrial deficiency in a sarcopenic cohort and patients with mitochondrial disease.

3.3 Methods

3.3.1 Cohort - clinical characteristics

Archived frozen muscle samples derived from either *post-mortem* tissue or clinical muscle biopsies (taken for diagnostic purposes) were assessed. Table 3.1 summarises relevant clinical information. The three aged control were assessed in order to quantify mitochondrial respiratory chain dysfunction accumulating with age. The young disease control (DC1) was previously investigated for suspected neuromuscular disease but showed normal muscle histology, oxidative enzyme histochemistry and normal respiratory chain biochemical activities (Kirby *et al.*, 2007); he was used as a "negative control" since mitochondrial dysfunction was not expected to be found. Patients P1 and P2 harboured the common m.3243A>G *MT-TL1* mutation and P3 multiple deletions in muscle (due to a mutation in a nuclear gene involved in mtDNA maintenance). P1 and P2 muscle biopsies' showed a mosaic COX deficiency which was challenging to visually classify, therefore they were used to quantify subjectivity of visual classification. P3 muscle biopsy also presented a mosaic COX deficiency, which was easy to visually classify, therefore he was used to validate the objective classification based on COX-I and SDHA immunodetection.

Subjects	Gender	Age	Genetic defect	Histochemical findings	Biopsy type (PM delay)
Aged controls					
AC1	Male	75y	n.r.	n.d.	PM (72h)
AC2	Female	76y	n.r.	n.d.	PM (48h)
AC3	Female	86y	n.r.	n.d.	PM (48h)
Disease control					
DC1	Male	4y	n.r.	Normal	Diagnostic (n.r.)
Patient with known mitochondrial disease					
P1	Female	40y	m.3243A>G MT-TL1 mutation;	>30% COX deficient, 10% RRF; also COX positive RRF	Diagnostic (n.r.)
P2	Male	53y	m.3243A>G MT-TL1 mutation;	45% COX deficient fibres; 20% RRF	Diagnostic (n.r.)
P3	Male	60y	Multiple mtDNA deletions;	20% COX deficient fibres; 7% RRF	Diagnostic (n.r.)

Table 3.1. Clinical information from subjects included in this study

Key: PM: *post-mortem*; n.d. = not determined; n.r.: not relevant; y: years old; RRF: ragged-red fibres.

3.3.2 Cryosectioning

Serial sections (10µm thickness) from transversely orientated muscle blocks were obtained as previously described in section 2.2.3 from chapter 2.

3.3.3 COX/SDH histochemistry

Individual and combined COX/SDH histochemistry was carried out according to the protocol described in section 2.2.5 from chapter 2.

3.3.4 Immunohistochemistry

Immunohistochemistry was performed using antibodies detecting subunits of mitochondrial respiratory chain complexes. Complex I abundance was detected using antibodies against either NDUFB8 or NDUFA13 subunits. These are nuclear-encoded subunits of complex I which are required for complex I assembly and electron transfer activity (Perales-Clemente *et al.*, 2010). To detect complex IV abundance we used an antibody to the mtDNA encoded complex IV subunit I (COX-I). To enable assessment of overall mitochondrial mass we used an antibody against the subunit A of the nuclear-encoded complex II or against porin (VDAC) (Mannella, 1998), commonly used as mitochondrial mass markers (Mahad *et al.*, 2009, Grunewald *et al.*, 2014).

3.3.4.1 Chromogen-based immunohistochemistry

Briefly, the sections were fixed in 4% PFA for 15min at RT and permeabilised in a gradient of methanol. Following TBST washes, the sections were incubated with 1% bovin serum albumin (BSA) for 30min and then incubated with the primary antibodies in a humidified chamber at 4°C overnight (Table 3.2). Following TBST washes, the sections were incubated with universal probe (for 30min at RT), and subsequently, incubated with polymer-HRP (for 30 min at RT) (MenaPath). Finally DAB was prepared and applied to the sections until satisfactory signal appeared. Sections were counter-stained with Mayer's Haematoxylin, dehydrated in ethanol gradient and cleared in histoclear before mounting in DPX.

3.3.4.2 Fluorescent-based immunohistochemistry

Briefly, the sections were fixed in 4% PFA for 3min and permeabilised in a gradient of methanol. Following TBST washes, sections were blocked with 1% normal goat serum (NGS) for 30min at RT and incubated with a primary antibody cocktail in a humidified chamber at 4°C overnight (Table 3.3). Subsequently to TBST washes, the sections were incubated with a cocktail of secondary antibodies for 1h at RT (Table 3.3). Finally, the sections were immersed in sudan black for 10 min at room temperature, to quench auto-fluorescence. Sections were washed in TBST and mounted in Prolong Gold mounting medium (Life Technologies).

3.3.5 Imaging

Brightfield and fluorescent images were acquired at 10x magnification (unless stated otherwise) using Zeiss Microscope (see section 2.2.7 from chapter 2). For the acquisition of fluorescent images, the exposure times for each channel were adjusted for each individual case.

3.3.6 Assessing mitochondrial respiratory chain dysfunction

3.3.6.1 Visual Classification of COX/SDH histochemistry

To assess mitochondrial dysfunction in different muscles from aged control AC3, muscle fibres reacted for COX/SDH histochemistry were visually classified using a stereological workstation with a modified light microscope (Olympus, Japan), motorized stage, CCD colour camera and stereology software (Stereo Investigator, MBF Bioscience, USA).

To assess the limitations of COX/SDH histochemistry (intra- and inter-observer variability), a third investigator imaged different areas of two biopsies and selected 100 muscle fibres for each patient. Both investigators 1 and 2 visually classified labelled muscle fibres in COX/SDH histochemistry images (at 20x magnification).

3.3.6.2 Visual classification of chromogen-based immunohistochemistry

To further explore the profile of abnormal fibres from aged control AC3, matching muscle fibres in: COX/SDH histochemistry, NDUFA13 immunohistochemistry and COX-I immunohistochemistry images, were visually classified into one of the following categories: ND (fibres presenting intermediate or absent COX activity and no detectable changes in complex IV abundance), NDUFB8 \downarrow /COX-I + (fibres with preserved COX activity and levels but down-regulated NDUFB8 levels), NDUFB8 \downarrow /COX-I \downarrow (fibres with preserved NDUFB8 but down-regulated COX activity and levels) or NDUFB8 \downarrow /COX-I \downarrow (fibres with down-regulated NDUFB8 and levels).

Table 3.2. Primary and secondar	y antibodies used for chromogen-based immunohistochemistr	y
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Antibodies	Host	Dilution	Company (Product number)
MTCOI (IgG2a)	Mouse	1:4000	Abcam (Ab14705)
SDHA (IgG1)	Mouse	1:2000	Abcam (Ab14715)
NDUFB8 (IgG1)	Mouse	1:300	Abcam (Ab110242)

Antibodies	Host	Dilution	Company (Product number)
Primary antibodies			
MTCOI (IgG2a)	Mouse	1:100	Abcam (Ab14705)
NDUFA13 (IgG2b)	Mouse	1:100	Abcam(Ab110240)
SDHA (IgG1)	Mouse	1:100	Abcam (Ab14715)
NDUFB8 (IgG1)	Mouse	1:100	Abcam (Ab110242)
Porin (IgG2b)	Mouse	1:100	Abcam (Ab14734)
Secondary antibodies			
Anti-mouse IgG2a Fluorescein – 488nm	Goat	1:300	Jackson IR Lab (115-095-206)
Anti-mouse IgG2b Rhodamine – 546nm	Goat	1:300	Jackson IR Lab (115-295-207)
Anti-mouse IgG1 Alexa fluor – 647nm	Goat	1:300	Life Technologies (A21240)
Anti-mouse IgG1 Rhodamine - 546nm	Goat	1:300	Jackson IR Lab (115-295-205)
Anti-mouse IgG2b Alexa fluor – 647nm	Goat	1:300	Life Technologies (A21242)
Anti-mouse IgG1 Alexa fluor – 488nm	Goat	1:200	Life Technologies (A21121)
Anti-mouse IgG2a Alexa fluor – 546nm	Goat	1:200	Life Technologies (A21133)
Anti-mouse IgG2b Alexa fluor – 633nm	Goat	1:200	Life Technologies (A21146)
Anti-mouse IgG2b Alexa fluor – 488nm	Goat	1:200	Life Technologies (A21141)
Anti-mouse IgG1 Alexa fluor – 546nm	Goat	1:200	Life Technologies (A21123)
Anti-mouse IgG2a Alexa fluor – 647nm	Goat	1:200	Life Technologies (A21241)
Anti-mouse IgG2a Biotin	Goat	1:200	Life Technologies (M32315)
Streptavidin – 647nm	Goat	1:100	Life Technologies (S31556)
Anti-rabbit IgG Alexa fluor 405nm	Goat	1:100	Life Technologies (A31556)

Table 3.3. Primary and secondary antibodies used for immunofluorescence

Key: Jackson IR Lab: Jackson ImmunoResearch Laboratories

3.4 Results

3.4.1 Challenges of COX/SDH histochemistry

In order to investigate the degree of mitochondrial dysfunction in the ageing muscle, three different muscles (biceps, diaphragm and psoas) from three aged controls (AC1, AC2 and AC3 (Table 3.1)) were studied. Serial sections from each muscle were assessed using COX/SDH histochemistry and chromogen-based immunohistochemistry detecting COX-I, SDHA and NDUFB8 (AC1: Figure 3.1, AC2: Figure 3.2 and AC3: Figure 3.3). Muscle fibres were visually classified based on COX/SDH histochemistry into one of the following three categories: COX-positive, COX-intermediate (int) and COX-deficient (neg) (Murphy *et al.*, 2012). Fibres were further classified based on immunohistochemistry of COX-I and NDUFB8 (see 3.3.6.2.). Throughout this assessment several problems and difficulties were encountered.

3.4.1.1 Underestimation of the overall mitochondrial dysfunction

All aged muscle biopsies showed some level of mitochondrial respiratory chain deficiency. Frequent abnormal fibre profiles (found in all biopsies assessed) included: fibres with absent COX activity and down-regulated COX-I and NDUFB8 (Figure 3.1, Figure 3.2 and Figure 3.3: fibres "1"), fibres with absent COX activity and COX-I immunoreactivity but preserved NDUFB8 (Figure 3.1, Figure 3.2 and Figure 3.3: fibres "2") or fibres with normal COX activity and COX-I immunoreactivity but down-regulated NDUFB8 (Figure 3.1, Figure 3.2 and Figure 3.3: fibres "2").

A deeper analysis was performed for aged control AC3. Stereological analysis of COX/SDH histochemistry revealed that the diaphragm was the most affected muscle (int: 4.5%, neg: 6.2%, n=674) followed by psoas (int: 3.6%, neg: 2.9%, n=100) and biceps (int: 1.1%, neg: 1.2%, n=4021) (Figure 3.4, left graphs). Following this, a population of COX deficient and intermediate fibres were selected in each muscle and further assessed for NDUFB8 and COX-I abundance. Immunohistochemistry showed that these muscles presented distinct features of mitochondrial dysfunction. The most common abnormal fibre profile in diaphragm and biceps was: absent COX activity/levels but preserved NDUFB8 (diaphragm: 55.3%, n=38 abnormal fibres, biceps: 40.8%, n=98 abnormal fibres) whereas in psoas it was: absent COX activity/levels (51.9%, n=27 abnormal fibres) (Figure 3.4, right graphs).



Figure 3.1: Mitochondrial dysfunction in biceps, diaphragm and psoas muscles from aged control 1. COX/SDH histochemistry and immunohistochemistry against NDUFB8 (complex I), SDHA (complex II) and COX-I (complex IV) were performed in serial sections obtained from biceps, diaphragm and psoas muscles. Selected muscle fibres demonstrate: (1) COX deficiency (COX-deficient fibres) with absent COX-I and NDUFB8 immunoreactivity, (2) COX deficiency (COX-deficient fibre) with absent COX-I immunoreactivity and normal NDUFB8 levels, (3) normal COX activity (COX-positive fibres) and COX-I levels but absent NDUFB8 immunoreactivity.



Figure 3.2: Mitochondrial dysfunction in biceps, diaphragm and psoas muscles from aged control 2. COX/SDH histochemistry and immunohistochemistry against NDUFB8 (complex I), SDHA (complex II) and COX-I (complex IV) were performed in serial sections obtained from biceps, diaphragm and psoas muscles. Fibres from both diaphragm and psoas muscle did not react for COX histochemistry leading to a false and widespread COX deficiency. Similarly, immunohistochemistry failed in both diaphragm and psoas muscles. Selected muscle fibres demonstrate: (1) COX deficiency (COX-deficient fibres) with absent COX-I and NDUFB8 immunoreactivity, (2) COX deficiency (COX-deficient fibre) with absent COX-I immunoreactivity and normal NDUFB8 levels and (*) absent COX activity (COX-deficient fibres) but intermediate levels of COX-I.



Figure 3.3: Mitochondrial dysfunction in biceps, diaphragm and psoas muscles from aged control 3. COX/SDH histochemistry and immunohistochemistry against NDUFB8 (complex I), SDHA (complex II) and COX-I (complex IV) were performed in serial sections obtained from biceps, diaphragm and psoas muscles. Selected muscle fibres demonstrate: (1) COX deficiency (COX-deficient fibres) with absent COX-I and NDUFB8 immunoreactivity, (2) COX deficiency (COX-deficient fibre) with absent COX-I immunoreactivity and normal NDUFB8 levels, (3) normal COX activity (COX-positive fibres) and COX-I levels but absent NDUFB8 immunoreactivity, (4) absent COX activity (COX-deficient fibres) with apparently normal levels of COX-I and NDUFB8, (5) absent COX activity (COX-deficient fibres) but intermediate levels of COX-I and (6) absent COX activity (COX-deficient fibres) and COX-I immunoreactivity with intermediate levels of NDUFB8.





According to these results, analysing mitochondrial dysfunction just based on COX/SDH histochemistry would underestimate the overall dysfunction. Indeed, approximately 20% of abnormal fibres (those with down-regulated NDUFB8, but normal COX activity/levels - representing 18.4% in biceps, 21.1% in diaphragm and 18.5% in psoas) would not be detected if COX/SDH histochemistry was not complemented with immunohistochemistry.

3.4.1.2 Inaccuracy of visual classification and the use of serial sections

The previous analysis of serial muscle sections highlighted several inconsistencies between COX histochemistry and COX-I immunohistochemistry. For example: fibres with absent COX activity but normal COX-I immunoreactivity (Figure 3.3: fibres "4"), fibres with absent COX activity but intermediate COX-I immunoreactivity (Figure 3.2: fibre "*" and Figure 3.3: fibres "5"), or fibres with intermediate or absent COX activity but no changes in complexes levels (Figure 3.4: right graphs "ND fibres").

One explanation for these inconsistencies could be the inaccuracy of visual assessment in detecting subtle changes in colour intensities (enzyme activity or protein levels). Fibres with normal COX activity might have been perceived as intermediate (leading to false intermediate fibres) or, alternatively, fibres with reduced COX activity might have been perceived as having preserved COX-I (leading to false normal COX-I fibres). Another possible explanation could be the use of serial sections. Combining information from serial sections to infer about a fibre profile is inaccurate since the activity and levels of complexes change along the fibre length (Matsuoka *et al.*, 1992, Murphy *et al.*, 2012). Therefore the information provided by each section should be specific to that 10um domain. For this particular study 4 serial sections were used, which means the inference about the activity and levels was performed across 40µm of the fibre length.

3.4.1.3 Subjectivity: intra- and inter-observer variability

Visual assessment of aged controls AC1 and AC3 diaphragm was particularly challenging. Both *post-mortem* muscles presented a high number of fibres looking pale blue and grey in COX/SDH histochemistry which were very difficult to classify. The boundaries between "normal", "intermediate" and "deficient" COX activity categories are defined by the observer, but inevitably, they can change between cases assessed, or over time.

To further illustrate how visual classification of muscle fibres based on COX/SDH histochemistry can be subjective and challenging we investigated muscle biopsies from two particular patients: P1 and P2 (Table 3.1). These biopsies presented a high proportion of COX intermediate fibres and the histochemical signal detected across muscle sections was less intense and variable. Sections were assessed using COX/SDH histochemistry and one hundred muscle fibres were selected to be visually categorised by two researchers, independently, into one of the four categories: COX positive, COX intermediate(+) (int(+)), COX intermediate(-) (int(-)) and COX deficient. Fibres were not randomly selected and therefore the results obtained are not representative of the overall respiratory deficiency in the patients.

Analysis of both patients P1 and P2 revealed marked differences in classification of fibres between the two investigators (Figure 3.5): both investigators agreed in the classification of COX-positive cells (P1: 40% and P2: 21%) but they markedly differed in remaining categories: both categories of intermediate and COX deficient fibres (Figure 3.5 A and B). Importantly, both investigators demonstrated a high degree of inconsistency. Investigator 1 underestimated COX deficiency in patient P1 and overestimated it in patient P2, whereas the opposite was true for investigator 2.



Figure 3.5. Inter- and intra-observer variability of visual classification.

COX/SDH histochemistry was performed in muscle sections obtained from patients (A) P1 and (B) P2. Left panels in (A) and (B) show representative images of COX/SDH histochemistry (20x). For each patient 100 muscle fibres reacted for COX/SDH histochemistry were selected and visually classified by two investigators. Bar graphs show the percentage of COX positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue) based on visual classification by an investigator 1 and 2.

3.4.2 Developing an objective classification using COX/SDH histochemistry

The following section describes the development of a new method to assess objectively the degree of mitochondrial respiratory chain dysfunction in single muscle fibres using COX/SDH histochemistry. In 2012, an objective assessment was developed using the intensity measurements of COX histochemistry, SDH histochemistry and NDUFB8 immunohistochemistry to determine the respiratory status of muscle fibres (Murphy *et al.*, 2012). The method developed here only use COX and SDH intensity measurements decreasing the complexity (and time) of image analysis and minimising problems associated with the use of serial sections.

3.4.2.1 New model rationale

The method uses COX and SDH intensity measurements to determine the percentage of COX activity in individual fibres; it was developed assuming the following premises:

- COX reaction measures the activity of complex IV, partially encoded by mitochondrial DNA, and SDH measures the activity of complex II, fully encoded by nuclear DNA.
- 2. SDH is a marker of mitochondrial mass and COX activity is proportional to SDH activity in a healthy cell.
- Within the linear period of incubation time, COX reactivity increases proportionally with COX activity (Figure 3.6, C and D), and therefore, COX reactivity is an outcome measure of COX activity (Murphy et al. 2012).

Therefore when COX and SDH histochemical signals are both intense (Figure 3.7B, scored as "++++") or proportionally reduced (Figure 3.7B, scored as "++)" COX activity is normal. As illustrated, the ratio between COX and SDH intensities equals 1 (Figure 3.7B). However when COX histochemical signal is reduced and SDH signal is preserved (Figure 3.7C, scored as "+++" or "+/-+"), COX activity is decreased according to the level of mitochondrial mass. In this scenario, the ratio between COX and SDH intensities equals approximately 0.5 (Figure 3.7C). In the absence of COX signal (Figure 3.7C scored as "-/++" or "-/+"), COX activity is entirely lost; according to this scenario the ratio equals 0 (Figure 3.7). We propose that the ratio between COX intensity and SDH intensity readings reflects the degree of COX activity in individual muscle fibres.



Figure 3.6. COX reactivity is an outcome measure of COX activity. (A,B) Both COX and SDH reactivity increase linearly within the "linear period" of incubation time; passed that point COX and SDH reactivity reach a maximum reactivity plateau. (C,D) During the "linear period" enzyme activity is proportional to COX and SDH reactivity/intensity.



Figure 3.7: Relationship between COX and SDH activities.

(A) COX histochemistry is represented in brown, SDH histochemistry is represented in blue. These colours range from light (-) to dark (++) according to the level of enzyme activity; lighter for low activity and darker for high activity. (B) Represents a scenario where COX activity is proportional to SDH activity, and so, COX activity is unaffected. (C) SDH activity is higher than the activity of COX, indicating that COX activity is reduced. (D) COX activity is lost in this muscle fibre.
3.4.2.2 Developing the objective classification

The objective classification was initially developed using a small sample of muscle fibres (19 muscle fibres from biceps of the aged control AC3, Table 3.1). Serial sections were assessed using individual and sequential COX/SDH histochemistry.

3.4.2.2.1 Optical density measurements (ODCOX, ODSDH)

The optical density (OD) of COX (ODCOX) and SDH (ODSDH) reactivity was measured in individual muscle fibres using image J. Both COX and SDH TIFF images were first converted into grey scale and the region of interest (ROI) was defined manually within individual muscle fibres using the freehand selection tool (Figure 3.8). Each new ROI was sequentially added (by pressing "t") in the ROI manager. Following this, the mean OD was measured for individual ROI. Grey scale range from 0 (darkest pixels) to 255 (brightest pixels).



Figure 3.8. Representative print-screen images showing the main steps of COX and SDH densitometry in individual fibres.

Individual (A) COX and (B) SDH histochemistry were converted into grey scale, (C) and (D) respectively, and then the region of interest (ROI) was traced within muscle fibres (red tracing). Areas 20, 21 and 22 were also sampled and measure the background noise (ODbackground). The software then automatically measured the optical density of each ROI.

3.4.2.2.2 Correction of ODCOX and ODSDH measurements

Having measured the OD of COX and SDH in individual muscle fibres, both ODCOX and ODSDH were normalised. This correction was necessary for several reasons:

- Re-scaling. The gray scale is an inverted scale with "0" representing the darkest pixels (high reactivity/activity) and "255" representing the brightest pixels (no reactivity/activity). However, according to the model (Figure 3.7), "0" indicated no COX reactivity/activity and "1" signified high COX reactivity/activity.
- 2. Correct for background noise. Fibres with no COX activity (although showing no COX reactivity) were not entirely white due to the background noise.
- Normalisation. According to the model, ODCOX/ODSDH from a normal and healthy fibre equals 1. Therefore 3 normal fibres were selected to define "normality": the standard levels of COX intensity and SDH intensity in healthy fibres.

ODCOX and ODSDH readings were therefore corrected proportionally, using the following normalisation equations:

CorrectedODCOX=|(ODCOX–ODbackground)/(ODCOX"darkest intensity"- ODbackground)| **CorrectedODSDH**=|(ODSDH–ODbackground)/(ODSDH"darkest intensity"- ODbackground)|

The "darkest intensity" was calculated by averaging the 3 lowest OD values of fibres, excluding ragged-red fibres (Figure 3.8C and D: fibres 11, 14 and 18). The "brightest intensity" was calculated by averaging 3 OD measurements of background – an area close to the section without fibres (ODbackground) (Figure 3.8C and D: areas 20, 21 and 22).

3.4.2.2.3 Calculating the relative percentage of COX activity

The ratio (correctedODCOX/correctedODSDH)*100 (yielding COX/SDH ratio, occasionally abbreviated to ratio) was calculated, providing a relative percentage of COX activity. Categories were arbitrary defined and fibres were classified into one of the following groups: COX deficient (\leq 25% COX/SDH ratio), COX intermediate (int, 25%< COX/SDH ratio <75%) and COX positive (COX/SDH ratio \geq 75%) groups. Since COX intermediate group encompassed a wide range of different levels of COX activity, this group was further subdivided into COX int(+), (50% \leq COX/SDH ratio <75%) and COX int(-) (25% < COX/SDH ratio <50%).



Fibre	Subjective classification	Ratio (%)	Objective classification
1	Neg	16	Neg
2	Neg	15	Neg
3	Pos	97	Pos
4	Int / Neg	37	Int(-)
5	Pos / Int	77	Pos
6	Neg	8	Neg
7	Neg	14	Neg
8	Int	31	Int(-)
9	Int/ Neg	21	Neg
10	Int/ Neg	41	Int(-)
11	Pos	89	Pos
12	Pos	90	Pos
13	Pos	118	Pos
14	Pos	105	Pos
15	Int	50	Int(+)
16	Pos	122	Pos
17	Pos	88	Pos
18	Pos	106	Pos
19	Pos	74	Int(+)

Figure 3.9. Objective classification of muscle fibres from aged control 3 according to the COX/SDH ratio.

Serial sections were reacted for combined COX/SDH histochemistry, individual COX histochemistry, individual SDH histochemistry and again, combined COX/SDH histochemistry. Top images show the combined COX/SDH histochemistry performed (A) at the beginning and (B) end (in the serial sections). (C) Table shows the subjective and objective classification of fibres. The visual method categorizes fibres into positive (pos), intermediate (int) and deficient (neg) fibres. The objective classification based on the COX/SDH ratio fall into four major classes: positive (pos, ratio \geq 75%), int(+) (50% ratio <75%), int(-) (25% ratio <50%) and deficient (neg, \leq 25% ratio).

3.4.2.2.4 Preliminary results

The objective classification based on the ratio of COX/SDH measurements was in agreement with the subjective classification (visual classification) (Figure 3.9). Visual classification is currently the only method available to analyse COX activity, and therefore, the only method

available to validate any new analysis. Both methods categorized fibres in the same groups (Figure 3.9, fibres 1 and 2). But importantly, the objective classification highlighted difficulties connected to the human eye error and improved the analysis (Figure 3.9, fibres 5 and 9).

3.4.2.3 Optimising the objective classification

3.4.2.3.1 Methods tested

Whilst examining larger number of muscle fibres to further validate the new objective assessment, a concern arose regarding the method of calculating COX activity. Deducting corrected ODCOX from ODSDH was recognised as an alternative: (correctedODSDH-correctedODCOX)*100 (yielding SDH-COX difference, and abbreviated occasionally to difference). Therefore both: COX/SDH ratio and SDH-COX difference were tested in order to determine which value was more accurate in identifying COX activity in single fibres. Furthermore, when other areas of the same biopsy were assessed, a second concern arose regarding the normalisation method. Consequently, different normalisation methods were tested in order to ensure the highest accuracy of this new method;

- Normalisation method 1: fibres chosen to normalise ODCOX and ODSDH measurements were those presenting the darkest COX intensities (lowest ODCOX values), excluding ragged red fibres (Figure 3.10 A, fibres 1, 2 and 3). Also, a normalisation equation was produced for each area covered (snap) from a given section (normalisation equation/area/section),
- Normalisation method 2: fibres chosen to normalise ODCOX and ODSDH measurements were also those presenting the darkest COX intensities, excluding ragged red fibres (Figure 3.10 B, fibres 1, 2 and 3). However, a unique normalisation equation was produced per section (normalisation equation/section),
- 3. Normalisation method 3: fibres chosen to normalise ODCOX and ODSDH measurements were those having the darkest COX and SDH intensities, respectively. Therefore fibres chosen to normalise ODCOX measurements (Figure 3.10 C, fibres 1, 2 and 3) may have been different from those normalising ODSDH measurements (Figure 3.10A, fibres 4, 5 and 6). Also, a unique normalisation equation was produced per section (normalisation equation/section).

XO2 2 3

A Normalisation method 1: normalisation equation/snap/section

B Normalisation method 2: normalisation equation/section



C Normalisation method 3: normalisation equation/section





Green squares highlight areas used to correct for the background (=0). Blue squares indicate the fibres used to establish the maximum intensity (=1). (A) In method 1: fibres used to normalise COX and SDH histochemistry are the same (fibres 1, 2 and 3). Also, a normalisation equation is produced for each snap (from a given section). (B) In method 2: fibres used to normalise COX and SDH histochemistry are also the same (fibres 1, 2 and 3). However one normalisation equation is generated for all snaps analysed. (C) In method 3: fibres used to normalise COX and SDH histochemistry; fibres 4, 5 and 6 to normalise SDH histochemistry). The normalisation equation is the same for all areas covered of the same section.

3.4.2.3.2 Methodology developed to compare the tested methods

Excel was used to compare the 6 different methods of determining COX activity (ratio versus difference each with the 3 normalisation method) with visual classification. Excel cells were conditionally formatted in order to be colour coded according to the COX/SDH ratio and SDH-COX difference values, and therefore, according to the category of fibre (Figure 3.11A): beige for COX positive, light beige for int(+), light blue for int(-) and blue for COX deficient.

In order to assess the sensitivity of the different methods tested, a scoring scale was developed to evaluate the agreement between the objective classification (OC) and the visual/ subjective classification (SC). Each method was given a score, as follows:

- Score 1 was given when subjective and objective classification agreed, categorizing fibres in the same group. For example: when both SC and OC classified a fibre as COX positive (Figure 3.11B, fibre 11: all methods tested) or when both classified a fibre as COX deficient (Figure 3.11B: fibre 1: ratio M1, M2 and M3).
- 2. Score 0 was given when objective classification categorised the fibre in the immediate flanking groups of subjective classification. For example: when SC classified a fibre as COX deficient but OC determined COX int(-) (Figure 3.11B, fibre 1: difference M1 and M2) or when SC classified a fibre as COX intermediate but OC determined COX positive (Figure 3.11B: fibre 15: difference M3). This decreased the impact of a wrong visual classification.
- 3. Score -1 was given whenever objective classification categorized the fibre in other groups than situation (1) and (2). For example when SC classified a fibre as COX deficient but OC classified it as int(+) (Figure 3.11B, fibre 1: difference M3), or the opposite: when SC determined COX positive but OC classified it as COX int(-) or deficient (not shown in Figure 3.11B).
- 4. Subjective classification did not discriminated between int(+) and int(-). Therefore score 1 was given to both COX int(+) and int(-) fibres when subjective classification classified a fibre as an COX intermediate, (Figure 3.11B, fibre 8: ratio M1, M2 and M3, difference M1 and M2). Rules (2) and (3) were applied on the following groups. For example score 0 was given when SC classified a fibre as COX intermediate but OC classified it as COX positive (Figure 3.11B, fibre 8: difference M3).
- 5. When visual classification was not easy, a range of 2 groups was determined, with score 1 being given to both groups. For example when SC classified a fibre as deficient/intermediate (Figure 3.11B, fibre 9) score 1 was given when OC classified it as deficient or int(-) (Figure 3.11B, fibre 9: ratio M1, M2 and M3) and score 0 was given when OC classified it as int(+) (Figure 3.11B, fibre 9: difference M3).

		В														
Ratio	Difference		Fibro	Subjective			Rat	io					Differ	ence		
(%)	(%)		Fibre	classification	М1	S	M2	S	M3	S	M1	S	M2	S	М3	S
100	0		1	Neg	16	1	16	1	20	1	72	0	67	0	42	-1
90	10		2	Neg	15	1	14	1	18	1	86	1	80	1	50	0
80	20		3	Pos	97	1	91	1	119	1	4	1	11	1	-16	1
75	24		4	Int/Neg	37	1	34	1	45	1	83	1	79	1	44	0
74	25		5	Pos / Int	77	1	72	1	94	1	22	1	24	1	3	1
60	30		6	Neg	8	1	8	1	10	1	121	1	112	1	73	0
50	49		7	Neg	14	1	14	1	18	1	108	1	101	1	64	0
49	50		8	Int	31	1	29	1	38	1	38	1	36	1	21	0
40	60		9	Neg/ Int	21	1	20	1	26	1	70	1	66	1	40	0
26	74		10	Neg/ Int	41	1	38	1	50	1	49	0	47	0	25	0
25	75		11	Pos	89	1	84	1	110	1	11	1	15	1	-6	1
20	80		12	Pos	90	1	85	1	111	1	10	1	15	1	-7	1
10	90		13	Pos	118	1	111	1	145	1	-10	1	-6	1	-16	1
0	100		14	Pos	105	1	99	1	130	1	-5	1	1	1	-16	1
			15	Int	50	1	47	1	62	1	41	1	40	1	19	0
			16	Pos	122	1	115	1	150	1	-17	1	-11	1	-23	1
			17	Pos	88	1	83	1	108	1	8	1	11	1	-4	1
			18	Pos	106	1	100	1	130	1	-6	1	0	1	-19	1
			19	Pos	74	0	69	0	90	1	16	1	18	1	4	1

Figure 3.11. Methodology developed to compare the different normalisation methods.

(A) Excel cells were colour coded according to the COX/SDH ratio or SDH-COX difference values, each colour corresponding to the category of fibres: COX-positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue). (B) Table shows for each fibre, the subjective classification and the objective classification based on ratio or difference value when calculated using the different normalisation methods (M1, M2 and M3 columns); cells containing values for ratio or difference were colour coded as shown in (A) to be easily visualized. The "S" columns show the score (-1, 0 or 1) given to each method if they agreed (or not) with the subjective classification.

3.4.2.3.3 Evaluation of different methods

Α

To evaluate the accuracy of each method in determining COX activity, muscle biopsies from aged controls AC1 (psoas), AC2 (biceps) and AC3 (biceps, diaphragm and psoas) were re-assessed (Table 3.1). Different areas of sections were imaged and only a selection of muscle fibres was visually classified. Following this, the same muscle fibres were objectively classified using the different methods.

Table 3.4 shows the counts of "1" scores given to each method - discriminated by muscle assessed or as a total. The method which reached the highest scores was the COX/SDH ratio with normalization method 3 (Table 3.4); 801 out of 849 muscle fibres scored "1". Also, it did not count any "-1" scores indicating absence of misclassifications (Table 3.4). By contrast, the difference (irrespective of the normalisation method) had consistently lower total scores "1" and some "-1" scores (Table 3.4). Unlike the ratio method, the objective classification of fibres

provided by the difference method detected fewer deficient fibres and most of the abnormal fibres were classified as intermediate (+) (Figure 3.12C).

The frequency distribution of both COX/SDH ratio and SDH-COX difference were plotted (Figure 3.12). Both distributions presented three distinct peaks that corresponded to the positive, intermediate and deficient groups of fibres (Figure 3.12A and B). However, the individual peaks of the COX/SDH ratio distribution were more symmetrical than the peaks from the SDH-COX difference distribution. Taken together, these results suggested that the COX/SDH ratio appears to be more accurate and sensitive in classifying fibres than the SDH-COX difference.

		Ratio			Difference		n
	M1	M2	M3	M1	M2	M3	(fibres)
"1" scores AC1 psoas	137	115	<u>139</u>	133	122	138	152
"1" scores AC2 biceps	206	208	<u>232</u>	199	202	188	236
"1" scores AC3 biceps	178	170	<u>191</u>	178	172	173	209
"1" scores AC3 diaphragm	85	<u>90</u>	<u>90</u>	82	88	86	91
"1" scores AC3 psoas	132	131	<u>151</u>	134	138	149	161
Total "-1" scores	0	3	0	2	3	9	849
Total "1" scores	738	714	<u>803</u>	726	722	734	

Table 3.4. Agreement between the objective classification and subjective classification when using different normalisation methods

M1, M2 and M3 indicate normalisation methods 1, 2 and 3 respectively; n(fibres), the total number of fibres assessed; total "-1" scores, the total number of "-1" each method counted in all analysed muscles; total "1" scores, the total number of "1" scores each method counted in all analysed muscles.



Figure 3.12. Objective classification given by the ratio and difference values when using the normalisation method 3.

Densitometry was performed biceps muscle from aged control 2 and corrected ODCOX and ODSDH readings were used to calculate either the COX/SDH ratio or SDH-COX difference. (A, B) Graphs show the frequency distribution of the (A) ratio and (B) difference values normalised by method 3 (M3). (C) Bar graph show the percentage of COX-positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue) based on the ratio (left bar) or difference (right bar), normalised by method 3.

3.4.3 Developing an objective classification using immunofluorescence

The following section describes the development of a new method to objectively assess the degree of mitochondrial respiratory chain dysfunction in single muscle fibres using fluorescence-based immunohistochemistry. This technique allows labelling multiple epitopes in a single section, avoiding the problems associated with the use of serial sections, and simplifying image analysis. Importantly, it allows investigating complex I levels in individual muscle fibres, overcoming the absence of histochemical techniques to measure complex I activity.

3.4.3.1 Double fluorescence-based immunohistochemistry

In order to assess the levels of complex IV and complex I in individual muscle fibres, double immunofluorescence of COX-I (FITC - 488nm, green) and NDUFA13 (Rhodamine - 546nm, red) (Table 3.3) was performed in a biceps muscle section obtained from aged control AC2 (Figure 3.14A).

3.4.3.1.1 Optical density measurements

The OD of COX-I (ODCOX-I) and NDUFA13 (ODNDUFA13) was measured in individual muscle fibres using image J (Figure 3.13). TIFF images were first opened individually and corrected to remove the background noise. This was achieved by increasing the threshold until the background value equalled zero. Subsequently, images from individual channels were imported as image sequence and the ROI was defined manually within individual muscle fibres using the freehand selection tool (Figure 3.13). The mean OD was recorded for all created individual ROI.



Figure 3.13. Representative print-screen images showing the main steps of COX-I and NDUFA13 densitometry in individual fibres.

Individual TIFFs of (A) COX-I and (B) NDUFA13 channels were imported as an image sequence and the region of interest (ROI) was traced within muscle fibres (red tracing).





Double immunofluorescence was performed in a muscle sections obtained from aged control AC2. Fluorescent detection was used to visualise: complex IV subunit I (COX-I) - green (Fluorescein – 488nm) and complex I subunit (NDUFA13) - red (Rhodamine – 546nm). (A) Shows a representative image of the double immunofluorescence. (B) Top panel shows muscle fibres selected for densitometry (n=100); lower panel shows the levels of COX-I and NDUFA13 in each muscle fibre. Selected muscle fibres (or plotted points) demonstrate: "blue" normal COX-I and NDUFA13 abundance, "red": down-regulated COX-I abundance, "green": down-regulated NDUFA13 abundance.

3.4.3.1.2 Preliminary data

A selection of 100 muscle fibres were assessed in terms of COX-I and NDUFA13 levels and both ODCOX-I and ODNDUFA13 from individual fibres were plotted (Figure 3.14B). The scatterplot showed 3 distinct populations of muscle fibres, which was consistent with the judgement of immunofluorescence by eye. Plotted points from "healthy appearing" muscle fibres (Figure 3.14B top panel: for example fibres marked "32", "83" and "92") were lining up above the extracted linear regression (Figure 3.14B lower panel: blue plotted points); in these fibres, COX-I and NDUFA13 were strongly and positively correlated/associated in a 1 to 1 proportion (Figure 3.14B lower panel: linear regression - y= 1.105x+ 1.08, R²=0.9). By contrast plotted points from fibres appearing green (Figure 3.14B top panel: fibres marked "60" and "84") appeared plotted above the linear regression (Figure 3.14B top panel: green plotted points); they presented 3 times less NDUFA13 than the expected, according to COX-I levels. Similarly, plotted points from fibres appearing red (Figure 3.14B top panel: fibres marked "17" and "30") appeared plotted below the linear regression (Figure 3.14B lower panel: red plotted points); these fibres presented 2 times less COX-I than the expected according to NDUFA13 level.

This analysis was incomplete as the abundance of complex I and IV subunits were not normalised to the mitochondrial content. This could be leading to the under-estimation of mitochondrial dysfunction. For example, this approach did not discriminate between healthy fibres with unaffected complex I and IV and abnormal fibres with both COX-I and NDUFA13 equally down-regulated to intermediate levels. Also it did not discriminate between fibres with no COX-I and normal NDUFA13 and fibres with no COX-I and intermediate NDUFA13 abundance (Figure 3.14B: red fibres/ red plotted points).

3.4.3.2 Triple fluorescence-based immunohistochemistry

Triple immunofluorescence was performed in order to improve the assessment of mitochondrial respiratory chain components in individual muscle fibres. The third variable assessed, mitochondrial mass, was crucial to assess the relative levels of COX-I and NDUFA13.

3.4.3.2.1 Developing the objective classification

A diaphragm muscle section obtained from aged control AC1 was assessed by triple immunofluorescence to detect COX-I (FITC - 488nm, green), NDUFA13 (Rhodamine - 546nm, red) and SDHA (Alexa fluor 647nm, blue) (Table 3.3) and the OD of each mitochondrial marker (ODCOX-I, ODNDUFA13 and ODSDHA) was measured in individual muscle fibres (imageJ) (Figure 3.15).

ODCOX-I and ODNDUFA13 were plotted against each other (Figure 3.15B: top graph). The scatterplots showed a positive association between complex I and complex IV abundance. The estimated linear regression showed that COX-I and NDUFA13 in "healthy appearing" fibres were moderately correlated in a 1.3 to 1 proportion, respectively (Figure 3.15B top graph: linear regression - y= 1.29x+ 3.17, R²=0.7). Moreover, the linear regressions from both ODCOX-I and ODSDHA plot (Figure 3.15B: middle graph) and ODNDUFA13 and ODSDHA plot (Figure 3.15B: lower graph) showed that, in "healthy appearing" fibres, both COX-I and NDUFA13 were positively correlated with SDHA. SDHA was associated in a 1 to 1 proportion with COX-I (y=0.93x + 0.53, R²=0.7) and in a 0.6 to 1 with NDUFA13 (y=0.61x + 0.30, R²=0.7).

With the added mitochondrial mass variable, it was possible to distinguish between healthy fibres with normal complex I and IV (Figure 3.15B: grey plotted points) and abnormal fibres with both COX-I and NDUFA13 equally down-regulated (Figure 3.15B: orange plotted points). It was also possible to distinguish among fibres with absent COX-I (Figure 3.15B middle panel: red plotted points) those presenting normal, intermediate or absent NDUFA13 (Figure 3.15B lower panel: red plotted points).

In order to investigate if ODCOX-I and ODSDHA could be used to calculate the percentage of COX protein levels, the ratio (ODCOX-I/ODSDHA)*100 (yielding COX-I/SDHA ratio) was calculated and muscle fibres were classified into one of the following classes: COX-I deficient (\leq 25% COX-I/SDHA ratio), int(-) (25%< COX-I/SDHA ratio <50%) and int(+) (50% COX-I/SDHA ratio <75%) and COX-I positive (COX-I/SDHA ratio \geq 75%) groups. Following this, fibres were visually assessed.

The objective classification based on the COX-I/SDHA ratio was in agreement with the subjective classification (visual classification, n=430 fibres) (Figure 3.16B). All fibres appearing COX-I deficient in triple immunofluorescence consistently showed a COX-I/SDHA ratio value lower than 25%. Similarly, fibres appearing normal always showed a COX-I/SDHA ratio higher than 75%. Unfortunately, fibres objectively classified as COX-I intermediate were more difficult to visually validate.





Triple immunofluorescence was performed in a muscle sections obtained from aged control AC1. Fluorescent detection was used to visualise: complex IV subunit I (COX-I) - green (Fluorescein – 488nm), complex I subunit (NDUFA13) - red (Rhodamine – 546nm) and complex II subunit A (SDHA) - blue (Alexa fluor 647nm). (A) Shows a representative image of the triple immunofluorescence. 430 muscle fibres were selected for densitometry. (B) The top graph plots ODCOX-I and ODNDUFA13 from individual muscle fibres, the middle graph plots ODCOX-I and ODSDHA, and the lower graph ODNDUFA13 and ODSDHA. Selected plotted points demonstrate: "red": down-regulated COX-I abundance, "orange": down-regulated COX-I and NDUFA13 abundance.



Subjective Ratio Objective Fibre classification classification (%) 1 Pos 97 Pos 7 Pos 106 Pos 12 Neg 13 Neg 13 Pos 126 Pos 14 Pos 91 Pos 5 16 Neg Neg 44 Pos 96 Pos 45 Pos 10 Neg Pos 77 46 Pos Int/ Pos 55 54 Int(+)57 9 Neg Neg 58 Pos 100 Pos 89 Pos 118 Pos 90 Neg 11 Neg 130 Pos 117 Pos 151 47 Int Int(-) 165 Neg 17 Neg 166 Pos 132 Pos 179 Int/ Neg 24 Neg

Figure 3.16. Objective classification of muscle fibres according to the COX-I/SDHA ratio.

(A) Shows one of the selected areas of the triple immunofluorescence assessed by densitometry. (B) The table shows the subjective classification and objective classification based on the ratio between ODCOX-I and ODSDHA measurements, from a sample of 19 fibres. The visual method categorized fibres into positive (pos), intermediate (int) and deficient (neg) fibres. The objective classification divided the fibres between 4 major classes: positive (pos, ratio \geq 75%), int(+) (50% \leq ratio <75%), int(-) (25%< ratio <50%) and deficient (neg, 25% \leq ratio). (C) Bar graph shows the percentage of COX-positive (beige), int(+) (light blue) or deficient (blue) based on the COX-I/SDHA ratio (n=430).

3.4.3.2.2 Validation of the new method

To investigate whether COX-I protein levels could be used to infer COX activity, COX/SDH histochemistry and triple immunofluorescence to detect COX-I (FITC - 488nm, green), NDUFA13 (Rhodamine - 546nm, red) and SDHA (Alexa fluor 647nm, blue) (Table 3.3) were performed in muscle sections obtained from a young disease control (DC1), an aged control (AC3) and a patient with a diagnosed mitochondrial disorder (P3) (Figure 3.17). Only COX-I levels were validated since there is no histochemical assay to validate NDUFB8 levels against. A selection of fibres was objectively assessed based on COX/SDH histochemistry (COX/SDH ratio) and immunofluorescence (COX-I/SDHA ratio or NDUFA13/SDHA ratio).

In both young disease and old control, the objective classification provided by the COX/SDH and COX-I/SDHA ratios presented slight differences (Figure 3.18A and B, left and middle bars). However, in the young disease control DC1 not all fibres assessed using COX/SDH histochemistry (n=306) were re-assessed using immunofluorescence (n=224); this was due to either lost areas (during cryostat sectioning) or folded areas. Also, in the old control AC2, the SDH histochemistry was not uniform across the section, with some areas more pale. These are believed to be tissue artefacts, and not a true decrease in SDH activity. By contrast, in patient P3 the objective classification provided by COX/SDH ratio (n=619) and COX-I/SDHA ratio (n=571) were in complete agreement (Figure 3.18C, left and middle bars). These results suggested that COX-I levels reflect COX activity, validating its use to infer mitochondrial respiratory chain dysfunction.

This new method allowed quantification of the level of complex IV and I dysfunction in single muscle fibres (Figure 3.18, middle bars). The mitochondrial patient showed the highest accumulation of muscle fibres with down-regulated COX-I (25.5% abnormal fibres, 18.2% deficient fibres, n=571) followed by the aged control (15.4% abnormal fibres, 5.8% deficient fibres, n=481) and the young disease control (0.4% abnormal fibres: all int(+), n=224) (Figure 3.18A, right bar). A similar trend was observed for complex I (Figure 3.18, right bars): the mitochondrial patient showed the highest percentage of fibres with down-regulated NDUFA13 (49% of abnormal fibres, 13.6% of deficient fibres, n=571, Figure 3.18C right bar), followed by the aged control (58.3% of abnormal fibres, 3.4% deficient fibres, n= 481, Figure 3.18B, right bar) and the young disease control (2.7% abnormal fibres: all int(+), n=224, Figure 3.18A, right bar) (Figure 3.18).



Figure 3.17. Comparing COX/SDH histochemistry with complex I, II and IV protein abundance in young, aged and diseased skeletal muscle.

COX/SDH histochemistry and triple immunofluorescence were performed in serial muscle sections obtained from young DS1, aged control 3 and patient P3. Fluorescent detection was used to visualise: complex IV subunit I (COX-I) - green (Fluorescein – 488nm), complex I subunit (NDUFA13) - red (Rhodamine – 546nm) and complex II subunit A (SDHA) – blue (Alexa fluor 647nm). DC1 is a paediatric control and therefore presents a reduced fibre size.





COX/SDH histochemistry and triple immunofluorescence were performed in serial muscle sections obtained from (A) young disease control (DC1), (B) aged control 3 (AC3) and (C) patient (P3). Graphs show the classification results from the objective classification based on COX/SDH ratio (left bars) and objective classification based on COX-I/SDHA (middle bar) and NDUFA13/SDHA (right bar) ratios. Fibres were classified into COX (activity/protein level) positive (beige), int(+) (light beige), int(-) (light blue), and deficient (blue) groups.

One surprising finding was the amount of fibres with intermediate NDUFA13 levels in the old control; this raised a concern: whether or not this down-regulation was real. In fact, the exposure time used to image NDUFA13 channel was extremely high and this channel showed a high level of background noise. This could account for the abnormally high complex I deficiency noted for this older adult, highlighting the need for further optimisation.

3.4.3.3 Optimising triple fluorescence-based immunohistochemistry

To optimise the assessment of mitochondrial respiratory chain components in individual muscle fibres, a triple immunofluorescence detecting COX-I and other mitochondrial markers, NDUFB8 and porin, was performed. NDUFB8 antibody binds its epitope with a higher affinity than NDUFA13, providing stronger signal; porin is a voltage-gated ion channel located in the outer mitochondrial membrane (Mannella, 1998), and it is commonly used to assess the overall mitochondrial mass (Mahad *et al.*, 2009, Grunewald *et al.*, 2014).

3.4.3.3.1 New mitochondrial markers

A diaphragm muscle section obtained from aged control AC1 (a serial section from that shown in Figure 3.15) was assessed using triple immunofluorescence to detect COX-I (FITC - 488nm, green), NDUFB8 (Rhodamine - 546nm, red) and porin (Alexa fluor 647nm, blue) (Table 3.3) and the OD of each mitochondrial marker (ODCOX-I, ODNDUFB8 and ODporin) was measured in individual muscle fibres (imageJ) (Figure 3.19).

ODCOX-I and ODNDUFB8 were plotted against each other (Figure 3.19: top graph). The scatterplot showed (as previously with NDUFA13) a positive association in "healthy appearing" fibres; COX-I and NDUFB8 were moderately correlated in a 1.3 to 1 proportion, respectively (Figure 3.19, top graph: y= 1.319x+ 0.195, R²=0.6). Moreover, both COX-I and NDUFB8 in "healthy appearing" fibres were positively correlated with porin: in a 3 to 2 proportion for COX-I (Figure 3.19, middle graph: y=1.500x, R²=0.7) and 1 to 1 for NDUFB8 (Figure 3.19, lower graph: y=1.0695x, R²=0.6). These results confirmed therefore that NDUFB8 antibody is stronger and provides better signal than NDUFA13 antibody.

To also validate the use of porin as a mitochondrial mass marker, matching fibres in COX-I, NDUFA13 and SDHA immunofluorescence (Figure 3.15) and COX-I, NDUFB8 and porin immunofluorescence (Figure 3.19) were first identified (Figure 3.20A and B). Following identification, their ODSDHA and ODporin were plotted together. The scatter of plotted points showed that porin was strongly correlated with SDHA, in a 2 to 1 proportion (Figure 3.20C, y=0.490x, $R^2=0.8$), confirming that porin can be used to infer about the overall mitochondrial mass in muscle fibres.

Consequently, the objective assessment of complex IV provided by the COX-I/SDHA ratio (normal: 83.7%, int(+): 8.6%, int(-): 2.1% and deficient= 5.6%) and the COX-I/porin ratio (normal: 88.0%, int(+): 3.0%, int(-): 4.0% and deficient= 5.0%) were not significantly different (Z test for two populations proportions, p<0.05). However, the objective assessment of complex I showed marked differences, especially among the normal and intermediate groups. The objective classification based on the NDUFA13/SDH ratio (normal: 10.7%, int(+): 73.4%, int(-): 12.4% and deficient: 3.5%) over-estimated mitochondrial dysfunction when compared to the objective classification based on the NDUFB8/porin ratio (normal: 88.0%, int(+): 4.0%, int(-): 6.0% and deficient: 2.0%).





Triple immunofluorescence was performed in a muscle sections obtained from aged control AC1. Fluorescent detection was used to visualise: complex IV subunit I (COX-I) - green (Fluorescein – 488nm), complex I subunit (NDUFB8) - red (Rhodamine – 546nm) and porin - blue (Alexa fluor 647nm). (A) Shows a representative image of the triple immunofluorescence. 200 muscle fibres were selected for densitometry. (B) Top graph plots ODCOX-I and NDUFB8 from individual muscle fibres, middle graph plots ODCOX-I and ODporin, and lower graph ODNDUFB8 and ODSDHA.



Figure 3.20. Correlation between SDHA and porin.

Triple immunofluorescence against (A) NDUFA13, COX-I and SDHA and (B) NDUFB8, COX-I and porin was performed in serial diaphragm muscle sections obtained from aged control AC1. (C) Graph plots the ODporin and ODSDHA measured in matching fibres (n=33). (D) Graphs show the classification results based on COX-I/SDHA and NDUFA13/SDHA (left double bars) and based on COX-I/porin and NDUFB8/SDHA (right bar) ratios. Fibres were classified into positive (beige), int(+) (light beige), int(-) (light blue), and deficient (blue) groups.

These results showed that NDUFB8 provided more signal than NDUFA13, when using the same exposure time (6.0s, Table 3.5). Also, they showed that porin provided more signal than SDHA, since SDHA required a longer exposure time than porin (5.0s to image SDHA and 2.4s to image porin). Still, the exposure times used remained high, especially for the NDUFB8 and porin channels.

Channel	Primary antibody	Exposure time	Primary antibody	Exposure time
FITC (488nm)	COX-I	1.7s	COX-I	1.7s
Rhodamine (546nm)	NDUFA13	6.0s	NDUFB8	6.0s
Cy (647nm)	SDHA	5.0s	porin	2.4s

Table 3.5. Exposure times used to image the different channels

3.4.3.3.2 New combination of secondary antibodies

Experimental optimisations were undertaken to decrease the exposure times needed to image the different channels and still achieve an acceptable signal (especially for NDUFB8 and porin channels). Serial diaphragm muscle sections obtained from aged control AC1 were assessed in terms of triple immunofluorescence, but different combinations of primary and secondary antibodies were used to detect COX-I, NDUFB8 and porin, as follows:

- 1. Combination 1: Alexa fluor488: NDUFB8, Alexa fluor 546: COX-I, Alexa fluor 633: porin
- 2. Combination 2: Alexa fluor 488: porin, Alexa fluor 546: NDUFB8, Alexa fluor 647: COX-I

All dyes (used so far) were replaced by the equivalent Alexa fluor (Table 3.3). These produce greater fluorescent output and are more photostable than the other spectrally similar conjugates. The concentration of the secondary antibodies was changed from 1:300 to 1:200, and sections were incubated for 2h at RT (instead of 1h). Also, muscle sections were pre-incubated with 5%NGS (instead of 1%NGS) for 1h at RT (instead 30min). One source of background signal is the non-specific binding of secondary antibodies to endogenous Fc receptors, therefore increasing the concentration and time of the protein block helps reducing unwanted background. Finally, all images were acquired at 20x instead of 10x. When using a higher magnification, the amount of light that penetrates through the objective is higher, therefore, the exposure time needed to obtain an optimal fluorescence, decreases.

The four changes made to the protocol had a positive impact on the exposure times needed to image sections (Figure 3.21: left and middle collumns). For example, the exposure time used for the 488nm channel was reduced from 1.7s to 300-500 ms. Furthermore, it was observed that the lowest exposure time needed to image NDUFB8 channel was achieved when the primary antibody was coupled with Alexa fluor 546 (ET(Alexa fluor 488)= 480ms; ET(Alexa fluor 546)= 180ms, Figure 3.21). Similarly, an acceptable exposure time to image porin was achieved when it was coupled with Alexa fluor 488 (ET(Alexa fluor 488)= 320ms; ET(Alexa fluor 647)= 3100ms, Figure 3.21).

The combination 2 tested (when COX-I, NDUFB8, porin were couple with Alexa fluor 647, 546 and 488 respectively) showed that the secondary antibody providing the weakest signal was Alexa fluor 647. A final optimisation was hence performed to amplify COX-I signal, using the complex biotin/streptavidin method. Briefly, streptavidin is a purified protein from *Streptomyces avidinii* bacteria which presents strong affinity (4 binding sites) to bind vitamin biotin. In this indirect method, a biotinylated secondary antibody links the primary antibody and streptavidin conjugated with 647 fluorophore.



Figure 3.21 Optimisation undertaken to decrease the exposure times needed to image the different channels (20x).

Triple immunofluorescence detecting COX-I, NDUFB and porin was performed in serial diaphragm muscle sections obtained from aged control AC1. Different combinations of primary and secondary antibodies were tested. Combination 1: NDUFB8/Alexa fluor 488 (green), COX-I/Alexa fluor 546 (red), porin/Alexa fluor 633 (purple). Combination 2: porin/Alexa fluor 488 (green), NDUFB8/Alexa fluor 546 (red), COX-I/Alexa fluor 647 (purple). Combination 3: porin/Alexa fluor 488 (green), NDUFB8/Alexa fluor 546 (red) and COX-I/biotin/Streptavidin 647 (purple). For this particular experiment, slides were counter-stained with DAPI.

Since several streptavidin proteins can bind biotin, this complex offers signal amplification. A serial diaphragm muscle section obtained from aged control AC1 was assessed using triple immunofluorescence detecting porin (Alexa fluor 488, green), NDUFB8 (Alexa fluor 546, red) and COX-I (biotin/Streptavidin 647). The protocol incorporated the previous optimisation with the following changes: the first incubation with secondary antibodies (Alexa fluor 488, Alexa fluor 546, biotinylated antibody) for 2h at RT and the second incubation with only Streptavidin conjugated with 647 for 1h at RT (Table 3.3) (Figure 3.21, right column: combination 3).In combination 2, the exposure time needed to image COX-I (ET=900ms) was almost threefold higher than the exposure time set to image porin (ET=320ms) (Figure 3.21 middle column). By contrast, in combination 3, the exposure time needed to image COX-I (ET=440ms) was lower than the exposure time set to image porin (ET=520ms) (Figure 3.21 right column). These results showed that the amplification of COX-I using the complex biotin/streptavidin proved to be efficient and lowered the exposure time needed to reach optimal fluorescence of COX-I signal.

3.5 Discussion

Mitochondrial defects are often challenging to quantify. The common mosaic pattern of deficiency restricts the use of several techniques to COX/SDH histochemistry and immunohistochemistry. This chapter highlights the problems associated with these assays and describes the development of a novel method to objectively assess mitochondrial respiratory chain function in individual muscle fibres.

3.5.1 Challenges of COX/SDH histochemistry

In order to quantify mitochondrial dysfuntion in the ageing skeletal muscle, different muscles obtained from healthy aged individuals were investigated for the activity and abundance of mitochondrial respiratory chain components. Consistent with previous studies (Cooper *et al.*, 1992), all muscles showed some level of mitochondrial dysfunction with both complexes I and IV affected. Interestingly, different muscles showed different degrees of mitochondrial pathology and different patterns. For example diaphragm muscle from aged control AC3 showed higher levels of mitochondrial deficiency than psoas or biceps. Also, deficiency in individual muscle fibres was either isolated (preserved NDUFB8 and down-regulated COX-I or preserved COX-I and down-regulated NDUFB8) or combined (both complexes down-regulated).

Whilst assessing mitochondrial function in these muscle samples, it became apparent that the methods used to assess mitochondria in human muscle sections had several limitations. For example, around 20% of muscle fibres had isolated complex I deficiency. These were not detected in COX/SDH histochemistry but were only identified using NDUFB8 immunohistochemistry. These results highlighted the importance of assessing complex I together with complex IV in order to have a real overview of the overall mitochondrial deficiency.

Also, visual classification showed a degree of subjectivity. The boundaries between "normal", "low" or "absent" activity/levels groups are difficult to establish and, inevitably, they change between days or even between sections. This intra-observer variability also arises from tissue-specific artefacts (such as uneven labelling) and limitations of the human eye. Indeed when two investigators visually assessed the same fibres reacted for COX/SDH, classification results differed markedly. The investigators counted the same number of cells in the COX positive group. However, a higher inter-variability was detected for the middle and lower categories, suggesting higher mis-classifications in these groups. Also, both investigators were internally inconsistent, over- and under-estimating mitochondrial dysfunction in samples from different patients.

Lastly, visual classification also showed some inaccuracy. A significant proportion of muscle fibres showed contradictory results between COX/SDH histochemistry and COX-I immunohistochemistry. For example some fibres demonstrated intermediate or absent COX activity and preserved COX-I levels. These inconsistencies could result from incorrect visual assessment of muscle fibres due to incapacity of human eye to detect subtle colour changes (while judging the activity or levels of complex IV). Alternatively, they could result from the use of serial muscle sections. Since COX respiratory chain deficiency is segmental (Matsuoka *et al.*, 1992, Elson *et al.*, 2002, Murphy *et al.*, 2012), the analysis of serial sections might be confounded by the changes in enzyme activity and levels along fibre length. Regardless of the underlying cause, these results showed that visual assessment and the use of serial sections were leading to wrong classification of fibres.

3.5.2 The objective classification based on COX/SDH histochemistry

The unreliable and biased results from visual classification stressed the importance of developing an objective and quantitative method to determine COX activity in single fibres. Murphy *et al.* had developed a method to determine objectively the respiratory status of

muscle fibres (Murphy *et al.*, 2012). However, this method is limited by several disadvantages. It is subject to inaccurate inference (Taylor and Levenson, 2006) due to the changing biochemical profile along the fibre length. It is also time consuming as individual muscle fibres have to be identified manually in each of the three tissue sections used. Additionally, the respiratory status value is not compared to a group of positive fibres, or a control group. Therefore, the intensities of individual COX and SDH histochemistry may vary between cases (due to tissue history, or uneven precipitation of chromogens (Choudhury *et al.*, 2010)) shifting the boundaries between normal and abnormal profiles. Finally, the respiratory status does not discriminate the individual levels/activity of complex I and complex IV.

To overcome the above limitations, a new assay was developed to assess mitochondrial respiratory chain deficiency. This approach used just COX and SDH intensity measurements to determine the relative percentage of COX activity in single muscle fibres. ODCOX and ODSDH were corrected for the background noise and normalised to internal controls (the ODCOX and ODSDH of normal appearing fibres). This normalisation approach has been used previously in other research fields and it takes advantage of reference structures present in the biological sample (Ronneberger *et al.*, 2008). Different muscle biopsies were assessed in order to evaluate which method - COX/SDH ratio or difference SDH-COX - was more reliable determining COX activity in individual muscle fibres. Results showed that the COX/SDH ratio was more accurate in classifying correctly fibres than the SDH-COX difference; the classification provided by the ratio agreed closely with visual classification (quantified as scores "1") and showed less false classifications (quantified as scores "-1"). These results demonstrated that the new objective classification allows accurate classification of fibres.

3.5.3 The objective classification based on immunofluorescence

The previous objective assessment improved the classification of muscle fibres, but still presented some lacunas, such as the lack of complex I assessment and the use of 2 serial sections. In order to overcome these, the use of a similar quantitative approach for protein levels was explored, using fluorescence-based immunohistochemistry. This approach presents several advantages. It allows multiple labelling using a single muscle section, avoiding the concerns associated with the use of serial sections: changing biochemical profile, time consuming analysis and variability in intensity across muscle sections due to variations in muscle slice thickness (due to un-even cutting) (Taylor and Levenson, 2006). Also, the image analysis is performed in an image stack (with the different channels superimposed). Therefore,

the region of interest delimiting fibres is the same for the different complexes investigated, and image analysis is largely simplified.

Given these advantages, double immunofluorescence was initially performed in order to assess complex I and IV levels simultaneously in individual muscle fibres. However, the importance of additional labelling of fibres with a mitochondrial mass marker became rapidly evident. This labelling is crucial to assess complex I and IV levels relative to the amount of mitochondria and differentiate fibres with normal complexes levels from those with downregulated levels. In order to validate the use of COX-I as a marker for assembled/functional complex IV, objective classifications using COX/SDH histochemistry and COX-I/SDHA were compared in three muscle samples: a young disease control, an aged control and a patient with known mitochondrial disease. Both COX/SDH and COX-I/SDHA ratio provided the same results when no uneven labelling was detected across muscle sections. This positive correlation between COX activity and COX-I immunoreactivity is consistent with previous studies showing a selective and restricted loss of COX-I abundance in COX deficient fibres (Oldfors *et al.*, 1992, Rahman *et al.*, 2000, Mahad *et al.*, 2009).

Furthermore, the assessment of these three muscle biopsies confirmed the sensitivity of the triple immunofluorescence assay. Assuming that somatic mutations in mtDNA accumulate with age, this assay should not detect any COX deficient fibres in the young disease control. By contrast a small proportion was expected to be detected in the aged control, and a higher proportion in the patient. The results were in line with this. The young disease control did not present any fibres with down-regulated COX-I levels whereas the aged control and the patient demonstrated 15% and 26% of fibres with down-regulated levels, respectively. In terms of complex I, there is no histochemical assay available to validate the use of NDUFB8 as a marker for functional complex I. However, it has been extensively used by others as a marker of assembled complex I (Perales-Clemente *et al.*, 2010; Murphy *et al.*, 2012). Finally, the use of porin as a marker of the overall mitochondrial mass was validated by showing that SDHA and porin were strongly and positively correlated (R²=0.8).

Finally, it is important to mention some concerns regarding the objective classification based on protein levels. In this approach, measurements were not normalised to internal controls (as performed for objective assessment based on COX/SDH histochemistry) though the objective classification based on COX-I/SDHA agreed entirely with visual classification. Also, the exposure times for image acquisition were adjusted to each case limiting the comparison between cases. This highlighted the need of further optimisation and refinement of the objective classification.

4.1 Introduction

The replacement of observer-based qualitative assessment by computer-based quantitative assessment is increasing in many fields (cancer, neurology, muscle and many other) (Ellis *et al.*, 2005, Taylor and Levenson, 2006, Arechavala-Gomeza *et al.*, 2010, Kodiha *et al.*, 2011). Computer-based quantification presents major advantages: it avoids human subjectivity by eliminating intra and inter-observer variation and allows higher accuracy and consistency (Thomson *et al.*, 2001, Kloppel *et al.*, 2008, Rizzardi *et al.*, 2012, Alvarenga *et al.*, 2013).

The novel method to assess mitochondrial function in muscle sections introduced in the previous chapter requires further optimisation to become a computer-based quantitative tool. A specific cell membrane marker has to be included in the immunohistochemical protocol in order to automate the detection of individual muscle fibres in tissue sections by imaging software. Several groups have adopted a similar approach by labelling sub-plasmalemmal proteins such as spectrin (Arechavala-Gomeza *et al.*, 2010, Taylor *et al.*, 2012) or dystrophin (Mula *et al.*, 2013) and using variety of imaging software platforms (Briguet *et al.*, 2004, Arechavala-Gomeza *et al.*, 2012).

Furthermore, objective classification of fibres necessitates refinement. In certain mitochondrial conditions a mosaic pattern of mitochondrial defect is observed, where dysfunctional cells coexist with their healthy counterparts containing normal levels of COX-I and NDUFB8. This mosaicism allowed normalization of individual OD measurements to "internal positive controls" (fibres appearing healthy, as previously described in chapter 3 for the objective assessment based on COX/SDH histochemistry) (Ronneberger *et al.*, 2008). In other conditions, however, muscle tissue shows wide-spread mitochondrial deficiency and normal fibres are absent. This stresses the importance of comparing patients and elderly individuals to a young control group.

4.2 Aim

The aims of this chapter were to:

- 1. Semi-automate image analysis,
- 2. Refine the objective assessment of individual muscle fibres,
- 3. Validating the novel assay by demonstrating it is reliable and reproducible.

4.3 Methods

4.3.1 Cohort clinical characteristics

Archived frozen muscle specimens derived from healthy controls, disease controls and twelve patients with clinically and genetically-characterised mitochondrial disease of either mtDNA or nuclear genetic origin were used. Table 4.1 summarises relevant clinical information. The healthy control samples were obtained during orthopaedic surgery; the five disease controls were previously investigated for suspected neuromuscular disease but showed normal muscle histology, oxidative enzyme histochemistry and normal respiratory chain biochemical activities (Kirby *et al.*, 2007). Patients P1, P12 and P11 were chosen as they harboured a specific (nuclear or mitochondrial) genetic defect affecting extensively either respiratory chain complex IV or I (P1 and P2) or both (P11). Remaining patients harboured single large-scale mtDNA deletion, multiple deletions (due to a mutation in a nuclear gene involved in mtDNA maintenance) or point mutations in mitochondrial encoded tRNAs. These patients were selected because they all (except P8 and P9) exhibited a clear mosaic pattern of COX deficiency in muscle tissue.

4.3.2 Cryo-sectioning

Serial sections (10µm thickness) from transversely orientated muscle blocks were obtained as previously described in section 2.2.3 from chapter 2.

4.3.3 COX/SDH histochemistry

Individual and combined COX/SDH histochemistry was carried out according to the protocol described in section 2.2.5 from chapter 2.

Subjects	Gender	Age	Genetic defect	Histochemical findings	Biopsy type
Healthy cor	itrols				
HC1	Female	20y	n.r.	Normal enzyme histochemistry	Surgery
HC2	Male	21y	n.r.	Normal enzyme histochemistry	Surgery
Disease con	trols				
DC1	Male	4у	n.r.	Normal enzyme histochemistry	Diagnostic
DC2	Male	18y	n.r.	Normal enzyme histochemistry	Diagnostic
DC3	Female	20y	n.r.	Normal enzyme histochemistry	Diagnostic
DC4		22y	n.r.	Normal enzyme histochemistry	Diagnostic
DC5	Female	38y	n.r.	Normal enzyme histochemistry	Diagnostic
Patients wit	t <mark>h known m</mark>	utation	s affecting complex I and IV		
P1	Male	1y	Compound heterozygous LRPPRC mutations;	Global COX deficiency	Diagnostic
P2 ^a	Male	20y	m.4175G>A (p.Trp290*) <i>MT-ND1</i> mutation;	COX-positive, RRF	Diagnostic
Patients acc	cumulating	single (or multiple) mtDNA deletions		
Р3	Female	39y	single, large-scale mtDNA deletion;	15% COX deficient fibres, 4% RRF	Diagnostic
P4 ^b	Female	43y	multiple mtDNA deletions;	>40% COX deficient fibres/RRF	Diagnostic
P5	Male	47y	multiple mtDNA deletions;	25% COX deficient fibres, 8% RRF	Diagnostic
P6#	Male	60y	multiple mtDNA deletions;	20% COX deficient fibres, 7% RRF	Diagnostic
Patients wit	th point mu	tations	in mitochondrial encoded tRN	As	
Ρ7	Female	25y	m.3243A>G <i>MT-TL1</i> mutation;	20% COX deficient, 8% RRF	Diagnostic
P8#	Female	40y	m.3243A>G <i>MT-TL1</i>	>30% COX deficient, 10% RRF; also COX positive RRF	Diagnostic
P9 [#]	Male	53y	m.3243A>G <i>MT-TL1</i> mutation:	45% COX deficient fibres, 20% RRF	Diagnostic
P10 ^c	Female	13y	m.5690A>G <i>MT-TN</i> mutation:	13% COX deficient, 5% RRF	Diagnostic
P11	Female	18y	Novel <i>MT-TP</i> mutation;	98% COX deficient, SDH-positive fibres	Diagnostic
P12	Male	33y	m.10010T>C <i>MT-TG</i> mutation;	>90% COX deficient fibres	Diagnostic

Table 4.1. Clinical information from subjects included in this study

Key: age = age when biopsied; y = years old; mtDNA = mitochondrial DNA; PEO = progressive external ophthalmoplegia; CPOE = chronic CPEO; BMI = body mass index; RRF: ragged-red fibres, n.r.: not relevant; a,b,c,d published cases: aGorman *et al.* (2015) in press, bP2O in Pitceathly *et al.* (2012), cP3 in Blakely *et al.* (2013); [#]cases assessed in chapter 3: current P6, P8 and P9 were P3, P1 and P2, respectively.

4.3.4 Fluorescence-based immunohistochemistry

Fluorescence-based immunohistochemistry (detecting NDUFB8, COX-I, porin and laminin α -1) (Table 4.2) was carried out according to the protocol described in section 3.3.4.2 from chapter 3, with minor changes highlighted in bold:

Following fixation and permeabilisation, sections were blocked with **5% NGS** for **1h at RT** and then incubated with a primary antibody cocktail (Table 4.2). Subsequently, the sections were incubated with a secondary antibody cocktail for **2h at RT** (anti-rabbit IgG Alexa fluor 405, anti-mouse IgG2b Alexa fluor 488, anti-mouse IgG1 Alexa fluor 546, and anti-mouse IgG2a biotinylated antibody, (Table 4.2)) following a final incubation with streptavidin 647 for **1h at RT** (Table 4.2)). Sections were washed in TBST and mounted in Vectashield hard set mounting medium (Vector laboratories).

Antibodies	Host	Dilution	Company (Product number)
Primary antibodies			
MTCOI (IgG2a)	Mouse	1:100	Abcam (Ab14705)
NDUFB8 (IgG1)	Mouse	1:100	Abcam (Ab110242)
Porin (IgG2b)	Mouse	1:100	Abcam (Ab14734)
Laminin α -1 (rabbit IgG polyclonal)	Rabbit	1:50	Sigma-Aldrich (L9393)
Secondary antibodies			
Anti-rabbit IgG Alexa fluor 405nm	Goat	1:50	Life Technologies (A31556)
Anti-mouse IgG2b Alexa fluor – 488nm	Goat	1:200	Life Technologies (A21141)
Anti-mouse IgG1 Alexa fluor – 546nm	Goat	1:200	Life Technologies (A21123)
Anti-mouse IgG2a Biotin	Goat	1:200	Life Technologies (M32315)
Anti-mouse IgG2a Alexa fluor – 488nm	Goat	1:200	Life Technologies (A21131)
Anti-mouse IgG2b Alexa fluor – 546nm	Goat	1:200	Life Technologies (A21143)
Anti-mouse IgG1 Biotin	Goat	1:200	Jackson IR Lab (115-065-205)
Streptavidin – 647nm	Goat	1:100	Life Technologies (S31556)

Table 4.2 Primary and secondary antibodies used for immunofluorescence

Key: Jackson IR Lab: Jackson ImmunoResearch Laboratories

4.3.5 Imaging

Brightfield and fluorescent images were acquired at 20x magnification using Zeiss Microscope (see section 2.2.7 from chapter 2).

4.4 Results

4.4.1 Automation of image analysis

In order to simplify image analysis and accelerate assessment of mitochondrial respiratory chain dysfunction in individual muscle sections, a policional antibody detecting laminin α -1 was included in the immunohistochemical protocol. As laminin is a major glycoprotein of the basement membrane (Sanes, 1982), its labelling marked the boundaries of individual muscle fibres and allowed automated object recognition by the imaging software IMARIS. To develop the new image analysis protocol using IMARIS, a section from patient P6 was labelled with a cocktail of antibodies recognising COX-I (biotin/streptavidin 647, purple), NDUFB8 (Alexa fluor 546, red), porin (Alexa fluor 488, green) and laminin α -1 (Alexa fluor 405, white) (Figure 4.1A).

Images acquired using a fluorescent microscope were opened in IMARIS as 16-bit czi files and segmentation was performed in order to detect individual muscle fibres (Figure 4.1B). 405 channel (labelling laminin α -1) was corrected for background noise using the option "background subtraction" (Figure 4.1 B1-B2). A first surface was created over 405 channel (Figure 4.1 B3-B4) and laminin was masked (Figure 4.1 B5). Following this, a second surface was created based on the masked 405 channel. As a result, multiple surfaces were formed over individual muscle fibres (Figure 4.1 B26). Unwanted areas were removed in a filtering step (Figure 4.1 B7), but an area of the section devoid of fibres was sampled (in order to measure the background). The mean intensity of 488, 546 and 647 nm channels from individual muscle fibres (ODCOX-I, ODNDUFB8 and ODporin) and the area devoid of fibres (OD488, OD549 and OD647) were automatically measured in a scale ranging from 0 (brightest pixels) to 65,535 (darkest pixels) intensity units (Figure 4.1 B8). For each muscle fibre ODCOX-I, ODNDUFB8 and ODporin were corrected for background signal by subtracting the mean OD647, OD546 and OD488, respectively.

4.4.2 Classification of fibres using COX-I/porin and NDUFB8/porin ratios

In order to develop an objective classification based on standard deviation limits, quadruple immunofluorescence was performed in muscle sections obtained from four disease controls (DC1, DC3, DC4 and DC5) and three patients (P1, P2 and P11) (Figure 4.3). These patients were selected for the following reasons: P1 showed isolated and wide-spread complex IV deficiency, P2 showed isolated and wide-spread complex I deficiency and P11 combined complex I and IV deficiencies. The ODCOX-I, ODNDUFB8 and ODporin were measured, corrected (subtracting

the background as described above) and the ratios (ODCOX-I/ODporin)*100 (yielding COX-I/porin ratio) and (ODNDUFB8/ODporin)*100 (yielding NDUFB8/porin ratio) were calculated.



Figure 4.1 Image analysis using IMARIS software.

(A) Representative images of quadruple immunofluorescence detecting COX-I, NDUFB8, porin and laminin α -1. (B) Representative screenshots showing main steps of segmentation: (1) Images were opened on IMARIS software as 16-bit czi files (2) Background noise of 405 channel was removed using "background subtraction" tool and the intensity of laminin signal was increased manually. (3-4) A first surface was created over 405 channel and used to create a (5) laminin mask. (6) A second surface was created over the masked 405 channel thereby filling the area of muscle fibres. (7) Using edit tool bar, unwanted areas such as background were removed. (8) The software measures automatically the optical density of the different channels in each muscle fibre.



Figure 4.2 Representative images of complex I and IV levels in skeletal muscle sections from controls and patients.

Quadruple immunofluorescence was performed in muscle sections and fluorescent detection was used to visualise: COX-I – purple (biotin/streptavidin 647nm), NDUFB8 – red (Alexa fluor 546nm), porin – green (Alexa fluor 488nm) and laminin α -1 – white (Alexa fluor 405nm). Representative images of respiratory-normal tissue (disease controls DC1, DC3, DC4 and DC5) and mitochondrial deficiency: compound heterozygous LRPPRC mutations (P1, widespread complex IV deficiency), m.4175G>A MT-ND1 mutation (P2, widespread complex I deficiency), and a novel MT-TP mutation (P11, widespread complex I and IV deficiency).

4.4.2.1 Control population

The control group was created by combining data from all control muscle fibres DC1 (n=447), DC3 (n=252), DC4 (n=318) and DC5 (n=289). The distribution of COX-I/porin was approximately symmetric (skewness = 0.37) but the distribution of NDUFB8/porin was right skewed (skewness = 1.1) (Figure 4.3 A and B: left graphs) and none of the distributions passed the normality tests (both Shapiro-Wilk and D'Agostino-Pearson's omnibus test), p<0.001).

The Box-Cox transformation (Box and Cox, 1964) identified the fourth root - x^(1/4) - and the negative reciprocal square root (-1/SQRT(x)) transformations as optimal to achieve normality of the COX-I/porin and NDUFB8/porin control data, respectively. This was confirmed using both visual inspection (Figure 4.3 A and B: right graphs) and statistical methods: kurtosis and skewness numerical measures of shape for COX-I/porinT (skewness= -0.03 and kurtosis= -0.40) (Geourge and Mallery, 1999) and D'Agostino-Pearson's omnibus test for NDUFB8/porinT (p>0.05).

A Frequency distribution of ratios COX/porin and COX/porinT from control group



B Frequency distribution of ratios NDUFB8/porin and NDUFB8/porinT from control group



Figure 4.3 Control population.

Graphs show the frequency distribution of (A) COX/porin (graph on the left) and COX/porinT (graph on the right) (B) NDUFB8/porin (graph on the left) and NDUFB8/porinT (graph on the right) ratios from the control population (DC1 (n=447), DC3 (n=252), DC4 (n=318) and DC5 (n=289)).

4.4.2.2 Classification of muscle fibres

The parameters: mean and standard deviations (SD) describing the distribution of COX-I/porinT and NDUFB8/porinT ratios in the control population (yielding meanCOX-I, meanNDUFB8, SDCOX-I and SDNDUFB8) were determined to calculate COX-I_sd and NDUFB8_sd for all fibres, as follows:

COX-I_sd = (COX-I/porinT – meanCOX-I)/SDCOX-I; **NDUFB8_sd** = (NDUFB8/porinT – meanNDUFB8)/SDNDUFB8

COX-I_sd and NDUFB8_sd are a measure of how much the COX-I/porinT ratio (or NDUFB8/porinT ratio) from a given fibre deviates from the meanCOX-I (or meanNDUFB8) of the control group. Following this, both COX_sd and NDUFB8_sd were used to classify fibres into different categories of COX-I and NDUFB8 levels, respectively. Three set of standard deviation limits defining the categories (positive, int(+), int(-) and deficient), were tested (Table 4.3). For example, in the 2nd set tested (Table 4.3), a fibre was classified as COX-I positive if COX-I_sd was higher than -2SDS; int(+) if this value ranged from -2SD to -3SD; int(-) if this value ranged from -3SD to -2SD; or deficient if this value was lower than -4SD.

	Positive	Int(+)	Int(-)	Deficient
Set of SD limits				
1 st set	> -3SD	<u>-3SD</u> to -4SD	<u>-4D</u> to -5SD	< <u>-5SD</u>
2 nd set	> -2SD	<u>-2SD</u> to -3SD	<u>-3SD</u> to -4SD	< <u>-4SD</u>
3 rd set	> -1SD	<u>-1SD</u> to -2SD	<u>-2SD</u> to -3SD	< <u>-3SD</u>

Table 4.3. Set of standard	deviation limits	tested to classify	fibres
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Key: underlined SD limits highlight the upper boundaries of the categories.

4.4.2.3 Preliminary results

The three sets of standard deviation limits were tested in both controls and patients. When - 3SD, -4SD and -5SD limits defined the upper boundaries of the int(+), int(-) and deficient categories (Table 4.3: 1st set), patient P1 showed a very mild COX-I deficiency. Only 31% of the fibres had intermediate levels of COX-I; remaining 69% showed preserved COX-I (n=486). These findings were not consistent with the known biochemical defect and sufficient to reject these standard deviation limits.

A LRPPRC mutations (P1)

			COX-I	levels						COX-I	levels		
		Pos	Int(+)	Int(-)	Neg	Total			Pos	Int(+)	Int(-)	Neg	Total
	Pos	29%	39%	29%	2%	100%		Pos	3%	26%	39%	31%	100%
NDUFB8	Int(+)	-	-	-	-	-	NDUFB8	Int(+)	-	-	-	-	-
levels	Int(-)	-	-	-	-	-	levels	Int(-)	-	-	-	-	-
	Neg	-	-	-	-	-		Neg	-	-	-	-	-
	Total	29%	39%	29%	2%	100%		Total	3%	26%	39%	31%	100%

B m.4175G>A *MT-ND1* mutation (P2)

			COX-I	levels		
		Pos	Int(+)	Int(-)	Neg	Total
	Pos	9%	-	-	-	9%
NDUFB8	Int(+)	5%	-	-	-	5%
levels	Int(-)	8%	-	-	-	8%
	Neg	78%	-	-	-	78%
	Total	100%	-	-	-	100%

			COX-I	levels		
		Pos	Int(+)	Int(-)	Neg	Total
	Pos	5%	-	-	-	5%
NDUFB8	Int(+)	4%	-	-	-	4%
levels	Int(-)	5%	-	-	-	5%
	Neg	86%	-	-	-	86%
	Total	100%	-	-	-	100%

C Novel *MT-TP* mutation (P11)

			COX-I	levels						COX-I	levels		
		Pos	Int(+)	Int(-)	Neg	Total			Pos	Int(+)	Int(-)	Neg	Total
	Pos	-	-	1%	2%	3%		Pos	-	-	-	-	-
NDUFB8	Int(+)	-	1%	1%	8%	10%	NDUFB8	Int(+)	-	-	-	2%	2%
levels	Int(-)	-	-	1%	22%	23%	levels	Int(-)	-	-	1%	9%	10%
	Neg	-	-	-	64%	64%		Neg	-	-	-	87%	87%
	Total	0%	1%	3%	96%	100%		Total	-	-	1%	99%	100%

Figure 4.4 Classification of muscle fibres based on the mean and standard deviation of COX-I/porinT and NDUFB8/porinT from the control population.

Tables show the percentage of fibres with positive, int(+ and -) and deficient COX-I (columns) and NDUFB8 (row) levels in patients (A) P1 (n=486), (B) P2 (n=417) and (C) P11 (n=559), when using -2SD, - 3SD and -4SD (left tables) or -1SD, -2SD and -3SD (right tables) limits to define the upper boundaries of the int(+), int(-) and deficient categories.

When the limits -2SD, -3SD and -4SD were used (Table 4.3: 2nd set), the objective classification detected patients P2 and P11 expected deficiencies. Yet, it only revealed a mild complex IV deficiency in patient P1; more fibres (68%) had intermediate levels of COX-I but only 2% of fibres showed no COX-I (Figure 4.4C left graphs).

The best approximation with the expected results was achieved when using the -1SD, -2SD and -3SD limits (Table 4.3: 3rd set). Indeed patient P1 showed wide-spread and isolated COX-I deficiency with only 3% of the fibres presenting preserved COX-I (int(+)= 26%, int(-)= 39%, deficient= 31%, n=486) (Figure 4.4A right graph). Also, patient P2 demonstrated wide-spread complex I deficiency with 86% of the fibres lacking NDUFB8 (n=417, Figure 4.4B right graph), and patient P11 displayed a combined complex I and IV deficiency with 87% of fibres devoid of COX-I and NDUFB8 (n=559, Figure 4.4C right graph). Unfortunately, when using these SD limits, several controls showed some degree of deficiency. For example, in disease control DC3: 11%
of muscle fibres presented intermediate levels of NDUFB8 (n=252); in control DC5: 39% of fibres showed intermediate levels of NDUFB8 and 34% of fibres exhibited intermediate levels of COX-I (n=289). The classification results from controls were incorrect, and therefore, these SD limits were also rejected.

These results showed that COX-I/porinT and NDUFB8/porinT ratios were not a good measure to assess COX-I and NDUFB8 abundance in individual muscle fibres; they were leading to misclassifications. Furthermore, the COX-I/porinT and NDUFB8/porinT did not provide any information about the overall mitochondrial mass (assessed by porin), missing an important aspect of this analysis. Therefore, another approach for the objective assessment was considered: using linear regressions of ODCOX-I and ODNDUFB8 (dependent variables) against ODporin (independent variable).

4.4.3 Optimizing NDUFB8 signal

In order to allow comparison of different muscle sections, the linear regressions of ODCOX-I and ODNDUFB8 (dependent variables) against ODporin (dependent variable) have to present zero intercepts. The following section describes the optimisation undertaken in order to achieve that.

4.4.3.1 Background correction using an area of the section devoid of fibres

In order to develop an objective classification using COX-I and NDUFB8 linear regressions, quadruple immunofluorescence was performed in muscle sections obtained from disease control DC5 and three patients (P6, P9 and P10). These patients were selected because their muscle biopsy had a mosaic pattern of COX deficiency. The optical densities: ODCOX-I, ODNDUFB8 and ODporin were measured and corrected (subtracting the background as previously described) and the linear regression of ODCOX-I against ODporin and ODNDUFB8 against ODporin were created (Figure 4.5).

COX-I linear regression from disease control DC5 (y=2.06x) had a y-axis intercept of zero, whereas NDUFB8 linear regression (y=0.81+325.8) did not, intercepting at y=325.8 (Figure 4.5A). Also, in patients P6, P9 and P10, fibres deficient for NDUFB8 (visually inspected) showed a low value of ODNDUFB8 recorded by IMARIS (Figure 4.5 B, C and D: left graphs). These results suggested that ODNDUFB8 was not correctly adjusted for the background.



Figure 4.5 COX-I and NDUFB8 versus porin signals background corrected using an area of the section devoid of fibres.

Graphs on the left plot the corrected ODCOX-I versus corrected ODporin and Graphs on the right plot the corrected ODNDUFB8 versus corrected ODporin from (A) disease control DC4 (n=147) and patients: (B) P6 (n=300), (C) P9 (n=240) and (D) P10 (n=300).

The signal measured as specific to the antibody binding was still combined with some residual background noise, explaining why NDUFB8 linear regression was shifted up (the baseline at y=325.8). This approach however, discriminated fibres with normal levels of porin from those with very high levels of porin (Figure 4.5D: fibres with a correctedODporin higher than 800 intensity units). This was not previously possible when assessing fibres based on the COX-I/porinT and NDUFB8/porinT ratios

4.4.3.2 Background correction using a no primary antibody control

There was a suspicion that the high background noise in NDUFB8 channel was being caused by non-specific binding of Alexa fluor 546 secondary antibody to endogenous Fc receptors. To explore this, quadruple immunofluorescence was performed in muscle sections obtained from two controls (HC1 and DC1) and two patients (P2 and P6). A no primary antibody control (NPC, incubated only with anti-laminin α -1 antibody) was included for each case. IMARIS image analysis was conducted for NPC sections from all four cases, and the background noise of different channels (measuring the non-specific binding of secondary antibodies, OD488, OD546 and OD647) was determined in individual muscle fibres. The optical densities ODCOX-I, ODNDUFB8 and ODporin were measured in cocktail antibody-labelled serial sections and values were corrected by subtracting the mean OD of the fluorescent channels: meanOD647, meanOD546 and meanOD488, respectively. The linear regression of ODCOX-I against ODporin and ODNDUFB8 against ODporin were created (Figure 4.6).

COX-I linear regressions from HC1 and DC1 showed an intercept near zero (HC1: y=1.89x+4.77, n=440; DC1: y=2.58x-24.6 n=1500, Figure 4.6A and B: left graphs) whereas NDUFB8 linear regressions crossed y axis at negative values (Figure 4.6A and B: right graphs). Surprisingly, in patients P2 and P6, fibres with low levels of porin showed negative values of ODNDUFB8 whereas fibres with high levels of porin presented some levels of ODNDUFB8 (Figure 4.6C and D: right graphs). This highlighted increased background signal especially in fibres with high mitochondrial content.





Graphs on the left plot the corrected ODCOX-I versus corrected ODporin and graphs on the right plot the corrected ODNDUFB8 versus corrected ODporin from (A) healthy control HC1 (n=440), (B) disease control DC1 (n=1500) and patients (C) P2 (n=570) and (D) P6 (n=780).

To explore this issue, all background measurements: OD488, OD546 and OD647 measured in individual fibres were plotted together (Figure 4.7). All cases showed low and constant background measurements of Alexa fluor 488 (HC1: meanOD488=39.2, Coefficient of variation: CV=1.0%; DC1: meanOD488=39.8, CV=1.8%'; P2: meanOD488=43.5, CV=5.4%; P6: meanOD488=41.0, CV=2.3%) (Figure 4.7: green plots) and Biotin/Streptavidin647 (HC1: meanOD647=48.0, CV=3.0%; DC1: meanOD647=50.0, CV=4.4%; P2: meanOD647=57.2, CV=10.7%; P6: meanOD647=50.3, CV=11.3%) (Figure 4.7: purple plots). However, Alexa fluor 546 presented high and extremely variable background measurements across the NPC (HC1: meanOD546=151.7, CV=12.0%; DC1: meanOD546=152.6, CV=9.7%; P2: meanOD546=135.0, CV=10.5%; P6: meanOD546=137.3, CV=16.3%) (Figure 4.7: red plots). The variability of OD546 was present in all cases but reached the highest level in control HC1 and patient P6 (Figure 4.7 A and D). These results demonstrated that the background measurement of Alexa fluor 546 was repeatedly high and that the signal-to noise ratio – which compares the level of NDUFB8 signal with the level of Alexa fluor 546 background noise, was likely to be very low.





Figure 4.7 Background noise from secondary antibodies measured across the NPC section.

Graphs plot the OD546 (red), OD647 (purple) and OD488 (green) (y axis) measured in individual muscle fibres (x axis) from (A) heathy control HC1 (n=240), (B) disease control DC1 (n=1200), and patients (C) P2 (n=400) and (D) P6 (n=380).

4.4.3.3 Optimization of NDUFB8 signal

In order to combat the low NDUFB8 signal and the excessive background emerging from the non-specific binding of Alexa fluor 546 antibody, several optimisation steps were carried out. HC1 was used for all experimental tests as it presented a highly variable Alexa fluor 546 background. For all conditions tested in the following section, immunofluorescence and a NPC were performed in serial sections, and the same areas of the muscle section (for both immunofluorescence and matching NPC) were imaged, where possible. Consequently, the same fibres were assessed allowing a more direct and accurate comparison. To be able to directly compare the different conditions, two measures were calculated: the signal-to-noise ratio (S/N) and the signal-to-noise range ratio (RS/RN):

S/N = meanODsignal/ meanODnoise

RS/RN = (95%ODsignal – 5%ODsignal) / (95%ODnoise – 5%ODnoise)

Where ODsignal corresponds to ODNDUFB8, ODCOX-I or ODporin, and the ODnoise corresponds to the respective channel/secondary antibody (OD546, OD647 and OD488). A higher S/N ratio means higher desired signal in comparison to the background noise. The signal-to-noise range ratio provides information about how far apart signal and noise distributions are; higher RS/RN means distant and non-overlapping distributions.

4.4.3.3.1 Titration of Alexa fluor 546 secondary antibody

Double immunofluorescence detecting NDUFB8 (Alexa fluor 546, red) and laminin α -1 (Alexa fluor 405) was performed in four serial sections from HC1 and different dilutions of Alexa fluor 546 secondary antibody: 1:200, 1:600, 1:1000 and 1:1500 were tested (Figure 4.8).

Increasing the dilution of Alexa Fluor 546 from 1:200, to 1:600, 1:1000 and 1:1500 did not have a positive impact on the S/N and RS/RN ratios, which remained lower than 1.2 (Figure 4.8). For all conditions tested, the distribution of ODNUFB8 (Figure 4.8: dark red) and OD546 (Figure 4.8: light red) measurements were not significantly separated. Particularly in dilutions 1:200, (meanODNDUFB8= 312.7, meanOD546= 331.4, Figure 4.8A), 1:1000 (meanODNDUFB8= 128.2, meanOD546= 157.5, Figure 4.8C) and 1:15000 (meanODNDUFB8= 99.8, meanOD546=108.8, Figure 4.8D), the distributions occasionally overlapped and inversed positions (OD546 > ODNUFB8).



Figure 4.8 Impact of increasing dilutions of Alexa fluor 546 antibody on the signal-to-noise ratio. Graphs plot the ODNDUFB8 (measured in immunofluorescently labelled section, dark red) and OD546 (measured in NPC section, light red) (y axis) from individual muscle fibres (x axis) (n=100) for (A) 1:200, (B) 1:600, (C) 1:1000 or (D) 1:1500 dilutions of Alexa fluor 546 secondary antibody. S/N: Signal/Noise, RS/RN: signal-to-noise range ratio.

4.4.3.3.2 Changes to the immunofluorescence protocol

Several changes to the immunofluorescence protocol were made in order to reduce the background noise of Alexa Fluor 546: sections were blocked with 10% NGS (instead of 5% NGS), incubated with secondary antibodies for 2h at 4°C (instead of 2h at RT) and were mounted with Prolong Gold (Life technologies) mounting medium (instead of Veactashield hard set) (Figure 4.9).

As shown (Figure 4.9), the background of Alexa Fluor 546 (meanOD546=114.6, CV=9.4) remained higher than the background of Alexa Fluor 647 (Figure 4.8: light purple plots, meanOD647=71.7, CV=5.6%) and Alexa Fluor 488 (Figure 4.8: light green plots, meanOD488=62.4, CV=4.1%) but it was constant across the NPC section. Also, these changes lead to a higher S/N and SR/NR ratios, with a greater difference between the signal of NDUFB8 and the background of Alexa546 (RS/RN=4.8) (Figure 4.8B).







Graphs plot (A) ODCOX-I (measured in immunofluorescently labelled section, dark green) and OD488 (measured in NPC section, light green) (B) ODNDUFB8 (dark red) and OD546 (light red) and (C) ODporin (dark purple) and OD647 (light purple) (y axis) measured in individual muscle fibres (x axis) (n=300). S/N: Signal/Noise, RS/RN: signal-to-noise range ratio.

4.4.3.3.3 Testing multiple quenching protocols

Multiple quenching protocols using sudan black and/or ammonia (known to quench tissue autofluorescence) were tested in order to explore if they could improve the S/N ratio. For sudan black quenching: sections were immersed in sudan black solution (0.5% in 70% ethanol) for 10min following the last incubation with antibodies. For ammonia quenching: sections were immersed in ammonia (0.5% in 70% ethanol) for 10 min prior to the protein blocking step. Quenching with ammonia decreased the signal of NDUFB8 and showed no major effect on the background OD546 (Figure 4.10A (no quenching): meanODNDUFB8=224.6, meanOD546=114.6; Figure 4.10B (ammonia): meanODNDUFB8=162.1, meanOD546=116.1); quenching with sudan black, decreased both ODNDUFB8 and OD546 (Figure 4.10C, meanODNDUFB8=136.2, meanOD546=83.8). The combination of ammonia and sudan black quenching protocols had a negative effect on both signal and noise (Figure 4.10D: meanODNDUFB8=112.4, mean OD546=79.6). Overall, quenching with either ammonia (S/N=1.5, RS/RN=2.1), sudan black (S/N=1.6, RS/RN=3.6) or both (S/N=1.5, RS/RN=2.8) caused a decline in both S/N and RS/RN ratios when compared with the non-quenched control (S/N=2.0, RS/RN=4.8) (Figure 4.10).



Figure 4.10 The impact of multiple quenching protocols on the NDUFB8 signal-to-noise ratio. Graphs plot ODNDUFB8 (measured in immunofluorescently labelled section, dark red) and OD546 (measured in NPC section, light red) (y axis) measured in individual muscle fibres (x axis) in sections (A) not subjected to any quenching protocol, (B) when quenched with ammonia, (C) when quenched with sudan black, and (D) when quenched with both ammonia and sudan black (n=300). S/N: Signal/Noise, RS/RN: signal-to-noise range ratio

4.4.3.3.4 Changing the combinations of primary and secondary antibodies

Since Alexa fluor 546 presented the highest background, multiple combinations of NDUFB8 with secondary antibodies were tested:

- 1. Alexa fluor 488: Porin, Alexa fluor 546: NDUFB8, Biotin/Streptavidin 647: COX-I
- 2. Alexa fluor 488: COX-I, Alexa fluor 546: porin, Biotin/Streptavidin 647: NDUFB8
- 3. Alexa fluor 488: NDUFB8, Alexa fluor 546: porin, Biotin/Streptavidin 647: COX-I

Testing new combinations of primary and secondary antibodies showed that coupling NDUFB8 with Biotin/Streptavidin 647 (S/N=2.7, RS/RN=14.3) provided the highest S/N ratio; also, distributions of signal and background showed a greater separation than when NDUFB8 was coupled with either Alexa fluor 546 (S/N=2.0, RS/RN=4.8) or Alexa fluor 488 (S/N=2.2, RS/RN=9.6) (Figure 4.11). Importantly, COX-I coupled with Alexa fluor 488 (S/N= 6.3, RS/RN=40.6) and porin coupled with Alexa fluor 546 (S/N= 4.7, RS/RN= 13.7) (Figure 4.12), also showed high S/N and RS/RN ratios validating this combination of antibodies as optimal.



C NDUFB8 coupled with Alexa Fluor 488



Figure 4.11 The impact of coupling NDUFB8 with different secondary antibodies on the signal-to-noise ratio.

Graphs plot (A) ODNDUFB8 signal (measured in immunofluorescently labelled section, dark red) and OD546 noise (measured in NPC section, light red), (B) ODNDUFB8 (dark purple) and OD647 (light purple) and (C) ODNDUFB8 (dark green) and OD488 (light green) (y axis) measured in individual muscle fibres (x axis) (n=300) . S/N: Signal/Noise, RS/RN: signal-to-noise range ratio.



Figure 4.12 COX-I and porin signal-to-noise ratios when coupled with Alexa fluor 488 and Alexa fluor 546 antibodies respectively.

Graphs plot (A) ODCOX-I signal (measured in immunofluorescently labelled section, dark green) and OD488 noise (measured in NPC section, light green), (B) ODporin (dark red) and OD546 (light red) (y axis) measured in individual muscle fibres (x axis) (n=300). S/N: Signal/Noise, RS/RN: signal-to-noise range ratio.

4.4.3.3.5 Testing different incubation times

In order to assess the impact of different incubation times and temperatures of secondary antibodies on the signal-to-noise ratio, quadruple immunofluorescence detecting COX-I (Alexa fluor 488, green), porin (Alexa fluor 546, red), NDUFB8 (Biotin/Streptavidin 647) and laminin α -1 (Alexa fluor 405, white) was performed in serial sections from HC1. Different incubation times of secondary antibodies were tested,

- Control condition: 1st incubation (secondary antibodies cocktail): 2h at 4°C; 2nd incubation (Streptavidin 647): 2h at 4°C
- 2. 1st incubation: 24h at 4°C, 2nd incubation: 2h at 4°C
- 3. 1st incubation: 24h at 4°C, 2nd incubation: 1h at RT
- 4. 1st incubation: 12h at 4°C, 2nd incubation: 1h at RT

Increasing the time of the 1st incubation from 2h to 24h at 4°C did not affect the signal of NDUFB8 but increased the background of Biotin/Streptavidin 647 (Figure 4.13A: meanODNDUFB8=376.2, meanOD647=102.2; Figure 4.13B: meanODNDUFB8=375.8, mean OD647=116.3). Decreasing the incubation of streptavidin 647 from 2h at 4°C to 1h at RT, had no impact on the background of Biotin/Streptavidin 647, which remained higher than the control condition, but increased the signal of NDUFB8 (Figure 4.13B: meanODNDUFB8=375.8, meanOD647=116.3; Figure 4.13C: meanODNDUFB8=411.9, mean OD647=115.0). Decreasing the time of 1st incubation from 24h to 12h at 4°C affected neither NDUFB8 signal nor the background (Figure 4.13C: mean ODNDUFB8=411.9, mean OD647=115.0; Figure 4.13D: mean ODNDUFB8=411.8, mean OD647=115.8). These results suggested that the incubation time of the biotinylated anti-IgG1 antibody affects the background of Alexa647 whereas the incubation of streptavidin influences the signal of NDUFB8. Regardless, increasing the incubation times of Alexa fluor 488, Alexa fluor 546 and Biotin (from 2h to 12h or 24h at 4°C) and performing the incubation of streptavidin647 for 1h at room temperature (instead of 2h at 4°C) provided lower S/N and RS/RN ratios when compared with the previously used protocol (Figure 4.13).



Figure 4.13 The impact of different incubation conditions (secondary antibodies) on the NDUFB8 signal-to-noise ratio.

Graphs plot ODNDUFB8 (measured in immunofluorescently labelled section, dark purple) and OD647 (measured in NPC section, light purple) when (A) all secondary antibodies were incubated 2h at 4°C, when (B) Alexa fluor 488, Alexa fluor 546 and anti-IgG1 Biotinylated were incubated for 24h at 4°C and streptavidin 647 2h at 4°C, when (C) Alexa fluor 488, Alexa fluor 546 and anti-IgG1 Biotinylated were incubated for 24h at 4°C and Streptavidin 647 1h at room temperature and when (D) Alexa fluor 488, Alexa fluor 546 and anti-IgG1 Biotinylated were incubated for 24h at 4°C and Streptavidin 647 1h at room temperature and when (D) Alexa fluor 488, Alexa fluor 546 and anti-IgG1 Biotinylated were incubated for 12h at 4°C and Streptavidin 647 1h at room temperature (n=300). RT: room temperature, S/N: Signal/Noise, RS/RN: signal-to-noise range ratio.

4.4.4 Classification of fibres based on COX-I and NDUFB8 linear regressions

4.4.4.1 The new objective assessment

Quadruple immunofluorescence, as previously optimised, was performed in serial muscle sections from a healthy control (HC2), disease controls (DC1, DC2, DC3) and mitochondrial patients (P1, P2, P6, P9, P10 and P11). A NPC section was included for each case.

4.4.4.1.1 Background correction

For each muscle fibre ODCOX-I, ODNDUFB8 and ODporin were corrected for background signal. ODCOX-I and ODporin values were corrected by subtracting the mean OD488 and mean OD546 measured in NPC, respectively. However, fibres with high mitochondrial mass exhibited

proportionally high level of background signal in the 647nm spectrum. Therefore a different background correction was required for that channel. Fibres were sorted into one hundred groups according to the ODporin percentile, and corrected using the mean OD647 of the matching ODporin percentile group from the NPC. This allowed correcting the ODNDUFB8 measurements according to the levels of mitochondrial mass; i.e. ODNDUFB8 values from fibres with very high levels of porin were corrected using the highest values of background.

4.4.4.1.2 Transformation of the corrected data

The Box-Cox transformation identified the log transformation as optimal to achieve normality of the background corrected data. This was confirmed using both visual inspection and normality tests (Shapiro-Wilk and D'Agostino-Pearson's omnibus test). Background corrected OD values were log transformed (yielding ODCOX-I_T, ODNDUFB8_T and ODporin_T) to normalize the data.

4.4.4.1.3 Control group

Initially, ODCOX-I_T versus ODporin_T and ODNDUFB8_T versus ODporin_T from all four control muscle biopsies (healthy and disease controls) were plotted together (Figure 4.14A). The combined data from individual controls clustered and were consistent, presenting similar individual linear regressions. Later on, the creation of control group was refined. The control group was created by simple random sampling of equal numbers of fibres from each of the control subject. The control with the lowest number of fibres was determined and used to set the number of fibres randomly chosen in remaining controls. This created a more balanced group with all controls carrying the same weight. From the selected fibres, ODCOX-I_T versus ODporin_T and ODNDUFB8_T versus ODporin_T were plotted (Figure 4.14).

Linear regressions of ODCOX-I_T and ODNDUFB8_T (dependent variables) against ODporin_T (independent variable) were performed, validated to ensure the residuals of the regression were normally distributed (Figure 4.15) and the standard error of estimate was determined; this enabled the estimate of deviation of COX-I and NDUFB8 levels in each fibre from the predicted level according to the porin level Figure 4.16.





Figure 4.14 Approaches tested to create the control group.

Initially, (A) all corrected ODCOX-IT versus corrected ODporinT (left graph) and all corrected ODNDUFB8T versus ODporinT (right graph) from controls were plotted together: HC2 (blue circles and trend line, n=226), DC1 (red circles and trend line, n=767) DC2 (green circles and trend line, n=595) and DC3 (black circles and trend line, n=128). (B) To get a balanced control group 128 fibres from each healthy and disease controls were randomly selected and both ODCOX-IT versus ODporinT (left graph) and ODNDUFB8T versus ODporinT (right graph) from selected fibres were plotted together. HC2 (blue circles and trend line, n=128), DC1 (red circles and trend line, n=128) DC2 (green circles and trend line, n=128) and DC3 (black circles and trend line, n=128).





4.4.4.1.4 Data analysis

The parameters (mean and standard deviation) describing the distribution of $ODporin_T$ in the control population (meanPorin, SDporin), as well as parameters describing the linear relationship between $ODCOX-I_T$ versus $ODporin_T$ and $ODNDUFB8_T$ versus $ODporin_T$ were used to determine the standard scores (Z-scores) for $ODporin_T$ (porin_z), COX-I ($COX-I_z$) and NDUFB8 ($NDUFB8_z$); based on the expected level of each, according to the porin level (Figure 4.16):

COX-I_z = $(ODCOX-I_T - predicted ODCOX-I_T)/(standard error of prediction of ODCOX-I_T)$ **NDUFB8_z** = $(ODNDUFB8_T - predicted NDUFB8_T) /(standard error of prediction of NDUFB8_T)$ **porin_z** = $(ODporin_T - meanPorin)/SDporin$

Fibres were classified into different groups relative to porin levels based on standard deviation limits (SD): fibres were considered to have "very low" levels of porin if porin_z was 3 SD below the mean of control group (reference value), "low" levels if porin_z was between 2 to 3 SD below the reference value, "normal" levels if porin_z was within 2 SD below and 2 SD above the reference value, "high" levels if porin_z was within 2 to 3 SD above and "very high" levels of porin levels if porin_z was 3 SD above the reference value. Fibres were similarly classified based on SD limits into groups of NDUFB8 and COX-I levels (normal if Z-scores > -3SD; int(+) if Z-scores between -3SD to -4.5SD; int(-) if Z-scores between -4.5SD to -6SD; deficient if Z-scores <<-6 SD). The upper SD boundary of COX-I and NDUFB8 deficient groups were defined by determining the highest NDUFB8_z and COX-I_z of red appearing fibres on quadruple immunofluorescence (staining for porin but with absent COX-I and NDUFB8) from patients P10 and P6. These limits were confirmed and validated when immunofluorescence was performed in a serial section on a different day for the purpose of assessing reproducibility of the technique. The lower SD boundaries of COX-I and NDUFB8 positive groups were defined by the distribution of the control group.

4.4.4.1.5 Graphing: MRC profiles

To have a better understanding of mitochondrial pathology, a novel graphical way to present the data (Figure 4.17 lower graph) was developed. This shows NDUFB8 and COX-I protein levels in conjunction with mitochondrial mass in individual muscle fibres. Each dot represents the measurement from an individual muscle fibre, colour coded according to its mitochondrial content (very low: blue, low: light blue, normal: light orange, high: orange and very high: red).



Figure 4.16 Deviation of COX-I and NDUFB8 levels in each fibre from the expected levels, according to porin level.

Graphs (A) plot corrected ODCOX-IT versus corrected ODporinT and (B) corrected ODNDUFB8T versus ODporinT (right graph) from control group (grey circles, n=512), and P6 (white circles, n=509).



Figure 4.17 Summary graphical outputs of the R programing supporting tool.

Bar graphs show the percentage of muscle fibres with positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue) abundance of COX-I (top left graph) and NDUFB8 (top middle graph), and the frequency distribution of porin_z highlights the range of porin_z and abundance per class. Lower and central graph presents in a comprehensive way: COX-I and NDUFB8 abundance in conjunction with the mitochondrial content in all the muscles fibres from an individual patient biopsy.

The position of dots along the axis determines the respective level of COX-I and NDUFB8. For instances, all dots (fibres) contained within the beige square have normal levels of COX-I and NDUFB8, clustering in the same area as the control population's muscle fibres. The dots located towards the left display down-regulated NDUFB8; the dots located towards the bottom present down-regulated COX-I. Dots contained within the blue square, have entirely lost COX-I and NDUFB8 proteins. Such graphical approach allows recognition of distinct patterns of mitochondrial respiratory chain dysfunction and enables precise quantification of the deficiency degree. This graphical representation will be, from now on, referred to as the mitochondrial respiratory chain (MRC) profile.

4.4.4.1.6 R programming

A supporting tool was written in R (Team, 2014) to automate the evaluation of the control population distributions of porin, NDUFB8, and COX-I, perform the linear regressions, calculate Z-scores for each of the proteins in each muscle fibre, and produce summary graphical outputs. The summary graphical output is a pdf file (Figure 4.17) showing the MRC graphs, and additionally, two bar graphs providing more details about complex I and IV and an histogram showing the frequency distribution of porin_z.

4.4.4.1.7 Preliminary results

All patients assessed showed varying proportions of COX-I and/or NDUFB8 deficiency and presented different patterns of mitochondrial deficiency (Figure 4.18). Patient P1 showed a severe and isolated complex IV deficiency, P2 showed a severe and isolated complex I deficiency and P11: a balanced down-regulation of both COX-I and NDUFB8 (Figure 4.18 A, B and F, respectively). Both patients P9 and P10 presented fibres with normal COX-I levels but down-regulated NDUFB8 that followed an asymmetrical trend towards down-regulation of both complexes (Figure 4.18 D and E). Patient P6 presented a population of fibres with normal COX-I but down-regulated NDUFB8 and a population of fibres with a gradual and balanced down-regulation for both complexes (Figure 4.18 C).



Figure 4.18 Mitochondrial respiratory chain expression profile linking complex I, complex IV and porin levels in patients with mitochondrial disease.

Patients with (A) isolated complex IV deficiency (LRPPRC mutations, P1, n= 424), (B) isolated complex I deficiency (m.4175G>A MT-ND1 mutation, P4, n = 1062), (C) multiple mtDNA mutations (P6, n= 509), (D) m.3243A>G MT-TL1 (P9, n=392), (E) m.5690A>G MT-TN mutation (P10, n= 315) and (F) novel MT-TP mutation (P11, n= 489) were assessed in terms of COX-I, NDUFB8 and porin protein levels. Each dot represents an individual muscle fibre, colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: light orange, high: orange and very high: red). Thin black dashed lines indicate the SD limits for the classification of fibres, lines next to x and y axis indicate the levels of NDUFB8 and COX-I respectively (beige: normal, light beige: int(+), light blue: int(-) and blue: deficient). Bold dashed lines indicate the mean abundance level of normal fibres.

4.4.4.2 Correlation between COX activity and COX-I levels

To assess the correlation between respiratory chain enzyme activity and mitochondrial protein levels, four different patients (P3, P5, P7 and P12) were investigated. These patients were carefully selected because their muscle biopsies showed a very clear pattern of COX/SDH reactivity allowing relatively simple classification of COX deficient and COX intermediate reacting fibres. Serial sections from these four patients were subjected to COX/SDH histochemistry and quadruple fluorescent immunohistochemistry. Muscle fibres were visually classified by an experienced investigator based on the COX/SDH enzymatic reactions into one of the four groups: COX positive, COX int(+), COX int(-) and COX deficient. Subsequently, the immunofluorescently labelled serial section was objectively classified based on COX-I and porin (Figure 4.19, Table 4.4).





(A) COX/SDH histochemistry (top) and quadruple immunofluorescence (bottom) detecting COX-I – green (Alexa fluor 488nm), NDUFB8 – purple (biotin/Streptavidin 647nm), porin - red (Alexa fluor 405nm) and laminin α -1 - white (Alexa fluor 405nm) were performed on serial muscle sections from patients P3, P5, P7 and P12. (B) Graphs show the results from visual classification (based on COX/SDH histochemistry) and objective classification (based on COX-I and porin immunodetection). Fibres were classified as COX (activity/protein abundance) positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue). Fibres counted (n=visual/immunodetection): P3 (n=1103/841); P5: (n=1395/1071); P7: (n=1887/1740) and P12: (n=956/769). Scale bars measure 50µm. Image taken from Rocha *et al.* (2015).

Datianta		Complex IV				
Patients		Pos	Int(+)	Int(-)	Neg	n
	VC	90.5%	1.5%	1.6%	6.3%	1103
P3 (Single mtDNA	ос	92.9%	2.1%	2.2%	2.7%	841
deletion)	E(VC-OC)	-2.4%	-0.6%	-0.6%	3.6%	
	p-value	0.06	0.34	0.33	<0.05	
	VC	67.0%	10.8%	7.5%	14.8%	1395
P5 (Multiple mtDNA	ос	69.3%	10.5%	6.0%	14.2%	1071
deletions)	E(VC-OC)	-2.3%	0.3%	1.5%	0.6%	
	p-value	0.21	0.81	0.14	0.69	
P7	VC	83.6%	4.3%	4.1%	8.0%	1887
(m.3243A>G	OC	82.2%	3.6%	2.2%	12.0%	1740
MT-TL1)	E(VC-OC)	1.4%	0.8%	1.8%	-4.1%	
	p-value	0.25	0.23	<0.05	<0.05	
	VC	13.1%	2.3%	2.0%	82.6%	956
P12	ос	11.6%	0.8%	0.7%	87.0%	769
MT-TG)	E(VC-OC)	1.5%	1.5%	1.3%	-4.4%	
	p-value	0.34	<0.05	<0.05	<0.05	

Table 4.4 Correlation of COX activity and COX-I levels

Key: VC = visual classification based on COX/SDH histochemistry; OC = objective classification based on quantitative immunofluorescence; E(VC-OC) = estimate for difference between VC and OC; Z test for 2 population proportions.

The difference between the objective classification (OC) based on COX-I levels and visual classification (VC) based on COX/SDH histochemistry was always lower than 5 percentage points (Table 4.4). In patient P5, the visual classification was consistent with the objective classification. Remaining patients showed some differences; visual classification overestimated COX deficient fibres by 3.6 percentage points in patient P3 (COX deficient: VC=6.3%, OC=2.7%), whereas it underestimated this group by 4.4 percentage points in patient P12 (COX deficient: VC=82.6%, OC=87.0%), and by 4.1 percentage points in patient P7 (COX deficient: VC=8.0%, OC=12.0%), as compared to objective classification.

4.4.4.3 Reliability of the technique

In order to evaluate the reliability of the method, quadruple fluorescent immunohistochemistry was performed on a muscle section from patient P4, and data was collected on two separate occasions by two investigators (Investigator 1: I1, Investigator 2: I2) at a 15-day interval. Data collection included imaging the muscle section (approximately the same area) and performing IMARIS analysis.

The analysis from the same muscle section provided comparable results when data was collected by two investigators (Figure 4.20, Table 4.5). Indeed, the objective classification results based on COX-I levels from I1 (pos= 67.7%, int(+)=4.7%, int(-)=2.8%, neg=24.9%, n=470) and I2 (pos= 71.6%, int(+)=1.5%, int(-)=2.3%, neg=24.6%, n=528) were almost identical; the greatest difference of less than 4 percentage points was noted for COX-I positive (p=0.18) and int (+) groups (p<0.05) (Figure 4.20A). For NDUFB8 levels, the results from I1 (pos= 65.7%, int(+)=6.0%, int(-)=4.7%, neg=23.9%, n=470) and I2 (pos= 62.9%, int(+)=3.8%, int(-)=4.0%, neg=29.4%, n=528) did not differ significantly though the maximum discrepancy within COX deficient fibres was 5.7 percentage points (p=0.04) (Figure 4.20B).





Quadruple immunofluorescence was performed in a muscle section taken from P4 and approximately the same area of the biopsy was assessed by investigator 1 (n = 528 fibres analysed) and investigator 2 (n = 470 fibres analysed). The assessment included imaging the selected area of the muscle section by each investigator on a separate occasion (15 days apart) and performing subsequent IMARIS analysis. Bar graphs show the percentage of fibres with normal (beige), int(+) (light beige), int(-) (light blue), and deficient (blue) levels of (A) COX-I and (B) NDUFB8, when assessed by investigators 1 and 2. Image taken from Rocha *et al.* (2015).

Dationt D4			COX-I / NDUFB8 levels			
Fatient F4		Pos	Int(+)	Int(-)	Neg	n
	Invest. 1	67.7%	4.7%	2.8%	24.9%	470
	Invest. 2	71.6%	1.5%	2.3%	24.6%	528
COX-I levels	E(I1-I2)	-3.9%	3.2%	0.5%	0.3%	
	p-value	0.18	<0.05	0.62	0.92	
	Invest. 1	65.7%	6.0%	4.7%	23.6%	470
	Invest. 2	62.9%	3.8%	4.0%	29.4%	528
NDOFB8 levels	E(I1-I2)	2.9%	2.2%	0.7%	-5.7%	
	p-value	0.35	0.11	0.59	0.04	

Table 4.5 Inter-observer variability of quadruple immunofluorescence.

Key: Invest. 1 = investigator 1; Invest. 2 = investigator 2; E(I1-I2) = estimate for difference between investigator 1 and investigator 2; Z test for 2 population proportions.

4.4.4.4 Reproducibility of the technique

In order to evaluate the reproducibility of the method, serial muscle sections from previously assessed five patients (P1, P2, P6, P10 and P11) were processed through the same methodological protocol (quadruple immunofluorescence, followed by muscle section imaging and IMARIS analysis) on two separate occasions ("day 1" and "day 2"), 2 months apart.

The analysis of serial muscle sections from same patients provided consistent results (Figure 4.21). Quantification of COX-I and NDUFB8 levels showed similar results between the two separate experiments for patients: P1, P2 and P10 (p<0.05, two proportions test). In patient P6, quantification of COX-I protein level did not show any difference between days whereas quantification of NDUFB8 showed on "day 2", a lower proportion of positive fibres (Day1: 63.4%, n=1589; Day 2: 54.4%, n=2400, p<0.001, two proportions test) and a higher proportion of int(+) (Day1: 12.4%, n=1589; Day 2: 19.0%, n=2400, p<0.001, two proportions test) and deficient fibres (Day1: 19.6%, n=1589; Day 2: 22.5%, n=2400, p=0.028, two proportions test). The estimated difference in the proportions of NDUFB8 positive, int(+) and deficient fibres between days was 8.96%, -6.64% and -2.89%, respectively. Similarly in patient P11, quantification of COX-I did not show any difference between days whereas quantification of NDUFB8 showed on "day 2" a slightly lower proportion of int(-) fibres (Day1: 4.4%, n=1012; Day 2: 2.2%, n=1689, p=0.002, two proportions test) together with a higher proportion of fibres with absent NDUFB8 (Day1: 94.7%, n=1012; Day 2: 97.2%, n=1689, p=0.002, two proportions test). The estimate difference in the proportions of int(-) and deficient fibres between days was 2.3% and -2.5%, respectively. The estimate difference between days is a measure of variability and is more valuable in assessing the reliability of immunofluorescence assay since with such high number of fibres analysed any small difference is statistically significant. Last but not least, the objective assessment of COX-I from all patients showed, again, a complete agreement with the visual classification from the Mitochondrial Diagnostics Service pathologist.

4.4.4.5 Advantage of the novel assay over COX/SDH histochemistry

In chapter 3 (section 3.4.1.3), patients P8 and P9 muscle biopsies' were used to illustrate how visual classification of muscle fibres can be subjective and challenging. That analysis revealed marked differences in classification of fibres between the two investigators (I1 and I2). However, since these biopsies were challenging to visually classify, there was some uncertainty regarding the validity of results.



Figure 4.21 Reproducibility of quadruple immunofluorescence.

COX-I and NDUFB8 protein levels were quantified in 2 different time points (Day 1, Day 2) using two serial muscle sections from patient with (A) LRPPRC mutations (P1, nday1= 424, nday2=1258), (B) m.4175G>A MT-ND1 mutation (P2, nday1=823, nday2=932), (C) multiple mtDNA deletions (P6, nday1=1589, nday2= 2400), (D) m.5690A>G MT-TN mutation (P10, nday1= 362, nday2= 1512) and (E) novel MT-TP mutation (P11, nday1=1012, nday2=1689). Bars indicate the percentage of muscle fibres with: normal levels of either COX-I or NDUFB8 (beige), int(+) (light beige), int(-) (light blue), and deficient (blue).

To illustrate how visual classification can lead to misleading classifications, serial muscle sections from P8 and P9 were assessed using quadruple immunofluorescence and the same 100 muscle fibres were objectively classified based on COX-I and porin levels (Figure 4.22).The objective classification showed that in patient P8 both investigators identified the majority of COX positive cells correctly (40% versus 42% using immunofluorescence) but differed in remaining categories and underestimated significantly COX deficient fibres (I1=28%, I2=36% and OC=50%). The objective classification showed that in patient P9, both investigators underestimated COX positive fibres (I1=22%, I2=20% and OC=37%), and overestimated COX deficient fibres (I1=49%, I2=29% and OC=33%), particularly investigator 1 who counted twice as many cells as revealed by the immunofluorescent technique. Both investigators were internally inconsistent (Figure 4.22).



Figure 4.22 Inter- and intra-observer variability of visual classification.

(A) COX/SDH histochemistry (top panels) and quadruple immunofluorescence (bottom panels) detecting COX-I (green), NDUFB8 (purple), porin (red) and laminin α -1 (white) were performed in two serial muscle sections obtained from patients P8 and P9. For each patient 100 muscle fibres reacted for COX/SDH histochemistry were selected and visually classified by two investigators. The same 100 fibres were objectively assessed using quadruple immunofluorescence. (B) Bar graphs show the percentage of COX-positive (beige), int(+) (light beige), int(-) (light blue) or deficient (blue) based on visual classification by an investigator 1 and 2 (first two bars) or objective classification of immunofluorescence (last bar). Image adapted from Rocha *et al.* (2015).

4.5 Discussion

This chapter describes a novel quadruple immunofluorescence, high-throughput and computer-based assay. It enables accurate quantification of key OXPHOS protein levels (complex I and complex IV) and a mitochondrial mass marker (porin) in individual muscle fibres, using a 10µm tissue section. The use of laminin to define cell boundaries enables semi-automatic quantification of a large number of muscle fibres. The analysis has minimal human intervention and is solely based on OD measurements. This reduces the variability and eliminates subjectivity and inconsistencies of results, which are common in currently available methods used to assess mitochondrial function in human muscle sections. This chapter also demonstrates that this technique is sensitive and reliable in detecting and quantifying focal mitochondrial deficiency in human muscle.

4.5.1 Advantages of the novel method

Previous studies have elegantly explored the involvement of multiple respiratory chain complexes using immunohistochemistry for the diagnosis and understanding of mitochondrial pathology (Tritschler *et al.*, 1991, Rahman *et al.*, 2000, Hanson *et al.*, 2002, De Paepe *et al.*, 2009, Mahad *et al.*, 2009), but only a few quantified the deficiency (Mahad *et al.*, 2009, Murphy *et al.*, 2012, Grunewald *et al.*, 2014). This novel quadruple immunofluorescent method quantifies accurately mitochondrial COX-I and NDUFB8 abundance in a single muscle section. Several optimisation steps were carried out in order to achieve that: by increasing the concentration of the protein blocking step, selecting the best combination of primary and secondary antibodies and by determining the optimal conditions and times of incubations. All conditions tested were carefully investigated by assessing the same group of fibres through densitometry, when possible. This allowed calculating S/N and RS/RN values) and not based on visual inspection. This together with the meticulous background signal correction approach guarantees an optimal detection of true COX-I, NDUFB8 and porin signals, which reflect the protein levels.

It was demonstrated that in cases of obvious COX deficiency, the visual classification of fibres was comparable with the objective classification based on the immunofluorescent technique. In these instances, COX-I protein levels correlated well with complex IV activity, validating the use of immunohistochemical techniques to assess mitochondrial biochemical profile in individual muscle fibres. However, in cases with a high proportion of COX-intermediate fibres or where histochemical signal detected was less intense and variable, visual classification of muscle fibres proved to be challenging and significantly differed between investigators. Also it significantly differed from the objective assessment confirming that visual assessment is prone to errors.

By contrast, this novel assay demonstrated to be subjectivity-devoid, reliable and reproducible. Reliability of quantification was tested through collecting data (image acquisition and IMARIS analysis) on two separate occasions by two investigators. Results showed that the magnitude of inter-observer variability of immunofluorescent technique was significantly low. The two investigators differed in objective classification by a maximum of 6 percentage points whereas the same two investigators had differed in their visual categorisation by as much as 20 percentage points. Reproducibility of quantification was formally tested by processing sections through the methodological protocol (quadruple immunofluorescence, image acquisition and IMARIS analysis) on two separate occasions. The difference in the classification of fibres between days was always within 9 percentage points. Additionally, the large numbers of experimental replicates carried out also supports the reproducibility of this technique; the controls demonstrated consistent and comparable levels of protein. Importantly, some patients demonstrated at least a small population of fibres that appeared normal and healthy, with Z-scores centred appropriately around the mean NDUFB8 and COX-I levels observed in the controls.

Due to high accuracy and precision, this novel assay quantifies focal mitochondrial deficiency in tissue muscle sections. Results from all patients (with genetically-characterized mitochondrial disease) assessed were consistent with the respective genetic mutation and histochemical findings. Interestingly, results also showed that different genetic defects might present different patterns of COX-I and NDUFB8 deficiency. A detailed analysis of these patients will be provided in the next chapter.

4.5.2 Technical methodology

A major problem in immunofluorescence assays is signal from non-specific binding of secondary antibodies. To overcome this, groups usually set an intensity threshold to identify positive cells (Brey *et al.*, 2003, Goedkoop *et al.*, 2005), which is itself subjective. Here, the intensities measured in the quadruple immunofluorescence were background corrected by subtracting the non-specific signal measured in the NPC. Despite that, the secondary antibody (lgG1 biotinylated) coupled with NDUFB8 showed a higher affinity in RRF, evident from the high fluorescence intensity from these fibres detected in the serial NPC section. This non-specific binding was associated with the anti-lgG1 secondary antibody, since exchanging the secondary antibody fluorophores lead to the switching of increased NPC signal to the alternate fluorophore (Alexa fluor 546).

To compensate for this effect and ensure the NDUFB8 values represented true signal, the ODNDUFB8 signal measured in the quadruple immunofluorescence was corrected according to mitochondrial mass. This approach was validated by the fact that without correction, ragged red fibres demonstrated constant basal levels of COX-I signal but ODNDUFB8 signal proportional to the ODporin. With correction the ODNDUFB8 in ragged red fibres was at a constant basal level, consistent with the COX-I levels and no increasing trend with ODporin was observed.

4.5.3 Potential applications

The novel quadruple immunofluorescent assay has a great potential to become broadly applied. It could play an important role in supporting the current diagnostic techniques, given the ability to detect complex I deficiency in individual muscle fibres. More investigation is required to evaluate if a specific mitochondrial defect demonstrates a unique pattern of respiratory chain dysfunction, as preliminary results from patients seem to indicate. Also, this assay is sensitive enough to detect and quantify subtle changes in protein abundance; it is therefore anticipated that it could be valuable in studying slowly progressing conditions, ageing, or assessing the impact of therapeutic interventions for mitochondrial diseases (Rocha *et al.*, 2015)

5.1 Introduction

Mitochondrial respiratory chain defects are found in a wide range of human pathologies. They can occur as a primary cause of disease - through genetic defects involving the mitochondrial or nuclear genome (Gorman *et al.*, 2015b) or as secondary when there are other prominent pathological processes such as inflammation (Oldfors *et al.*, 1992) or degenerative features Reeve (Reeve *et al.*, 2008).

Mitochondrial diseases are a group of disorders which are clinically heterogeneous. Some affect a single organ whereas others are multi-systemic (usually affecting central nervous and muscular systems). Skeletal muscle is commonly involved, either exclusively or as a predominant feature in multisystem phenotypes (Taylor *et al.*, 2004, McFarland *et al.*, 2010), due to both mitochondrial and nuclear defects. Mitochondrial myopathies are progressive and clinical features include chronic progressive external ophthalmoplegia, rhabdomyolysis, muscle fatigue and severe proximal weakness (Pitceathly and McFarland, 2014).

Both mitochondrial and nuclear genetic defects can lead to either isolated enzyme deficiencies (affecting one complex of the mitochondrial respiratory chain) or combined deficiencies (affecting multiple complexes). Isolated complex I deficiencies are the most common OXPHOS defects accounting for up to a third of all OXPHOS disorders (Loeffen *et al.*, 2000), followed by isolated complex IV deficiencies (Diaz, 2010). These are generally caused by mutations in either structural genes (mitochondrial or nuclear genes encoding subunits of OXPHOS complexes) or nuclear-encoded factors required for the proper assembly of OXPHOS complexes. Combined deficiencies are generally caused by large-scale deletions in the mtDNA, point mutations in mitochondrial-encoded tRNAs or rRNAs required for mitochondrial protein synthesis (Chinnery *et al.*, 2000). Furthermore, they can also arise by mutations in nuclear genes encoding proteins involved in mtDNA maintenance, mitochondrial transcription and translation, import of nuclear-encoded proteins or RNAs into the mitochondria or mitochondrial biogenesis (Smits *et al.*, 2010).

Current diagnostic algorithms for investigating and diagnosing mitochondrial disease rely on histochemical and biochemical assessment of OXPHOS activities in clinically-affected tissues

(Kirby *et al.*, 2007). Biochemical assays measure the activity of each individual OXPHOS enzyme complex (complex I–V) in homogenised muscle tissue (Kirby *et al.*, 2007). Although very useful for identifying wide-spread mitochondrial defects, they have several limitations. They require substantial quantities of muscle (>50mg) and they may fail to detect subtle OXPHOS deficiencies, especially when only a subset of muscle fibres are affected.

The histochemical assessment of cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) activities are standard methods to assess mitochondrial function in tissue muscle sections (Old and Johnson, 1989, Sciacco and Bonilla, 1996). Although valuable for detecting the mosaic pattern of mitochondrial deficiency, the classification of muscle fibres is subjective, qualitative and prone to inter-observer variability (Choudhury *et al.*, 2010, Rizzardi *et al.*, 2012). Furthermore, there are no histochemical assays to investigate other OXPHOS complexes, namely complex I.

5.2 Aims

The aims of this chapter were to:

- 1. Characterise the MRC profile of individual muscle fibres in a broad range of primary and secondary mitochondrial genetic defects.
- 2. Validate the use of quadruple immunofluorescence as a tool to diagnose and investigate the underlying pathogenic mechanisms of mitochondrial diseases.

5.3 Methods

5.3.1 Cohort clinical characteristics

Forty five patients with clinically and genetically-characterised mitochondrial disease were studied: two patients with a specific genetic defect affecting only one respiratory chain complex (isolated complex I or complex IV deficiency); seventeen patients with single-large scale mtDNA deletion; five patients with autosomal disorders of mtDNA maintenance leading to the generation of multiple mtDNA deletions in muscle; fifteen patients with point mutations in mitochondrial-encoded tRNAs; five patients with mutations in nuclear genes involved in mitochondrial RNA processing translation (*ELAC2, GFM2*), mitochondrial RNA import (*PNPT1*) and mitochondrial protein import (*GEFR*). Five patients with suspected mitochondrial disease were also assessed for diagnostic purposes. Table 5.1 summarises relevant clinical information.

The healthy control sample was obtained during orthopaedic surgery (HC: female, 20 years old). The three disease controls were previously investigated for suspected neuromuscular disease but showed normal muscle histology, oxidative enzyme histochemistry and normal respiratory chain biochemical activities (Kirby *et al.*, 2007). Approval for this research was granted by the Newcastle and North Tyneside Local Research Ethics Committees (reference 09/H0906/75).

5.3.2 Cryo-sectioning

Serial sections (10µm thickness) from transversely orientated muscle blocks were obtained as previously described 2.2.3 from chapter 2.

5.3.3 Immunofluorescence

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Fluorescence-based immunohistochemistry was carried out according to the protocol described in section 3.3.4.2 from chapter 3, with minor changes highlighted in bold:

Following fixation and permeabilisation, sections were blocked with **10% NGS** for **1h at RT** and then incubated with a primary antibody cocktail (Table 5.2). Subsequently, the sections were incubated with a secondary antibody cocktail for **2h at 4°C** (anti-rabbit IgG Alexa luor 405, **anti-mouse IgG2a Alexa fluor 488 nm**, **anti-mouse IgG2b Alexa fluor 546 nm**, and **anti-mouse IgG1 biotinylated antibody**, Table 5.2) following a final incubation with **streptavidin 647 for 2h at 4°C** (Table 5.2). Sections were washed in TBST and mounted in **Prolong Gold** (Life Technologies). A NPC was processed for each muscle sample used in the study.

Table 5.1 Patients cohort details

Patients	Sex	Age	Clinical information	Genetic defect	Biochemical findings	Mutation load
Patients v	with kno	own mut	ations affecting complex I and IV specifically			
P1	М	1y	Encephalopathy, seizures, hypotonia, developmental delay, central apnoeas	Compound heterozygous LRPPRC mutations (manuscript in preparation)	Isolated CIV deficiency in muscle	n.d.
P2ª	М	20y	Exercise-induced muscle weakness and lactic acidosis	m.4175G>A (p.Trp290*) <i>MTND1</i> mutation	Isolated CI deficiency in muscle	90%
Single, lar	ge-scale	e mtDNA	deletion			
Р3	F	22y	CPEO	Deletion size ~ 7.1 bp Breakpoints: 8543- 15672	n.d.	7.0%
P4	М	25y	Ptosis, CPEO, some fatigable weakness. Ultra peculiar patient	Deletion size ~ 4.7 kb Breakpoints: 10946-15598	n.d.	85%
Р5	F	25y	CPEO	Deletion size ~3.0 kb (breakpoints not mapped)	n.d.	n.d.
P6	F	26y	CPEO, mitochondrial myopathy	Deletion size ~ 4.9 kb Breakpoints: 10747-15598	n.d.	65%
P7	F	29y	CPEO, bilateral ptosis	Deletion size ~ 4.4 kb Breakpoints: 8929-13301	n.d.	53%
P8	F	36y	CPEO, myopathy	Deletion size ~ 5.0 kb (breakpoints not mapped)	n.d.	79%
Р9	М	39y	CPEO, myopathy	Deletion size ~ 4.1 kb Breakpoints: 11262- 15375	n.d.	72%
P10	F	39y	CPEO, diplopia	Deletion size ~ 7.5 kb Breakpoints: 7130-14628	n.d.	28%
P11	F	40y	CPEO, muscle atrophy, weakness, retinal pigmentary changes, increased resting lactate	Deletion size ~ 2.3 kb Breakpoints: 12113-14421	n.d.	87%
P12	F	41y	CPEO, myopathy	n.d.	n.d.	42%
P13	F	43y	CPEO, myopathy	Deletion size ~ 5.0 kb Breakpoints: 8482- 13460	n.d.	78%
P14	F	44y	CPEO, myopathy	Deletion size ~ 4.2 kb Breakpoints: 9486-13723	n.d.	81%

P15	F	47y	Ptosis, CPEO, jaw aching with chewing	Deletion size ~ 2.3 kb Breakpoints: 12112- 14412	n.d.	76%
P16	F	48y	CPEO, myopathy	Deletion size ~ 4.2 kb Breakpoints: 9498-13739	n.d.	39%
P17	F	61y	CPEO	n.d.	n.d.	n.d.
P18	Μ	61y	CPEO	Deletion size ~ 5.0 kb Breakpoints: 8482- 13460	n.d.	3%
P19	F	74y	CPEO	Deletion size ~ 4.9 kb Breakpoints: 7205-12090	n.d.	34%
Autosom	nal disor	ders of r	ntDNA maintenance			
P20 ^b	М	36y	Severe PEO, ptosis, proximal muscle weakness, facial weakness, scapulae winging, low BMI, hypogonadism and osteoporosis.	multiple mtDNA deletions; autosomal recessive p.(Thr144lle) and p.(Gly273Ser) <i>RRM2B</i> mutations	n.d.	n.d.
P21 ^c	F	43y	Severe PEO, asymmetrical ptosis, proximal and distal muscle weakness, ataxia, SNHL, facial weakness, low BMI, leukoencephalopathy and depression	multiple mtDNA deletions; autosomal recessive p.(Arg186Gly) and p.(Thr218Ile) <i>RRM2B</i> mutations	n.d.	n.d.
P22	М	47y	CPEO, bilateral ptosis	multiple mtDNA deletions; autosomal dominant p.(Asp104Gly) <i>SLC25A4</i> mutation	n.d.	n.d.
P23	F	54y	CPEO, ptosis, muscle weakness	autosomal dominant p.(Gln458His) PEO1 mutation	n.d.	n.d.
P24	Μ	60y	CPEO, bilateral ptosis	multiple mtDNA deletions; nuclear basis unresolved	n.d.	n.d.
Point mu	tations	in mitoc	hondrial-encoded tRNA leucine 1 (<i>MT-TL1</i>)			
P25	F	25y	Exercise intolerance, ptosis	m.3243A>G MT-TL1 mutation	n.d.	n.d.
P26	F	38y	Mild exercise intolerance, diabetes	m.3243A>G MT-TL1 mutation	n.d.	n.d.
P27	F	40y	Exercise intolerance, mild deafness	m.3243A>G MT-TL1 mutation	n.d.	n.d.
P28	F	42y	Epilepsy, bilateral sensorineural hearing loss, diabetes, gastrointestinal complications	m.3243A>G MT-TL1 mutation	Isolated CI deficiency in muscle	52%
P29	F	43y	Mild exercise intolerance, diabetes	m.3243A>G MT-TL1 mutation	n.d.	n.d.

P30	F	47y	Modest exercise intolerance	m.3243A>G MT-TL1 mutation	n.d.	n.d.
P31	М	53y	CPEO	m.3243A>G MT-TL1 mutation	n.d.	74%
P32	М	76y	Muscle pain and weakness	m.3243A>T <i>MT-TL1</i> mutation	n.d.	53%
Point mu	tations i	in other	mitochondrial-encoded tRNAs			
P33 ^d	F	13y	CPEO, ptosis, proximal myopathy	m.5690A>G MT-TN mutation	n.d	35%
P34	F	35y	Mild weakness	m.14709T>C <i>MT-TE</i> mutation	n.d.	n.d.
P35	F	58y	Myopathy	m.14709T>C <i>MT-TE</i> mutation	n.d.	87%
P36	F	18y	Seizures, deafness, retinopathy	Novel m.16021_16022detCT <i>MT-TP</i> mutation (unpublished)	n.d.	95%
P37	М	33у	Mitochondrial myopathy	m.10010T>C <i>MT-TG</i> mutation	CI, CIII and CIV deficiency in muscle	89%
P38	М	63y	Pure exercise intolerance, prominent exertional dyspnea	m.5543T>C <i>MT-TW</i> mutation	n.d.	n.d.
P39	М	4y	Developmental delay, progressive white matter loss on brain MRI; raised serum and CSF lactates	(unpublished) Novel m.7483C>A <i>MT-TS1</i> anticodon mutation	Severe CI and CIV defects	83%
Mutation	is in oth	er nucle	ar genes			
P40	М	18y	Myopathy, cardiomyopathy, cataracts	p.(Arg192Gly) and p.(Arg194His) GFER variants	Severe CI, CIII and CIV defects	n.d.
P41	М	26y	CPEO, ptosis, cataracts, cardiomyopathy	p.(Arg67Glyfs*83) and p.(Arg196Cys) GFER variants	Evidence of CI deficiency and mild CIV	n.d.
P42	М	27у	CPEO, ptosis, cataracts, cardiomyopathy	p.(Arg67Glyfs*83) and p.(Arg196Cys) GFER variants	Evidence of Cl deficiency and mild CIV defect	n.d.
P43	F	4y	Cardiomyopathy and developmental delay; normal serum lactate	Recessive c.297-2_297delinsTG, p.? and p.(Arg781His) <i>ELAC2</i> variants	Mild CI defect	n.d.
P44	F	4y	Developmental delay, deafness, brain MRI changes and elevated serum lactate levels	Novel p.(Arg136His) <i>PNTPT1</i> variant	Mild CIV deficiency (UCLH)	n.d.

P45	М	6у	IUGR, developmental delay, symmetrical high signal changes in caudate and cerebellar dentate nucleus	p.(Glu213Argfs*3) and p.(Arg190Gln) GFM2 variants	Mild CI and CIV defects	n.d.
Patients v	vith sus	pected	mitochondrial disease investigated for diagnostic purpose	15		
P46	F	4у	Myopathy, deafness	Not known	n.d.	n.d.
P47	F	18y	Myopathy; limb girdle weakness, raised CK	Not known	n.d.	n.d.
P48 M 24y Lipid storage myopathy, neuropathy, endocrinopathy, elevated lactates		Lipid storage myopathy, neuropathy, endocrinopathy, elevated lactates	Not known	Low CI, CII and CIV activities in muscle	n.d.	
P49	F	39y	Fatigue, GI problems, diabetes, sensory and autonomic neuropathy; serum lactates elevated	Not known	n.d.	n.d
P50	F	46y	Axonal neuropathy, liver and renal abnormalties, deafness, raised serum lactates	Not known	Low CI, CII, CIII and CIV activities in muscle	n.d.

Key: age = age when biopsied; y = years old; mtDNA = mitochondrial DNA; PEO = progressive external ophthalmoplegia; CPEO = chronic CPEO; BMI = body mass index; IUGR = intrauterine growth restriction; CK = creatine kinase; MRI = magnetic resonance imaging, GI = gastrointestinal, n.d. = not determined, ^{a,b,c,d} published cases: ^aGorman et al. (2015), ^bP19 and ^cP20 in Pitceathly *et al.* (2012), ^dP3 in Blakely *et al.* (2013).

Antibodies	Host	Dilution	Company (Product number)
Primary antibodies			
MTCOI (IgG2a)	Mouse	1:100	Abcam (Ab14705)
NDUFB8 (IgG1)	Mouse	1:100	Abcam (Ab110242)
Porin (IgG2b)	Mouse	1:100	Abcam (Ab14734)
Laminin α -1 (rabbit IgG polyclonal)	Rabbit	1:50	Sigma-Aldrich (L9393)
Secondary antibodies			
Anti-rabbit Alexa Fluor 405 nm	Goat	1:200	Life Technologies (A31556)
Anti-mouse IgG2a Alexa Fluor 488 nm	Goat	1:200	Life Technologies (A21131)
Anti-mouse IgG2b Alexa Fluor 546 nm	Goat	1:200	Life Technologies (A21143)
Anti-mouse IgG1 biotin	Goat	1:200	Jackson IR Lab (115-065-205)
Streptavidin Alexa Fluor 647 nm	Goat	1:100	Life Technologies (S31556)

Table 5.2 Primary and secondary antibodies used for immunofluorescence

Key: Jackson IR Lab = Jackson ImmunoResearch Laboratories

5.3.4 Imaging

Brightfield and fluorescent images were acquired at 20x magnification using Zeiss Microscope (see section 2.2.7 from chapter 2).

5.3.5 Densitometry measurements

Fluorescent images were analysed using IMARIS software (Bitplane). Segmentation was performed in order to measure the OD from individual muscle fibres (ODCOXI, ODNDUFB8 and ODporin or OD₄₈₈, OD₅₄₆ and OD₆₄₇), as described in section 4.4.1 from chapter 4.

5.3.6 Data analysis

Data analysis (background correction, creation of control group, and calculation of Z scores for porin, NDUFB8 and COX-I) and classification of fibres was performed as previously described in chapter 4 (see section 4.4.4.1).

5.4 Results

Quadruple immunofluorescence was performed in muscle sections from forty five patients with genetically-determined mitochondrial disease (P1-P45) in order to characterise their MRC phenotype. Muscle sections from five patients with suspected mitochondrial disease (P46-P50) were also investigated and included in this chapter to show the potential of this technique as a diagnostic tool.

5.4.1 MRC profile from patients with known mutations affecting complex I or IV activities specifically

Patient P1 (compound heterozygous LRPPRC mutations, Figure 5.1A) showed isolated COX-I deficiency with 99.8% of fibres down-regulated for COX-I and 99.6% of fibres with preserved NDUFB8 levels. Patient P2 (m.4175G>A MT-ND1 mutation, Figure 5.1B) showed isolated NDUFB8 deficiency with 88.1% of fibres with down-regulated NDUFB8 and 99.9% of fibres with preserved COX-I levels. These patients demonstrated different MRC profiles –an "I" shape in patient P1 (Figure 5.1A) and a "—" shape in P2 (Figure 5.1B). These results are in agreement with the biochemical defects assessed by spectrophotometric enzyme assay from the diagnostic laboratory (Table 5.3).



Figure 5.1 MRC profile linking complex I, complex IV and porin levels from patients showing isolated complex I or IV deficiency.

Patients with: (A) isolated complex IV deficiency (compound heterozygous LRPPRC mutations, P1, n = 1258), (B) isolated complex I deficiency (m.4175G>A MT-ND1 mutation, P2, n = 1062) were assessed in terms of COX-I, NDUFB8 and porin protein levels. Each dot represents the measurement from an individual muscle fibre, colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: light orange, high: orange and very high: red). Thin black dashed lines indicate the SD limits for the classification of fibres, colour lines next to x and y axis indicate the levels of NDUFB8 and COX-I respectively (beige: normal, light beige: int(+), light blue: int(-) and blue: deficient). Bold dashed lines indicate the mean expression level of normal fibres, which cluster in the same area as the control population's muscle fibres.
Patients	Complex I/CS	Complex II/CS	Complex III/CS	Complex IV/CS
P1 (<i>LRPPRC</i> mutations)	0.062	0.096	0.472	0.242
P2 (m.4175G>A <i>MT-ND1</i>)	0.001	0.070	0.634	1.216
Control (n=25)	0.104±0.036	0.145±0.047	0.554±0.345	1.124±0.511

Table 5.3 Activity of the respiratory chain complexes measured in muscle homogenates

Enzyme activities are expressed as nmols NADH oxidised.min⁻¹.unit citrate synthase (CS)⁻¹ for complex I, nmols DCPIP reduced.min⁻¹.unit citrate synthase⁻¹ for complex II and the apparent first-order rate constant (K).sec⁻¹.unit citrate synthase⁻¹ for complexes III and IV (x 10³). Control values are shown as mean±standard deviation. DCPIP = 2,6-dichlorophenolindophenol.

5.4.2 MRC profile from patients with single large-scale mtDNA deletions

All patients harbouring single, large-scale mtDNA deletions (P3-P19: Figure 5.2, Figure 5.3 and Figure 5.4, Table 5.4) showed decreased levels of both complex IV and complex I. However, 3 distinct MRC profiles were detected, and therefore patients were grouped accordingly. The first group of patients (8 out of 17 patients) showed both complexes I and IV equally and simultaneously down-regulated (Figure 5.2, Table 5.4) – the MRC graphs showed a " / " shape. This contrasted with the second group of patients (6 out of 17) which showed a " Γ " MRC shape – with an early and more pronounced involvement of complex I over complex IV (Figure 5.3, Table 5.4). The third group of patients (3 out of 17) presented a ")" MRC shape – with a slightly more pronounced and earlier involvement of complex IV deficiency (Figure 5.4, Table 5.4).





Plots show complex I and IV expression profile from (A) P3 (n = 1027), (B) P4 (n = 631), (C) P7 (n = 1228), (D) P8 (n = 322), (E) P13 (n = 853), (F) P14 (n = 609), (G) P16 (n = 942) and (H) P18 (n = 388). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).



Figure 5.3 MRC profile from patients harbouring single, large-scale mtDNA deletions that show a specific pattern of complex I more affected than complex IV.

Plots show complex I and IV expression profile from (A) P5 (n = 322), (B) P6 (n = 579), (C) P9 (n = 272), (D) P11 (n = 1118), (E) P15 (n = 606) and (F) P17 (n = 283). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).



Figure 5.4 MRC profile from patients harbouring single, large-scale mtDNA deletions that show a specific pattern of complex IV more affected than complex I.

Plots show complex I and IV expression profile from (A) P10 (n = 841), (B) P12 (n = 283) and (C) P19 (n = 779). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

		C	OX-I leve	ls		NDUFB8 levels					n
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR	
Group 1	: comple	exes I and	IV equal	ly down-i	regulated						
P3	92.6%	4.3%	2.7%	0.4%	7%	93.1%	4.9%	1.8%	0.3%	7%	1027
P4	53.7%	6.0%	3.8%	36.5%	46%	52.5%	6.0%	5.4%	36.1%	48%	631
P7	92.7%	4.4%	1.3%	1.6%	7%	96.3%	1.9%	1.1%	0.7%	4%	1228
P8	42.5%	5.4%	3.1%	49.0%	58%	39.2%	5.4%	4.1%	51.3%	61%	388
P13	51.3%	6.8%	4.9%	36.9%	49%	48.7%	4.6%	4.5%	42.3%	51%	853
P14	46.0%	16.3%	7.1%	30.7%	54%	48.3%	11.0%	7.9%	32.8%	52%	609
P16	81.7%	2.5%	1.5%	14.2%	18%	79.2%	3.6%	2.0%	15.2%	21%	942
P18	96.4%	1.2%	0.6%	1.8%	4%	94.4%	1.5%	1.5%	2.7%	6%	388
Group 2	2 : earlier	and more	e pronou	nced invo	lvement	of comple	ex I				
P5	92.9%	0.9%	0.9%	5.3%	7%	46.3%	7.8%	7.5%	38.5%	54%	322
P6	75.3%	6.4%	5.7%	12.6%	25%	38.2%	6.2%	3.5%	52.2%	62%	579
Р9	69.1%	7.4%	5.5%	18.0%	31%	31.6%	2.2%	5.5%	60.7%	68%	272
P11	62.3%	10.8%	6.2%	20.8%	38%	7.0%	4.7%	4.3%	84.0%	93%	1118
P15	81.4%	6.9%	3.1%	8.6%	19%	31.7%	6.8%	5.6%	55.9%	68%	606
P17	89.0%	1.4%	0.0%	9.5%	11%	83.4%	2.5%	2.1%	12.0%	17%	283
Group 3	: earlier	and more	e pronou	nced invo	lvement	of comple	ex IV				
P10	92.6%	2.3%	2.3%	2.9%	7%	95.6%	2.6%	1.7%	0.1%	4%	841
P12	80.2%	7.1%	2.5%	10.2%	20%	91.2%	1.4%	1.1%	6.4%	9%	283
P19	81.5%	2.1%	2.3%	14.1%	19%	88.4%	2.2%	2.2%	7.2%	12%	779

Table 5.4 Quantification of both COX-I and NDUFB8 deficiency in patients with single large-scale mtDNA deletion

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg, DS = deletion size.

To explore the underlying genetic causes of the distinct MRC profiles, the sizes of individual mtDNA deletions within each group were plotted together. Interestingly, the different groups of patients tended to harbour slightly different deletion sizes with bigger deletions in patients from group 1 and 3 (Figure 5.5), suggesting a possible correlation between the observed MRC profile and the nature of the genes removed.



Figure 5.5 Deletion size among the different groups of patients. Graph shows the size of the deletion from patients belonging to group 1, 2 and 3. Each dot corresponds to a patient. Dashed lines show the average deletion size in each group.

A deeper analysis was performed in order to determine if the number and type of genes removed could explain the different MRC profiles found between the groups. As most patients (7 patients from group 1, 4 patients from group 2, and 2 patients from group 3) had the deletion breakpoints mapped, the genes removed by the deletion were determined for each patient, using the mitomap website tool (Table 5.5).

Patients within the same group presented a similar pattern of genes removed. In group 1, individual mtDNA deletions consistently removed 5-6 tRNAs genes, 4-5 complex I genes and 1 complex IV gene, whereas complex III and V genes were only occasionally removed. In group 2, deletions tended to be smaller, removing 3-4 tRNAs genes and 3-4 complex I genes; one complex III gene was occasionally removed and complex IV genes were consistently preserved. In group 3, deletions tended to be larger, removing 8-5 tRNAs genes, 4-2 complex I genes, 2 complex V genes and all complex IV genes. Patient P4 was excluded from this analysis. This patient was studied in the past by two other PhD students, who detected some inconsistencies and requested a repeated sequencing of the mtDNA genome. This has not been performed to date.

			(Group	L				Gro	up 2		Gro	up 3
	Р3	P4	P7	P13	P14	P16	P18	P6	Р9	P11	P15	P10	P19
MT-COI												x	x
tRNA serine 1												x	x
NC nucleotides												x	x
tRNA aspartic acid												х	x
MT-COII												х	x
NC nucleotides												х	x
tRNA lysine												x	x
NC nucleotides												x	x
MT-ATP8	х			х			х					x	x
MT-ATP6	х		х	х			x					x	x
MT-COIII	х		х	х	х	х	х					x	x
tRNA glycine	х		х	х	х	x	x					x	x
MT-ND3	х		х	х	х	х	х					x	x
tRNA arginine	х		х	х	х	х	х					x	x
MT-ND4L	х		х	х	х	х	х	х				x	x
MT-ND4	х	Х	х	х	х	х	x	х	х	х	x	x	x
tRNA histidine	х	Х	х	х	х	х	x	х	х	х	x	x	
tRNA serine2	х	Х	х	х	х	х	x	х	х	х	x	x	
tRNA leucine2	х	Х	х	х	х	х	x	х	х	х	x	x	
MT-ND5	х	Х	х	х	х	х	x	х	х	х	x	x	
MT-ND6	х	Х						х	х	х	x	x	
tRNA glutamic acid	x	Х						х	х				
NC nucleotides	x	Х						х	х				
(MT-CYB)	x	Х						х	х				
	I						1	I				I	1
tRNAs removed	6	4	5	5	5	5	5	4	4	3	3	8	5
CI genes removed	5	3	4	4	4	4	4	4	3	3	3	5	2
CIV genes removed	1	-	1	1	1	1	1	-	-	-	-	3	3
CIII genes removed	1	1	-	-	-	-	-	1	1	-	-	-	-
CV genes removed	2	-	1	2	-	-	2	-	-	-	-	2	2
	I						I	I					I
Mutation load (%)	7.0	85	56	78	81	39	3.0	65	72	87	76	28	34
%DR (COX-I) fibres	7.4	46	7.3	49	54	18	3.6	25	31	38	19	7.4	19
%DR (NDUFB8) fibres	6.9	48	3.7	51	52	21	5.6	62	68	93	68	4.4	12

Table 5.5 Genes removed by the deletion in patients from group 1, 2 and 3.

NC nucleotides = non-coding nucleotides; %DR (COX-I) fibres = percentage of fibres with down-regulated levels of COX-I; %DR (NDUFB8) fibres = percentage of fibres with down-regulated levels of NDUFB8.

Moreover, patients lacking the same mitochondrial genes not only belonged to the same group but also the degree of deficiency was consistent with the mutation load. For example, in group 1, P14 showed a higher mutation load (81%) and higher levels of deficiency (54% fibres with down-regulated COX-I; 52% fibres with down-regulated NDUFB8) than P16 (39% mutation load; 18% fibres with down-regulated COX-I; 21% fibres with down-regulated NDUFB8). Similarly in group 2, P11 had a higher mutation load (87%) and higher levels of deficiency (38% fibres with down-regulated COX-I; 93% fibres with down-regulated NDUFB8) than P15 (76% mutation load; 19% fibres with down-regulated COX-I; 68% fibres with down-regulated NDUFB8).

To further explore if mutation load correlated with degree of deficiency, the percentage of fibres with down-regulated COX-I and NDUFB8 (i.e. the sum of int(+), int(-) and deficient fibres) was plotted against the mutation load for each group. In group 1, where both complex I and IV are equally down-regulated, both percentage of muscle fibres with down-regulated COX-I (y=1.5x+2, R²=0.9) and percentage of muscle fibres with down-regulated NDUFB8 (y=1.5x-0.5, R²=0.9) showed a strong correlation with the mutation load (Figure 5.6). In group 2, where complex I is more affected than complex IV, the percentage of muscle fibres with down-regulated NDUFB8 showed a strong correlation (y=0.6x+29, R²=0.9) with the mutation load whereas the percentage of muscle fibres with down-regulated COX-I only showed a weak correlation (R²=0.3). The linear regression of NDUFB8 deficiency, however, did not present a zero intercept. These results suggest that in group 2 the mutation load might be predicted by multiple independent variables of NDUFB8 and COX-I deficiency. Due to the low number of patients in group 3, it was not possible to investigate the correlation between mutation load and degree of deficiency in this particular group.



Figure 5.6 Correlation between mitochondrial respiratory chain deficiency and mutation load in patients from group 1.

Graphs plot the (A) percentage of fibres with down-regulated COX-I and (B) percentage of fibres with down-regulated NDUFB8 against the percentage of mutated mtDNA relative to wild-type mtDNA (mutation load) measured in muscle homogenates.

5.4.3 MRC profile from patients with mutations in nuclear genes involved in in mtDNA maintenance

Patients diagnosed with a nuclear-driven disorder leading to the accumulation of multiple mtDNA deletions in muscle (P20-P24, Figure 5.7, Table 5.6) showed a greater proportion of fibres deficient in complex I than complex IV. All five patients presented a similar MRC profile: a discrete population of fibres that showed decreased NDUFB8 expression without concomitant complex IV deficiency and a further, distinct population of muscle fibres, in which subunits of complexes I and IV were equally down-regulated (Figure 5.7). It is interesting to notice that patients with the SLC25A4 (P22) and PEO1 (P23) mutations showed an almost identical MRC profile - "Z" shape (Figure 5.7 C and D) -, which was slightly different from the MRC profile detected in patients with the RRM2B mutation (P20 and P21) and the unresolved nuclear defect (P24) - "7" shape (Figure 5.7 A, B and E). In both P22 and P23.



Figure 5.7 MRC profile from patients harbouring multiple mtDNA deletions in muscle.

Plots show complex I and IV expression profile from (A) P20 (n = 526), (B) P21 (n = 528), (C) P22 (n = 1071), (D) P23 (n = 1118) and (E) P24 (n = 2400). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

5.4.4 MRC profile from patients with mutations in mitochondrial-encoded tRNA leucine 1 (MT-TL1)

All patients with a mutation in *MT-TL1* gene (Figure 5.8, Table 5.7) – either a transition A>G (P25-31) or a transversion A>T (P32) - presented a similar profile of deficiency: a distinctive " Γ " shape. In all: complex I was much more severely affected than complex IV and was always affected prior to complex IV deficiency.



Figure 5.8 MRC profile from patients with the m.3243A>G *MT-TL1* mutation.

Plots show complex I and IV expression profile from (A) P25 (n = 1328), (B) P26 (n = 615), (C) P27 (n=499), (D) P28 (n = 1441), (E) P29 (n=212), (F) P30 (n = 741), (G) P31 (n = 918) and (H) P32 (n = 1042). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

	-						-		· ·		
		С	OX-I leve	ls				n			
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR	
P20	60.1%	3.4%	3.8%	32.7%	39.9%	49.2%	3.4%	4.4%	43.0%	50.8%	526
P21	71.6%	1.5%	2.3%	24.6%	28.4%	62.9%	3.8%	4.0%	29.4%	37.1%	528
P22	69.0%	10.2%	6.3%	14.5%	31.0%	62.0%	13.6%	14.4%	10.0%	38.0%	1071
P23	72.4%	5.1%	3.4%	19.1%	27.6%	70.6%	14.8%	11.7%	2.9%	29.4%	1118
P24	71.2%	5.2%	2.7%	21.0%	28.8%	54.4%	19.0%	4.1%	22.5%	45.6%	2400

Table 5.6 Quantification of COX-I and NDUFB8 deficiency in patients with multiple mtDNA deletions

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg.

Table 5.7 Quantification of	COX-I and NDUFB8	deficiency in patients	s with m.3243A>G	point mutation
in MT-TL1				

		C	OX-I leve	ls		NDUFB8 levels					n
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR	
P25	83.7%	2.5%	1.8%	12.0%	16.3%	73.7%	3.1%	1.5%	21.7%	26.3%	1328
P26	82.8%	2.0%	3.9%	11.4%	17.2%	74.5%	1.0%	1.8%	22.8%	25.5%	615
P27	69.1%	11.0%	9.4%	10.4%	30.9%	48.5%	3.4%	2.8%	45.3%	51.5%	499
P28	90.6%	4.4%	1.8%	3.2%	9.4%	64.6%	3.3%	2.4%	29.6%	35.4%	1441
P29	99.1%	0.0%	0.0%	0.9%	0.9%	97.2%	0.5%	0.9%	1.4%	2.8%	212
P30	89.1%	3.0%	1.3%	6.6%	10.9%	78.8%	7.4%	1.9%	11.9%	21.2%	741
P31	67.0%	16.7%	7.0%	9.4%	33%	30.6%	5.7%	11.8%	52.0%	69.4%	918
P32	89.6%	2.9%	1.8%	5.7%	10.4%	68.0%	3.0%	3.8%	25.1%	32.0%	1042

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg.

5.4.5 MRC profile from patients with mutations in other mitochondrial-encoded tRNAs

Patients with mutations in the mitochondrial-encoded asparagine and glutamic acid tRNAs: P33 (m.5690A>G *MT-TN* mutation, Figure 5.9A), P34 and P35 (m.14709T>C *MT-TE* mutation, Figure 5.9B and C) presented a pattern of deficiency similar to patients with m.3243A>G *MT-TL1* mutation. Complex I deficiency was higher than complex IV deficiency, however in these patients (P33, P34 and P35) there was more evidence of complex IV involvement. By contrast, patients with mutations in the mitochondrial-encoded proline, glycine, tryptophan and serine1 tRNAs: P36 (Novel *MT-TP* mutation, Figure 5.9D), P37 (m.10010T>G *MT-TG* mutation, Figure 5.9E), P38 (m.5543T>C *MT-TW* mutation, Figure 5.9F) and P39 (m.7483C>A *MT-TS1* mutation, Figure 5.9G) all demonstrated a severe deficiency of both complexes. These results are in agreement with the spectrophotometric enzyme assays carried out by the diagnostic laboratory (P37 and P39, Table 5.1). Interestingly, the patient harbouring the *MT-TW* mutation demonstrated a different pattern of complex IV deficiency. Here, complex IV appeared to plateau at a much higher level than reported for other mitochondrial-encoded tRNA point mutations.

The degree of pathogenicity of different tRNA mutations was also explored where such data was available. This was performed by comparing the observed degree of deficiency caused by different mutations, only when mutated mtDNA was present in a similar proportion relative to wild type mtDNA (Table 5.8). For instance, the m.14709T>E *MT-TE* mutation led to a milder COX-I and NDUFB8 deficiency (P35: 11%, 17%, respectively) than the m.10010T>C *MT-TG* mutation (P37: 88%, 89%, respectively) or m.7483C>A *MT-TS1* mutation (P39: 100%, 100%, respectively) when the mutation load was within the same range (83-89%). Also, m.7483C>A *MT-TS1* (P39) mutation led to a similarly severe degree of COX-I and NDUFB8 deficiency as seen in the novel *MT-TP* mutation (P36: 100%, 100%, respectively), though at a lower mutation load.



Figure 5.9. MRC profile from patients with mutations in other mitochondrial-encoded tRNAs.

Plots show complex I and IV expression profile from patients with (A) m.5690A>G MT-TN mutation (P33, n = 1512), (B-C) m.14709T>C MT-TE mutation (B: P34, n = 1782; C: P35, n = 1510), (D) novel MT-TP mutation (P36, n = 1012), (E) m.10010T>G MT-TG mutation (P37, n = 769), (F) m.5543T>C MT-TW mutation (P38, n = 447) and (G) m.7483C>A MT-TS1 mutation (P39, n = 447). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

		С	OX-I leve	ls			NC	UFB8 lev	vels		ML	n
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR		
P33	91.5%	1.1%	1.2%	6.2%	8.5%	81.2%	5.9%	3.6%	9.3%	18.8%	35%	1512
P34	74.1%	5.7%	4.8%	15.5%	25.9%	58.0%	3.3%	6.3%	32.5%	42%	n.a.	1782
P35	89.4%	3.2%	2.3%	5.2%	10.6%	83.8%	4.6%	5.4%	6.3%	16.7%	87%	1510
P36	0.5%	1.1%	2.5%	95.9%	99.5%	0.4%	0.5%	4.4%	94.7%	99.6%	95%	1012
P37	11.6%	0.8%	0.7%	87.0%	88.4%	13.5%	2.2%	1.3%	83.0%	86.5%	89%	769
P38	13.6%	5.1%	3.6%	77.6%	86.4%	14.3%	15.2%	26.4%	44.1%	85.7%	n.a.	447
P39	0.1%	0.0%	0.4%	99.6%	99.9%	0.1%	0.0%	0.4%	99.6%	100%	83%	1894

Table 5.8 Quantification of COX-I and NDUFB8 deficiency in patients with other point mutation in mitochondrial-encoded tRNAs.

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg, ML= mutation load, n.a. = not assessed.

5.4.6 MRC profile from patients with mutations in other nuclear genes

Patients with mutations in *GFER* gene (P40, P41 and P42) showed down-regulation of complexes I and IV (Figure 5.10A, B and C) (**Table** 5.9). This was consistent with the biochemical findings reported by the diagnostic laboratory (Table 5.1). Interestingly, P41 presented an additional population of fibres, showing complex I more affected than complex IV, which was not evident in patients P40 and P42. The patient with the novel *PNPT1* mutation (P44) showed both complexes affected, with a higher involvement of complex IV (Figure 5.10E, **Table** 5.9). Patients with *ELAC2* (P43) and *GFM2* (P45) mutations showed isolated and mild complex I and complex IV deficiency, respectively (Figure 5.10D and F, **Table** 5.9). These results were, however, not consistent with the spectrophotometric enzyme analysis, which reported the contrasting findings (Table 5.1).



Figure 5.10 MRC profile from patients with different nuclear genetic defects.

Plots show complex I and IV expression profile from patients with GEFR mutations: (A) P40 (n = 524), (B) P41 (n = 627), (C) P42 (n=685), and other nuclear DNA mutations including (D) ELAC2 mutations in P43 (n = 2191), (E) a novel PNPT1 mutation in P44 (n=797) and (F) GFM2 mutations in P45 (n = 1784). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

		С	OX-I leve	ls		NDUFB8 levels					n
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR	
P40	40.6%	24.0%	19.3%	16.0%	59%	13.4%	28.4%	33.8%	24.4%	87%	524
P41	28.1%	23.6%	24.2%	24.1%	71%	12.0%	20.4%	32.2%	35.4%	88%	627
P42	54.7%	26.0%	13.0%	6.3%	45%	40.7%	26.6%	19.1%	13.6%	59%	685
P43	99.9%	0.1%	0.0%	0.0%	0.1%	37.8%	52.3%	9.5%	0.4%	62%	2191
P44	19.8%	22.7%	15.6%	41.9%	80%	62.6%	10.8%	13.2%	13.4%	37%	797
P45	0.1%	11.9%	69.8%	18.2%	99.9%	99.8%	0.0%	0.1%	0.1%	0.2%	1784

Table 5.9 Quantification of both COX-I and NDUFB8 deficiency in patients with other nuclear genetic defects

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg.

5.4.7 MRC profile from patients with suspected mitochondrial disease investigated for diagnostic purposes

All diagnostic patients presented combined OXPHOS defects, with both complex I and IV affected (Figure 5.11, Table 5.10), confirming mitochondrial disease diagnosis. In patients P46, P49 and P50, the decline in both complexes was simultaneous (Figure 5.11A, D and E). This pattern contrasted with those found in patients P47 and P48. Patient P47 showed a polarised pattern of deficiency with a complete absence of complex I in all muscle fibres, and some evidence of complex IV deficiency (Figure 5.11B). Patient P48 presented a wider range of deficiency, from fibres with more evidence of complex I or complex IV deficiency to fibres with both complexes I to IV equally down-regulated (Figure 5.11C).



Figure 5.11 MRC expression profile from patients with suspected mitochondrial disease. Plots show complex I and IV expression profile from (A) P46 (n = 2918), (B) P47 (n = 417), (C) P48 (n = 811), (D) P49 (n = 1126) and (E) P50 (n = 1140). Each dot represents an individual muscle fibre colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: beige, high: orange and very high: red).

		С	OX-I leve	ls				n			
	Pos	Int(+)	Int(-)	Neg	%DR	Pos	Int(+)	Int(-)	Neg	%DR	
P46	75.0%	4.0%	2.7%	18.2%	25%	75.0%	4.4%	3.0%	17.6%	25%	2918
P47	70.7%	28.8%	0.5%	0.0%	29%	0.0%	0.0%	0.2%	99.8%	100%	417
P48	15.7%	35.1%	23.6%	25.6%	84%	1.1%	5.1%	12.7%	81.1%	98.9	811
P49	74.5%	15.5%	7.9%	2.1%	26%	83.5%	10.8%	4.2%	1.5%	17%	1126
P50	63.6%	22.9%	9.0%	4.5%	36%	79.5%	13.4%	6.2%	0.9%	21%	1140

Table 5.10 Quantification of both COX-I and NDUFB8 deficiency in patients with suspected mitochondrial disease

Key: n = number of fibres quantified, %DR = percentage of muscle fibres with down-regulated protein levels, either int(+), int(-) or neg.

5.5 Discussion

This chapter shows that different mitochondrial genotypes – both mtDNA and nuclear DNA driven - exhibit distinctive signatures of mitochondrial dysfunction distinguishable by their MRC profiles. This highlights the great potential for this technique not only as a diagnostic tool but also as a means to study mechanisms of disease.

5.5.1 Patients with isolated complex deficiencies

The patient harbouring compound heterozygous *LRPPRC* mutations (P1) showed isolated COX deficiency, in agreement with the spectrophotometric measurements performed in muscle homogenates (Table 5.3). The nuclear-encoded LRPPRC protein is involved in translation and/or stability of COX mRNA transcripts derived from mtDNA and is required for the assembly of complex IV (Xu *et al.*, 2004). It is therefore not surprising that mutations in *LRPPRC* gene result in a wide-spread deficiency of complex IV (Merante *et al.*, 1993, Mootha *et al.*, 2003, Debray *et al.*, 2011).

The patient carrying the m.4175G>A *MT-ND1* truncating mutation (P2) (Gorman *et al.*, 2015a) showed an isolated complex I deficiency, consistent with the biochemical studies in muscle (Table 5.3). *MT-ND1* is a mitochondrial gene that encodes the most conserved structural subunit of complex I (Fearnley and Walker, 1992, Kirby *et al.*, 2004). Several other pathogenic mutations have been reported in this gene all associated with a decline in fully assembled complex I and ultimately leading to a complex I-specific biochemical defect (Howell *et al.*, 1991, Huoponen *et al.*, 1991, Kirby *et al.*, 2004, Swalwell *et al.*, 2011), mirroring what we observed in our patient.

5.5.2 Patients with single large-scale mtDNA deletion

Single large-scale deletions of mtDNA (Holt *et al.*, 1988) are a common cause of adult and paediatric mtDNA related mitochondrial disease. They are considered sporadic events that arise during replication of mitochondrial genome. Although the location and size of the deletion may vary, the majority of patients harbour the common 4977bp deletion (Schon *et al.*, 1989). A variable number of genes encoding subunits of complex I, III, IV and V, rRNAs or tRNAs are removed by the deletion leading to multiple OXPHOS defects (Pitceathly *et al.*, 2012a).

All patients with single, large-scale mtDNA deletions assessed in this study (P3-P19) showed a combined complex I and IV deficiency. This is consistent with previous findings (Schröder *et al.*, 2000) and with the removal of several mitochondrial-encoded structural subunits and mt-tRNAs by the deletion. However, these patients showed differences in the MRC profiles suggesting differential rates of COX-I decline in comparison to NDUFB8. Patients were therefore classified in three groups according to the MRC profile. The majority of patients (group 1) presented a simultaneous loss of both complex I and complex IV. By contrast, in some patients (group 2) complex I deficiency preceded complex IV deficiency, whereas in others (group 3) complex IV deficiency preceded complex I deficiency. These results are in agreement with previous reports of either combined complex I and complex IV deficiency, or isolated complex I deficiency (Holt *et al.*, 1988, Yamamoto *et al.*, 1991, Oldfors *et al.*, 1992).

The different MRC defects reflected a relationship between the site of deletion and the type of genes removed, as previously reported (Grady *et al.*, 2014). In all three groups, tRNA genes and complex I genes were removed. However, the number of deleted complex IV differed between patients: in group 1 only one complex IV gene was removed, in group 2 all complex IV genes were preserved and in group 3 all complex IV genes were deleted.

The MRC defect also correlated with the mutation load. In group 1, where complexes I and IV are equally affected, the percentage of fibres with down-regulated COX-I and NDUFB8 strongly correlated with the mutation load. In group 2, the correlation was stronger for NDUFB8 than for COX-I deficiency, consistent with the more severe defect of complex I in these patients. Previous studies have provided contradictory results demonstrating either significant (Goto *et al.*, 1990, Fassati *et al.*, 1994, Schröder *et al.*, 2000, Gellerich *et al.*, 2002), weak (Yamamoto *et al.*, 1991, Laforet *et al.*, 1995) or no (Kiyomoto *et al.*, 1997) correlation between the percentage of COX deficient fibres and the degree of mtDNA heteroplasmy. One explanation for these inconsistencies could be the assessment of all single deletions patients together. In such studies it was assumed that single deletion patients were a homogenous group. The

present study has, however, shown the opposite and stressed the importance of analysing patients separately, according to the MRC profile. Another possible explanation could be the use of COX/SDH histochemistry. This technique does not allow accurate quantification of COX deficiency, and therefore might not have revealed the true extent of COX deficiency in such studies. Moreover the present study highlighted the relevance of taking into consideration complex I deficiency, which is the major predictor of the mutation load in a subset of patients. Patients with single large-scale deletions present a wide spectrum of clinical phenotypes and it has been shown that the percentage and location of the deletion (Lopez-Gallardo *et al.*, 2009) or the size of the deletion, with regards to the number of tRNA genes removed, (Yamashita *et al.*, 2008) are predictive of the syndrome. It would be interesting to explore a larger cohort of patients and assess whether the pattern of complex I and complex IV deficiency could also be indicative of distinct clinical features.

5.5.3 Patients with mutations in nuclear genes involved in mtDNA maintenance

Several nuclear-encoded proteins are directly or indirectly responsible for mtDNA maintenance and regulation of mtDNA copy number. The helicase Twinkle, encoded by *PEO1*, (Spelbrink *et al.*, 2001) is an essential mtDNA replisome component and an example of a protein directly involved in mtDNA replication (Longley *et al.*, 2010). Autosomal dominant and recessive mutations have been reported in this gene leading to multiple mtDNA deletions (Spelbrink *et al.*, 2001) and depletion (Sarzi *et al.*, 2007), respectively. The p53R2 subunit, encoded by *RRM2B* (Tanaka *et al.*, 2000), and ANT1, encoded by *SLC25A4*, (Neckelmann *et al.*, 1987) supply dNTPs to mitochondria and are examples of proteins indirectly involved in mtDNA replication. Mutations in these genes were reported to cause a shortage or imbalance of the dNTP pool thereby interfering with mtDNA replication. Consequently, they can lead to mtDNA depletion (Bourdon *et al.*, 2007, Bornstein *et al.*, 2008) and generation of multiple deletions (Kaukonen *et al.*, 2000, Tyynismaa *et al.*, 2009).

Patients with mutations in nuclear genes which regulate maintenance of mtDNA demonstrated a similar pattern of mitochondrial deficiency, regardless of the underlying molecular genetic defect. Two populations of muscle fibres were detected: one showed a simultaneous and equal loss of both complexes I and IV, whereas the other presented complex I predominantly affected. The presence of fibres with complex I deficiency is consistent with reports of the loss of structural genes of complex I in the deleted molecules (Pitceathly *et al.*, 2012b). All patients shared a similar pattern of deficiency, however some slight differences were observed. Patients with *SLC25A4* (P22) and *PEO1* (P23) mutations showed a "Z" shape MRC profile, whereas patients with *RRM2B* mutations (P20 and P21) demonstrated a "7" shape MRC profile. These findings were surprising because similarities would be expected between *SLC25A4* and *RRM2B* mutations as both encoded-proteins are ultimately involved in the same pathway controlling the mitochondrial dNTP pool. The observed resemblance could reflect the different mode of inheritance: *SLC25A4* and *PEO1* mutations are dominantly inherited whereas *RRM2B* mutations are recessively inherited.

Also, though speculative, the pattern of deficiency in *SLC25A4* and *PEO1* mutations seemed milder than that caused by *RRM2B* mutations. In the former, both COX-I and NDUFB8 Z scores plateaued at a higher level than in the latter mutations. This is in agreement with previous studies that demonstrated that autosomal recessive mutations such as *RRM2B* lead to more severe clinical phenotypes (Pitceathly *et al.*, 2011, Pitceathly *et al.*, 2012b).

5.5.4 Mutations in mitochondrial-encoded tRNAs

Pathogenic mutations in mitochondrial-encoded tRNAs are associated with multiple OXPHOS defects that can arise from multiple deleterious effects such as defective transcription termination, defective tRNA maturation, impaired tRNA post-transcription modifications, conformational alteration of tRNA, reduced tRNA stability, decreased amynoacylation, reduced interaction with the mitoribosome and disturbed codon reading. These effects lead to an overall decline in the rate of mitochondrial protein synthesis, usually affecting multiple OXPHOS complexes (Abbott *et al.*, 2014).

5.5.4.1 Mitochondrial-encoded tRNA leucine 1 (*MT-TL1*) point mutations

The m.3243A>G mutation in *MT-TL1* gene (Goto *et al.*, 1990) is one of the most common mutations, affecting the structure and function of the tRNA^{Leu(UUR)}. This mutation was shown to induce decreased aminoacylation rate, reduced tRNA stability, and reduced binding capacity of the mutant tRNA to mitoribosomes, underlying the decreased mitochondrial protein synthesis (Chomyn *et al.*, 2000, Yasukawa *et al.*, 2000, Sasarman *et al.*, 2008). Also the loss of the post-transcriptional taurine modification (at the first (wobble) position of the anticodon) which is required for accurate translation has been reported (Yasukawa *et al.*, 2000). This loss was

shown to lead to a severe decoding defect of leucine at UUG codons and misincorporation of leucine at non-cognate codons (Jacobs, 2003, Kirino *et al.*, 2004).

Patients with the common m.3243A>G MT-TL1 (P25-P31), were shown to have combined complex I and IV deficiencies, as previously reported (Koga et al., 2000). Interestingly, the pattern of mitochondrial deficiency - a " Γ " shape - suggests that complex IV deficiency was only acquired after complex I deficiency was established, and that the deficiency is smoothly graduated from normal to deficient levels of both complexes. These results are in agreement with some observations of a primarily complex I defect caused by m.3243A>G MT-TL1 mutation (Morgan-Hughes et al., 1995, James et al., 1996, Fornuskova et al., 2008). ND6 mRNA presents an unusually high density of UUG codons when compared with the remaining mitochondrial-encoded proteins and therefore is more prone to be affected by the decoding defect (Kirino et al., 2004, Sasarman et al., 2008). This explains the marked reduction of ND6 translation reported in several studies (Kirino et al., 2004, Sasarman et al., 2008). Although most patients present with maternally inherited deafness and diabetes (MIDD), the m.3243A>G MT-TL1 mutation is associated with a variety of clinical phenotypes ranging from the severe MELAS syndrome to milder single tissue presentations) (Nesbitt et al., 2013). It would be interesting to explore if the MRC phenotype detected in muscle is able to provide greater insight into the underlying mechanisms causing this variability.

The patient carrying a transversion mutation at this site - m.3243A>T *MT-TL1* - (P32) showed the same pattern of mitochondrial deficiency seen with m.3243A>G *MT-TL1*. This observation is interesting because the transversion mutation has been reported to induce a higher deleterious effect on translation (Shaag *et al.*, 1997), severely reducing aminoacylation efficiency to approximately 1% wild-type level, whereas the common transverse mutation has a more moderate negative effect (Sohm *et al.*, 2003).

5.5.4.2 Other rarer mitochondrial-encoded tRNAs point mutations

Unlike the m.3243A>G mutation, other mutations in mt-tRNAs are relatively rare and have only been described in single or few families to date (Mitomap website).

The mutations studied in *MT-TN* (P33) and *MT-TE* (P34 and P35) genes, encoding the mitochondrial aspargine and glutamic acid tRNAs respectively, showed the same pattern of OXPHOS deficiency as seen in the *MT-TL1* mutations, namely complex I deficiency preceding complex IV deficiency. The m.5690A>G *MT-TN* variant (occurring within the anticodon stem)

has only been reported in one patient, and was suggested to disrupt the tRNA secondary structure (Blakely *et al.*, 2013). The m.14709T>C *MT-TE* variant (occurring within the anticodon loop) has been previously investigated using biochemical and immunohistochemical studies in muscle which revealed a reduction of complex I and IV activities and levels (Hanna *et al.*, 1995, Hao *et al.*, 1995, Mancuso *et al.*, 2005). Our investigations resulted in similar findings for patients P34 and P35, who had the same genetic defect. Additionally, measurements of activity of mitochondrial respiratory chain complexes in fibroblasts from six patients with this defect have shown a severe reduction in complex I activity and a mild decrease in complex IV activity (Perucca-Lostanlen *et al.*, 2002, McFarland *et al.*, 2004). This further supports the finding that complex I deficiency precedes complex IV deficiency. The proposed molecular mechanisms underlying this translation defect include a decoding defect of glutamate at the GAA/GAG codons and/or reduced steady state levels of amynoacylated tRNA^{Glu} (Perucca-Lostanlen *et al.*, 2002), though it has been suggested that mutated tRNA still have some residual function.

The mutations studied in *MT-TG* (P37) and *MT-TP* (P36) genes, encoding the mitochondrial glycine and proline tRNAs respectively, showed a simultaneous and severe decline in both complex I and complex IV protein levels. The m.10010T>C *MT-TG* variant is located in the D stem, and biochemical studies in muscle also showed a decrease in both complexes I and IV activities (Bidooki *et al.*, 1997, Nishigaki *et al.*, 2002). The *MT-TP* variant with a 2 base pair deletion reported, is located in the middle of the acceptor stem and has never been identified to date. Point mutations in this region have been detected in other mt-tRNA, and were suggested to impair both tRNA secondary structure and binding of amynoacil-tRNA synthases (Blakely *et al.*, 2013). A similar effect could explain the severe complex I and IV defects seen in patient P36. The patient carrying the novel m.7483C>A *MT-TS1* mutation (P39), also exhibited a severe complex I and IV deficiency, with all fibres lacking complex I and IV. However, this patient is unusual because the variant he harbours is located in one of the anticodon bases. Mutations occurring at this site tend to destroy the tRNA function and are likely to be incompatible with embryogenesis and development (Suzuki *et al.*, 2011).

A third distinctive pattern was seen in the *MT-TW* mutation (P38), affecting the mitochondrial tryptophan tRNA. A severe deficiency was observed in both complex I and complex IV, however the deficiency in complex IV appeared to plateau at a much higher level than observed in other mt-tRNA point mutations. Also, fibres with complex IV deficiency showed a wide and gradual range of complex I deficiency. These results suggest that the relative rate of decline in complex I and complex IV protein levels varies considerably from fibre to fibre for

this mutation, an effect not observed in other mt-tRNA point mutations. The 5543T>C *MT-TW* variant (located in the anticodon loop) has only been reported in few patients (Anitori *et al.*, 2005, Alston *et al.*, 2011), and biochemical studies in muscle reported severe deficiencies of complexes I and IV (Alston *et al.*, 2011), similar to what we detected in muscle samples from patient P38.

For all mt-tRNAs mutations, future investigations are required to correlate the observed MRC profile with heteroplasmy levels. Furthermore, it would be interesting to explore associations between the MRC profile detected for each of these mt-tRNAs mutations and the relative amounts of the composite amino acids within each of the mitochondrial-encoded complex I and IV proteins.

5.5.5 Mutations in other nuclear genes

Nuclear genetic defects can cause mitochondrial disease not only by impairing mtDNA maintenance as described previously, but also by affecting other mechanisms such as mitochondrial translation, protein and RNAs translocation across mitochondrial inner and outer membranes or proteins promoting mitochondrial biogenesis (Ylikallio and Suomalainen, 2012).

The mitochondrial Disulphide Relay System Protein (GFER), is located in the mitochondrial intermembrane space and is involved in the translocation of several proteins relevant to COX biogenesis and TIM chaperones across the mitochondrial outer membrane (Di Fonzo *et al.*, 2009, Fischer and Riemer, 2013). Three patients with *GFER* mutations (P40, P41 and P42) were assessed, and all showed both complexes equally affected. However, in patient P41, a higher range of complex I and IV deficiency was observed. Mutations in *GFER* gene have been previously reported (Di Fonzo *et al.*, 2009, Calderwood *et al.*, 2015). The lowest stability of the mutated protein was shown to affect the protein content of the mitochondrial intermembrane space, including some proteins involved in COX biogenesis. This was shown to ultimately lead to a either moderate complex IV deficiency in muscle and fibroblast, or combined complex I and IV deficiency in patients P40, P41 and P42, and it is possible that *GFER* mutations affect the import of components or assembly factors essential for not only complex IV, but also complex I.

The polynucleotide phosphorylase (PNPT1 or PNPase) is a component of the import machinery also localised in the mitochondrial intermembrane space. It is involved in the transport of nuclear-encoded RNAs into mitochondria (5S rRNA, RNase P RNA, MRP RNA and other tRNAs) which are required for proper replication and transcription of mtDNA (Wang *et al.*, 2010, Mimaki *et al.*, 2012). The patient with *PNPT1* mutations assessed here (P44) showed a combined complex I and IV deficiency, with complex IV more affected. Two other studies reported mutations in this gene. These showed a disturbed capacity of the mutant protein to assemble into higher-order trimeric PNPase complexes, impairing mitochondrial RNA import (Vedrenne *et al.*, 2012, von Ameln *et al.*, 2012). A complex IV deficiency has been reported in liver homogenate, and a translational defect has been detected in fibroblasts due to a decreased import of 5S rRNA to mitochondrial (Vedrenne *et al.*, 2012). This study concluded similar findings for patient P44, who clearly presents a translation defect.

ELAC2 gene encodes mitochondrial tRNase Z, an endonuclease that participates in the maturation of the long polycistronic transcripts by cleaving the 3'end of tRNAs. The presence and stability of tRNase Z is crucial for the efficient synthesis of mitochondrial-encoded proteins (Rossmanith and Karwan, 1998, Brzezniak et al., 2011). The patient with ELAC2 mutations (P43) assessed in the present study showed an isolated complex I deficiency, consistent with previous reports of reduced complex I activities in muscle homogenates (Haack et al., 2013). However, the pattern of deficiency found in this patient was slightly different from the one found in the patient carrying the MT-ND1 mutation (P2) who also showed an isolated complex I deficiency. In P2, a subset of fibres still showed normal levels of complex I, consistent with a threshold effect theory. It is possible to speculate that in the remaining normal fibres, the levels of mutated mtDNA had not reached the threshold to express a biochemical defect. By contrast, in the MRC graph from ELAC2 patient P43 the cloud of muscle fibres were all leftshifted, suggesting that all muscle fibres were equally affected by the mutation. The impaired activity of the mutated RNase Z was shown to lead to a 400-fold increase in levels of 3'end unprocessed mt-tRNAs precursors (Brzezniak et al., 2011, Haack et al., 2013) and ultimately to a reduced synthesis of mtDNA-encoded OXPHOS subunits (Haack et al., 2013). The reason why this mutation affects specifically complex I is still not known. It is, however, possible to speculate that the 3'end processing defects could predominantly affect complex I since this complex has a higher number of mitochondrial-encoded subunits and therefore more complex I mRNA transcripts are required to be translated.

The mitochondrial elongation factor G2 (mtEFG2, encoded by *GFM2* gene) has a central role in ribosome recycling (Tsuboi *et al.*, 2009). Surprisingly, the patient with this genetic defect (P45)

only showed a mild complex IV deficiency, with no evidence of a complex I defect. Also, in the MRC graph from this patient, the cloud of muscle fibres were all down-shifted suggesting that all fibres were equally affected by the mutation, similar to what was observed in patient P43 with the *ELAC2* mutations. These results were not in agreement with the biochemical studies which detected an additional mild complex I deficiency. Two possible causes could underlie this discrepancy. Firstly, the mild complex I deficiency could be real and was not revealed by this assay. Alternatively, the results from this assay are real and some error occurred throughout the biochemical experiment. Since this mutation does not occur in a structural gene, the first hypothesis is less probable. It is important to mention that only mutations occurring in structural genes and affecting solely the activity of the complexes might not be detected by this assay. These are uncommon mutations that do not disturb complex assembly.

5.5.6 Diagnostic patients

Patients with suspected mitochondrial disease are still undergoing investigation and have not been genetically characterised. However, it is possible to narrow-down the underlying cause based on the MRC profiles obtained in this study.

Since all patients showed multiple OXPHOS defects, mutations in mitochondrial and nuclearencoded structural genes or assembly factors of OXPHOS complexes are excluded. Furthermore, point mutations in leucine, aspargine and glutamate mitochondrial-encoded tRNAs (characterised by left-rotated "L" shape MRC profile) or multiple mtDNA deletions (characterized by "Z" or "7" shape MRC profile) are also excluded.

This leaves the diagnosis of single large-scale mtDNA deletion, point mutations in tRNAs leading to equal loss of both complexes, or point mutations in nuclear-genes involved in pathways such as transcription, translation or import of proteins/rRNAs into mitochondria. For patient P46, it is possible to speculate a diagnosis of single large-scale mtDNA deletion, since this patient presents a MRC profile very similar to a group of patients with this defect.

5.6 Conclusions

This work has provided important insights into the nature of the defects in patients with mitochondrial disease. It has shown how this new assay has important implications in terms of diagnosis of mitochondrial disease (Figure 5.12). It allows detection of complex I deficiencies at

the single fibre level. Also, since different genotypes exhibit different MRC profiles, this tool can support the current diagnostic setting. Figure 5.12 only references MRC profiles from common genetic defects but illustrates how valuable this assay is in helping screening the underlying genetic cause based on the MRC profile of diagnostic patients.



Figure 5.12 MRC profiles from common primary and secondary mitochondrial genetic defects.

Chapter 6. Therapeutic effect of a six months endurance training program in patients with mitochondrial myopathy

6.1 Introduction

Adult mitochondrial myopathies are frequently caused by heteroplasmic mtDNA mutations resulting from either single large-scale mtDNA deletions or point mutations in either tRNAs or protein coding genes (Schaefer *et al.*, 2008, Gorman *et al.*, 2015). These genetic defects in mtDNA often impair oxidative phosphorylation and, subsequently, cellular ATP production in muscle (Mariotti *et al.*, 1994, Pallotti *et al.*, 2004). As a consequence, patients usually present with exercise intolerance, whereby trivial levels of activity which are easily tolerated by healthy individuals result in muscle weakness, fatigue, lactic acidosis and tachycardia (Haller *et al.*, 1978, Haller *et al.*, 1989).

The spectrum of exercise intolerance in mitochondrial myopathies varies from mild to debilitating (Taivassalo *et al.*, 2003). Exercise intolerance is associated with a decreased capacity of working muscle to extract available oxygen from arterial blood. By contrast, cardiovascular capacity to deliver oxygen is usually preserved and comparable to healthy subjects (in the absence of significant cardiac muscle involvement). The degree of exercise intolerance exhibited by patients was shown to correlate directly with the severity of impaired muscle oxygen extraction, which itself is proportional to the mutation load (Taivassalo *et al.*, 2003).

Exercise intolerance promotes a sedentary lifestyle, which leads to further downregulation of muscle oxidative phosphorylation, enhancing muscle disability. Given the lack of effective therapeutic treatments, exercise training has been explored as a means to improve mitochondrial respiratory chain function and ultimately improve quality of life (Taivassalo and Haller, 2005).

6.1.1 Endurance training in healthy population

Regular and sustained endurance training taken up by a healthy individual was shown to induce adaptation of the cardiovascular and skeletal muscle systems in order to increase exercise capacity and utilisation of oxygen by muscle. Cardiovascular adaptations enhance the delivery of oxygen and nutrients to working muscles by increasing cardiac output (total volume of blood pumped by the left ventricle per minute), respiratory rate (the number of inspiration/expiration cycles per minute), and muscle vasodilatation (relaxation of blood vessels) (Saltin, 1986, Cox, 1991). Muscle vasodilatation is crucial to redistribute and increase blood flow to areas of high energy demand, such as active muscles.

Increased capillary density and mitochondrial oxidative capacity in skeletal muscle allow for more efficient extraction of oxygen from blood in this tissue (Holloszy, 1967, Holloszy and Booth, 1976). The increase in mitochondrial oxidative capacity is generated through mitochondrial biogenesis. Several intracellular signals (calcium, AMP) produced during the acute phase of exercise cause the transient activation of PC1- α (Pilegaard et al., 2003). This in turn activates transcription of both mitochondrial and nuclear DNA leading to an increased synthesis of mitochondrial proteins, and therefore, to higher overall mitochondrial activity and content (Holloszy, 1967). As a result, the metabolic efficiency of skeletal muscle is improved due to an increase in fatty acid oxidation and reduced carbohydrate oxidation, or glycolysis (Henriksson, 1992).

Conversely, the cessation of exercise (known as deconditioning or detraining), has been shown to cause a reduction in cardiovascular fitness (as measured by a fall in cardiac output due to reduced blood volume), and a decrease in capillary density, in mitochondrial activity and in mitochondrial content (Coyle et al., 1984, Coyle et al., 1985, Saltin, 1986, Neufer, 1989). The consequent reverse in training-induced adaptations leads to a higher carbohydrate oxidation and glycolysis, and therefore to muscle fatigue and a decline in work capacity (Neufer, 1989).

6.1.2 Endurance training in mitochondrial myopathies

In the past decade several studies have consistently reported significant beneficial effects of endurance training in patients with mitochondrial myopathy. Early studies performed by Taivassalo *et al.* in 1996 and 1998 showed an increase in aerobic capacity, (by 71%, 30%, respectively) and muscle oxidative capacity (by 2.8 fold, 60%, respectively) in patients with mitochondrial myopathy following a 14 and 8 week training program (3-4 times a week, 20-30min per session). Also, blood lactate concentrations decreased (by 50%, 30%, respectively) and both fatigue and exercise performance improved (Taivassalo et al., 1996, Taivassalo et al., 1998). These results demonstrated that this form of exercise was well tolerated and could benefit patients presenting with this condition.

Subsequent studies using cycling and treadmill exercise regimens further confirmed these findings (Taivassalo et al., 1999, Siciliano et al., 2000, Cejudo et al., 2005, Voet et al., 2010). Both Cejudo *et al.* and Voet *et al.* went further, and designed a combined endurance and resistance exercise programme which resulted in a significant increase in both endurance and muscle strength of participants.

In 2001, Taivassalo *et al.* further explored the mechanisms underlying the improved physiological responses (Taivassalo et al., 2001). They showed an improved ability of muscle to extract available oxygen (by 20%) and confirmed higher muscle oxidative capacity in patients with mitochondrial myopathy following 14 weeks of aerobic training (cycling 3-4 times a week, 30-40 min per session). The rise in cytrate synthase (CS – enzyme of the TCA cycle commonly used as a mitochondrial mass marker) and SDH activity by 50% and 40%, respectively, indicated an increase in mitochondrial proliferation. Also, biochemical studies showed an increase in the activity of the respiratory chain complexes affected by the mutation, with the exception of two patients with complex III defects. Unexpectedly, analysis of mitochondrial mutation load revealed a significant increase in the percentage of mutant relative to wild-type mtDNA after completion of the training program in 9 out of the 12 patients assessed. This raised concerns regarding the safety of exercise (Taivassalo et al., 2001).

Later studies, however, did not detect changes in overall mutation load after 12 or 14 weeks of endurance exercise (Jeppesen et al., 2006, Taivassalo et al., 2006, Jeppesen et al., 2009), or after 14 weeks or 12 months of deconditioning (Taivassalo et al., 2006, Jeppesen et al., 2009). Also, further studies have found only a mild increasing trend in the activity of mitochondrial respiratory chain complexes and no changes to the percentage of COX deficient fibres after aerobic exercise. Deconditioning of 8 or 14 weeks was shown to induce a loss of the physiological and biochemical adaptation to pre-training values, and consequently, a reduction in muscle capacity for oxidative phosphorylation (Jeppesen et al., 2006, Taivassalo et al., 2006).

The majority of studies have exclusively addressed the effects of short-term exercise regimens ranging from 8-14 weeks duration. Jeppensen *et al.* was the only group evaluating and comparing the effects of short-term (3 months, cycling 5 times a week, 30-40 min per session) and long-term exercise (12months, cycling 3 times a week, 30 min per session), with a period of 12 or 18 months detraining in between, in a group of four patients with mitochondrial myopathy (Jeppesen et al., 2009). They demonstrated a consistent beneficial effect to training, with an increase in oxidative capacity by 23%. Also, no adverse effect on the mtDNA mutation load was observed over the 24 months period. Despite these promising results, further studies

assessing larger cohorts of patients are required, as well as more insight regarding the focal proportion and distribution of wild-type and mutated mtDNA within individual muscle fibres.

6.2 Aims

The aim of this chapter was to investigate whether exercise training provides a long-term, effective and safe therapy for mitochondrial myopathy, by addressing:

- 1. The impact of exercise on the levels of OXPHOS complexes in individual muscle fibres
- 2. The impact of deconditioning on the levels of OXPHOS complexes in individual muscle fibres

6.3 Methods

6.3.1 Cohort clinical characteristics

Twenty two patients with clinically and genetically-characterised mitochondrial disease (13 patients with single-large scale mtDNA deletion and 9 patients with mtDNA point mutations), with ability to exercise and that consented for four biopsies were recruited for this multicenter exercise trial. Ethical approval, patient information sheet and consent form (including a brief description of the procedures) can be found in Appendix.

These patients have been previously characterised in chapter 5, and were attributed a new number in the present chapter. Relevant information from chapter 5 is summarised in table Table 6.1. Healthy and disease control muscle biopsy samples were also included in this study (Kirby *et al.*, 2007).

6.3.2 Endurance training program

Initial assessment of patients, training and deconditioning assessments, and biopsies were performed in the Neuromuscular Centre, Institute for Exercise and Environmental Medicine and at the University of Texas Southwestern Medical Centre, in Dallas.

Patients (Chapter 5)	MRC profile	Patients	Sex	Age	Clinical information	Genetic defect	Mutation load	Endurance training program
Single, larg	ge-scale m	tDNA deleti	on					
P4	Group 1	P1	М	25y	Ptosis, CPEO, some fatigable weakness. Ultra peculiar patient	Deletion size ~ 4.7 kb Breakpoints: 10946-15598	85%	Group B
Р5	Group 2	P2	F	25y	СРЕО	Deletion size ~3.0 kb (breakpoints not mapped)	n.d.	Group A
P6	Group 2	Р3	F	26y	CPEO, mitochondrial myopathy	Deletion size ~ 4.9 kb Breakpoints: 10747-15598	65%	Group B
P8	Group 1	P4	F	36y	CPEO, myopathy	Deletion size ~ 5.0 kb (breakpoints not mapped)	79%	Group A
Р9	Group 2	Р5	Μ	39y	CPEO, myopathy	Deletion size ~ 4.1 kb Breakpoints: 11262- 15375	72%	Group B
P11	Group 2	P6	F	40y	CPEO, muscle atrophy, weakness, retinal pigmentary changes, increased resting lactate	Deletion size ~ 2.3 kb Breakpoints: 12113-14421	87%	Group A
P12	Group 3	P7	F	41y	CPEO, myopathy	n.d.	42%	Group A
P13	Group 1	P8	F	43y	CPEO, myopathy	Deletion size ~ 5.0 kb Breakpoints: 8482- 13460	78%	Group A
P14	Group 1	Р9	F	44y	CPEO, myopathy	Deletion size ~ 4.2 kb Breakpoints: 9486-13723	81%	Group B
P15	Group 2	P10	F	47γ	Ptosis, CPEO, jaw aching with chewing	Deletion size ~ 2.3 kb Breakpoints: 12112- 14412	76%	Group B
P16	Group 1	P11	F	48y	CPEO, myopathy	Deletion size ~ 4.2 kb Breakpoints: 9498-13739	39%	Group A
P17	Group 2	P12	F	61y	CPEO	n.d.	n.d.	Group B
P18	Group 1	P13	М	61y	СРЕО	Deletion size ~ 5.0 kb Breakpoints: 8482- 13460	3%	Group A
Point mut	ations in m	iitochondria	l enco	ded tRN	As			
P25	n.r.	P14	F	25y	Exercise intolerance, ptosis	m.3243A>G MT-TL1 mutation	n.d.	Group B

Table 6.1 Clinical information from subjects included in this study

P26	n.r.	P15	F	38y	Mild exercise intolerance, diabetes	m.3243A>G MT-TL1 mutation	n.d.	Group B
P27	n.r.	P16	F	40y	Exercise intolerance and mild deafness	m.3243A>G MT-TL1 mutation	n.d.	Group B
P29	n.r.	P17	F	43y	Mild exercise intolerance, diabetes	m.3243A>G MT-TL1 mutation	n.d.	Group B
P30	n.r.	P18	F	47y	Modest exercise intolerance	m.3243A>G MT-TL1 mutation	n.d.	Group A
P34	n.r.	P19	F	35y	Mild weakness	m.14709T>C <i>MT-TE</i> mutation	n.d.	Group B
P35	n.r.	P20	F	58y	Myopathy	m.14709T>C <i>MT-TE</i> mutation	87%	Group B
P37	n.r.	P21	Μ	33у	Mitochondrial myopathy	m.10010T>C MT-TG mutation	89%	Group A
P38	n.r.	P22	Μ	63y	Pure exercise intolerance, prominent exertional dyspnea	m.5543T>C MT-TW mutation	n.d.	Group A

Key: age = age when biopsied; y = years old; mtDNA = mitochondrial DNA; PEO = progressive external ophthalmoplegia; CPEO = chronic CPEO; Patients (chapter 5) = corresponding ID number of patients in chapter 5, MRC profile = only relevant for patients with single large-scale deletion which were classified into 3 groups according to the MRC profile; group 1 = patients showing both complexes I and IV equally and simultaneously down-regulated; Group 2 = patients showing an early and more pronounced deficiency of complex I over complex IV; Group 3 = patients showing a slightly more pronounced and earlier deficiency of complex IV over complex I; n.d. = not determined; n.r. = not relevant.

Patients were randomly assigned into group A or group B and underwent periods of activity and inactivity (Figure 6.1). Patients assigned to Group A engaged in 6 months endurance training at baseline, and detrained (only monitoring daily activity levels) for the following 6 months. Patients assigned to Group B performed no specific training for the first 6 months (only monitoring the activity levels), and engaged in endurance training for the following 6 months. At the end of the first year, patients were encouraged to carry on or resume regular endurance exercise according to the training effects on their cardiac and muscle functions. Patients advised to continue training were followed up with heart rate and activity monitors. A final evaluation was performed at the end of the exercise program (24months).

The exercise training period comprised of 100 sessions over a period of 26 weeks on a stationary cycle. Each session lasted 30 min for the first 13 weeks and 40 min for the following 13 weeks, and exercise was performed at an intensity corresponding to 70%-80% of maximal heart rate. Both heart rate and exercise duration were continuously monitored using a chest band and a watch.



Figure 6.1 Endurance training program.

Patients were randomly assigned into group A and group B. (A) Patients in Group A engaged 6 months of endurance training at baseline, and detrained for the following 6 months. (B) Patients in Group B did not perform training during the first 6 months and engaged endurance training for the following 6 months. At the end of the first year, patients either carried on or resumed regular endurance exercise.

6.3.3 Muscle biopsies

Needle muscle biopsies were collected from the same *vastus lateralis* of patients at baseline (1st), 6 months (2nd), 12 months (3rd) and 24 months (4th) of the exercise trial program. Subsequently, frozen samples were sent from the centres in Dallas to the Mitochondria research group in Newcastle. The trial was still ongoing at the time these experiments were performed, therefore not all biopsies had been collected and sent. For this reason only 1st, 2nd and 3rd muscle biopsies from a selection of patients were assessed in this chapter.

6.3.4 Cryo-sectioning

Serial sections (10µm thickness) from transversely orientated muscle blocks were obtained as previously described in section 2.2.3 from chapter 2.

6.3.5 Immunofluorescence

Fluorescence-based immunohistochemistry was carried out according to the protocol described in section 5.3.3 from chapter 5.

6.3.6 Imaging

Brightfield and fluorescent images were acquired at 20x magnification using Zeiss Microscope (see section 2.2.7 from chapter 2).

6.3.7 Densitometry measurements

Fluorescent images were analysed using IMARIS software (Bitplane). Segmentation was performed in order to measure the OD from individual muscle fibres (ODCOXI, ODNDUFB8 and ODporin or OD₄₈₈, OD₅₄₆ and OD₆₄₇) as described in section 4.4.1 from chapter 4.

6.3.8 Data analysis

Data analysis, involving background correction, creation of control group, and calculation of Z scores for porin, NDUFB8 and COX-I, was performed as previously described in chapter 4 (see section 4.4.4.1).

The effects of exercise and no specific training, or detraining, were assessed using two different approaches. Both present advantages and disadvantages which will be discussed at the end of this section.

In the first approach, biopsies were compared based on the distribution of COX-I and NDUFB8 Z scores. For that, Z scores from 1st, 2nd and 3rd biopsies were separately plotted as box plots, as illustrated in Figure 6.2. Top and bottom of the box are the 1st and 3rd quartiles; the horizontal line within the box indicates the median. The whiskers represent the 5th and 95th percentiles. Results were interpreted based on the median value, whereby an increase represents a positive effect. For example, the graph shown in Figure 6.2 demonstrate that exercise training, which took place between 1st and 2nd biopsies, led to an overall increase in COX-I Z scores.



Figure 6.2: Example of a box-plot showing the COX-I Z scores from 1st, 2nd and 3rd muscle biopsies.

In the second approach, the 1st, 2nd and 3rd biopsies were compared based on COX-I and NDUFB8 levels, rather than the Z scores distribution. To quantify levels of these complexes, fibres were classified into 2 groups based on Z scores. The first, yielding "upper" class, encompassed fibres with Z scores greater than or equal to -4.5SD. The second, yielding "lower" class, included fibres with Z scores lower than -4.5 SD. The reason why fibres were classified only into 2 groups was to simplify the analysis. In short, the upper class contains the positive and int(+) fibres as classified using the previous 4 group system from earlier chapters, whereas the lower class contains the int(-) and negative fibres. The percentage of fibres in COX-I and NDUFB8 upper and lower classes was calculated and results were presented in a table as

illustrated in Table 6.2. In addition, the percentage change in COX-I or NDUFB8 levels between 1st and 2nd biopsies or 2nd and 3rd biopsies was calculated as follows;

% change(COX-I or NDUFB8) = % of fibres in (COX-I or NDUFB8) upper class (2^{nd} biopsy) - % of fibres in (COX-I or NDUFB8) upper class (1^{st} biopsy). The %change between 1^{st} and 2^{nd} biopsies quantifies the effect of exercise in group A and the effect of no specific training in patients from group B.

% change(COX-I or NDUFB8) = % of fibres in (COX-I or NDUFB8) upper class (3^{rd} biopsy) - % of fibres in (COX-I or NDUFB8) upper class (2^{nd} biopsy). The %change between 2^{nd} and 3^{rd} biopsies quantifies the effect of deconditioning/detraining in group A and the effect of exercise in patients from group B.

			COX-I levels		I			
	Biopsy	Upper class	Lower class	%change	Upper class	Lower class	%change	n
	1st	2.8%	97.2%		4.6%	95.4%		218
P21	2nd	12.4%	87.6%	9.6%	15.7%	84.3%	11.1%	769
	3rd	4.1%	95.9%	-8.2%	5.5%	94.5%	-10.3%	750

Table 6.2 Example of quantification of COX-I and NDUFB8 levels

A positive value reflects an increase in the percentage of fibres in the upper group and therefore an increase in COX-I/NDUFB8 levels. By contrast, a negative value indicates a decrease in the percentage of fibres in the upper group due to a decline in COX-I/NDUFB8 levels. For example, the results shown in Table 6.2 demonstrate that detraining, which took place between 2nd and 3rd biopsies, induced a decline in COX-I and NDUFB8 levels.

These two approaches to analysing data were considered for the several reasons. The quantification of COX-I and NDUFB8 levels allows detection of changes in the percentage of fibres in the upper class. However, it has the disadvantage of not quantifying precisely the changes occurring in the lower class as the range of Z scores in this group is very wide (varying from -4.5SD up to -20SD). So, a deficient fibre with a Z score of -18SD that slightly improves following exercise to a value of -10SD, will still be classified in the negative group. Therefore, slight changes in the lower group may not be detected. The use of box plots to visualise data has the advantage of indicating subtle changes in the distribution of Z scores. Although very useful, the interpretation of results may be more complex in some cases, due to the amount of information conveyed by the graphs, which present medians, quartiles, and 5th and 95th percentiles.

6.4 Results

6.4.1 Patients with single large-scale mtDNA deletions

6.4.1.1 Group A

6.4.1.1.1 Distribution of COX-I and NDUFB8 Z scores

Patients P2, P4, P6, P7, P8, P11 and P13 were assigned to group A, and engaged exercise training at baseline. The distribution of COX-I and NDUFB8 Z scores in 1st, 2nd and 3rd biopsies are shown in Figure 6.3.

Patients in this group responded differently to periods of endurance exercise and detraining. Patients P6 and P8 exhibited a clear increase in COX-I and NDUFB8 Z scores following training and showed a clear decline in both Z scores after 6 months of detraining (Figure 6.3 C and E). These changes were recorded by respective increases and decreases in median, and 1st and 3rd quartile values, in addition to a narrowing of the Z scores distribution in P8. P13 also displayed an increase in both COX-I_z and NDUFB8_z following training. However, following the detraining period this patient showed an increase in COX-I_z, as seen in P6 and P8, though NDUFB8_z was shown to decrease (Figure 6.3G). This indicates a partial benefit of exercise to patient P13, with a positive effect only observed on complex I.

By contrast, exercise training induced a decline in COX-I_z, with no major changes to NDUFB8_z, in patient P4, and a decline in both COX-I_z and NDUFB8_z in P11. Both patients showed a clear increase in COX-I and NDUFB8 Z scores following the detraining period (Figure 6.3 B and F), meaning that P4 and P11 benefited more from the deconditioning phase.

Finally, patients P2 and P7 showed contradictory results (Figure 6.3 A and D). Exercise training induced an increase in COX-I Z scores, and a decline in NDUFB8 Z scores in both patients. The 6 months of detraining resulted in a decline in both Z scores for P2, whereas P7 saw an increase in both Z scores following this period. This means that exercise was partially beneficial for P2 (positive for COX-I, negative for NDUFB8) and had adverse effects on P7.


Figure 6.3 Box plots of COX-I and NDUFB8 Z scores of individual muscle fibres in 1st, 2nd and 3rd biopsies from patients with single large-scale mtDNA deletions assigned to group A (continues on the next page).

Left graphs plot COX-I Z scores, right graphs plot NDUFB8 Z scores from patients (A) P2, (B) P4, (C) P6, (D) P7, (E) P8, (F) P11 and (G) P13, which were assessed at baseline (left white box plot), following 6 months of endurance exercise (middle blue box plot), and following 6 months of deconditioning (right white box plot). The box indicates the upper and lower quartiles, and the median is represented by a horizontal black line within the box. Whiskers indicate the 5th and 95th percentiles.











G Patient P13



		COX-I levels			I			
	Biopsy	Upper class	Lower class	%change	Upper class	Lower class	%change	n
	1st	93.8%	6.2%		54.0%	46.0%		322
P2	2nd	99.0%	1.0%	5.2%	45.3%	54.7%	-8.8%	296
	3rd	93.5%	6.5%	-5.5%	46.7%	53.3%	1.5%	92
	1st	47.9%	52.1%		44.6%	55.4%		388
P4	2nd	45.6%	54.4%	-2.4%	42.3%	57.7%	-2.3%	781
	3rd	54.1%	45.9%	8.5%	51.3%	48.7%	9.1%	896
	1st	73.1%	26.9%		11.7%	88.3%		1118
P6	2nd	75.3%	24.7%	2.2%	19.9%	80.1%	8.2%	231
	3rd	68.3%	31.7%	-7.0%	12.2%	87.8%	-7.7%	262
	1st	87.3%	12.7%		92.6%	7.4%		283
P7	2nd	91.4%	8.6%	4.1%	93.3%	6.7%	0.8%	105
	3rd	93.9%	6.1%	2.4%	96.3%	3.7%	3.0%	163
	1st	58.1%	41.9%		53.2%	46.8%		853
P8	2nd	66.1%	33.9%	8.0%	62.4%	37.6%	9.2%	242
	3rd	55.4%	44.6%	-10.7%	50.4%	49.6%	-12.0%	399
	1st	84.3%	15.7%		82.8%	17.2%		942
P11	2nd	71.3%	28.7%	-13.0%	70.5%	29.5%	-12.3%	268
	3rd	80.1%	19.9%	8.8%	79.6%	20.4%	9.1%	803
	1st	98.1%	1.9%		98.4%	1.6%		365
P13	2nd	97.6%	2.4%	-0.5%	95.9%	4.1%	-2.5%	338
	3rd	98.5%	1.5%	0.9%	96.6%	3.4%	0.7%	592

Table 6.3 Quantification of COX-I and NDUFB8 levels in patients with single mtDNA deletions assigned to group A

Key: %change = difference in the percentage of muscle fibres in COX-I/NDUFB8 upper classes from 1^{st} to 2^{nd} biopsy and from 2^{nd} to 3^{rd} biopsy. Blue highlights the biopsy taken following the 6 months period of exercise.

6.4.1.1.2 Quantification of the change in COX-I and NDUFB8 levels

The results from the quantification of COX-I and NUFB8 levels (Table 6.3) were, overall, in agreement with the observed changes to the distribution of COX-I and NDUFB8 Z scores. Patients P6 and P8 exhibited an increase in the percentage of fibres in COX-I and NDUFB8 upper classes - hence an increase in COX-I and NDUFB8 levels - following the 6 month period of endurance training (Table 6.3, 2nd biopsy % change). After 6 months of detraining, both patients showed a clear decline in COX-I and NDUFB8 levels (Table 6.3, 3rd biopsy % change).

Endurance training caused a decline in COX-I and NDUFB8 levels in P4 and P11, and an increase in both complexes levels following the detraining period in these patients. In patient P13, exercise training induced a decline in NDUFB8 levels, with no major effects in COX-I levels. The detraining period induced a slight increase in both complexes in this patient.

Endurance exercise in patients P2 and P7 yielded interesting results demonstrating a positive impact on complex IV levels, as revealed by an increase in COX-I levels, alongside a negative

effect on complex I. In patient P2, this adverse effect was due to a decline in NDUFB8 levels post exercise. Patient P7 actually showed an increase in NDUFB8 levels following the training phase (%change (NDUFB8) = 0.8%), however this increase was less pronounced than that recorded following detraining (%change (NDUFB8) = 3.0%). Detraining also caused contradictory results in both patients: however here a negative impact on complex IV was noted, while complex I seemed to be positively affected. Patient P7 demonstrated an increase in COX-I levels following the detraining phase (%change (COX-I) = 2.4%), however this was less pronounced than the increase recorded in the post-exercise period (%change (COX-I) = 4.1%), creating an overall negative impact of detraining on COX-I levels.

6.4.1.2 Group B

6.4.1.2.1 Distributions of COX-I and NDUFB8 Z scores

Patients P1, P3, P5, P9, P10 and P12 were assigned to group B and only initiated endurance exercise following a 6 month period of no specific training. The distribution of COX-I and NDUFB8 Z scores in 1st, 2nd and 3rd biopsies are shown in Figure 6.4.

Patients P3, P5, and P10 exhibited a decline in COX-I and NDUFB8 Z scores following the period of no specific training and showed a clear increase in both Z scores after 6 months of exercise training (Figure 6.4 B, C and F). The box plots from these patients suggest that they all benefited from the exercise training. Similarly, patient P12 showed a slight decline in both Z scores following the no specific training period, and P9 showed an increase following the training period (Figure 6.4 D and F). Unfortunately, the 2nd and 3rd muscle biopsies from patients P9 and P12, respectively, presented a high degree of freezing artefacts not suitable for appropriate assessment.

By contrast, patient P1 recorded an increase in COX-I_z and NDUFB8_z following the no specific training period. They also showed a decline in Z scores for both complexes after the 6 months period of endurance training (Figure 6.4A), suggesting that exercise training had an adverse effect.



Figure 6.4 Box plots of COX-I and NDUFB8 Z scores of individual muscle fibres in 1st, 2nd and 3rd biopsies from patients with single mtDNA deletions assigned to group B (continues on the next page). Left graphs plot COX-I Z scores, right graphs plot NDUFB8 Z scores from patients (A) P1, (B) P3, (C) P5, (D) P9, (E) P10 and (F) P12, which were assessed at baseline (left white box plot), following 6 months of no specific training (middle white box plot), and following 6 months of endurance training (right blue box plot). The box indicates the upper and lower quartiles, and the median is represented by a horizontal black line within the box. Whiskers indicate the 5th and 95th percentiles.

D Patient P9









6.4.1.2.2 Quantification of the change in COX-I and NDUFB8 levels

The results from the quantification of COX-I and NUFB8 levels (Table 6.4) were in complete agreement with the observed changes in COX-I and NDUFB8 Z scores.

In patients P3, P5 and P10, six months of no specific training caused a decline in COX-I and NDUFB8 levels, indicated by a negative percentage change (Table 6.4; 2^{nd} biopsy % change) (except for P3 which showed no major changes). By contrast, the six months endurance training induced an increase in both complexes levels in all of the patients (Table 6.4, 3^{rd} biopsy % change). Similarly, endurance exercise induced an increase in COX-I and NDUFB8 in patient P9, and no training caused a decline in patient P12. By contrast, patient P1 showed an increase in the levels of both complex I (%change (NDUFB8) = 5.9%) and complex IV (%change (COX-I) = 6.4%) following the no specific training phase, whereas endurance training induced a decline in mitochondrial function represented by a decrease in levels of both complexes (%change (COX-I) = -8.9%; %change (NDUFB8) = -6.7%).

			COX-I levels			NDUFB8 levels			
	Biopsy	Upper	Lower	%change	Upper	Lower	%change	n	
	1st	62.2%	37.8%		59.3%	40.7%		609	
P1	2nd	68.6%	31.4%	6.4%	65.2%	34.8%	5.9%	207	
	3rd	59.7%	40.3%	-8.9%	58.5%	41.5%	-6.7%	631	
	1st	81.7%	18.3%		44.4%	55.6%		579	
Р3	2nd	79.8%	20.2%	-1.8%	38.8%	61.2%	-5.6%	263	
	3rd	82.4%	17.6%	2.5%	45.5%	54.5%	6.7%	409	
	1st	76.5%	23.5%		33.8%	66.2%		272	
Р5	2nd	76.2%	23.8%	-0.3%	29.2%	70.8%	-4.6%	130	
	3rd	88.8%	11.2%	12.7%	61.5%	38.5%	32.3%	143	
	1st	-	-		-	-		-	
Р9	2nd	40.1%	59.9%	-	39.9%	60.1%	-	419	
	3rd	44.5%	55.5%	4.4%	41.3%	58.7%	1.5%	670	
	1st	92.0%	8.0%		47.5%	52.5%		301	
P10	2nd	88.3%	11.7%	-3.7%	38.4%	61.6%	-9.1%	606	
	3rd	93.4%	6.6%	5.2%	40.2%	59.8%	1.7%	122	
	1st	90.5%	9.5%		85.9%	14.1%		283	
P12	2nd	89.5%	10.5%	-0.9%	81.8%	18.2%	-0.9%	220	
	3rd	-	-		-	-		-	

Table 6.4 Quantification of COX-I and NDUFB8 levels in patients with single mtDNA deletions assigned to group B

Key: %change = difference in the percentage of muscle fibres in COX-I/NDUFB8 upper classes from 1^{st} to 2^{nd} biopsy and from 2^{nd} to 3^{rd} biopsy. Blue highlights the biopsy taken following the 6 months period of exercise.

6.4.1.3 Summary of results

To summarise, the impact of endurance exercise on patients with single large-scale mtDNA deletions was variable (Table 6.5). Two approaches of analysis (distribution of Z scores and quantification of complexes levels) were considred to interpret the data.

In five patients (P3, P5, P6, P8 and P10), exercise had a positive effect on COX-I and NDUFB8 levels, when compared to periods of no specific training. Both approaches of analysis also detected a beneficial effect of the training period for patient P9; however, as the 1st biopsy of this patient was not appropriate for analysis, it was not possible to assess the impact of no specific training on this patient. Since it cannot be excluded that no specific training would cause a more pronounced increase than that observed with exercise, results are not conclusive. In contrast, P1, P4, and P11 showed an adverse response to the 6 months period of endurance exercise. Patient P12 also revealed a decline in complexes levels, however, as the 3rd biopsy was also not appropriate for analysis, results are not conclusive. Interestingly, patients P2, P7 and P13 showed contradictory results regarding the effects on individual complexes. In patients P2 and P7, exercise demonstrated a positive impact on complex IV but adverse impact on complex I. Whereas in P2, both analysis approaches were in agreement, this was not the case for P7. Therefore, I had to make a judgement for this particular patient, which was based on the increase in COX-I levels. Patient P13 showed the opposite effect with an adverse impact on complex IV but positive impact on complex I levels.

This group of single deletion patients is very heterogenous. It includes patients from both genders over a broad age range (25 to 61 years old), with biopsies displaying different MRC profiles and variable mtDNA mutation loads. Further analysis was therefore performed in order to explore if this heterogeneity could underlie the variability found in results. However, no association was found between gender and the impact of exercise. The three male patients assessed responded differently to the training programme reacting either positively (P5), partially positively (P13) or adversely (P1). The same mixed response was observed for female patients. Also, no link was found between age and effect of exercise; P3 (26years old) and P10 (47years old) presented beneficial reactions to exercise; P2 (25years old) and P13 (61years old) showed partial benefit; P1 (25year old) and P11 (48 years old) responded negatively to training. Furthermore, there was no correlation found between the response to exercise and the MRC profile of these patients (assessed in chapter 5). Patients showing a positive response to exercise were found in both group 1 (MRC profile: complexes I and IV equally affected, P8 and P10) and group 2 (MRC profile: complex I more affected than complex IV, P6, P3 and P5). Also, the patients showing contradictory results in COX-I and NDUFB8 levels were found in

group 1 (P13), group 2 (P2) and group 3 (MRC profile: complex IV more affected; P7). Interestingly, patients responding negatively to exercise were exclusively from group 2 (P1, P4 and P11). Finally, no correlation was found between the response to exercise and the mutation load. Patients showing an improvement of mitochondrial function presented variable mutation load (ranging from 65% to 87%) and patients showing deterioration (P1, P4 and P11), harboured mutation loads of 85%, 79% and 39%, respectively.

	Sex	MRC	Effect of exercise on complex IV			Effect of	f exercise on co	mplex I
	(Age)	Profile	Z scores	COX-I levels	Summary	Z scores	COX-I levels	Summary
Exercis	e trainin	g: group A						
P2	F (25)	Group 2	+	+	+	-	-	-
P4	F (36)	Group 1	-	-	-	-	-	-
P6	F (40)	Group 2	+	+	+	+	+	+
P7	F (41)	Group 3	-	+	+	-	-	-
P8	F (43)	Group 1	+	+	+	+	+	+
P11	F (48)	Group 1	-	-	-	-	-	-
P13	M (61)	Group 1	-	-	-	+	-	+
Exercis	e trainin	g: group B				-		
P1	M (25)	Group 1	-	-	-	-	-	-
P3	F (26)	Group 2	+	+	+	+	+	+
P5	M (39)	Group 2	+	+	+	+	+	+
Р9	F (44)	Group 1	+ (?)	+ (?)	n.c.	+ (?)	+ (?)	n.c.
P10	F (47)	Group 2	+	+	+	+	+	+
P12	F (61)	Group 2	- (?)	- (?)	n.c.	- (?)	- (?)	n.c.

Table 6.5 Effect of exercise in complexes IV and I levels in patients with single large-scale mtDNA deletions

Key: age = age when biopsied, years old; group 1 = patients showing both complexes I and IV equally and simultaneously down-regulated; Group 2 = patients showing a more pronounced deficiency of complex I than complex IV; Group 3 = patients showing a slightly more pronounced deficiency of complex IV; n.c. = not conclusive.

6.4.2 Patients with point mutations in mitochondrial-encoded tRNAs

6.4.2.1 Group A

6.4.2.1.1 Distribution of Z scores for COX-I and NDUFB8

The distributions of COX-I and NDUFB8 Z scores in 1st, 2nd and 3rd biopsies are shown in Figure 6.5. All patients assigned to group A (P18, P21 and P22) showed an increase in COX-I and NDUFB8 Z scores following endurance training (Figure 6.5A-C). This was recoded as an increase in the 1st quartile, the median value and/or the 3rd quartile. Patients P18 and P21 demonstrated a decline in both Z scores following 6 months of detraining (Figure 6.5A and B). These results suggest that exercise training was beneficial for these patients. Unfortunately, P22's 3rd muscle section was lost during the immunofluorescence protocol.





Left graphs plot COX-I Z scores, right graphs plot NDUFB8 Z scores from patients (A) P18, (B) P21 and (C) P22, which were assessed at baseline (left white box plot), following 6 months of endurance exercise (middle blue box plot), and following 6 months of deconditioning (right white box plot). The box indicates the upper and lower quartiles, and the median is represented by a horizontal black line within the box. Whiskers indicate the 5th and 95th percentiles.

6.4.2.1.2 Quantification of the change in COX-I and NDUFB8 levels

The results from the quantification of COX-I and NUFB8 levels (Table 6.6) were in complete agreement with the observed changes in COX-I and NDUFB8 Z scores. Patients P18, P21 and P22 exhibited an increase in COX-I and NDUFB8 levels following 6 months of endurance exercise (Table 6.6, 2nd biopsy, % change), and a decline in COX-I and NDUFB8 levels following detraining (Table 6.6, 3rd biopsy, % change, except P22).

		COX-I levels			Γ			
	Biopsy	Upper class	Lower class	%change	Upper class	Lower class	%change	n
	1st	90.6%	9.4%		83.6%	16.4%		128
P18	2nd	92.0%	8.0%	1.4%	86.2%	13.8%	2.6%	741
	3rd	88.5%	11.5%	-3.5%	82.3%	17.7%	-3.9%	1200
P21	1st	2.8%	97.2%		4.6%	95.4%		218
	2nd	12.4%	87.6%	9.6%	15.7%	84.3%	11.1%	769
	3rd	4.1%	95.9%	-8.2%	5.5%	94.5%	-10.3%	750
P22	1st	17.6%	82.4%		19.5%	80.5%		677
	2nd	30.9%	69.1%	13.3%	30.5%	69.5%	11.0%	220
	3rd	-	-	-	-	-	-	-

Table 6.6 Quantification of COX-I and NDUFB8 levels in patients with point mutations assigned to group A

Key: %change = difference in the percentage of muscle fibres in COX-I/NDUFB8 upper classes from 1^{st} to 2^{nd} biopsy and from 2^{nd} to 3^{rd} biopsy. Blue highlights the biopsy taken following the 6 months period of exercise.

6.4.2.2 Group B

6.4.2.2.1 Distribution of COX-I and NDUFB8 Z scores

The distribution of COX-I and NDUFB8 Z scores in 1st, 2nd and 3rd biopsies are shown in Figure 6.6. All patients assigned to group B (P14, P15, P16, P17, P19 and P20) showed a decline in COX-I and NDUFB8 Z scores (except P14) following 6 months of no specific training. This decline was evident from the decrease in recorded quartiles values, and in some cases from the widening of the Z scores distributions (P15 and P16). Patient P14 was the only exception as this particular case showed a general increase in both COX-I and NDUFB8 Z scores, where median, 1st and 3rd quartiles shifted up (Figure 6.6A).

In patients P14, P15 and P19, endurance training caused an increase in both COX-I (or no significant change in P19) and NDUFB8 Z scores (Figure 6.6 A, B and E). This was recorded through the increase in the quartile values or through the narrowing of distributions. This suggests that P14, P15 and P19 benefited from the training program.

A m.3243 A>G *MT-TL1* (P14)





Left graphs plot COX-I Z scores, right graphs plot NDUFB8 Z scores from patients (A) P14, (B) P15, (C) P16, (D) P17, (E) P19 and (F) P20, which were assessed at baseline (left white box plot), following 6 months of only daily base activity with no specific training (middle white box plot), and following 6 months of endurance training (right blue box plot). The box indicates the upper and lower quartiles, and the median is represented by a horizontal black line within the box. Whiskers indicate the 5th and 95th percentiles.











In patient P16, exercise caused an increase in COX-I_z and decline in NDUFB8_z as measured by a drop in the median level (Figure 6.6C), whereas in P20 the exercise period induced a decline in COX-I_z and an increase in NDUFB8_z (Figure 6.6F). For these particular patients, results indicate that the training programme was partially beneficial. In patient P17, endurance training caused a decline in both COX-I and NDUFB8 Z scores (Figure 6.6D), meaning that exercise had adverse effects on this patient.

6.4.2.2.2 Quantification of the change in COX-I and NDUFB8 levels

The results from the quantification of COX-I and NUFB8 levels are shown in Table 6.7. All patients with point mutations assigned to group B, with the exception of P14 and P17, showed a decline in COX-I and NDUFB8 levels following 6 months of no specific training (Table 6.7, 2nd biopsy % change). Patient 14 showed no major changes to NDUFB8 levels following the no specific training period, and Patient P17 recorded no impact on COX-I levels (%change < 0.5%).

By contrast, endurance exercise induced an increase in the level of both complexes in patients P14 and P15 (Table 6.7, 3^{rd} biopsy % change). Patient P16 showed an increase in the levels of COX-I (%change (COX-I) = 6.7%), while no major effect on NDUFB8 levels was observed, as the decline observed post exercise (%change (NDUFB8) = -5.8%) was similar to the decline recorded following no specific training (%change (NDUFB8) = -5.2%).

In patients P19 and P20, endurance exercise had an indirect positive effect. Following endurance exercise, both patients exhibited a decline in COX-I and/or NDUFB8 levels less pronounced than the decline recorded previously for periods of no specific training. For instance, in patient P19 the %change in COX-I and NDUFB8 levels after no training declined by - 3.2% and -3.0%, respectively. However, following exercise the decline recorded for COX-I levels was attenuated to -1.6% and NDUFB8 levels recorded a slight increase of 0.9%. Similarly, in patient P20 the %change in COX-I and NDUFB8 levels after no training was -1.1% and -4.2%, respectively, whereas after exercise, the decline was attenuated to -0.8% and -0.8%, respectively.

In patient P17 the training period did not have a major impact on COX-I levels. However it induced a decline in NDUFB8 levels (%change (NDUFB8) = -2.3%), that surpassed the change occurring following periods of no specific training (%change (NDUFB8) = -0.8%).

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			COX-I levels			NDUFB8 levels			
	Biopsy	Upper class	Lower class	%change	Upper class	Lower class	%change	n	
	1st	87.2%	12.8%		77.8%	22.2%		906	
P14	2nd	85.7%	14.3%	-1.4%	77.7%	22.3%	-0.1%	1740	
	3rd	88.5%	11.5%	2.8%	80.4%	19.6%	2.7%	1749	
	1st	84.7%	15.3%		75.4%	24.6%		615	
P15	2nd	83.1%	16.9%	-1.6%	71.6%	28.4%	-3.9%	985	
	3rd	86.5%	13.5%	3.4%	80.3%	19.7%	8.7%	542	
	1st	81.1%	18.9%		56.7%	43.3%		799	
P16	2nd	75.2%	24.8%	-6.0%	51.5%	48.5%	-5.2%	499	
	3rd	81.8%	18.2%	6.7%	45.7%	54.3%	-5.8%	396	
	1st	99.1%	0.9%		97.6%	2.4%		212	
P17	2nd	98.9%	1.1%	-0.1%	96.8%	3.2%	-0.8%	1376	
	3rd	98.9%	1.1%	0.0%	94.5%	5.5%	-2.3%	274	
	1st	74.9%	25.1%		60.8%	39.2%		1782	
P19	2nd	71.7%	28.3%	-3.2%	57.8%	42.2%	-3.0%	972	
	3rd	70.1%	29.9%	-1.6%	58.7%	41.3%	0.9%	906	
	1st	92.6%	7.4%		88.3%	11.7%		1510	
P20	2nd	91.5%	8.5%	-1.1%	84.1%	15.9%	-4.2%	1001	
	3rd	90.7%	9.3%	-0.8%	83.3%	16.7%	-0.8%	852	

Table 6.7 Quantification of COX-I and NDUFB8 levels in patients with point mutations assigned to group B

Key: %change = difference in the percentage of muscle fibres in COX-I/NDUFB8 upper classes from 1^{st} to 2^{nd} biopsy and from 2^{nd} to 3^{rd} biopsy. Blue highlights the biopsy taken following the 6 months period of exercise.

6.4.2.3 Summary of results

The impact of endurance exercise on patients with point mutations in different mitochondriaencoded tRNAs is shown in Table 6.8. In five patients (P14, P15, P18, P19 and P21), the six months of endurance exercise led to an improvement of mitochondrial function as measured by an increase in levels of COX-I and complex I. The same was found for P22 however, as the effect of no specific training was not assessed, results are not conclusive for this patient. In two of the patients (P16 and P20), exercise showed a partial positive effect, improving one of the complexes but decreasing the other. Finally, only patient P17 showed an adverse response to exercise.

Like the single deletion patients, this group of patients displayed some heterogeneity in gender, age and, specifically for this group, genotype. Unfortunately, as only one patient showed an adverse response to exercise, it is more difficult to correlate these variables and the results obtained. However it is possible to make some observations. The age does not seem to predict the impact of exercise as P17 and P18, carrying the same mutation

(m.3243A>G *MT-TL1*), both females and of the same age, responded differently to the exercise regimen. Regarding gender, no males were affected adversely, however, the majority of patients were females (7 out of 9). Finally, the genotypes *MT-TL1* and *MT-TE* do not seem to be a predictor of the effect of the training program. For example, patients P19 and P20, carrying the m.14709T>C *MT-TE* mutation, showed both an improvement and a deterioration of mitochondrial function, respectively, as assessed by the complexes levels. Similarly, patients P16, P17 and P18, all carrying the m.3243A>G *MT-TL1* mutation, all females and within the same age range (40, 47 and 47 years old) demonstrated variable responses to exercise.

Table 6.8 Effect of endurance exercise in complexes IV and I levels in patients with point mutation in mt-tRNAs

	Sex	Genotype	Effect of exercise on complex IV			Effect of	f exercise on co	mplex I
	(Age)		Z scores	COX-I levels	Summary	Z scores	COX-I levels	Summary
Exercis	e trainin	g: group A						
P18	F (47)	MT-TL1	+	+	+	+	+	+
P21	M (33)	MT-TG	+	+	+	+	+	+
P22	M (63)	MT-TW	+ (?)	+ (?)	n.c.	+ (?)	+ (?)	n.c.
Exercis	e trainin	g: group B						
P14	F (25)	MT-TL1	+	+	+	+	+	+
P15	F (38)	MT-TL1	+	+	+	+	+	+
P16	F (40)	MT-TL1	+	+	+	-	No effect	-
P17	F (47)	MT-TL1	-	No effect	-	-	-	-
P19	F (35)	MT-TE	No effect	+	+	+	+	+
P20	F (58)	MT-TE	-	+	-	+	+	+

Key: age = age when biopsied, years old; genotype = tRNA gene affected by the mutation

6.5 Discussion

Patients with mitochondrial myopathy frequently experience exercise intolerance, triggering a vicious cycle of sedentary lifestyle and muscle disability (Taivassalo and Haller, 2005). It is established that 14 weeks of endurance exercise increases aerobic capacity and quality of life of patients with mitochondrial disease (Taivassalo *et al.*, 1996, Taivassalo *et al.*, 1998). However less is known about the impact of long term exercise and more insights are needed into the underlying mechanisms of improved physiological responses. Quadruple immunofluorescence was used to assess muscle biopsies from patients with single large-scale mtDNA deletions and point mutations in mitochondrial encoded tRNAs that engaged in different training programmes. Muscle biopsies were obtain before the trial, after 6 months of endurance exercise and following 6 months of no specific training (only performing daily activities). Although results presented here are preliminary, this chapter shows that patients

with mitochondrial disease respond differently to endurance exercise. Three responses were found: an increase in complex I and IV; a decrease in both complexes; and an opposing effect with an increase in one complex but decline in the other. Due to the presence of freezing artefacts in most muscle biopsies, the effect of exercise on the overall mitochondrial mass was not studied.

6.5.1 The effect of endurance exercise

Previous studies have explored the changes in the activity of respiratory complexes from patients with mitochondrial disease undergoing endurance exercise. They found inconsistent results, reporting either an increase in the activity of the complexes affected by the mutation (Taivassalo *et al.*, 2001), or only mild increasing trends in activity with no changes in the percentage of COX deficient fibres (Jeppesen et al., 2006, Taivassalo *et al.*, 2006, Jeppesen *et al.*, 2009). The accuracy of the quadruple immunofluorescence assay allowed quantitative assessment of the subtle changes in complexes levels before and after periods of endurance training and periods of no training, which could not be detected using current biochemical or histochemical methodologies. Although some of the changes recorded in patient's biopsies were very subtle, it was recognised that they had practical significance. Furthermore, as the mutation load and mtDNA copy number have a direct impact on the activity and levels of respiratory complexes (Murphy *et al.*, 2012), the results revealed by quadruple immunofluorescence can be considered a good outcome measure of the mutational load in single fibres and ultimately, an outcome measure of the effects of exercise.

The effect of exercise on patients with single large-scale mtDNA deletions was variable. Indeed, in five patients (out of 10), exercise showed beneficial effects increasing the levels of complex I and IV. In three patients no training was more beneficial, leading to an increase in complexes I and IV levels, whereas exercise caused a decline. Interestingly in three of the patients, exercise showed contradictory results, with a positive impact on one complex but negative effect on the other. The possible underlying causes of the variability of results were further explored. There was no significant association found between the response to exercise and the gender, age or mtDNA mutation load of the patient. Also, the effect of exercise did not seem to correlate with the MRC profile, though patients responding negatively to exercise were only found in profile group 2, characterised by an earlier and more pronounced deficiency of complex I. Further investigations with a larger cohort of patients balanced in terms of MRC profile are needed to confirm this observation. The same was found for patients with point mutations in mitochondrial-encoded tRNAs. In five patients (out of nine), exercise caused an improvement in mitochondrial function, either through an increase in complex I and IV levels or through the attenuation of the decline in both complexes recorded following periods of no specific training. Two patients showed divergent results, whereas one patient showed an adverse response to exercise, with a decline in levels of both complexes. Yet again, no correlation was found between the response to exercise and the gender, age or genotype of the patient. However, further investigations with a larger and gender-balanced cohort of patients are needed.

Two patients with single deletions (P9, P12, group B) and one patient with a point mutation (P22, group A) showed no conclusive results. Although results were more convincing in some patients (P22), this chapter has shown the importance of considering effects from periods of training and no training when addressing the impact of exercise. For example in patient P7, although both periods of training and detraining led to an increase of COX-I and NDUFB8 levels, exercise attenuated that increase. By contrast, in patients P19 and P20, both periods of no training and endurance exercise led to a decline in COX-I and NDUFB8, despite attenuation of the decline following exercise. Without the assessment of the isolated effect of no training, it would be mistakenly concluded that exercise was beneficial for P7 and deleterious for both P19 and P20. In patients P9, P12 and P22 information regarding one of the effects was missing, and therefore it was not possible to assess the overall impact of exercise on these patients.

6.5.2 Weaknesses of this study

The work presented in this chapter was limited by the lack of information about the patients. A detailed report on the training intensity, patient's commitment and/or other factors such as different degrees of sedentary behaviour prior to the trial are crucial. These could help understand some of the variability recorded in patient's results. Furthermore, it would be interesting to correlate the results provided by the immunofluorescence assay with mtDNA mutation load, mtDNA copy number and assembly and function of respiratory complexes for each biopsy. On a larger scale, it would also be interesting to correlate the present results with the findings on skeletal muscle structure and function, as patients were assessed through magnetic resonance imaging (MRI) and spectroscopy (MRS) at the four time points.

Furthermore, not all patients recruited for the study were assessed. Forty patients participated in this study, 20 with single large-scale mtDNA deletions and 20 with point mutations in mitochondrial encoded tRNAs. Further investigations are therefore required to assess the remaining patients. Hopefully, this will provide more insight into the underlying causes of the different responses to exercise. Also, further studies are needed to assess the fourth biopsy taken from each patient. This will allow investigation of the impact of exercise over a period of two years - with exercise being performed for 12 (group A) or 18 months (group B). Finally, it should be mentioned that results need careful interpretation. It cannot be excluded that some of the differences revealed in this study may result from variations in the biopsy site. Perhaps a future study should aim to quantify the variability found in biopsies collected from the same muscle at different sites. Since this variability may also change with mutation load, different patients with different heteroplasmy levels should be evaluated. This would determine an expected percentage of variability for different mutation loads, which would then be taken into account when comparing different biopsies of the same patient.

Last but not least, another weakness of this study relates to the method of analysis. The approach used to calculate the Z scores requires further optimisation. At present, one Z score (for each variable: COX-I, NDUFB8 and porin) is calculated based on the control population parameters and attributed to each muscle fibre. However, since the control population is heterogeneous, several simulations of control groups should ideally be performed. This would allow calculating multiple Z scores for each fibre to assign an average Z score together with a confidence interval. As a result, the percentage change in COX-I and NDUFB8 levels between biopsies will be determined with a higher degree of precision. Furthermore, the data analysis approach also requires improvement. The present study used a combination of Z scores distributions and classification of fibres into broad categories (upper and lower classes) to describe the changes occurring in mitochondrial function with exercise. However, a more accurate analysis should be developed. Ideally this would feature an analysis based on the distribution.

Mitochondrial diseases are a group of heterogeneous disorders characterised by an impairment of the OXPHOS system and the skeletal muscle is commonly affected (Taylor *et al.*, 2004, Kirby *et al.*, 2007). When the mtDNA is involved, muscle often exhibits a particular feature: a mosaic pattern of COX deficiency (Johnson *et al.*, 1983, Sciacco *et al.*, 1994). Since only a subset of fibres is affected, deficiency is challenging to quantify. This research study was therefore aimed to (1) develop a novel assay to assess mitochondrial function in human skeletal muscle, which was then used to (2) test its use in improving the diagnosis of patients with mitochondrial disease and (3) evaluate the effectiveness of possible therapeutic approaches.

7.1 Main findings

The novel quadruple immunofluorescent assay presented in this thesis is an objective computer-based technique that allows accurate quantification of the two most commonly affected respiratory complexes - I and IV (Jackson *et al.*, 1995). Furthermore, the new graphical representation developed (MRC graphs) convey information about the biochemical profile of individual muscle fibres (dictated by the levels of complex I, complex IV and the mitochondrial mass). This graphical output offers the opportunity to answer multiple questions such as: which complex(es) is/are affected by the mutation; Is the defect symmetrical (affecting equally both complexes), slightly asymmetrical (affecting preferentially one), or markedly asymmetrical; Is the deficiency uniform across fibres or multiple populations of deficient fibres are found; Is the defect nuclear (affecting equally all fibres) or mitochondrial (affecting only a subset of fibres) driven?

In the context of studying disease mechanisms, forty-five genetically-characterised patients were assessed using the quadruple immunofluorescence assay. This work showed evidence that groups of patients harbouring the same mitochondrial or nuclear mutation manifest a similar MRC profile, with the exception of single large-scale mtDNA deletions. Interestingly, in the later, mutations show three different MRC profiles depending on the nature of the genes removed. Furthermore, this work provided information on how deficiency progresses at the single fibre level for different genetic defects. For example, in patients carrying the common m.3243A>G *MT-TL1* mutation, demonstrated that complex IV deficiency only develops after

complex I deficiency is established. By contrast, the assay showed that deficiency in patients with single mtDNA deletions progress through different paths depending on the nature of the mtDNA deletion; deficiency can affect complexes I and IV either simultaneously or asynchronously (with complex I preceding complex IV deficiency, or the opposite). Patients with multiple mtDNA deletions display a unique feature: two populations of fibres with distinct biochemical features.

The different biochemical signatures found in different genetic defects highlights the great diagnostic value of this technique. To further illustrate this, five patients with suspected mitochondrial disease were also assessed. Quadruple immunofluorescence assay allowed quantifying complex I deficiency in three of the patients where there was not enough muscle tissue to perform the biochemical studies. But importantly, it allowed narrowing-down the probable underlying genetic cause from all of these patients. This is novel and extremely valuable since such inference cannot be done using existing diagnostic tools. Currently, COX/SDH histochemistry just provides a yes/no answer and biochemical studies are only capable of detecting isolated or multiple deficiencies in cases where the defect is marked or severe.

Last but not least, the sensitivity of this technique allows detection of subtle changes, making the assessment of complexe levels revealed by this assay an important outcome measure in clinical trials aimed at improving muscle oxidative capacity. In that respect, twenty-two patients that engaged in a different endurance training programme were assessed. Quadruple immunofluorescence allowed the detection of small changes in respiratory chain levels in all patients' biopsies, which would not be possible applying alternative methods.

7.2 Future work

This research has raised several questions and has opened several routes for future investigations. One route includes further characterisation of common genetic defects causing mitochondrial disease. In these instances, it will be interesting to explore the MRC profile from the m.3243A>G *MT-TL1* (Goto *et al.*, 1990) and from other common point mutations, such as the m.8344A>G *MT-TK* (Shoffner *et al.*, 1990). This will allow investigating how the MRC profile correlates with the genotype and the clinical phenotype of patients. For the single large-scale mtDNA deletion patients, it will be crucial to investigate a bigger cohort of patients with CPEO in order to validate that the MRC profile reflects the nature of the individual mtDNA deletion. Additionally, future assessment of patients with KSS syndrome (Moraes *et al.*, 1989) will

identify if the MRC profile can be indicative of different clinical features. Finally, further mutations in nuclear genes that lead to the generation of multiple mtDNA deletions need to be studied in order to validate their common MRC profile. Other routes of investigations include the use of this technique to study: disease progression of common genetic defects, investigate mitochondrial dysfunction in other conditions such as secondary mitochondrial diseases and ageing, assess the impact of therapeutic approaches (not exclusively exercise training programs) in patients with mitochondrial diseases and assess the impact of endurance training programs in delaying sarcopenia.

Lastly, quadruple immunofluorescence also allows the opportunity for further developments. Future work encompasses the development of an in-house software. This will accelerate image analysis and improve sampling of fibres. This is important since the current software used (IMARIS) does not allow the quantification of sub-sarcolemmal accumulation of mitochondria (one of the hallmarks of mitochondrial dysfunction). Also, when the laminin labelling is not entirely uniform, the segmentation is more laborious and more editing is needed to separate merged surfaces. Therefore tailoring the software to the present needs will overcome these technical concerns. More progress remains to be made regarding the data analysis. This includes refining the way Z scores are calculated so an average Z score with a confidence interval are assigned to each fibre. In addition, the analysis and quantification of changes in studies where multiple biopsies from same patients are assessed requires further improvement. Finally, the potential of making the quadruple immunofluorescence assay into a quintuple immunofluorescence assay is large. A secondary antibody conjugated with 750 fluorophore is commercially available and will allow detection of a fifth epitope - either a subunit of complex III or V. Future work is required to select the two primary antibodies capable of detecting complex III or V deficiencies.

7.3 Final conclusion

This work has upgraded the methods of investigating mitochondrial function in human skeletal muscle, filling the following existing gaps: the absence of a histochemical assay to evaluate complex I, the subjectivity/inaccuracy associated with COX/SDH histochemistry, and the failure to detect subtle changes in complexes activity and levels. Using this novel assay, this work provided more insights into the nature of several genetic defects, and showed evidence that it can be broadly applied - in the current diagnostic setting and in future clinical trials.

8.1 Renewal of ethics approval

UT Southwestern Medical Center

eIRB System

 From:
 Ahamed Idris Institutional Review Board Chairperson IRB - 8843

 To:
 Ronald Haller , Mary Childers , Elaine Salinas

 Date:
 June 4, 2015

 Re:
 Continuing Review Approval

 IRB Number:
 CR00010951 /STU 092010-077 Number:

 Title:
 Exercise Adaptations in Mitochondrial Myopathy: Therapeutic Implications

Documents: Project Summary and all smart form attachments

The UT Southwestern Institutional Review Board (IRB) reviewed the above-referenced research study at a convened meeting of the full board on May 27, 2015. Having met all applicable requirements, the research study is approved for continuation for a period of 12 months. The approval period for this research study begins on June 4, 2015 and lasts until May 26, 2016. Please note, the consent form was not considered for re-approval because enrollment is closed to new participants.

The research study cannot continue beyond the approval period without continuing review and approval by the IRB. In order to avoid a lapse in IRB approval, the Principal Investigator must apply for continuing review of the protocol and related documents before the expiration date. A reminder will be sent to you approximately <u>90</u> days prior to expiration of research study approval.

If you have any questions related to this approval letter or about IRB policies and procedures, please telephone the IRB Office at 214-648-3060 .

General Instructions

To maintain IRB approval in good standing, please observe the following requirements:

- Obtain prior IRB approval for any modifications including addition of new recruiting materials, changes in research personnel or site location, sponsor amendments or other changes to the protocol or associated documents. Only those changes that are necessary to avoid an immediate apparent hazard to a subject may be implemented without prior IRB approval.
- 2. Report all adverse events, protocol violations, and study closures promptly to the IRB.
- 3. Make study records available for inspection. All research-related records and documentation may be inspected by the IRB for the purpose of ensuring compliance with UT Southwestern policies and procedures and federal regulations governing the protection of human subjects. The IRB has authority to suspend or terminate its approval if applicable requirements are not strictly adhered to by all research study personnel.

Warning: This is a private message for authorized UT Southwestern employees only. If the reader of this message is not the intended recipient you are hereby notified that any dissemination, distribution or copying of this information is STRICTLY PROHIBITED.



University of Texas Southwestern Medical Center Institutional Review Board

> 5323 Harry Hines Boulevard Dallas, Texas 75390-8843 phone: 214-648-3060 fax: 214-648-2171

The University of Texas Southwestern Medical Center at Dallas Institute for Exercise and Environmental Medicine, Presbyterian Hospital at Dallas

CONSENT TO PARTICIPATE IN RESEARCH

TITLE OF STUDY: Exercise Adaptations in Mitochondrial Myopathy: Therapeutic Implications (Patients)

SPONSOR: National Institute of Health (NIH)

INVESTIGATORS:	PHONE # OFFICE	NIGHTS & WEEKENDS
 <u>Ronald G. Haller, M.D.</u> <u>Marta Newby, R.N.</u> <u>Qi Fu, M.D.</u> <u>Lauren Phillips, M.D.</u> Lydia Sharp, M.D. 	(214) 345-4617 (214) 345-4655 (214) 345-8125 (214) 345-7112 (214) 345-7112	<u>(214) 352-7395</u> (214) 693-6144

INVITATION: You are invited to participate in this research because you have been diagnosed with a mitochondrial myopathy (a disease of the muscle) due to a mutation in mitochondrial DNA (the genes contained within mitochondria). This research study is aimed at identifying the effects of regular exercise training and of not exercising regularly upon mitochondrial (small structures within cells where oxygen is used and carbon dioxide produced in the process of energy production) function and on levels of normal versus mutant mitochondrial DNA. We are also interested in determining whether you have evidence of dysfunction of the autonomic nervous system (the part of the nervous system which controls such functions as blood pressure, heart rate, sweating, and digestion).

NUMBER OF PARTICIPANTS: It is anticipated that 108 subjects will screened for this study. Forty-eight patients and 20 controls will be enrolled.

PURPOSE: The purpose of this study is to determine the ability of exercise training to improve muscle exercise performance and muscle mitochondrial function and to determine the effects of stopping exercise in patients with muscle mitochondrial disorders due to mutations in mitochondrial DNA. Every muscle cell contains thousands of mitochondria and each mitochondrion contains 2-5 copies of DNA. Your muscle mitochondria contain a mixture of normal and abnormal (mutant) DNA. When levels of mutant mitochondrial DNA greatly outnumber the level of normal mitochondrial DNA in skeletal muscle, the muscle cells are not able to produce energy that is needed for normal physical activity. This results in symptoms of decreased exercise ability and abnormal fatigue that may be associated with shortness of breath and rapid heart rate during minimal levels of activity. Decreased exercise capacity may also be seen in individuals who are healthy but very physically inactive. Exercise training is well known to increase exercise ability in healthy individuals by stimulating increases in mitochondria and circulatory capacity that improve muscle and heart function. This research is being done because we want to determine whether these normal changes will also occur in response to exercise training in muscle of patients with muscle energy defects due to mitochondrial DNA mutations and result in improved exercise ability and quality of life in such patients. A secondary purpose of this study is to determine whether patients affected with mitochondrial myopathy demonstrate evidence of autonomic dysfunction. The autonomic system affects many aspects of the body, including regulation of heart rate, blood pressure, sweating, and digestion. Some patients with autonomic dysfunction experience exercise intolerance and easy fatigue, and a few studies have suggested that symptoms of autonomic dysfunction can be seen in some patients with mitochondrial disease. This research is

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being done because we want to determine whether there is any evidence of autonomic dysfunction in people who are known to have mitochondrial myopathy.

PROCEDURES

SCREENING: The study doctor or nurse will ask you questions on the phone about your health, medications you take, and any surgical procedures you have had. If you qualify for the study you will be scheduled for five days of exercise testing at the Neuromuscular Disease Center of the Institute for Exercise and Environmental Medicine at Presbyterian Hospital of Dallas or at the University of Texas Southwestern Medical Center.

Group Assignment: If the study doctor believes that you qualify to participate in this research, you will participate in a two year exercise training program. This program contains two groups. One group of subjects will exercise regularly for 6 months and stop regular exercise and resume normal activity for 6 months. The other group will continue their regular level of activity without regular exercise for 6 months and then will exercise regularly for 6 months. At the end of one year, both groups will be encouraged to exercise regularly. Whether you will start with regular exercise or not will be made by a process similar to flipping a coin.

Treatment: Your exercise training treatment includes 6 months of about 100 training sessions at 70-80% of your maximum heart rate. During the first 3 months you will exercise on a stationary bike for 30 minutes per session. During the second 3 months you will exercise 40 minutes per session. The training will be done at a gym of your choice and supervised by an exercise physiologist and physician.

Evaluations during the research:

The following tests will be repeated during the study to determine the benefit and safety of exercise training.

Day 1: Medical history and physical examination Health questionnaire Physical activity questionnaire Maximal cycle exercise with blood sampling (using an arm venous cathether, a small thin plastic tube inserted in an arm vein) and measurement of cardiac output (the amount of blood pumped by the heart).

- Day 2: Autonomic function testing (performed during one of the four exercise evaluations) (1.5 hours)
- Day 3: Moderate (submaximal) cycle exercise with blood sampling using a venous cathether
- Day 4: Moderate (submaximal) cycle exercise with blood sampling using a venous cathether Receive individualized instructions for exercise training Get familiar with the equipment (2-4 hours)

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Day 5: Maximal cycle exercise with blood sampling using a venous catheter and measurement of cardiac output Muscle biopsy (less than a size of a pea) (3-4 hours)

You will be evaluated according to the above schedule 4 times: during the initial evaluation, at 6 months, at 1 year and at the end of 2 years. Less then than half of a cup of blood will be taken during each of the four periods of exercise evaluation. Less than 2 tablespoons of blood will be taken during autonomic function testing.

EVALUATIONS DURING THE RESEARCH:

If you decide to participate in this two year study you are committing to 4 muscle biopsies, approximately 60 hours of testing and having two cups of blood drawn. You will undergo the following tests.

Aerobic Exercise Tests

You will be asked to exercise continuously from 5-40 minutes at several workloads on a stationary bicycle to measure the effects of exercise stress on your heart, lungs and muscles. Measures will be obtained at rest (before exercise) and during exercise, and include breathing through a mouthpiece (similar to the apparatus used in snorkeling), and the continual monitoring of your heart rate and blood pressure. You will exercise at both moderate and high intensity levels, and may stop the exercise at your own will or at the instruction of the qualified physician who will directly supervise the test. You will undergo this test three times on three separate days at the beginning, at 6 months, at 1 year and at the end of two years.

Cardiac Output (the amount of blood pumped by the heart in a minute)

We will measure the amount of blood the heart is pumping at rest and during exercise by analyzing the air you breathe. You will breathe through a mouthpiece connected to a bag full of air and a small concentration of harmless gases, including helium and acetylene for 15-20 seconds. There is no known risk to anyone doing this procedure.

Arm Venous Catheters and/or needle stick for single blood sample (Peripheral)

A thin, soft, plastic tube (catheter), will be inserted into an arm vein during each day of exercise testing to avoid repeated punctures during studies that require several blood samples or injections. Samples of blood will be removed from a vein in your arm and will be used for various analyses. A maximum of 130 ml (about a half of a cup) of blood will be withdrawn during the entire week and this will not affect your health or well-being. You will have the catheter placed for 3 days. 2 days about 60ml will be removed and 10 ml removed on the third day. During the autonomic function testing, an additional 20 ml of blood will be removed. Usually this procedure is done without difficulty, but occasionally, a bruise results. You may experience discomfort or bleeding, or feel dizzy and faint. There is a very small risk of infection and a still smaller risk of a blood clot or breakage of the catheter. The likelihood of any complications is very remote when the procedure is carried out by trained personnel and proper equipment is used.

Muscle Biopsy

This procedure will allow us to obtain a small sample of muscle from your thigh for examination of muscle histology (as examined under a microscope) and biochemistry One or two small pieces (totaling

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about 450 milligrams or approximately the size of a pea) are obtained. This is done by numbing about a 1/4 inch spot on your thigh with an injection of Lidocaine, a local anesthetic similar to Novocain used in the dentist's office. A nick in the numbed skin will be made with a scalpel; a special sharp pencil-shaped tube will be inserted a short distance into the thigh and the small piece of muscle is removed. No stitches are required to heal the opening. A very small scar may result. Rarely, slight bleeding occurs and even more rarely, requires a larger incision to find and tie off the bleeding vessel. After the Lidocaine effect wears off, the thigh will be sore for 1-2 days and usually does not interfere with normal activities. Tylenol is given for the discomfort. Strenuous exercise should be avoided for 1-2 days. Infection is a very rare complication but can be effectively treated should it occur by your PCP (Primary Care Physician) or Dr. Haller with antibiotics.

Exercise training

This consists of participation in a two year program consisting of periods of regular exercise training and periods during which you will not perform regular exercise training. During the exercise phases of the study, you will be asked to ride a stationary bicycle 3 to 5 times a week up to a maximum of 40 minutes per training session. The level of exercise you will be asked to perform will be based on 70-90% of your maximal heart rate. You will be given a Polar Heart Rate Monitor to take home and use during training sessions. You will be instructed as to how to use this monitor which will record your heart rate throughout your exercise sessions. There is little or no risk to healthy individuals during exercise at submaximal target pulse rates. In patients with skeletal muscle disease there is the possibility of muscle pain and cramping. You should reduce your exercise level if you experience muscle pain or exceed your target heart rate. If muscle pain persists or muscle cramping occurs or if you should experience chest pain, dizziness, or irregular heartbeats you should stop exercising at once and promptly notify the investigators.

During the phase of the study where you are not exercising you will be asked to resume the level of activity that you did before participating in this research project. In order to help us determine that level of activity, we will ask you to maintain an activity diary and to wear a monitor for 2-6 weeks every 6 months that measures physical activity levels. The monitor is similar to a pager and is worn at the waist. Data will be sent to our laboratory and downloaded.

SF-36 Health Survey

At the time of your initial visit and during follow up visits, you will be asked to complete a questionnaire that will provide insight into your assessment of your level of health and well-being.

Autonomic Function Testing

- (1) Height, weight, baseline sitting blood pressure and heart rate
- (2) Peripheral IV placement in laying down position
- (3) Apply EKG (Electrocardiogram) leads and blood pressure cuffs to arm and finger
- (4) Lay flat for at least 10 minutes, then draw 1 tube of blood
- (5) Breathe at rate of 12 breaths per minute for 3 minutes, and then 6 breaths per minute for 1
- minute while measuring heart rate and blood pressure responses (6) Blow into a mouthpiece at 40 mmHg for 20 seconds while measuring heart rate and blood
- (6) Blow into a mouthpiece at 40 mmHg for 20 seconds while measuring heart rate and blood pressure

(7) After resting for 10 minutes, proceed with 10 minute stand test: You will be asked to lay flat and quietly on an exam table for an additional 10 minutes, and then you will be asked to stand

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up straight for 10 minutes. During this time, your heart rate and blood pressure will be measured and you will be asked how you feel. 1 tube of blood will drawn from the IV 5 minutes after you stand up. (8) Peripheral IV will be removed

INVESTIGATIONAL PROCEDURES:

While it is well known how regular exercise training and habitual inactivity affect skeletal muscle and the heart in healthy persons, the effect of these different activity patterns in patients with muscle mitochondrial disorders is poorly understood. In particular, the effects of regular physical activity or inactivity upon muscle mitochondrial function in patients who have mutations in mitochondrial DNA is not known. The tests that will be performed before and after exercise training or stopping regular exercise training will enable us to determine how your heart, muscles, and muscle mitochondria respond and will provide a basis on which to advise patients in the future about the benefits or risks of regular exercise or habitual inactivity.

RISKS AND DISCOMFORTS:

Aerobic Exercise Tests:

Exercise rarely causes any problems in normal subjects but the test may in patients with known or hidden heart disease cause chest pain, dizziness, or bouts of irregular heart rhythm. There is a very slight risk of a heart attack occurring during or after such exercise but this risk is no different than if you were performing such exercise on your own at home. Your pulse, blood pressure, and electrocardiogram (EKG) will be recorded during the test. A recent report on risks of maximal dynamic exercise testing indicated no significant complications in a population of more than 300,000 normal subjects. The risk of death in a series of 700,000 tests in patients with heart disease was less than 1/40,000 test and the risk of serious complication 1/10,000 tests.

Muscle Biopsy:

A very small scar may result. Rarely, slight bleeding occurs and even more rarely, requires a larger incision to find and tie off the bleeding vessel. Infection is a very rare complication but can be effectively treated, should it occur, by your PCP or Dr. Haller with antibiotics.

Throughout this experiment, multiple procedures may be performed. Individually each procedure should not cause you much discomfort, however it is possible that you may experience discomfort due to the sum of these procedures. The protocol is designed to reduce the likelihood of this occurring by minimizing the duration of each procedure. Nevertheless, if you experience a degree of discomfort which is greater than your expectations, we will stop the procedure(s) at your request. All tests that are to be performed have safely been used in both healthy individuals and patients with muscle disorders. Throughout the tests, you will be closely monitored by Dr. Haller or Dr. Heinicke and by a highly skilled research nurse.

Autonomic function tests:

Rarely patients who undergo autonomic function testing will experience symptoms of presyncope or syncope. Most of the time, patients tolerate these tests very well. Throughout the tests, you will be closely monitored by Dr. Haller, Dr. Phillips, or Dr. Sharp and by a highly skilled research nurse.

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Pregnancy test: A pregnancy test will be performed for any woman who is able to have children and wishes to participate in this research. A pregnancy test may be repeated later. A study doctor will ask for the date when a woman's last monthly period started.

Avoiding pregnancy: You should ask your study doctor about the effective means to avoid becoming pregnant during this research. If you change your method of avoiding pregnancy during the research, you must notify your study doctor promptly.

Pregnancy during participation in this research: If you become pregnancy during this research, you must tell your study doctor immediately. Your participation in the research will stop.

UNFORESEEN RISKS: A previously unknown problem could result from your participation in this research. There could be an interaction between exercise and medications you take (prescribed or over-the-counter). It is not possible to estimate the chances of such problems or how serious the problems could be.

How you can help reduce some of the risks: During your participation in this research, the study doctor and investigators will watch closely to determine whether there are problems that need medical care. It is your responsibility to do the following:

- · Ask questions about anything you do not understand.
- Keep appointments.
- · Follow the study doctor's instructions.
- · Let your study doctor know if your telephone number changes.
- Tell your study doctor before you take any new medication even if it is prescribed by another doctor for a different medical problem.
- Tell your regular doctor about your participation in this research.
- · Talk to a family member or friend about your participation in this research.
- Carry information about the research in your purse or wallet.

What to do if you have problems: If you have problems, such as unusual symptoms or pain, at any time during your participation in the research, your study doctor can recommend treatment. Please report the problem to your study doctor promptly. Telephone numbers where any of the investigators may be reached are listed on the first page of this form. One of the investigators will be available to answer your questions about the study at any time.

If you suddenly have a serious problem (such as difficulty breathing) or severe pain, go to the nearest hospital emergency room, or call 911 (or the appropriate emergency telephone number in your area). Tell emergency personnel about your participation in this research. Ask them to telephone your study doctor immediately.

POSSIBLE BENEFITS

Benefit to you: This study may improve symptoms associated with impaired energy metabolism in your muscles and result in overall improved functional capacity. While your medical problem may get better, it also could possibly get worse. We cannot guarantee that you will benefit from participation in this research.

Benefit to other people with disorders of muscle energy metabolism: Knowledge gained from this study is expected to provide guidance with respect to the advisability of regular exercise to other people with mitochondrial myopathies. Additionally, we will learn more about whether autonomic nervous system dysfunction is seen in patients with mitochondrial myopathy

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In these ways, information gained from this research could lead to improved medical care for these patients. However, we will not know whether there are benefits to these other patients until all of the information obtained from this research has been collected and analyzed.

ALTERNATIVES TO PARTICIPATION IN THIS RESEARCH: You do not have to participate in this research to receive care for your mitochondrial myopathy. Please ask your study doctor as many questions as you wish. The doctor's answers to your questions could help you decide whether to participate in this research. If you decide to participate in research now, and later change your mind, you may stop your participation in the research at any time.

THE STUDY DOCTOR'S DECISION TO STOP YOUR PARTICIPATION: Your study doctor or the sponsor may stop your participation in this research without your permission under any one of the following conditions:

- Your medical problem becomes worse.
- Your study doctor believes that participation in the research is no longer safe for you.
- Your study doctor believes that other treatment may be more helpful.
- The sponsor stops the research for the safety of the participants.
- The sponsor cancels the research.
- · You are unable to keep appointments or to follow your study doctor's instructions.

PROCEDURES AFTER STOPPING PARTICIPATION IN THIS RESEARCH: If you, the study doctor, or the sponsor stops your participation in the research, it is your responsibility to do the following:

- Let your study doctor know immediately that you wish to withdraw from the research.
- Return to the research center for tests that may be needed for your safety.
- Return all study equipment.
- Discuss your future medical care with your study doctor and/or your regular doctor.

PAYMENT TO TAKE PART IN THIS RESEARCH: You will not be paid to undergo exercise training as part of this research. However, certain expenses that you may incur during the study may be paid for, such as gym fee, travel or accommodations.

If you are an employee of UT Southwestern, tax will be deducted from the payment given to you for your participation in the research. UT Southwestern, as a State agency, will not be able to make any payments to you for your participation in this research if the State Comptroller has issued a "hold" on all State payments to you. Such a "hold" could result from your failure to make child support payments or pay student loans, franchise taxes, etc. Should this occur, UT Southwestern will be able to pay you for your participation in this research after you have made the outstanding payments, and the State Comptroller has issued a release of the "hold."

COSTS TO YOU: There will be no charge for any of the procedures performed during the study. There are no funds available to pay for lost time away from work and other activities, lost wages, or child care expenses. Expenses related to standard medical care for mitochondrial myopathy are your responsibility (or the responsibility of your insurance provider or government program).

COMPENSATION FOR INJURY: Compensation for an injury resulting from your participation in this research is not available from the University of Texas Southwestern Medical Center at Dallas or the Institute for Exercise and Environmental Medicine of Presbyterian Hospital, Dallas. You retain your legal rights during your participation in this research.

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VOLUNTARY PARTICIPATION IN RESEARCH: You have the right to agree or refuse to participate in this research. If you decide to participate and later change your mind, you are free to discontinue participation in the research at any time.

Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. Refusal to participate will not affect your legal rights or the quality of health care that you receive at this center.

NEW INFORMATION: Any new information which becomes available during your participation in the research and may affect your health, safety, or willingness to continue in the research will be given to you.

"RECORDS OF YOUR PARTICIPATION IN THIS RESEARCH: You have the right to privacy. Any information about you that is collected for this research will remain confidential as required by law. In addition to this consent form, you will be asked to sign an "Authorization for Use and Disclosure of Protected Health Information for Research Purposes."

CERTIFICATE OF CONFIDENTIALITY: To help us further protect your privacy, the investigators have obtained a Confidentiality Certificate from the Department of Health and Human Services (DHHS).

With this Certificate, the investigators cannot be forced (for example by court subpoena) to disclose research information that may identify you in any Federal, State, or local civil, criminal, administrative, legislative, or other proceedings. Disclosure will be necessary, however, upon request of DHHS for audit or program evaluation purposes.

You should understand that a Confidentiality Certificate does not prevent you or a member of your family from voluntarily releasing information about yourself or your involvement in this research. Note however, that if an insurer or employer learns about your participation, and obtains your consent to receive research information, then the investigator may not use the Certificate of Confidentiality to withhold this information. This means that you and your family must also actively protect your own privacy.

If the investigator suspects child, elder or disabled person's abuse, they will report such concerns to proper authorities as required by law.

Finally, you should understand that the investigator is not prevented from taking steps, including reporting to authorities, to prevent serious harm to yourself or others.

YOUR QUESTIONS: Ronald Haller, M.D. is available to answer your questions about this research.

The Chairman of the IRB is available to answer questions about your rights as a participant in research or to answer your questions about an injury or other complication resulting from your participation. You may telephone the Chairman of the IRB during regular office hours at 214-648-3060.

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YOU WILL HAVE A COPY OF THIS CONSENT FORM TO KEEP.

Your signature below certifies the following:

- You have read (or been read) the information provided above.
- You have received answers to all of your questions.
- You have freely decided to participate in this research.
- · You understand that you are not giving up any of your legal rights.

Participant's Name (printed)			
Participant's Signature	Date	Time	AM/PM
Name of person obtaining consent (printed)			
Signature of person obtaining consent	Date	Time	AM/PM

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